Ectomycorrhizae of Sessile Oak (*Quercus petraea* (Matt.) LIEBL.): Their Distribution, Abundance and Aluminum Content with Respect to Limed and Unlimed Regions of Merzalben Forest District 04/0705, Palatinate Forest, Rheinland-Pfalz, Germany

Dissertation

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Hiermit versichere ich, die vorliegende Arbeit selbständig und nur mit den angegebenen Hilfsmitteln angefertigt zu haben.

Datum:

Unterschrift:

Original Hypothesis

"The sessile oaks in a limed forest will exhibit greater seasonal diversity and abundance of their symbiotic mycorrhizal species in rootlets extracted from three soil horizons (A, B, and C); mycorrhizal root tips will have less evidence of incorporated aluminum; and whole roots will have quantitatively less aluminum present than in similar unlimed regions of oak forest." (Wilson, 1999).

ABSTRACT

A. General Mycorrhizal Abundance and Diversity

Ectomycorrhizal oak tree roots from three soil horizons (0-10 cm, 30-40 cm and 50-60 cm) from unlimed and limed observation plots in the Rheinland Palatinate Forest in Germany were sampled spring and fall 1999 and 2000. In the unlimed probes, the mycorrhizal diversity was lower than in the limed probes, however contrary to expectations, the relative abundance of each mycorrhizal species and the total fine root biomass was higher in the unlimed samples for every horizon and season. In addition, liming dramatically affected the seasonal mycorrhizal dominance hierarchy. Liming dramatically reduced the abundance, but increased the diversity of mycorrhizae implying long-term subterranean K-selection stress.

B. Aluminum and Soil Depth

The second focus of this study is to localize and quantify potentially toxic aluminum within symbiotic ectomycorrhizae and the fine host roots of stressed sessile oaks growing in soils affected by acid precipitation and compare them to ectomycorrhizal roots from

lime treated soils in an adjacent oak forest. Histochemical and mineral analysis of the mycorrhizal tips determined that each species was independent but characteristic in its aluminum uptake with some species acting as barriers while others allowed or even promoted aluminum movement into adjacent fine root cells. At 0-10 cm depth, samples from the unlimed soils (pH 3.53-3.79) usually had little or no aluminum present, while surprisingly, many samples from the limed soils (pH 4.24 - 4.47) exhibited enhanced aluminum uptake and sequestration. There was no significant difference in aluminum uptake at 30-40 and 50-60 cm depths between the plots but the aluminum content tended to increase relative to soil depth.

C. Morphological localization of Aluminum

Aluminum freed into the acidified soil solution, and taken up by the highly absorptive ectomycorhizae on fine roots, was either deposited outside the walls or incorporated into the mycorrhizal sheath and/or filtered into the hartig net and stopped, or passed freely into the corresponding root cells. ***The mode of aluminum binding or blocking in the ectomycorrhizae was extremely species specific.*** In general, mycorrhizae on the limed plots incorporated more aluminum into their cell walls, and as a result less unbound aluminum was translocated into the fine roots than in comparative species from the unlimed forest soils. If aluminum passed the mycorrhizal barrier, it was apoplastically translocated through the cortical zone up to the endodermal barrier. Once in the cortical zone, the heaviest accumulation areas were generally adjacent to the endodermal barrier. Aluminum did not accumulate in the endodermal, hypodermal or phloem cell walls, except in rare instances. These cells seemed to be barriers to translocation. In

meristematic areas where the endodermis had not yet formed, or in regions where secondary roots disrupted the endodermal barrier, aluminum entered the stele. Once in the stele, aluminum targeted the xylem and was strongly incorporated into the lignified walls. Aluminum, which has been previously found in the leaves and hardwood of oaks was most likely translocated to these regions via the xylem flow.

D. Bound verses Unbound Aluminum

Bound aluminum content was higher in mycorrhizal roots from limed probes but the free unbound aluminum content was reduced in comparison to the unlimed probes. In combination with the fluorescence observations, liming was associated with increased aluminum retention in the mycorrhizal tissues and reduced translocation to the root proper. Initially one might consider this to be a benefit to the tree, but accumulation of toxic aluminum is definitely not of benefit to the mycorrhizae. A new question arises then. If there is a prolonged drought or other negative environmental impact, then what will happen to all the aluminum bound in the mycorrhizae that are so intricately associated with the fine roots ? Will it dissipate back into the soil or will it be released suddenly and directly into the fine roots ?

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SECTION A: INTRODUCTION

A1-1-1: *Quercus petraea*

Quercus petraea (Mattuschka) LIEBL (Syn: *Quercus sessilis* ERH; *Quercus sessiflora* SALIB.) is commonly known as the Traubeneiche or Wintereiche in Germany (Schütt et al, 1992), British oak (Romagnoli & Codipietro, 1996), Sessile or Durmast Oak in England (Lewington & Streeter, 1993), and Chêne sessile (Timbal & Aussenac, 1996). It is a tree of enormous economic value because of its fine hard durable timber (Van Dersal, 1938). *Quercus petraea* is a member of the beech family Fagaceae, genus Quercus, subgenus Lepidobalanus (white oak), section Robur and it is closely related to *Q. pubescens, Q. robur, Q. pyrenaica, Q. macranthera and Q. frainetto* (Schütt et al, 1992) all members of the rugged white oak group with similar smooth lobed-leaf morphology (Encylopaedia Britannica, 1973) (Figure A1-1-1). Hybridization can occur between *Quercus petraea* and *Q. pubescens* to produce *Q. robur* and likewise between *Q. petraea* and *Q. robur* to produce *Q. robur x rosacea* a characteristically intermediate offspring and furthermore, *Q. robur* being very versatile and can hybridize with *Q. ilex* to produce *Q. robur x turneri* (Schütt et al, 1992). All this implies both close genetic ties and temporal evolution which may be possibly translated into similarities in the dynamic ectomycorrhizal relationships. Furthermore, if the loss of ectomycorrhizal diversity due to environmental stressors affects the health and vigor of one host species, then it is quite probable that the same detrimental changes in fungal biodiversity would also affect other related species in possibly geographically distant regions. This study is an initial attempt at monitoring the vigor and diversity of symbiotic root fungi of *Quercus petraea* which may help us to understand the far reaching effects of negative environmental changes.

Differentiation of common oak species can be made by leaf size, margins, vein distribution and petiole length (Figure A1-1-1). Because of natural variation in leaf morphology, a more accurate identification is made by including other growth factors such as twig and bud morphology, tree height, crown shape, bark, wood grain and geographic location (cf: Porkorný & Tousová 1986; Lohmann, 1997; Hecker, 1998).

Examples of leaves and acorns of *Quercus petraea* collected from selected trees from the study area in Merzalben Forest District 04/0705 are presented in Figure A1-1-2.

Generally, most oaks do not produce acorns until about twenty years of age and often require a century or more to be fully mature with an average life-span of 200 to 300 years (Comstock, 1922). Sessile oak can grow to a height of 20 to 30 meters (max. 40) with a diameter of 2 meters, and can live up to 800 years with an average life expectancy of 435 years (Schütt et al, 1992). The average life expectancy which has declined dramatically over the last half century has been attributed to a variety of diseases, climatic stresses and pollution.

A1-1-2: *Quercus petraea* Distribution in Europe

Quercus petraea is one of the approximately 600 oak species found world wide (Schütt et al., 1992). About ten different oak species are native to the European continent (Schütt et al., 1992), and of these, pedunculate oak (*Quercus rubor* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) predominate Central Europe (Schütt et al., 1992, Timbal & Aussenac, 1996). (Figure A1-1-3). Sessile and pedunculate oaks are differentially adapted to their primary habitats. While sessile oak (winter oak) tolerates severe winters and occurs naturally on elevated plains, hills and mountains up to 700 meters above sea level, most pedunculate oaks (summer oaks) favor relatively milder winters and prefer lowland habitats (Schütt et al, 1992). The sessile oak of the Palatinate Forest is considered to be autochthonous, while most pedunculate oak stands in Rheinland-Palatinate were nursed from seeds of unknown origin, with some seeds derived from late budding subpopulations imported from Slovenia (Maurer and Tabel, 1995). The late budding varieties were introduced because they are resistant to late frosts and to several parasites such as oak tortrix species and winter moth species. The quality of wood is generally better when from mixed stands of beech and oak than from pure stands (Timbal and Aussenac, 1996). It is likely that the predominance of these species in European forests is not entirely natural but the direct result of anthropogenic selection. The distribution of Sessile oaks in Germany is shown in Figure A1-1-4. .)

A1-1-3: *Quercus petraea* in Merzalben

The oak forest region under consideration is located approximately 7.5 kilometers SSE of Trippstadt, in Rheinland-Pfalz in Merzalben Forest District 04/0705, a long term ecological research station under the jurisdiction of the Forstliche Versuchsanstalt Rheinland-Pflaz. (Figure A1-1-5). At approximately 523 to 591 meters in elevation (Fig. A1-1-5 and GPS), the specific area of study is a mixed forest of primarily *Quercus petraea* interspersed with common European beech (*Fagus sylvatica* L.) and a minor population of Scott's pine (*Pinus sylvestris* L.) (Schütt et al, 1992). The general distribution of trees in the vicinity of the research station is shown in Figure A1-1-6. Figure A1-1-7 shows the actual station with mature tree canopy shading the forest floor. Mounting concern has been expressed over the rapid demise of all oak species throughout Germany, with many trees living only to 125 to 150 years of age, or less than half of their expected life span and others showing symptoms of severe stress (Block, 1999, pc). In 1989, an area of approximately 60 square meters outside of the fenced research facility was limed in an effort to counteract the damaging effects of acid rain (Block, 1999, pc). The oak forest beyond this region was left unbuffered. Figure A1-1-8 is a schematic diagram showing the actual distribution of *Quercus petraea* and *Fagus sylvatica* in the selected limed and unlimed forest zones studied. In Rheinland - Pflaz, oak trees (Eiche), which comprise 17 % of the forested area, (Kronauer, 1999), are under severe stress.

Figure A1-1-1: Six common European Oak leaves shown at 50% their original relative sizes. Each can be identified by their leaf morphology and dendrological facts (Schütt et al, 1992, p. 246). A = *Quercus petraea* (leaf length, L: 6-16 cm; petiole length, P:.8-30 mm). B = *Quercus pubescens* (L: 5-10 cm, : 7-10 mm). C = *Quercus robur* (L: 5-15 cm, P: 2-13 mm). D = *Quercus frainetto* (L: 10-20 cm, P: 6-10 mm). E = *Quercus macranthera* (L: 6-18 cm, P: 1-2 cm). F = *Quercus pyrenaica* (L: 6-17 cm, P: 5-15 mm).

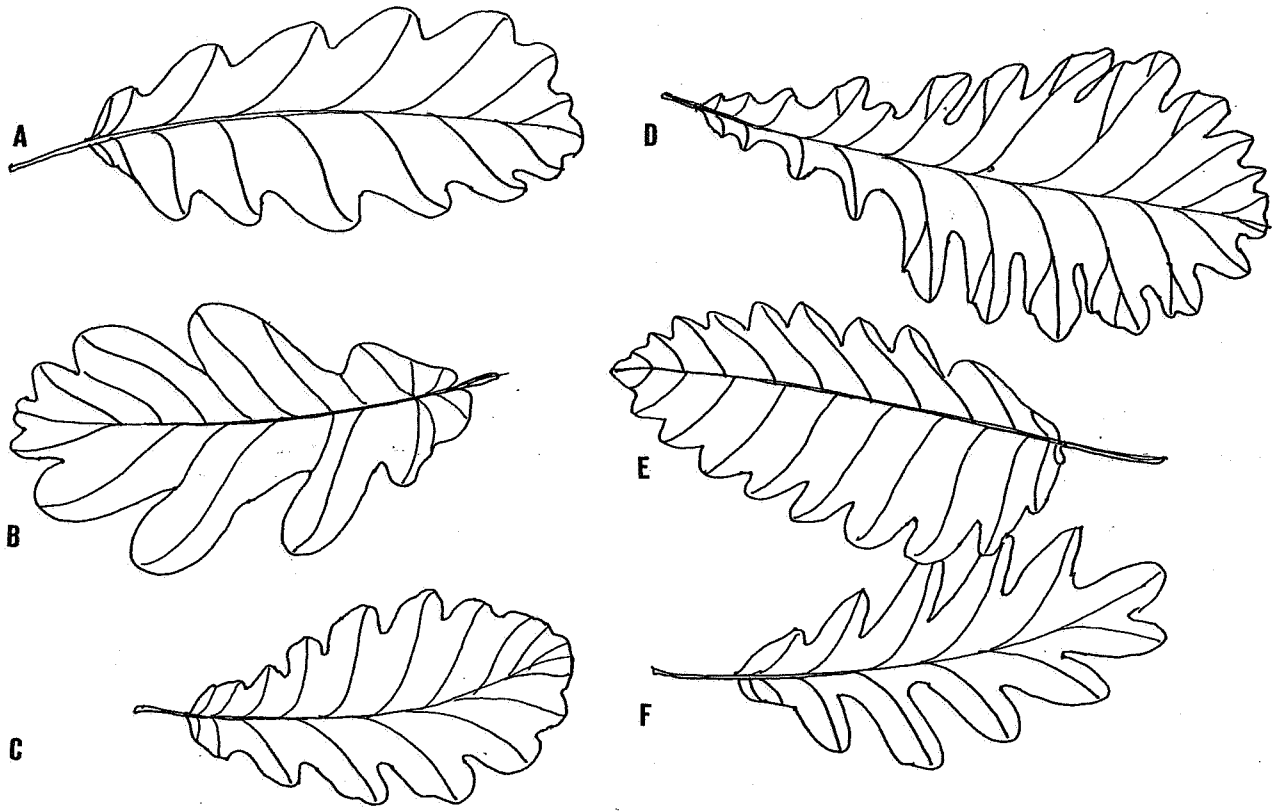


Figure A1-1-2: *Quercus petraea* (Matt.) LIEBL. (Sessile Oak) leaves and acorns collected from Merzalben Forest District 04/0705 in fall 2000. Actual size shown. The leaves show minor insect damage and some peripheral cell necrosis. A = Leaf surface. B = Underside of leaf. C = Acorns in left to right sequence from freshly dropped to previously dropped, with the last showing signs of germination.

A

B

C

Figure A1-1-3: Natural distribution of the two dominant European oak species; *Quercus petraea,* which is more limited in range, and the more wide-spread *Quercus robur.* Drawn with information provided by Schütt et al (1992, p. 437).

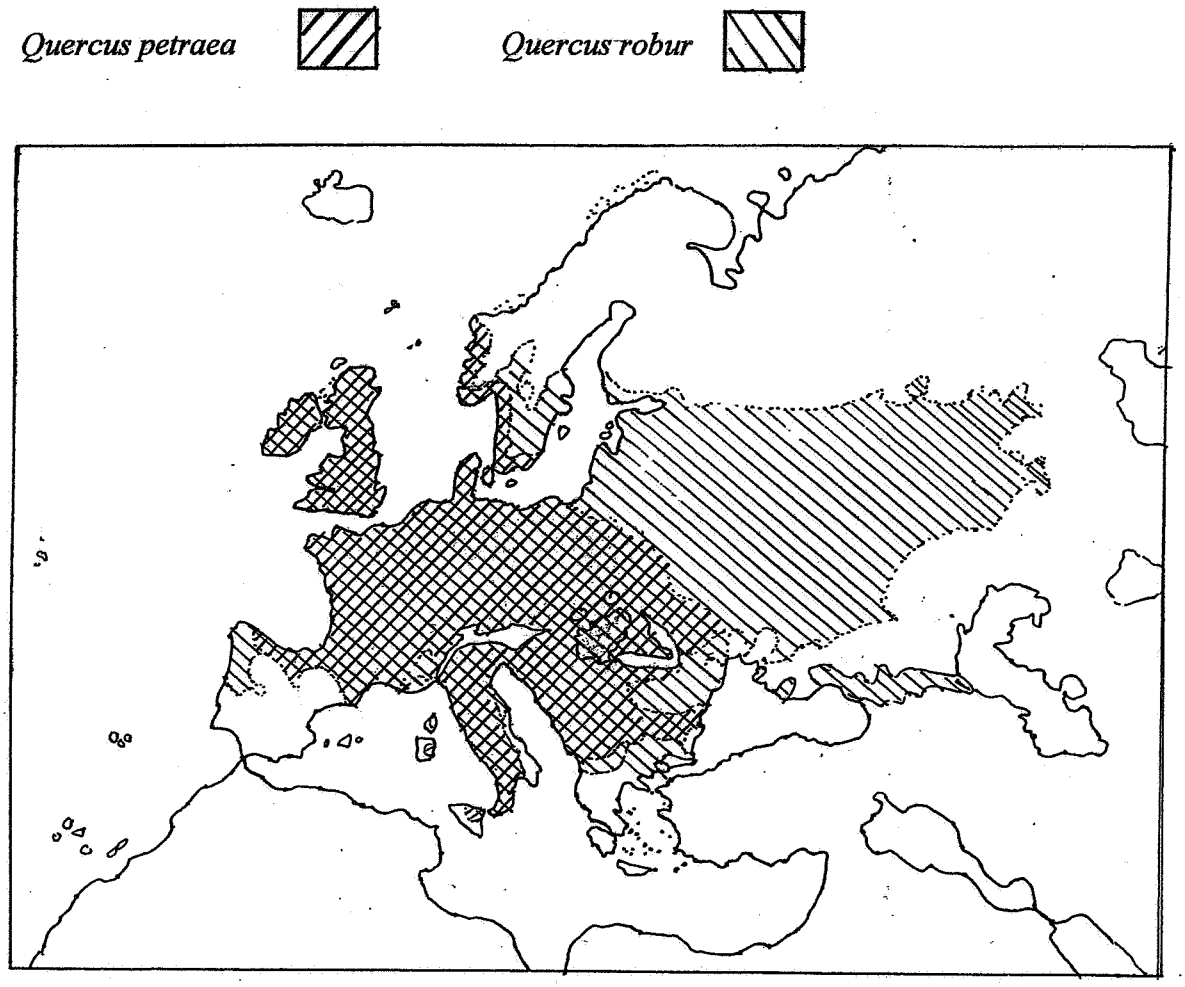


Figure A1-1-4: Density distribution of *Quercus petraea* in Germany in 2002. Darker shading implies denser distribution. The area under study is marked *. Map derived from the German forestry school (www.baumschule.de). GIF image 550x720 pixels.

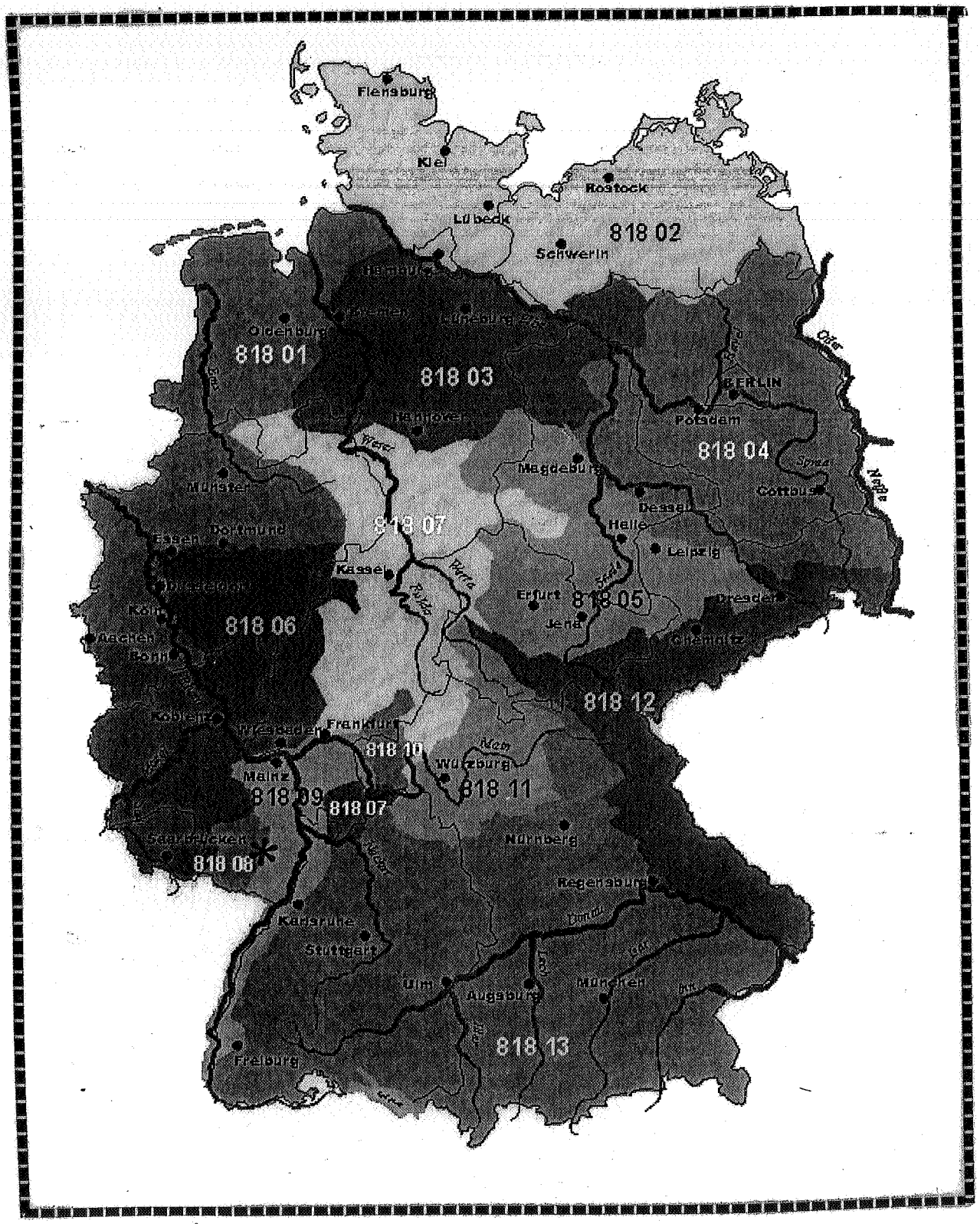


Figure A1-1-5: Detail of Pfälzerwald topographic map. The map has been altered to show access roads to Merzalben Research Station 04/0705 (■) at approximately 07°48' longitude, 49°16' latitude and 523 meters altitude. Map source: Landesvermessungsamtes Rheinland-Pfalz, Ausgabe1994.

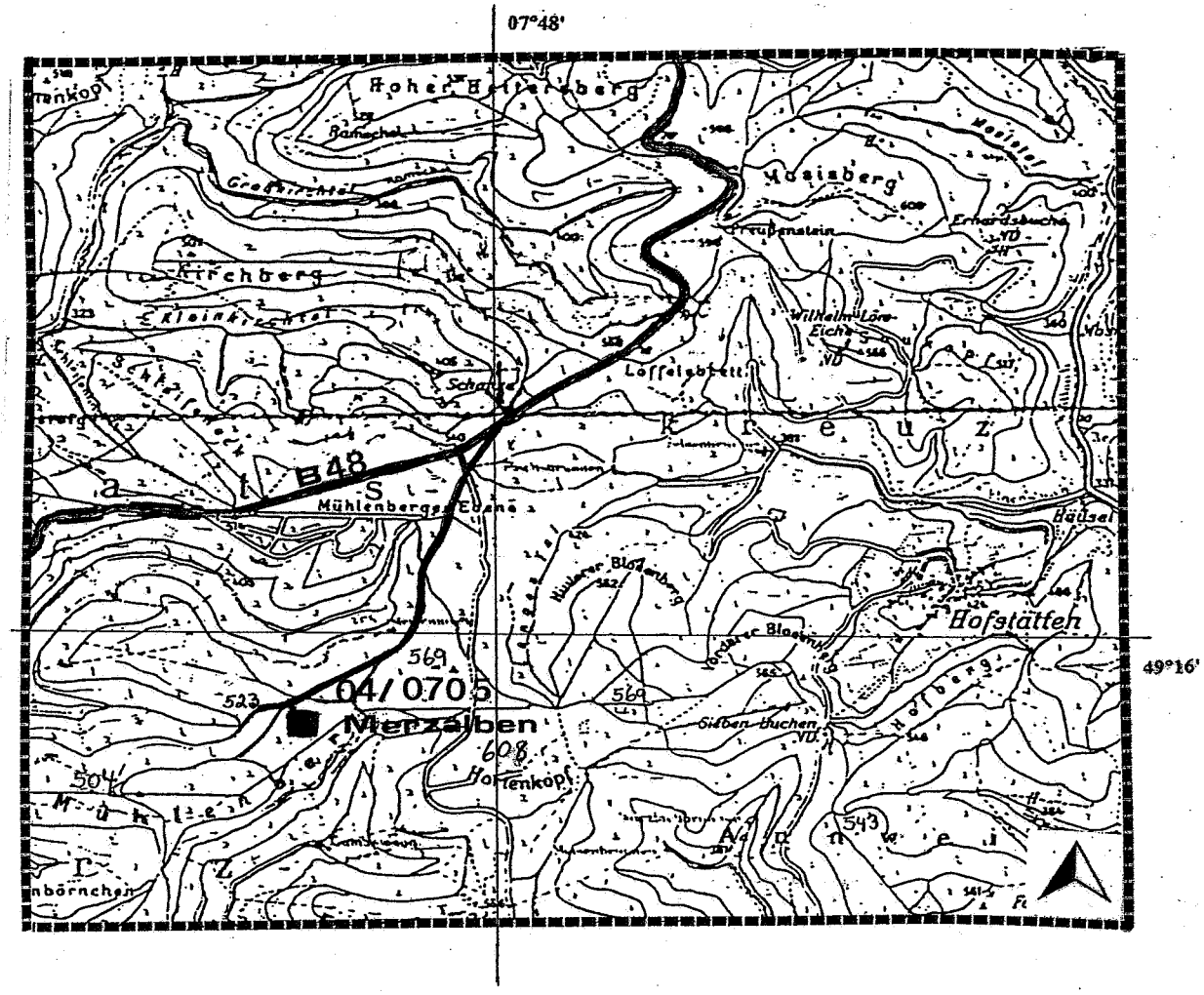


Figure A1-1-6: Tree distribution in Merzalben Forest Research Area 04/0705. The station has a mix of *Quercus petraea* (sessile oak), *Fagus sylvatica* (European beech) and randomly scattered *Pinus sylvestris* (Scott's pine). The shaded area of approximately 60 square meters was limed in 1989

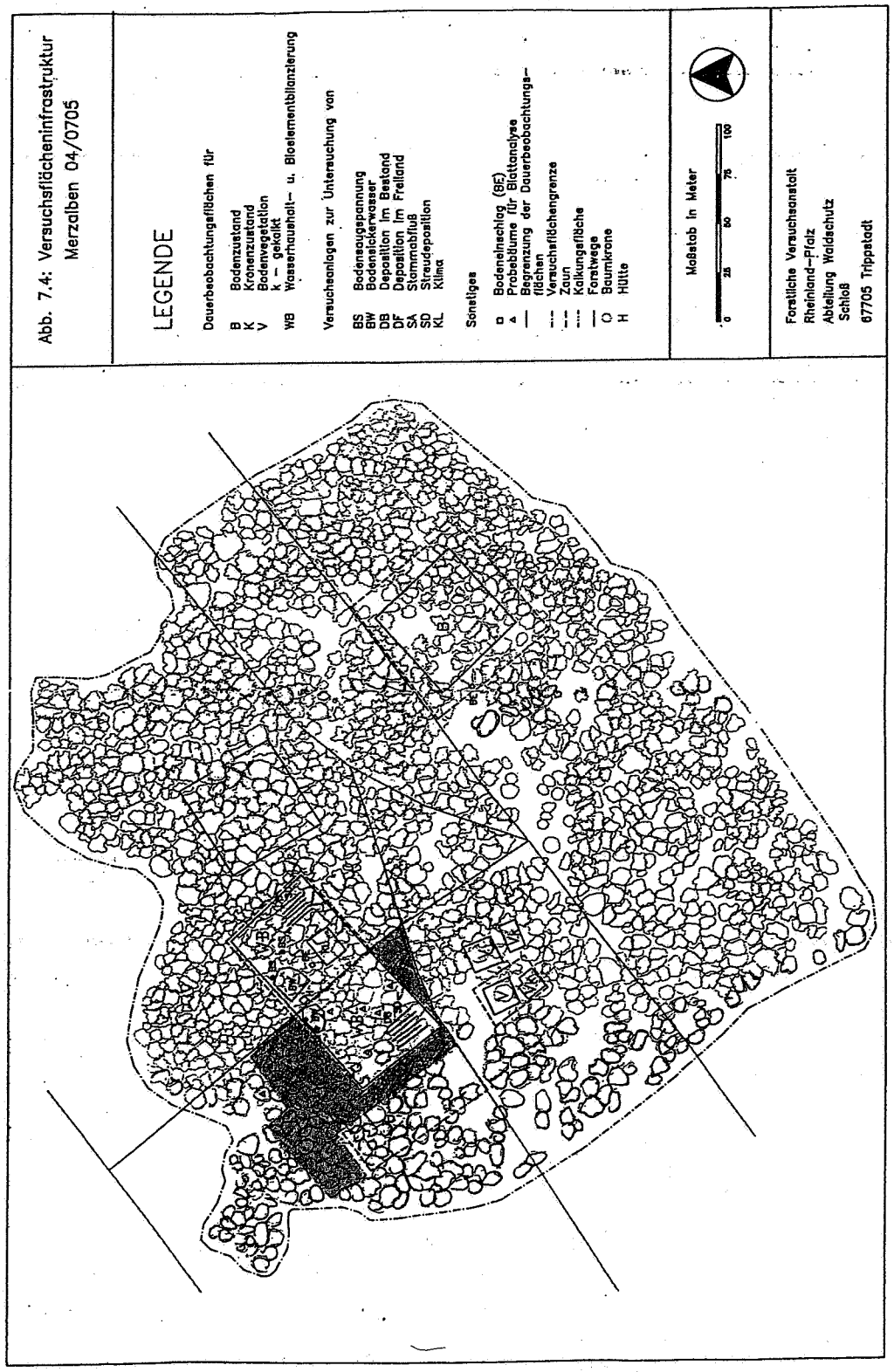
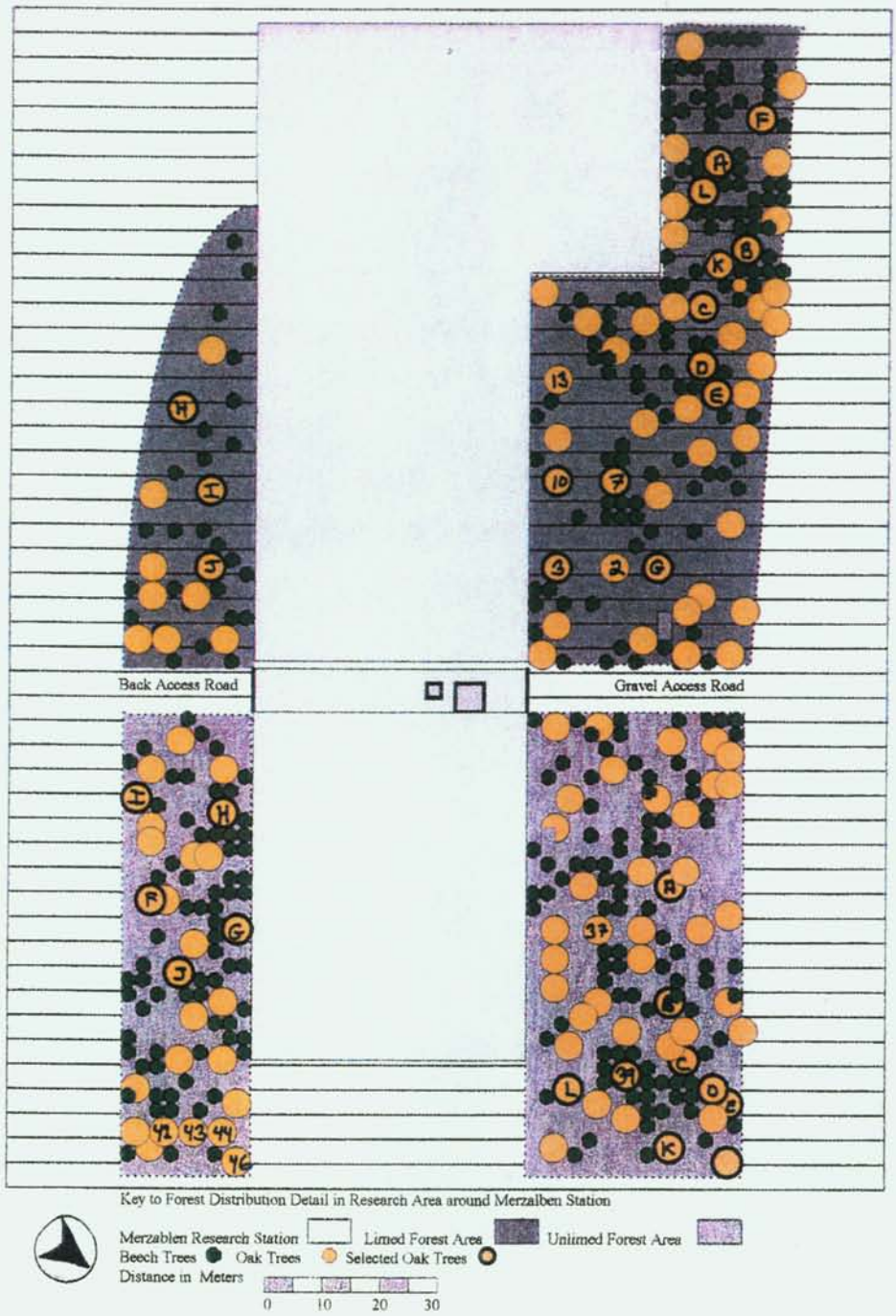


Figure A1-1-7: Area of study outside of the enclosed research station at Merzalben Forest District 04/0705 (Photo A). Individual trees were painted with identifying letters in both the limed and unlimed areas at the beginning of the examination period (Photo B). Both photographs show the general distribution of trees providing the forest floor with a shaded canopy which is ideal for mycorrhizal growth.

A. Research Station, Merzalben Forest District 04 / 0705, Rheinland - Pfalz, Germany

B. Limed area of Merzalben Oak Forest with test trees marked

Figure A1-1-8: Schematic diagram of Merzalben research area showing the distribution of *Fagus sylvaticus* (European beech = small green dots) and *Quercus petraea* (Sessile oak = large yellow dots) in both the unlimed (light gray) and limed (dark gray) plots. The circle sizes represent the relative diameter of the tree trunks at one meter from the base. Selected oaks were lettered in spring 1999. Numbered oaks were from a previous study and are included here for reference only. Selected trees were a minimum of 5 feet from adjacent trees. Each selected tree has an average diameter of approximately 60 cm.



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A1-2: Growth Parameters

A1-2-1 Ideal Growth Parameters

Although sessile oak is competitively successful in diverse natural environments, it has limitations in its growth strategies. The following is a brief listing of some pertinent growth features which may affect Sessile oak growth and survival.

* Sessile oak prefers well drained , acidic soils (Lewington & Streeter, 1993).

* Germinating seedlings growing in direct sunlight have larger root collar diameter, but saplings require somewhat less sunlight. Under nursery netting or in full sunlight, oak saplings had similar crown and stem length (Gross et al, 1996).

* Mature Sessile oaks are light demanding trees. Their crowns usually extend above those of beech in a canopy (Timbal and Aussenac, 1996).

* The quality of wood is better when from mixed stands of beech and oak than from pure oak stands (Timbal and Aussenac, 1996).

* *Q. petraea* and *Q. robur*, are the most cold resistant (-30 ^{o}C) oak species (Timbal and Aussenac, 1996).

* *Q. petraea* and *Q. robur* grow on southerly aspects of the Pyrenees and the French Alps, but only *Q. petraea* reaches the highest altitudes (1300 m) (Timbal and Aussenac, 1996).

* In Germany, *Q. petraea* can be found growing at 600 m (Harz), 700 m (Bayern), 900 m (Bayern Alpine forest), and 1,200 m (Southern Alps) (Schütt et al, 1992).

* In spring, at higher elevations, both sessile and pedunculate oaks are sensitive to late spring frosts due to early bud burst, but overall, *Q. petraea,* has a very slightly

lower optimal moisture requirement (600-800 mm yr^{-1}) and lower temperature optimal (11°C). (Timbal and Aussenac, 1996).

* When turgid, sessile oaks have an average water potential of -3.0 MPa, stomatal conductance of 950 mmol m^{-2} s^{-1} and maximum photosynthesis rate of 17 µmol m^{-2} s^{-1}, which is nearly identical to that of *Q. rubor* but overall, the latter species is more embolism prone and drought sensitive (Timbal and Aussenac, 1996).

* Sessile oaks, on certain stands, seemed more drought resistant than pedunculates, and where the damage was severest on both, the percentage of dead fine roots was the highest on the pedunculate oaks (Thomas and Hartman, 1996).

* Mediterranean oaks are more resistant to summer drought than either *Q. petraea* or *Q. robur* with complete stomatal closure when the predawn water potentials near -3.5 to 4.0 MPa, while for sessile oak, the stomatas close when the predawn water potentials are about -1.8 to 2.0 MPa. (Epron et al, 1993; Epron & Dreyer, 1996, Ridolfi et al, 1996).

* The optimal mineral nutrition for adult oaks including *Q. petraea* is estimated to be: N (2-2.5%), P (0.15-0.2 %), K (0.8-1.3%), Ca (0-0.8%), Mg (0.2%) (Bonneau, & Delmas, 1995, Bonneau, 1986).

* Nutrient requirements for *Q. rubor* are generally higher than those of *Q. petraea* (Lévy et al , 1992).

* Once mature, white oaks produce an annually maturing acorn mast which is an important nutritional source for forest birds, squirrels, deer (Van Dersal, 1938) and wild pigs (Lewington & Streeter, 1993).

* While the summer leaves and bark can be used in stock feed, they both contain tannic acid and can be potentially toxic especially in the spring or when from very young trees (Van Dersal, 1938). The trees have a natural protection from predators.

* Aromatic fungal fruiting bodies associated with oak and their subsurface mycelial mats can provide direct nutrition to the denizens of the forest as well as indirectly decomposing toxic litter to release stored nutrients which can be reutilized by the tree (Lewington & Streeter, 1993).

Summary:

In comparison to other oak species, Sessile oak is very well adapted to high elevation environments. Sessile oak generally requires abundant solar radiation but cooler temperatures, and is well adapted to low moisture levels as long as the two previous requirements are met. In warmer climates, the declining drought tolerance of sessile oak, as related to its delayed stomatal closure, may be a major range-limiting factor. It can survive well in dry to moist soils of a sandy, slightly acid nature with average to low nutrient complements and it can compete successfully in a variety of mixed forests in intracontinental regions however, reforestation needs to occur in areas that meet solar, mineral and hydric nutrition needs. To adequately meet these needs appropriate fungal innoculums may be essential. Although recognized, here is very little known about these symbiotic fungal requirements.

A1-2-2 Growth Parameters in Merzalben Forest District

From the parameters given above, the growing region of Merzalben is nearly ideal. The elevation of the research site Merzalben 04 / 0705 in the Rheinland-Pfalz Forest District in Germany was 523 to 591 m, so although a high elevation forest, it was not at the extreme. The actual research facility was at the crest of a hill but from the topographic maps it is evident that there is much undulation in the vicinity with many southerly exposed slopes for better sunlight exposure. In the mature mixed beech /oak forest at Merzalben, while the beech crowns occasionally extended beyond the oaks, with their

broader crowns, and larger girths, the oaks predominantly occupied the canopy space. In 1988, the beech trees had an average height of 18 m and diameter of 10.1 cm while the average oak was 27.5 m high with a diameter of 40.1 cm (Block, 1993). Despite the fact there are over 1000+ beech trees per hectare compared to 200+ oaks per hectare, it is not likely that light accessibility is a current stressor for photosynthesis in the mature oaks. Oak seedlings germinated well, especially in the fenced region where they were protected from animals seeking the tender shoots but the light requirements for replacement seedlings may be low due to the rather full canopy.

The podsoils horizons present at Merzalben are suitable for the preferential survival of the deep-rooted oak species which predominates the area. The surface litter layer is 1-5 cm deep. The soil at 0-10 cm depth, in the A horizon, is a sandy humus, at 30-40 cm depth, the B horizon is sandy with a minor component of heavily oxidized humus, and at 50-60 cm depth, the soil is a typical C horizon which is sandy with numerous stones and rocks. In a preliminary study of these aluminosilicate soils, it was found that the uppermost A horizon, under a thin litter layer, supported a good diversity of symbiotic mycorrhizal species and that the abundance of mycorrhizal species decreased dramatically in the B and C horizons.

The beechs currently average 90-100 years of age while the oaks average 170 to 197 years of age (cf Block, 1993). In the last 25 years both have exhibited signs of the wide-spread stress affecting trees throughout Europe. In 1993 it was estimated that 16% of the oaks over 60 years of age were seriously stressed. There are many possible causes for this continent wide problem (Dreyer & Aussenac, 1996), which will be discussed shortly, but the current leading theory involves soil acidification due to the effects of acidic precipitation. According to the Forestry Department in Trippstadt, it is likely that this acidification has caused heavy mineral elements, such as potentially toxic aluminum, to leach into the soil and through mechanisms poorly understood, be taken up into the trees. The most likely site of uptake is at the mycorrhizal - root interface. That is the subject of this study.

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A1-3: Role of Ectomycorrhizae in Forests

Fungi can be used as indicators of tree health (Cripps, 2001; Old 2002).

A1-3-1-1: Endomycorrhizae and Ectomycorrhizae

Over 80% of plant families are mycorrhizal, the symbiotic relationship being a rule in nature and not an exception (Malloch et al, 1980). According to Hawkins and George of the Institute of Plant Nutrition, Hohenheim University, Stuttgart, Germany, shrub and tree species are usually colonized by ectomycorrhizal fungi most of which are basidiomycetes (ectomycorrhizal) rather than the arbuscular (endomycorrhizal) forms (Glomales and Zygomycetes) which are more common on the fine roots of grasses and non-woody vegetation. In both cases however, the basis of the symbiosis is an exchange of carbohydrates (plant to fungus) and minerals (soil to fungus to plants).

Many mycorrhizae seem to be obligate biotrophs, requiring a plant host to complete their life cycles. Most ectomycorrhizae however are also symbiotic. This is especially true in forest systems where the tree provides carbohydrates (Smith & Read, 1997) and the fungi benefit trees by augmenting inorganic nutrient and water uptake, and provide protection from bacterial and other fungal pathogens, and affect heavy metal uptake (Fogel, 1980).

A1-3-1-2: Mineral Uptake

In grasses, fine mycorrhizal roots take up much more phosphate than non-mycorrhizal roots (George et al, 1995) and can enhance water (George & Marschner, 1996) and other mineral uptake (George et al, 1994; Marschner & Dell, 1994). It is generally accepted that forest mycorrhizae also improve water and mineral uptake (Timbal & Aussenac, 1996; Molina et al, 1992; Allen. 1991), especially P and N (Cripps 2001) in their host roots. Fertilizers are generally antagonistic to fungal colonization in invitro studies (Cripps, 2001). In light of this fact, it will be interesting to take particular note of the effect of liming upon mycorrhizal selection in the Merzalben forest.

A1-3-1-3: Heavy metals

It has been generally assumed that the function of ectomycorrhizae with respect to nutrition and tree growth is highly dependent upon the fungal species involved (Agerer et al, 1994). Many species of ectomycorrhiza are inhibited by low pH and heavy metal content (Harris and Jurgensen, 1977, Hung and Trappe, 1983, McCreight and Schroeder 1982) while others are believed to ameliorate the effects of heavy metals in plants (Hartley et al, 1997). In a recent study it was determined that ectomycorrhizal fungi are crucial to the survival of pioneering aspens growing on Al, Cu, Fe, and Zn contaminated, sandy, acidic soils of smelter-impacted sites in the area of Butte-Anaconda, Montana (Cripps 2001). Many of the metal tolerant mycorrhizae [*Thelephora terrestris*, *Laccaria laccata*, *Laccaria proxima*, *Hebeloma mesophaeum*, and *Inocybe lacera*] seemed to be nonspecific with regard to the host plant but rather "early colonizers" of coal spoils with potential value in reclamation sites (Cripps 2001). An effort will be made in this study to differentiate between "acid tolerant/intolerant" and "Aluminum sensitive/resistant" mycorrhizal species.

A1-3-2-1: Ectomycorrhizal Morphology

According to Cripps (2001), ectomycorrhiza (EM) produce fungal hyphae that remain external to the root cells producing a mantle over the root tips, but the hyphae can also grow between the epidermal, hypodermal and cortical cells forming an intercellular network referred to as a Hartig net where nutrient exchange occurs. The hyphae however do not penetrate deeply into the cortex or past the pericycle into the stele. Unlike vesicular arbuscular mycorrhizae (VAM), ectomycorrhizae also do not form haustoria which penetrate into the cells forming tight arbuscule/membrane connections for efficient exchange of nutrients. (Since most grasses grow on nutrient rich soil, this is a logical adaptation.) In contrast, forest ectomycorrhizae tend to produce extraradical fungal strands that proliferate into the (nutrient poor) soil, dramatically increasing the nutrient exchange surface area. Extending the root system, and enhancing the uptake of inorganic nutrients, particularly P and N greatly benefits the tree (Cripps 2001). Figures A1-3-1 and A1-3-2 show general examples of mycorrhizal and non-mycorrhizal roots.

Figure A1-3-1: Cross-section of a simple root compared to a mycorrhizal root. Modified from Steffens et al (1994). The simple root is on the left, the ectomycorrhizal fungal mantle with its emanating hyphae is on the right.

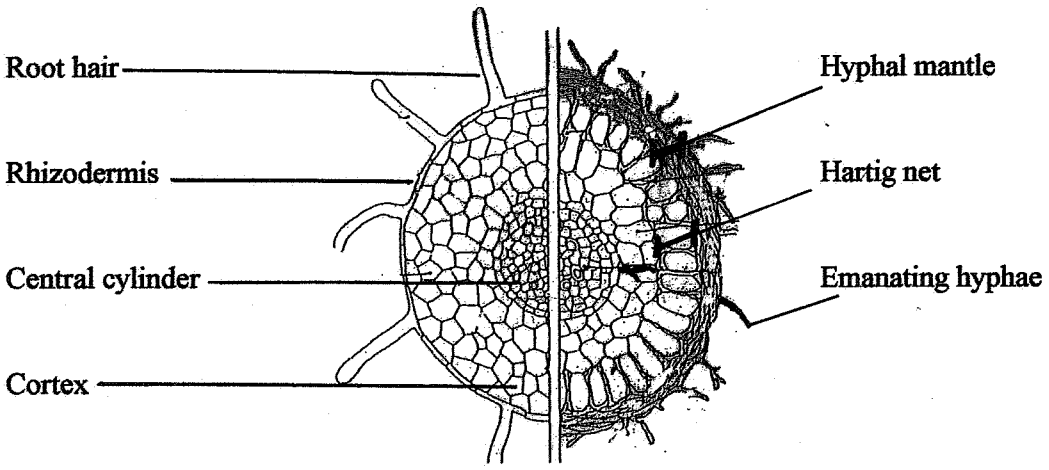
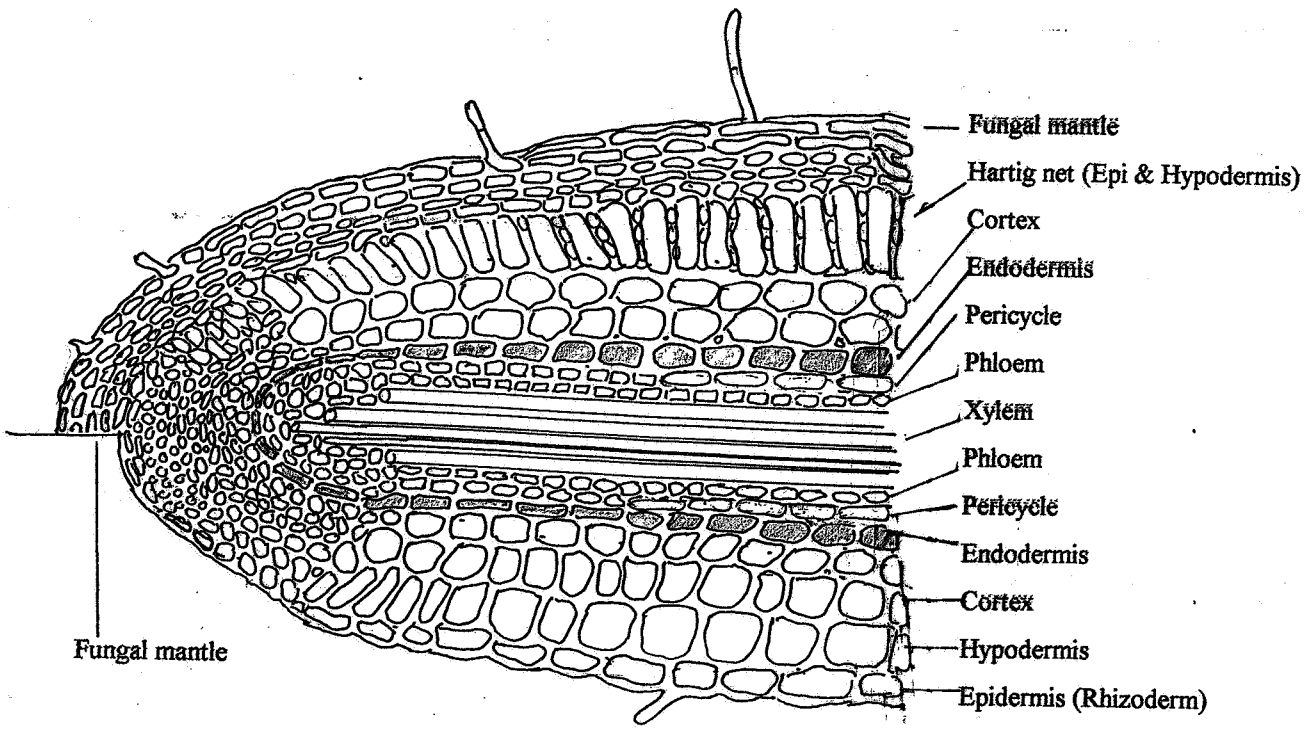


Figure A1-3-2: Long-section of an oak root with normal morphology below and **Cenococcum geophilum** above. Rhizodermis cells are protected and modified in the presence of fungal hyphae and overall surface area is increased. At the very tip there is cell modification but little penetration.



In addition to Cripps description, Agerer (1987) stated that some mycorrhiza produce extramantle hyphae that extend into the soil matrix forming special cord-like extensions referred to as rhizomorphs. This and other species-specific variations will be addressed in the section concerning identification, abundance and distribution of mycorrhizae.

A1-3-2-2: Mycorrhizal Induced Changes in Tree Morphology

Individual species of mycorrhizae seem to have definite effects on the morphology of seedlings, although it is not known how the entire mycorrhizal complement in a natural system affects the overall appearance or physiology of a mature tree. *Boletus piperatus* presence doubled the number of Aspen roots but did not form mycorrhizae (Cripps 2001). *Boletus pipeatus* possibly indirectly increased nutrient transfer but it is likely that the pre-invasion hormone release of fungal IAA (indole acetic acid) may be as important to the stimulation of root biomass as it is to establishment of mycorrhizal symbiosis (Cripps 2001). Pure *Cenococcum* strains well incorporated into the root tips can cause Aspen seedlings to etiolate (long thin stems, petioles and pale leaves); while *Tricholoma* strains produced seedlings with wide, dark green leaves (Cripps 2001). In this study we noted the ability of the extremely common *Cenococcum* to tolerate both alkaline and acid soils that inhibited many other fungi. This species, which can also alter root morphology, may be a crucial survival link in stressed environments.

A healthy complement of mycorrhizal species may be essential, with each contributing to the overall tree vitality in its own special capacity. In inoculation experiments, pure strains of mycorrhizae may behave differently than in a species-rich natural rhizosphere. Aspen seedlings inoculated with non-native fungi had a higher mortality rate (*Philoderma croceum* 10%, *Cenococcum graniforme* and *Pisolithus tinctorius* 20%) than those inoculated with native fungi (*Amanita muscaria, A. Pantherina, Paxillus vernalis, Tricholoma scalpuratum* : 0% mortality), although even native fungi in pure stains can be detrimental (*Inocybe lacera* 100% mortality) (Cripps, 2001). So, besides tree-fungal selection, native fungal-fungal interactions/competition may be essential for seedling survival and possibly also for mature tree vigor.

A1-3-2-3: Host Induced Changes in Mycorrhizal Morphology

Conversely, tree species may have a marked effect upon mycorrhizal morphology. Variations in the ectomycorrhizal morphology of the various species of *Lactarius* may be due to the different possible host interventions (*Fagus, Picea, Pinus, Quercus*) (Palfner & Agerer, 1996, Brand & Agerer, 1986). The ectomycorrhiza *Russula ochroleuca* associated with *Abies alba, Betual pendula, Carprinus betulus, Fagus sylvatica, Larix decidua, Picea abies, Pinus sylvestris* and *Quercus robur,* although very similar in basic fungal morphology (emanating hyphae, and piles of ochre-yellow granules in mantle cells), exhibits host-dependent differences in the type of ramification, shape, dimensions, occurrence of tannin cells, shape and orientation of cortical cells and depth of Hartig net. (Pillukat & Agerer, 1991). The same may be true of other genuses of ectomycorrhizae. Just as mycorrhizae may be altered by their hosts, as we will find out in this report, the host roots can be variously altered by the mycorrhizae.

A1-3-3: Mycorrhizae and Fungal Fruiting Bodies

The Pinaceae (pine family), Betulaceae (birch family), Salicaceae (willow and aspen family), Fagaceae (Beech and Oak family) and Mytraceae (Eucalyptus family) are primarily ectomycorrhizal and associate mostly with basidomycete fungi, which produce mushrooms as reproductive structures (Malloch et al, 1980). Mushrooms produced by mycorrhizal fungi can be observed successionally near host trees at certain times of the year depending upon the ambient weather conditions and can be evidence of mycorrhizal presence in the rhizosphere (Last et al, 1987). However, the abundance of fungal sporocarps is not necessarily indicative of the predominance of a fungus in the soil and on the plant roots (Cripps, 2001). Nor does the presence of fruiting bodies mean that ectomycorrhizal species absolutely must be present on the roots. Not all mushroom producing fungi are mycorrhizal and many fleshy fungi may be saprophytic, parasitic or mutualistic in other ways (Pilz and Molina, 1996).

The symbiotic vegetative state of mycorrhizae can preclude the formation of fruiting bodies if the tree provides adequate nutrition, and outside stressors are within normal

tolerance ranges that do not stimulate fruiting. In the lab, we found that *Xerocomus chrysenteron,* which can form fruiting bodies in the wild, remained viable but mycorrhizal on extracted root/soil cubes for up to 2 years at 10^0C in dark controlled temperature chambers as long as adequate moisture and ventilation was provided. Since it is possible to culture some of the known mycorrhizae in the lab on artificial mediums, it is quite possible for some species to be facultative or ephemeral in their root associations as long as their nutritional needs are met in the litter, humus or mineral soil layers and never produce fruiting bodies. Some mycorrhiza may never produce surface fruiting structures (i.e.-Truffles, Tuber sp.) or may reproduce within the soil primarily by spore ball production (*Cenococcum geophilum*). So, many ectomycorrhizal species may be abundantly present and rarely or never produce fruiting bodies.

The identification and naming of mycorrhiza is often dependent upon tracing the mycelial strands directly to a known fruiting body and vica versa (Agerer 1987-1998). However, since the presence of mycorrhizae does not mean that fruiting bodies must be found in the same area, this process can be problematic. The attempt to trace mycorrhizal strands to possible fruiting structures can therefore be fruitless. Identifications were therefore made here using intrinsic macro and microscopic morphological features, such as those presented in Agerer's guide.

A1-3-4: Ectomycorrhizal Species Associated with European trees.

According to Schütt et al (1992) symbiotic mycorrhizal associations with higher plants were first recognized by Von Frank in 1885 and currently, the most well known of these are Basidiomycetes which can form symbiotic associations with 250 tree species in Europe. Over 1300 Basidiomycete species in 58 genuses have been recognized to have strong specificity's with certain tree species and of the 58 genuses, the most well known include : Boletus, Russula, Lactarius, Cantharellus, Tricholoma, Inocybe, Hebeloma, Cortinarius, and Amanita (Schütt et al, 1992, pp. 149, 300).

Some basidiomycete species are very specific (i.e.- Rhizopogon species and Douglas fir) while others are wide-spectrum symbionts forming associations with needle and broadleaf trees, (i.e.- *Amanita rubescens, Cantharellus cibarius, Paxillus involutus, Russula ochroleuca, and Xerocomus badius.*) (Schütt et al, 1992, pp. 149, 300). The genus *Gomphidius* appears restricted to pines (Cripps, 2001), *although a few tips were rarely found on our oak specimens.* *Suillus* occurs mainly on conifers, and *Lactarius controversus* is restricted to aspen and birch (Cripps, 2001). Clearly some tree-fungal selection has been occurring. *Amanita* species may be "crossover" species in succession processes from coniferous to aspen forests (Cripps, 2001). Mycorrhizal species can be specific to certain tree species, although further research may determine if they are truly exclusive, especially in mixed forests.

In the Ascomycetes group there are about 10 genuses but they primarily form symbiotic associations with shrubby plants and the Zygomycetales is primarily represented by Endogone and Glomus species (Schütt et al, 1992, pp. 149, 300).

A single tree can support numerous species of mycorrhizal fungi simultaneously or in dynamic succession. Douglas fir (*Pseudotsuga menziesii*) can form mycorrhizae with over 2000 species of fungi (Trappe, 1977) while white alder (Alnus spp.) is limited only to a few (Brunner et al, 1992). Aspen (*Populus termuloides* Michx.) is predominantly ectomycorrhizal (54 species, primarily in the Cortinariaceae family) with rare associations with arbuscular mycorrhiza (Cripps 2001).

A1-3-5: Oak Ectomycorrhizae

Oak species are considered to be strongly mycorrhizal in nature with the mycorrhiza providing increased water and mineral uptake and bactericidal secretions that protect the roots and improve longevity of the primary roots and promote the development of secondary roots with the mycorrhizae receiving from the plants carbohydrates and amino acids (Schütt et al, 1992, pp. 149, 300).

Sessile oaks are generally considered to be intermediate between real pioneers (pines and birches) and shade tolerant species (beech and fir), but because of their economic importance, they have been preferentially forested to the detriment of other species. (Timbal and Aussenac, 1996). These pioneering (or introduced) oak hardwoods form mycorrhizae with many of the same fungal species as the climax conifer or beech species in the shifting climax communities (Molina et al, 1992). Plants less dependent upon mycorrhizae tend to invade new environments quickly providing competition for oak seedlings, while obligate symbionts like oak integrate slowly, especially if the mycorrhizal innoculum is low (Allen, 1991). Mother trees left behind after selective cutting or coppicing that retain active mycorrhizae on their roots can provide the necessary innocula for the next oak seedling generation in reforestation projects (Molina et al, 1992). As a result, it is expected that some cross-over of mycorrhizal species from conifers to beech to oak (and backwards) will occur. A climax oak forest that has usurped a coniferous forest site is likely to have mycorrhizal "residuals". A listing of mycorrhiza fungi associated with oak from this study, and in the literature, can be found in **Appendix 1**. In this listing, alternate known hosts will be identified.

A1-3-6: Ectomycorrhizae in Merzablen Forest District

Preliminary observations indicated that the Merzalben Forest was rich in fungal fruiting bodies and that the subsurface rhizosphere contained abundant mycorrhizae. Approximately 62 oak mycorrhizal species were collected from the Merzalben Forest Research site. Of these 49 samples were reviewed for general abundance, distribution at various soil depths in both the unlimed and limed forest, and aluminum localization. Aluminum, bound and unbound mineral content was quantified for 4 species and for all the root samples. Despite the fact that presence of a fruiting body is not necessarily representative of the mycorrhizal population, a random review of the fruiting bodies in the Merzalben forest district was made. Since no definitive relationship could be discerned between the mycorrhizal presence and the appearance of the fruiting structures, these results will be presented only in **Appendix 2**. The mineral content and appearance of ectomycorrhizal roots may be a primary indicator of tree health.

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A1-4-1: General Damage Classifications

Defoliation has been the primary tool used to determine a decline classification. Even so, there is no commonality of rating. According to Thomas and Hartman (1996) a "declining" oak stand typically shows at least 5 damaged trees per hectare (Severe: > 60% leaf loss and crown die back; Moderate: <60% leaf loss). A healthy stand may have 0-25% leaf loss but show no other visible signs of debilitation or injury. Causin et al (1996) used the following derived classification system: Class 0: <20% defoliated (non-declining) ; Class 1: 20-45%; Class 2: 45-70%; Class 3: >70% defoliation, (Müller and Bosshard, 1986) with an upgrading to the next class if crown dieback, or leaf yellowing was evident (Keizer, 1993). Oak et al (1996) published an oak decline risk rating profile for the southeastern United States in which they determine the probability of decline based upon a myriad of pre-disposing factors, including: slope gradient, soil depth class, and oak basal area. clay content of soil, depth of soil to the impermeable layer, height of red oak at 50 years of age, height above sea level, direction of the slope gradient, prevailing age of dominant and codominant oaks, incidence of existing declining and dead oaks, number of trees over 12.7 cm dbh. They assigned values to each of the factors to ultimately determine the risk probability (R^2) but could not establish definitive co-relations between sites and concluded oak decline events must be influenced by additional parameters. They did not estimate potential climatic or biotic stresses, nor take into account pollution or nutritional factors.... an overwhelming task. There is no simple answer to predicting and avoiding oak forest decline.

In Germany, trees are assigned a damage classification of -1 (dead) to 0 (healthy) to 1 (some damage) to 2 (increasing damage) to 3 (very poor health) to 4 (severe damage). The damage classification is based upon several parameters which include: crown size and shape as measured in winter, light penetration in summer, leaves lost to insects, fruiting strength or loss, and by the Roloff number (Block, 1995, Schröck, 1994; Schröck et al, 1999; MUF, 1999). Roloff numbers can vary from 0 (good) to 3 (poor) or 0 to 8 (poor) through a relative combination of general or fine growth details (Schröck, 2003, pc).

A1-4-2: General Damage Classes in the Merzalben Forest

In Rheinland - Pfalz, oak trees which comprise 17 % of the forested area (Kronauer, 1999), are under severe stress. In 1984, only 8% oaks were in damage classes 2 to 4 (7% =class 2; 1% = classes 3 & 4), but by 1999, 45% of the oaks were damage class 2, and 5% classes 3& 4 (Kronauer,1999), with many trees living only to 125 to 150 years, or less than half of their expected life span (Dr. Block, 1999, p.c.).

According to the analysis provided by Hans Werner Schröck of the FAWF (Forschungsanstalt für Waldökologie und Forstwirtschaft Rheinland-Pfalz) (Appendix 3), since 1998, 3 trees in the limed zone had died, one of which was in our study group. In the unlimed forest, 15 trees had died in the same time period. One of those trees was also in our study area. Except for the trees that died, and one tree in the unlimed forest which was in damage class 3, the trees in both the unlimed and limed zones were in damage class 2. The health status of 52 trees in the unlimed, and 32 trees in the limed forest was determined by a summer 2002 survey of the light penetration of the crowns, and by a winter 2003 survey of the individual crown structures. In the limed forest, approximately 88% of the trees had < 25% light penetration, which is considered to be very good. In the remaining 22%, the light penetration did not exceed 35%. In contrast, in the unlimed forest, only 20% of the trees had < 25% light penetration, 45% of the trees had > 35% penetration, and of these, 16% had > 45% penetration, which is considered to be very bad. The conclusion drawn was that the trees in the limed forest were in better overall health in comparison to those in the unlimed forest.

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A2: Global Climatic Impact on Forests

A2-1: Why are the oaks suddenly over the last 20 years declining and dying?

It has been generally proposed that with increasing acidity of the soil due to acid precipitation, aluminum ions freed into solution are made available to the rhizosphere and can affect tree health (Block, 1999, p.c.). Since mycorrhizal roots are the primary nutritional access points for oak trees, the ultimate goal of this project was to determine if the mycorrhizae were controlling Al access into the oak vascular system. And, if so, which species were significantly assisting uptake, and which were effectively blocking it.

Any single organism, or group of similar organisms, can act as indicator species, but only if there is a fairly concrete idea of how it (or they) fit (s) into the interactive forest complex. To assist interpretation, information concerning the known natural distribution of the subterranean mycorrhizae was gathered (Appendices 1 & 2). After an extensive search, it must be acknowledged that information concerning historic variation in the mycorrhizal populations was sparse indeed. To accurately portray the impact of the findings of this research project, it is essential, at the very minimum, to place the mycorrhizae within the context of the known environmental and climatic factors.

A2-2: Global Thermal Pollution

According to the International Panel for Climate Change (IPCC) Report, since 1861, the global average surface temperature has risen 0.6 ± 0.2 degrees (95% confidence level), with the two greatest warming periods occurring, first from 1910 to 1945, and second from 1976 to 2000, with the 1990's decade being the warmest, and 1998 the warmest year in instrumental record (IPCC, 2001). In addition, it was stated that from tree rings, ice cores, corals and historic records in the Northern Hemisphere, the temperature increase over the last 140 years is likely to have been the largest in the last 1000 years.

This temperature rise can be directly correlated to the explosion in human population and the rise in anthropogenic heat-generating activities. Based upon similar or increased human activity, within the next century, older climatic models predicted an estimated

global temperature rise of 1.0 to 3.5 ^{0} C by 2100 (USDA-FS, 2001). According to a number of newer climatic models, the estimated average global surface temperature will, more likely, rise 1.4 to 5.8 ^{0} C in the same time period (IPCC-SRES, 2001). In Europe, this may alter continental climate resulting in increasingly dry summers and aggravated droughts in some areas and extreme precipitation events in others. In addition, fewer cold and frost days and more moderate seasonal changes will probably alter traditional growing zones, and increase the risk of exotic insect, and fungal migration and infestation.

A2-3: Greenhouse Gases

Greenhouse gases are termed such because they are thought to contribute to the overall global warming effect observed in the last century. They include, but are not limited to: carbon dioxide, methane, nitrous oxide, halocarbons, sulfur dioxide, and ozone. All of these will be briefly discussed with respect to their known roles in forest deterioration. Carbon dioxide is a long-lived greenhouse gas which contributes greatly to the warming trends. It is estimated that CO_2 has a Forcing Value (warming ability) of 1.46 Wm^{-2} (Watts per meter squared), which because of its molecular concentration in the atmosphere, is greater than that of all the other greenhouse gases combined (IPCC, 2001). While CO_2 is a potentially severe problem, increased within physiological ranges, it may actually contribute to forest photosynthesis. On the other hand, SO_2 and NO_x both directly contribute to acid precipitation (USDA FS-SRS & SR, 2001).

A2-3-1: Carbon Dioxide

According to the International Panel for Climatic Change (IPCC, 2001), since 1750, atmospheric CO_2 concentration has increased by 31%. In the last 20 years, the global rate of CO_2 production was about 1.5 ppm (0.4%) per year, a rate unprecedented in the last 20,000 years! It was estimated that 67 % of the increase during the last 20 years was due to fossil fuel combustion and emissions, and the remaining 33 % was due to land-use change, especially deliberate deforestation (Miller, 1992). Once released, CO_2 remains in the atmosphere up to 500 years (Miller, 1992). Using models created by the Special Report on Emission Scenarios (SRES) of the IPCC (2001), it was predicted that given the

current rate of production, CO_2 concentrations will rise from 540 to 970 ppm by 2100 (90 to 250% above the base-line concentration of 280 ppm in the year 1750). To drop the rate and cumulative concentration of CO_2 in the next century, man must drastically reduce fossil fuel consumption and initiate massive reforestation to create a terrestrial CO_2 sink.

A2-3-2: Methane

Since 1750, the atmospheric concentration of methane (CH_4) has increased by 1060 ppb (151%), the highest in 420,000 years, with about 50% of the emissions being attributed to human activity (fossil fuels, cattle, rice paddies, and landfills) (IPCC, 2001), and the remainder coming from oxygen-poor swamps, bogs, marshes and other wetlands (Miller, 1992, p.286). Methane, which constitutes approximately 18% of the greenhouse gases, is about 25 times more effective at warming the atmosphere than CO_2 and can remain in the troposphere for 7 to 10 years (Miller, 1992). It is unclear how this gas may affect elevated forests.

A2-3-3: Halocarbons

The International Panel on Climate Change determined that since 1995, the atmospheric concentrations of certain greenhouse and ozone-depleting halocarbons ($CFCl_3$ and CF_2Cl_2) is either slowing increasing or decreasing in response to the Montreal Protocol, however, their industry substitutes (CHF_2Cl, CF_3CH_2F, PFCs and SF_6) are increasing in concentration. It is too soon to determine how these new derivatives will affect the ozone layer, but theoretically, stratospheric ozone should recover from the original halocarbons during the 21st century. This may be optimistic since according to Miller (1992) Chlorofluorocarbons (CFC's) which contribute 14% of the greenhouse gases, can remain in the atmosphere 60 to 400 years depending upon the type and have 10,000-20,000 times more impact per molecule on global warming that each CO_2 molecule has.

A2-3-4: Nitrous Oxide

According to the IPCC (2001) nitrous oxide (N_2O) has increased by 46 ppb (17%) since 1750, the highest in a least the last 1000 years, with about one third from human sources

(agricultural fertilizers, chemical industry , livestock wastes, sewage treatments, and biomass burning). A total input from all nitrogen forms (HNO_3, NH_3, NH_4, NO, NO_2, NO_3) of 3 -10 kg/ha-yr is considered to be within a normal acceptable range for most wilderness areas (Fox et al, 1989). Based upon an admittedly limited data base, Fox et al (1989) estimated that an increase in N deposition in excess of 10 -15 kg/ha-yr for all nitrogen forms would result in significant changes species composition and ecosystem chemical balance.

According to Fox et al (1989), Nitrogen saturation occurs when nitrate (NO_3) and ammonium (NH_4) concentrations exceed biological demand in susceptible (acidified) forest soils. Nitrogen in the ammonium forms rarely significantly accumulates because of biological uptake by plants, grazers, decomposers and nitrifying bacteria, and as such it is more of a growth limiting factor since many forests are nitrogen deficient and would actually benefit from N input. Non-the-less, when excessive nitrogen soil levels occur they seem to affect Ca /Al ratios by decreasing Ca uptake, leaching cations and promoting aluminum toxicity due to the acid-base balance shift (USDA-FS, SRS & SR, 2001). In addition, changes in plant carbon allocation due to nitrification may alter their defensive processes and predispose the (trees) to insect or fungal attack (Fox et al, 1989). Soil microbial nitrification processes (ammonia to nitrite to nitrate) may ultimately increase the complement of nitrate byproduct, which once leached, may degrade surface or groundwater unless it encounters anaerobic conditions where bacteria can further reduce the nitrate to N_2O gas (denitrification) thus reducing deleterious effects (Fox et al, 1989). So although N_2O alone is not considered detrimental to forests, it is an indicator of overall higher Nitrogen inputs. Nitrogen levels have increased to stress levels over the last 70 years in some German deciduous (Eichhorn & Paar, 1992), and coniferous (Leisen et al, 1990) forests.

According to the US Forest Service (USDA-FS, SRS & SR, 2001), in high elevation areas of the North Carolina Pedimount area, the general prediction is that the sensitive oak-pine forests could respond with reduced growth rates, vigor and accelerated mortality over

the next 20 years, with many areas receiving an expected 10-30% increase in the already near-saturation forest floor nitrogen content. The southern USA hardwood forests are considered to be less sensitive to nitrogen deposition than spruce-fir forests because of a greater base cation nutrient content due to delayed harvesting, lower nutrient demand, and faster soil Nitrogen cycling. As long as the soil pH remains within physiologically safe limits, the damage directly due to nitrification should be limited. However, the USDA - FS is anticipating the present-day European scenario.

A2-3-5: Ground-level Ozone

According to the USDA-FS-SRS & SR (2001), ground-level ozone (smog) is created when atmospheric NO_x and volatile organic compounds (VOC) interact under certain weather conditions such as warm temperatures, stationary high pressure system, low humidity, and low windspeed. Forests at the most risk are those in close proximity to biogenic emission sources such as morning automobile traffic and coal-fired energy sources and high elevation forests. As the gases rise and cool they can contribute to cloud formations. "Mountains are frequently bathed in clouds which may result in an atmospheric loading of 3 to 10 times greater than valley or lowland deposition and in addition, mountain forests receive a higher dose of ozone (and possibly UV-B on clear days) than low elevation forests" (USDA-FS-SRS & SR, 2001).

According to the IPCC (2001), tropospheric ozone has increased 36% since 1750. Over the last century, ambient O_3 increased world -wide from 0.02 - 0.04 to 0.04 - 0.06 ppm, and by most estimates will continue to increase anywhere from 0.5% to 2% per annum depending upon location and time of year (USDA-FS-SRS & SR, 2001). According to a screening procedure developed by the Rocky Mountain Forest and Range Experimental Station using a range of wilderness areas, forests experiencing an 35 ppb (0.035 ppm) annual O_3 should be capable of maintaining their integrity; however with an increase to 55 ppb (0.055 ppm) during the growing season with occasional ozone values peaking at 110 ppb (0.11 ppm / hour), net photosynthetic productivity will decline (Fox et al, 1989). The recently published SUMO6 Exposure Index indicates that for most vegetation, the threshold value of O_3 concentration is 0.06 ppm-hrs (USDA-FS-SRS & SR, 2001). Below

this level most vegetation should be able to resist the detrimental cumulative effects of ground-level ozone.

According to a summary of tree dieback written by Little (1995), excessive ozone can bleach (oxidize) chlorophyll from needles. Although it has not been thoroughly investigated, ozone can also directly affect leaves of deciduous trees reducing photosynthetic ability, foliage production and retention. Given similar O_3 levels, immature and mature hard woods have more growth loss than softwoods in southern US plantations (USDA- FS -SRS, 2001)

A2-3-6: Stratospheric Ozone

Despite the fact that we create ozone on the ground, from 1979 to 2000, serious depletions in the stratospheric ozone layer have been recorded (IPCC, 2001). These depletions are associated with chlorofluorocarbons and NO_x gases which have also reached the cold, low pressure stratosphere zones and act to disrupt the O_3 (Miller, 1992). Ozone produced at ground level (smog), rises slowly in the atmosphere from the troposphere to accumulate in the "stratospheric ozone blanket" at about 17 to 26 kilometers above sea level, providing protection from damaging UV-B radiation (Miller, 1992, p.81, 298-301).

Little (1995) states that a thinning of the stratospheric ozone blanket can lead to UV-B damage which causes curling and browning of broad leaves causing premature fall similar to drought-induced damage. According to Little, as the Ozone layer thins, and the Dobson units decline, UV-B exposure rises at a rate of about two to one. According to Satellite readings by the National Oceanic and Atmospheric Administration (NOAA) for the last 16 years, the July noontime stratospheric ozone concentration in West Virginia, averaged 330 Dobson units. A large drop (to 278) in ozone coverage in 1995 coincided with massive leaf damage. If accurate, this implies a 30% increase in UV-B exposure. After ruling out other possible causes, the US Forest Service lab in Morgantown WV is currently trying to confirm if the damage was UV-B directed. If confirmed, it will be the first example of direct evidence of UV-B damage to broadleaf trees. (Little, 1995).

A2-4-1: Global Precipitation Patterns

The IPCC-SRES (2001) states that satellite data show that there has been approximately a 10% loss in snow cover since 1960, and ground base observations indicate that there has been about a two week reduction in annual duration of lake and river ice cover in the mid and high latitudes of the Northern Hemisphere. In addition, there has been widespread retreat of mountain glaciers in non-polar regions as well as a 40% decline in Arctic sea-ice thickness during late summer and early fall. Tide gauge data indicate a global rise in average sea level between 0.1 and 0.2 meters over the last century. If the warming trend continues, a local warming of 3°C would completely melt the Greenland and destabilize the West Antarctic ice sheets in fewer than 1000 years, raising sea levels 7-10 meters. For the next 100 years however a more modest rise in sea level is predicted of 0.09 to 0.88 meters, resulting in changing local and distant rainfall patterns with increased precipitation in some areas and prolonged drought in others, along with warmer winters.

According to the IPCC (2001), global fluctuations in rainfall patterns have occurred in the 20th century. Precipitation has increased by 0.5 to 1.0 % over most of the (>30^{0}N) latitudes with a corresponding 2% increase in cloud cover, and a 2-4 % increase in storm activity. In the equatorial zones, rain fall has risen from 0.2 to 0.3 % over the tropical land areas (10^{0}N to 10^{0}S), but data for the southern hemisphere and over the oceans was insufficient for the IPCC to establish long-term trends. Conversely, precipitation has dropped about 0.3% in regions from 10^{0}N to 30^{0}N with an increase in the frequency and intensity of droughts in some regions. The trend to continental drying and increasing drought events is expected to continue into the next century in select areas.

A2-4-2: Acid rain - SO_2 & Al

The National Acid Precipitation Assessment Program (NAPAP, 1990) concluded that the vast majority of trees in the US and Canada are not in decline (as they currently are in Europe), however the stress imposed by acidic precipitation in combination with other factors may contribute to the decline especially in the higher reaches of the Appalachian Mountains. High elevation forests, especially those with extensive "acidified" cloud cover are at the greatest risk. In north west Britain, acid precipitation was found to reduce

the bark buffering capacity of *Quercus petraea*, increasing its acidity and adversely affecting associated bark epiphytes such as *Lobaria pulmonaria* and the moss *Isothecium myosuroides* (Farmer et al. 1991). *Quercus alba* and *Quercus rubra* oaks growing near coal-fired utility sites were negatively correlated to SO_2 emissions with respect to their associated lichens, which had a lower total bark cover, lower species richness, and more pollution - tolerant species than in remote sites (Muir and McCune, 1988). These studies imply surface damage due to acid rain, but as we will see, although the visible problems are important, the subsurface acidification of the roots and their associated mycorrhizae are probably more significant.

According to Fox et al (1989), a loading of 3 kg/ha per annum of sulfur could be offset by weathering and would be acceptable in areas where soils are deep or well supplied with bases. They further estimate that the approximate 19 μeq/l of soluble S produced by this load would be close to the background levels and not result in significant mobilization of aluminum ions. However an excess of 20 kg/ha/yr in most areas would be unacceptable unless the system contained free $CaCO_3$, and even so, the concentration of solubilized S would exceed 125 μeq/l, and this concentration is in the range where aluminum mobilization might occur.

The Southern Forest Resource Assessment (USDA-FS-SRS & SR, 2001) Report states that although sulfur in small quantities is an essential plant nutrient, excessive deposition has been shown to provide a stimulus to mobilize aluminum into soil solutions and to affect nutrient cycling by leaching ions, such as calcium and magnesium. Acid rain contributes to long term nutrient loss especially from soils with low buffering capacities and "recent research .... suggests acid rain can induce soil aluminum toxicity in some cases" where dissolved aluminum interferes with the uptake of calcium and other root functions (USDA-FS, 2001). Since the 1950's, historical tree ring chemistry studies have shown an increase in the Al / Ca ratio, suggesting calcium loss coinciding with aluminum accumulation (Bondietti and McLaughlin, 1992), possibly indicating a competitive inhibition of calcium uptake by aluminum. In Amance State forest in France, 60 year old Pedunculate oaks heartwood increased in both Al and N and decreased in P, K, and Mg

but did not change in average Ca concentration (Lévy et al, 1996). These results were consistent with augmented nitrification and soil acidification trends due to atmospheric deposition (Thimonier et al, 1992). Calcium concentrations are important because they typically increase in stem and branch wood as growth increases conferring resistance to mechanical stresses (Bondietti and McLaughlin, 1992).

A2-4-3: Acid Measurements

Sulfur, nitrogen (USDA FS-SRS & SR, 2001) and chloride (Cunningham & Saigo, 1995) can be used as indicators of acidic precipitation. There are two basic methods of directly determining how acidic an area is. The first is by calculating the acid neutralization capacity (ANC) of the soil or roots using mineral analysis results, and the second is by direct measurement of the pH of the water.

Acid Neutralizing Capacity can be defined as the sum of the base cations (Ca^{+2}, Mg^{+2}) minus the sum of the strong acid anions (SO_4^-, NO_3^-, Cl^-) in a water sample if the concentrations of organic acids and aluminum are insignificant (Fox et al, 1989). It is assumed that the base cations (Ca^{+2}, Mg^{+2}) can neutralize soil acids, increase the pH, and reduce aluminum toxicity (and maybe solubility). Potassium (K) and sodium (Na) can also affect the balance and are often incorporated into the formula by researchers (Fox et al, 1989). When the ANC falls below zero, the area is considered to have become acidic.

Using Henriksen's (1979) empirical nomograph for lake acidification, a given fresh water source can be classified as acidic (pH<4.7), transitional (pH 4.7-5.3) or bicarbonate dominated (pH >5.3). However, any water sample below pH 5.2 is often considered to be dangerously acidic (Fox et al, 1989).

A2-5: General Climate & Weather Patterns - Summary

Over the last 100 years the global climate has altered, most likely due to anthropogenic influences. Major weather disturbances have increased in frequency and intensity in recent years. Aside from immediate damage done by severe storms, additional mortality occurs when stands are weakened by: acidified rain which alters soil pH and solubilizes

toxic metals, warmer summer temperatures which induce repeated droughts, warmer winter temperatures which lower snow cover and which can reduce frost tolerance. Debilitated trees may then be more susceptible invasion by opportunistic and pathogenic insect and fungi. Once disease organisms take hold, they can spread to new ranges causing extensive damage, especially in monoculture forests. Other possible biotic and abiotic causes for sudden oak death are described in Appendix 4.

A2-6: General Climate & Weather Patterns in Merzalben Forest District

At 49 °N (Fig. A1-1-5), Merzalben is likely to fall into the areas of increased temperatures (1.4 - 5.8 °C), cloud cover (>2%) precipitation (>1%) and storm activity (>2-4%) over the next century, according to the IPCC predictions (2001). But since weather patterns and global climatic changes are very difficult to predict and extremes are likely, it is quite possible that Merzalben will also experience extensive drought periods similar to the most recent one (1979-2000). Either extreme, or wide fluctuations, will not benefit the forest.

According to the National Oceanic and Atmosphere Administration - National Climatic Data Center (NOAA- NCDC), during the specific time period of this study, in 1999 the "May global land temperatures were > 0.3 ^{0}C above average (1880-1998).... and.... global precipitation was < 2.5 mm below average in May" (NOAA-NCDC, June 1999), while "in parts of central and northern Europe, September 1999 was the warmest of this century with recorded anomalies in Germany exceeding 8^{0}F above the long-term average. Global land temperatures for November were the warmest on record." (NOAA-NCDC, Dec. 1999). The year 2000 was the 6th warmest year on record (1999, 1998,1997, 1995, 1990 were warmer), averaging 1-2 C warmer in the European area and the third wettest year on record (1.65 inches above normal). (NOAA-NCDC, Jan. 2001). This corresponds to the general observation that spring and fall 1999 were extremely warm and dry and spring and fall 2000 were extremely wet during the mycorrhizal harvest periods.

Merzalben is a high elevation forest with frequent cloud cover, and so is at high risk for ozone-induced problems, acid deposition and excessive UV-B exposure on clear days.

Estimates of the Ozone, Sulfur dioxide and Nitrogen dioxide deposition from 1988 to 1998 are summarized in Table A2-6 from information provided by the Waldzustansbericht für Rheinland-Pfalz (1999). From 1988 to 1998, the Sulfur deposition has been declining over all the Rheinland-Pfalz region from the 1985 highs of >200 $\mu g/m^3$ to 1998 lows of 4-7 $\mu g/m^3$ which is very good news. The Nitrate levels undergo annual fluctuations with summer high points ($\cong$ 15-30 $\mu g/m^3$) and winter lows (< 10 $\mu g/m^3$). The nitrate levels fluctuate but do not seem to be declining. The ozone levels on the other hand seem to be slowly rising. Ozone readings for the same region from other sources were generally a little higher than shown in Table A2-6. According to Block (1993), the annual average O_3 $\mu g/m^3$ readings were: 1988 : 74 1989 : 77, 1990 : 80, 1991 : 78. In 1995 the O_3 concentration in Merzalben averaged 67 $\mu g/m^3$ (BMELF, 1997). Hortenkopf forest station (608 m above sea level), which is a few miles from Merzalben in the Pfälzerwald (Figure A1-1-5) on November 1, 2000 averaged 16-27 $\mu g/m^3$/hr ozone, while low lying Kaiserlautern (233-261 m) averaged 1-3 $\mu g/m^3$/hr (Landsamt Umweltschutz, 2000). This last example is provided to demonstrate that ozone deposition is generally higher in high elevation forests than it is in low lying regions.

Table A2-6: Average annual deposition of Immission Products in Merzalben in $\mu g/m^3$. [Adapted from the Waldzustandsbericht für Rheinland-Pfalz (1999)] .

	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998
SO_2	11	10	16	14	11	8	6	6	7	6	4
NO_2	14	15	11	9	14	16	10	14	17	13	10
O_3	64	66	69	67	64	64	64	63	66	73	74

For our intents and purposes, our adjacent forest research plots (unlimed and limed) in Merzalben will be considered to be approximately equivalent with respect to ozone (in low clouds) and UV-B exposure. Although it must be acknowledged that it is precisely because of atmospheric problems that the forests in this area are in trouble. Both areas are

under acid stress (Block, 1999, p.c.) and both are rated to have many trees in damage class 2, with the unlimed forest containing some trees in damage class 3 (Appendix 3).

In 1988, at 0-5 cm depth the averaged pH ($CaCl_2$) acidity of the soil was pH 3.5 and at 10-30 cm depth it was pH 4.3, with an overall average of pH 3.9 for the Merzalben region (BMELF, 1997). The mineral contents of the soil and the soil solution, but not the roots, have been previously measured and are given in Appendix 5. In this study, current soil acid levels were measured and root mineral analysis was done to determine root Acid Neutralizing Capacity (ANC). The ratios of Al to Ca will be reviewed to determine if Aluminum is displacing Calcium in the unlimed and/or the limed forest regions. Individual mycorrhizal species will be tested to determine their relative abilities to promote, inhibit or block aluminum translocation to the xylem in the unlimed acid region compared to the limed, less acidic region.

A2-7: Merzalben Fungal Fruiting Bodies & Stress

In the event of tree loss, it is expected that saprophytic species would appear in larger numbers to fulfill their role in recycling forest nutrients. In the survey of surface fruiting bodies several saprophytic genuses were present (*Bisporella, Collybia, Corolius, Daedalea, Hydropus, Hypholoma, Mycena, Pluteus, Psathyrella, Sterum, Tyromyces, Xylaria*) and one saprophitic / parasitic form (*Armillaria*) on fallen branches and tree stumps (Appendix 2C). Some species were found only on the limed *plot (Daedalea quercina, Hydropus atramentosus, Mycena filopes, Mycena lactea, Mycena rosella, Mycena sepia, Psathyrella atrolaminata, Sterum rugosum, Xylaria hypoxylon, Xylaria longipes*) and some were found only on the unlimed *plot (Corolius versicolor, Hypholoma sublateritum, Mycena aetites, Mycena inclinata, Pluteus dietrichii, Psanthyrella hydrophila, Tyromyces albellus, Armillaria bulbosa, Armillaria mellea, Armillaria ostoyae-malformed)*. The distribution may have been accidental for some of the species, a fault of the timing of the survey for example, but it is interesting to note that the greatest diversity of potentially pathogenic Armillaria species was present on the unlimed plot. The presence of a variety of Armillaria species, along poor crown growth and tree loss in

the unlimed zone (Appendix 3), supports the perception that the limed forest was in an overall healthier state.

Debilitated, but still living trees, may become susceptible to pathogens of various sorts, including opportunistic insects and fungi (Appendix 4). In Merzalben, the incidence of insect and moth damage has been variable with peaks in 1988 and 1996 (Schröck et al, 1999), but is not considered to be the primary cause of forest debilitation.

In the natural flora of the forest soil, opportunists are kept in check by the presence of competing species (Appendix 1 and 2). The loss of fungal symbionts due to soil acidification and metal toxicity would leave the roots susceptible to invasion by unsympathetic parasitic and opportunistic fungi. It is difficult to say at this point exactly which mycorrhizal species would be essential to the good health of the trees in an oak forest, but it will be shown that liming did affect the distribution of fungal symbionts.

A2-8: Merzalben Mycorrhizae & Stress

"In the early 1980's, disturbing reports appeared of rapid forest declines in both Europe and North America .... West German foresters estimated in 1982 only 8 percent of their forests showed air pollution damage. By 1983, some 34 % of the forest was affected, and in 1985, more than 4 million hectares (about half the total forest) were reported to be in a state of decline .... this complex phenomena probably has many contributing factors, but air pollution and deposition of atmospheric acids are thought to be the leading causes of forest destruction ... high-altitude forests are subject to especially intense doses of these acids because clouds saturated with pollutants tend to hang on mountain tops .... " (Cunningham and Saigo, 1995, p.386). Cunningham goes on to say that toxic elements, such as Al, may be solubilized by acidic groundwater and that " fungi that form essential mutualistic associations with tree roots may be damaged by acid rain". The effects of chronic exposure of trees to air pollutants may not be visible for many decades and so "Waldsterben" (forest death), (especially in higher elevations) may be acting as an early warning system that many tree species at lower elevations are at risk (Miller, 1992).

In an altered environment it would be expected that previously less successful members of the natural flora or perhaps introduced mycorrhizal species may supplant the lost fungi. As a result, changes in the fungal hierarchy, abundance, diversity and succession series should be evident. In this study, a comparison will be made between the unlimed and limed high-elevation forest to determine if such a change has occurred. One further step will be taken to determine if these changes (which did occur) are in any way associated with the uptake of aluminum from the unlimed and limed-acid soils.

In a stable, unchanging or very slowly changing environment, selection pressures generally result in a lower biodiversity but increased species strength through energy efficient adaptation, slow growth and maintenance of carrying capacity (K). This adaptation form is generally referred to as K - selection. In a rapidly changing, stressed environment, energy is diverted into rapid growth (R), enhanced reproduction and increased biodiversity. This second general adaptation pattern is referred to as R - selection. In a summary provided by Miller (1992), characteristics of the two types of reproductive strategies are outlined.

In very general terms the K-strategists tend to fewer, larger young and larger, longer-lived adults, with stable population numbers while R-strategists tend to have more, smaller young and smaller, shorter-lived adults with wildly fluctuating population numbers. It would be expected that most oak-mycorrhizal species would tend to be between these two extremes in environments experiencing moderated climatic changes. It was expected that mycorrhizal species in response to environmental stresses would show signs of selection which would alter their abundance and diversity within the unlimed and limed forest regions. What was not expected was that they would show such distinct signs of K (unlimed) and R (limed) reproductive strategies.

Stresses are seldom attributable to single events, but rather a culmination of many simultaneous or consecutive events with cumulative effects. We will look at several possible stressors in the general introductory overview but will concentrate upon the effects of pH and aluminum mobility within the report. Since both research areas are

within the same microclimatic environment (adjacent plots), and at the same elevation, it could be assumed that any obvious trends to either K or R selection strategies are then the direct result of the liming treatment.

Overcoming a short term stress (summer drought, early frost) may involve fungal surface or subsurface fruiting body and spore formation, or at the minimum hyphal stasis or dormancy, and fungal succession. In overcoming long-term or multiple stresses (acid precipitation, thermal climatic change, altered precipitation patterns), concurrent threats to species vitality may involve displacement of the dominant fungal species with numerous contenders. During either transition time, the host will most likely suffer. If none of the long term contenders or short term succession species can overcome the negative effects of the changes, or if the host weakens excessively, additional biotic (pathogens) or abiotic (heavy metal) stresses may finish the job with the death of the host tree species. In a mono or a biculture forest setting this might mean the rapid loss of large tracts of susceptible forest or at the very minimum sudden oak death in individual trees.

Besides altering our definition of what a climax forest really is, artificial selection and stand maintenance may diminish genetic diversity and interfere with natural forest succession and protective biodiversity which could be potentially disastrous in the event of extreme local or global climatic changes. This would be especially true if essential species of mycorrhizae have been adversely affected. Persistent or sudden extinction of essential fungal symbiants may have insidious negative effects upon the health and vigor of their host trees. Extremely little is known about the subsoil fungal hierarchy and succession processes.

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A3: Aluminum In Forest Soils

A3-1: Introduction - General Impact of Acid Rain

Aerosolized input of sulfur and nitrous oxides released from fossil fuel combustion is believed to be the main source of acid precipitation affecting industrialized countries, US, Canada, and western Europe, leading to a sharp increase in soil acidification, and the solubilization of soil aluminum (Appanna & St. Pierre, 1996). It has been estimated that (world wide) 40% of the arable surface soils and up to 70% of the potential crop soils are subject to acidification and Al solubilization (Haug, 1984). According to Haug (1984), Aluminum was implicated as early as 1918 as the primary cause of root retardation in barley and rye plants grown on acidic soil. In addition, acidic subsoils are more easily leached, depriving them of water and minerals making the deep roots more prone to the effects of drought and nutrient deprivation (Haug, 1984).

Throughout Europe, 73% of the natural ecosystems have been stressed by acid deposition, and in 1992 it was estimated that the fallout ranged from <1 to 3 keq H^+/ha·a (Isermann and Isermann, 1995). From 1850 to 1987, the cumulative acid deposition in the area of West Germany averaged 370 kmol·ha^{-1} with the highest deposition in high elevation coniferous forests, especially on exposed slopes; while low elevation, rural deciduous forests experienced the lowest acid accumulations (Ulrich, 1989, p.9-10). It was estimated that between 1.6 and 5.3 kmol acid units per hectar per year may actually reach the soils (Ulrich, 1983), implying strong crown and understory foliage filtering.

From 1988 to 1992, the Pfälz forest stand selected for this study averaged an annual soil proton deposition of only 0.83 kmol/ha (Block, 1995). Nevertheless, the earth at Merzalben was acidified up to 60 cm depth with a pH of 4.4 (Block, 1995). Aluminum first becomes soluble at $\leq$ pH 5 (Foy, 1978, 1992). The suspicion was that Aluminum freed into the soil may be the underlying cause of sudden oak death and general forest die-back (Block, 1999, pc). The purpose of this study was to look at the role of ectomycorrhizae, as the first-line defense against potentially toxic Aluminum uptake.

The deposition of atmospheric acids into the groundwater may cause agrilaceous minerals (such as Al) to be soluabilized into potentially toxic forms (Al^{3+}) (Cunningham and Saigo, 1995) Subsequently, the chemical equilibrium of the soil can be altered resulting in reduced growth and extensive mortality of sensitive forest species (Petereson, 1995).

In General, when the pH drops below 5, Al can precipitate phosphate within plant tissues, leading to P deficiency (Bollard, 1983). Aluminum may also bind to meristem DNA and so interfere with cellular division (Horst et al, 1983). Laser micromass spectrometry and EDAX techniques have revealed that, in non-mycorrhizal roots, Ca is displaced by Al in cortical wall structure, possibly affecting elongation (Godbold et al 1988). Proton toxicity is assumed to occur if the molar ratio of Ca^{2+}/H^{+} becomes ≤ 0.1 and Al toxicity is most likely to occur if the ratio of $(Ca + Mg + K) / Al \leq 1$ (Sverdrup and Warvinge, 1993). Aluminum is known to slowly translocate from the roots, via the xylem, to the heartwood of a tree through a long term detoxification process (Lévy et al, 1996). Aluminum toxicity symptoms include damage to root caps and similar restricted growth in plant shoots with mottling and necrosis of leaves (Bollard, 1993).

In forest soils, the humic top soils contain minor Fe and Al buffer systems that can accommodate short term, mild or intermittent acidification in the range of pH 2.8 to 3.8 (Ulrich, 1989). Between pH 3.8 and 4.2 an Al buffer range also exists (Notwotny et al, 1998). According to Ulrich (1989), in deeper soil, aluminum hydroxide can buffer a portion of the acid deposited within the pH range of 4.0 to 4.4, but as the acid front moves downwards the Al is again released to its freely soluble, mobile and potentially toxic Al^{3+} forms with the highest accumulations of Al occurring in the subsoil at 60 cm depth.

Liming of agricultural soils, to counteract acidification and improve nutrition of seasonal crops, is now a common practice world wide. Liming of forest soils is slowly becoming more common. In Germany, forest soil liming has been done extensively to counteract the effects of acidification, enhance nutrient cycling, reduce Al toxicity, correct Mg deficiencies and improve forest productivity (Ulrich 1986, 1989). Since the 1950's more than one million hectars of damaged forest in Germany nation-wide have been limed with

an average of 3 tons per hectar (BLF, 1988). Between 1953 and 1965 more than 100,000 ha of southwestern Germany's Black Forest were limed, occasionally with phosphate and magnesium fertilization accompanying the liming with the primary goal of improving the humus form of the topsoil layers (Aldinger, 1987). Kiesert and dolomite liming trials expanded from primarily coniferous forests to select, very small, deciduous forest plots over the last few decades (Zoettl, 1990).

In 1989, a small area of approximately 60 square meters adjacent to the Merzalben Research station, was limed with 3 ton/ha of dolomite lime to counteract the effects of acidification (Block, 1999, p.c.). The biogeochemical impact of this liming trial on the ectomycorrhizal community in comparison to an unlimed zone is the focus of this study.

In the 1970's most liming trials in the coniferous forests were discontinued because not only did many forests not show signs of improved growth but critical questions arose related to the ecological side-effects of liming and a "new type of forest damage" (Huettl and Zoettl, 1993). In the 1990's reports of the 10 to 20 year effects of liming began appearing (Zoettl, 1990, Huettl and Zoettl, 1993). These reports did not address mycorrhizal contributions but mostly measured soil or water mineral content and biomass of whole fine root systems. In the trials described by Zoettl (1990), the expected compensations from liming were not realized. Liming: promoted shallow root growth but with reduced total biomass (Hildebrand and Schack-Kirchner, 1990); increased the risk of drought damage; delayed Mg remediation efforts (Zoettl, 1987); promoted monovalent and divalent cation leaching (Huettl and Zoettl, 1993); and contributed to "acidic pushes" into deeper soil horizons (Hildebrand, 1990). The initial short term (1-2 year) boost in pH, growth and vigor noted in many stands was often followed by a net volume loss in the decades following liming (Huettl and Zoettl, 1993). After liming, Al soil content often declined. Anderson and Persson (1988) concluded that heavier, more frequent, applications of lime were required to impede Al leaching to the ground and surface water.

Ectomycorrhizae can assist in the liberation of water and critical nutrients in the soil exchange system and can promote their subsequent translocation into the root (Lewington

and Streeter, 1993). The presence of bound and free Al has been detected in fresh mycorrhizal Norway spruce root cross sections using Morin, a fluorescent chemical marker (Notwotny et al, 1998). In this study, using Morin, aluminium was localized within oak root-fungal complexes isolated from acidified untreated and lime-treated forest soils. As will be demonstrated, Al acumulation was augmented within limed roots. This phenomenon may contribute to the "new type of forest damage" noted by Heuttl and Zoettl. The role of Al at the soil-root interface must be illucidated to determine if Al loss from limed soil was just due to leaching, or also related to excessive uptake at the roots.

A3-1-1: General Soil Composition

Of all the minerals in the earth's crust, silica is the most abundant followed by aluminum. The average Continental Crust concentration of various minerals (SiO_2 = 60.2 %, Al_2O_3 = 15.2%, Fe_2O_3 = 2.5%, FeO = 3.8%, MgO = 3.1%, CaO = 5.5%, Na_2O = 3%, K_2O = 2.9%) in sedimentary, granite and basaltic layers has been defined (Press & Siever, 1985, p.388). According to Haug (1984), Aluminum is the primary constituent of most common rocks except limestone and sandstone, from which it is easily leached, and while Aluminum occupies 7.5% of the crust of the earth by weight, with an average bound concentration of 81,000 ppm (1 ppm ≅ 37μM), the mineral content in specific rocks can vary considerably. Appendix 5-1 gives a summary of the approximate average abundance of some minerals (including Al) in various rocks prior to weathering. As we will see in the following discussion, this metal is highly reactive and so rarely found free after weathering, but rather bonds easily to form composites such as Aluminosilicates, and less abundantly, aluminum oxides which are the primary ore forms (Haug, 1984). Aluminum can also form complexes with organic phosphates, humic acids and ligand chelators which are found not only in the soil but also on some cell surfaces and within cells.

A3-1-2: Aluminosilicates

A3-1-2A: Zeolites

Zeolites, which form one of the major classes of aluminosilicates (sandy soils), are characterized by their tetrahedral subunits which link together to form an anionic network

which contains 2,5 - 9 openings into which water molecules and cations can be accommodated (Haug, 1984). In this capacity, aluminum-silica complexes can help hold water, contribute to soil building through mineral retention, and potentially aid in recovery processes through absorption and sieving of various elements. Some types of zeolites, depending on their Al and Si orientation within the framework, can be used to trap radioactive materials from nuclear waste, but in heavily polluted soil, zeolites can be deactivated by fouling molecules plugging up the shape-selective pores and reducing the surface activity (Haug, 1984).

A3-1-2B. Zeolite-like Aluminum Phosphates

Organic phosphates, comprising 30-70% of the total soil phosphate, can form strong bonds with aluminum (Foy, 1992). In the weathering process, as phosphates are released, it is likely they are deposited on the silicate clay surface to subsequently bond to the Al within the crystalline tetrahedral Al-Si skeleton previously mentioned (Haug, 1984). Over time, zeolite-like aluminum phosphates ($AlPO_4$) form with the same tetrahedral structure as the alumino-silicates but now containing strictly alternating aluminum and phosphate ions, all covalently bound to oxygen, effectively neutralizing the oxygen and within the porous lattice produced, water molecules and charge balancing cations, may find refuge, while others (including solubilized aluminum) are transported along the surface interfaces to the roots (Haug, 1984).

A3-1-2C. Phyllosilicates

Summarized from Haug (1984), the phyllosilicate (layered) clays include kaolinite, monmorillonite and vermiculite which can form tetrahedral ($Si_4O_6(OH)_4$) or octahedral sheets ($Si_4O_6(OH)_4)_2$ which may contain Al, Fe or Mg ions trapped in the Lewis acid-base sites. In a tetrahedral sheet, if Al is substituted for Si, the layered silicate will have a permanent negative charge near this point and generate a Lewis base site while the sandwiched space contains a corresponding acid site. These combined Lewis acid-base sites vary in density and have been viewed as primitive multienzyme complexes of low selectivity. The Lewis base site will bond with any cation (acid) structure, non-selectively, thus contributing to the „sticky" nature of clay and its ability to hold ions, minerals and

charged proteins. In addition, the Lewis base sites have catalytic properties. When alkaline (OH^-) ions in the clay inner layer are replaced with water (H_2O) at the AlO_4 site, the released OH^- ions are eventually transformed by redox processes into monatomic oxygen (O^-) which in turn can oxidize hydrocarbons (paraffins) attached to the silicate surfaces to form fatty acids and eventually CO_2. This basic soil respiration process requires the presence of water and oxygen gas in excess of the hydrogen ion concentration. Imbalances, such as excessive hydrogen ion input (via acid rain), reduced oxygenation (via microfauna death), squeezing water out (via compaction by logging vehicles), or dehydration (via edge-effect temperature gradients) will disrupt normal soil respiration processes (Haug, 1984).

According to Haug (1984), monmorillonites, in the presence of high densities of water, contain hydronium (H_3O^-) ions in the interlayer regions which can hydrolyze organic proteins and peptides releasing ammonium and forming smaller charged peptide subgroups which can bind at the Al-Lewis sites in the crystalline network. Once bound, the hydronium ions can further degenerate the peptides releasing amino acids, and their constituent parts (carboxyls and amines). The released amines (NH_3^-) in the presence of hydronium can form ammonium (NH_4) and H_2O end products (Haug, 1984). Excessive concentrations of hydrogen ions, forming hydroniums, can drive the process and possibly have the same protein degenerative abilities within cell walls or on membranes within the lysosomes in other organelles which complex with aluminum potentially increasing the levels of toxic ammonium within the cells, or at the very minimum, disrupting cellular processes. Although untested, ammonium accumulation would in part explain the damaging effects of aluminum within cells.

A3-1-3: Aluminum Oxides

A3-1-3A. Dehydrated Aluminum Oxides

Aluminum oxides are classified according to their dehydration characteristics, number of layered hydroxyl groups, surface functional groups, electron transfer sites, and Lewis and Bronsted acid sites (Haug, 1984). Examples of Aluminum oxides include : Gibbsite

($Al(OH)_3$ Crystalline clay), Diaspore ($AlO(OH)_2$), Norstrandite ($Al(OH)_1$ di-octahedral layers), Alumina ($Al_2O_3^+$) and Aluminates (AlO_2^-) (Haug, 1984). Alumina ($Al_2O_3^+$) and aluminate (AlO_2^-) ions, bound to resins, can complex with D-fructose enhancing its conversion from D-glucose in in-vitro aldose-ketose reactions (Haug, 1984).

A3-1-3B: Hydrated Aluminum Oxides

Hydrated Al $(H_2O)_6^{3+}$ has a hydrolysis constant of pK = 5.0, which is about the same strength as acetic acid and as such the hydrated Al oxides can act as Bronsted acids, and undergo acid-base reactions involving the transfer of protons (Haug, 1984). At the Al-oxide aqueous interface, numerous hydroxyls carry a pH dependent negative charge, which can be balanced by cation absorption and transfer and while monovalent cations can be easily absorbed, divalents, aromatics and complex organic acids can only be absorbed with more specificity (Haug, 1984). In this way the Al-oxide complex have a significant soil-building action but more importantly, Alumina soil compounds ($Na_2(OH)Al_2O_4$) can act as planar superconductors, with oxygen molecules acting as bridges along the conductivity plane, rapidly moving ions over their hard surface during diffusion (Haug, 1984). On the surface of other oxide compounds, equidistant lattice-terminal hydroxyls can form a two dimensional plane along which excess protons are delocalized, mobilized and rapidly conducted (Haug, 1984). This mobilization may be important for nutrient uptake by plants, and during the leaching process. This so called „Proton conduction plane" or „proton wire" may also occur in biological systems (i.e.-membrane pores), if additional arrays of hydroxyl or perhaps amino groups are present (Haug, 1984). Limited Al incorporation within cell membranes may then assist with nutrient uptake, whereas excessive aluminum incorporation may result in a loss of control and thus interfere profoundly with normal cellular functions.

A3-2: Aluminum Weathering

Sandy soils, in advanced stages of weathering, will release metals at various rates depending upon a variety of factors including: stone blend, particle size, pH of the soil bathing solution, frost, drought, rain, runoff , wind patterns, microbial action, fungal and

plant exudates. Very generally, the smaller the particle size, the greater the surface-volume area, the faster the weathering, however, the degree of release is also highly dependent on the stone composition. Chemically, there are three general weathering reaction mechanisms: Hydrolysis, Chelation and Oxidation / Reduction.

According to Press & Siever (1985, p.104,108,112), iron and Al oxides tend to be very stable minerals under weathering conditions. The common feldspar component of granite (orthoclase), which is high in Al, will form kaolinite when weathered with acidic water. The silica (SiO_2) and some anion products leached away from the rocks create fissures and erosions that can promote faster erosion, eventually forming kaolinite clay. The Al however is often still tightly bound (Formula A3-2-1) (Press & Siever, 1985). Under extreme weather conditions (heavy tropical rains), kaolinite clay can further dissolve leaving a solid residue of gibbsite $Al(OH)_3$ (aluminum hydroxide) which is a major component of bauxite, a primary source of Al for manufacturing (Press & Siever, 1985).

Formula A3-2-1: Dissociation of Feldspar

$$2KAlSi_2O_8 + H_2O + 2(H^+ + Cl^-) \rightarrow Al_2O_3 2SiO_2 2H_2O + 4SiO_2 + 2K^+ + 2Cl^-$$

All this is to say that aluminum is not easily soluabilized and leached. Weak carbonic acid in "normal" acid rain does little to contribute to the alumino-silicate weathering process but stronger sulfuric acids promote more rapid, noticeable damage releasing Aluminum and Potassium ions (Formula A3-2-2), (Press & Siever, 1985).

Formula A3-2-2: Dissociation of Kaolinite

$$2KAlSi_2O_8 + 4H^+ + 4\,H_2O \rightarrow Al^{+3} + 3H_4\,SiO_4 + K^+$$

When completely dissolved at pH 4.5, an alkaline feldspar will release Aluminum ions. The free Al^{+3} ions can form strong complexes with organic anions (L^{n-} = ligands) such as oxalate or citrate (Formula A3-3-3), (Landeweert et al, 2001).

Formula A3-3-3: Aluminum-ligand formation

$$Al^{+3} + L^{n-} \rightarrow Al\,L^{3-n}$$

Aluminum, iron and other ions, including Calcium (Appendix 5-2 to 5-5) can be weathered by a inorganic acids, organic acids and water through forming or altering chemical complexes. Razzaghe & Robert (1979) determined that Al, Fe, Mg, Si and K could be weathered from phlogopit via complexing with a large variety of inorganics, strong, medium and mild organic acids and water but overall they determined that in comparing all of the leaching substrates, strong organic acids (Oxalic, Tartaric and Salicylic acids) which operate within the initial pH range of 3.1 to 3.48 had the greatest total average mineral extraction ability (Appendix 5-6, 5-7). Organic acids in the bulk soil are generally too low to accelerate mineral weathering but the production of specific organic acids by microorganisms (bacteria, fungi, lichens and plant root tips) within the microenvironment of the rhizosphere at certain pH levels can, influence the release of minerals from the soil to the bathing solution (Landeweert et al, 2001). The primary organic chelators of trivalent Al and Fe ions released by roots, bacteria and fungi are oxalate, citrate and malate (Jones, 1998). Oxalic acid is the strongest with the ability to form complexes with K, Ca, Mg, Mn, Zn, Cu, Al and Fe (Gadd, 1999). Oxalic acid is commonly produced in large quantities by a variety of fungal species (Dutton et al, 1996) . Although probably toxic to different fungi, oxalic acid may have a variety of functional roles from metal detoxification, to altering host cell pH, to increasing hyphal penetration into plant tissues (Dutton et al, 1996).

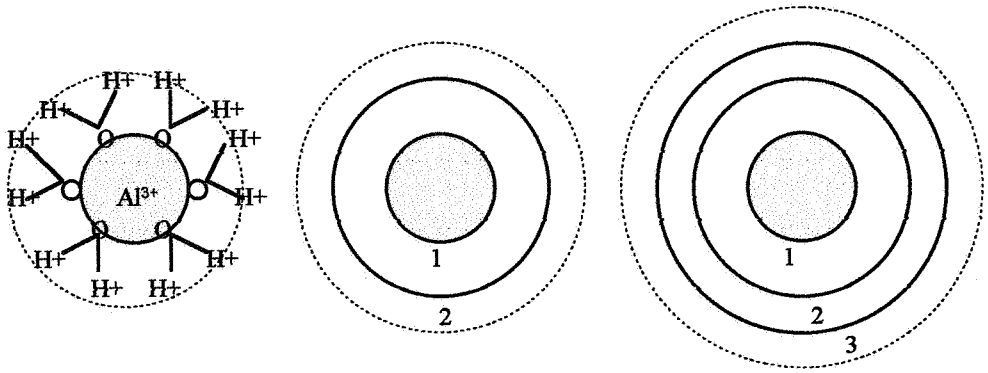
A3-3: Aluminum Solubilization

A3-3-1: Hydrated Aluminum

Aluminum first becomes soluble at $\leq$ pH 5 and it is precisely when Al is solubilized, below pH 5, that most susceptible plants begin to exhibit root die back symptoms (Foy, 1978, 1992). According to Haug (1984), when hydrated, the trivalent Al ion is surrounded by up to 3 hydration shells with the number and arrangement of the water and hydroxyl groups highly dependent upon the ambient pH and soil moisture. The primary hydration shell contains 6 water molecules ($Al(H_2O)_6^{3+}$) at low pH, with the water protons directed away from the Al ion. The secondary hydration shell consists of 12 to 31 water molecules dependent upon temperature and external competing ionic interactions. The

outermost tertiary hydration shell, in well hydrated soils, is more ill defined and dynamic forming gradual variable transitions into the microenvironmental solutions as the complex diffuses along an interface (Diagram A3-2-1). Due to the intense proton charge over the shell surface, the hydration capacity of Al is high relative to other cations. As the hydrogen ion concentration increases, soil hydroxyls (OH-) are transformed into water molecules which form the hydration rings around Al, (and other ions) solubilizing and mobilizing them in the mass flow (Haug, 1984). The most significant feature of this process is the "piggyback" mobilization of other ions.

Diagram A3-3-1 : Hydrated Aluminum. Aluminum in acid solutions can form a primary hydration shell consisting of 6 water molecules, and depending upon the surrounding microenvironment, a secondary shell consisting of 12-31 water molecules, or a tertiary shell containing a variable quantity of water molecules may form.



Primary Secondary Tertiary

A3-3-3: Mononuclear Aluminum Hydrolysis Products

In acid pH, Al $(H_2O)_6^{3+}$ and its byproducts are found, and in alkaline pH aluminate Al $(H_2O)_2$ $(OH)_4^-$ hydrolysis forms are mainly present (Haug, 1984). The possible Aluminum hydrolysis species present in hydrated soils at various pH levels are depicted in Table A3-2-3 based upon work by Baes & Mesmer (1976). Baes and Mesmer (1976) noted that at pH <3 the Al $(H_2O)_6^{3+}$ monomer species is primarily present, and as the Aluminum concentration in the soil rises from 10 to 60 μM, the Al $(H_2O)_5^{2+}$ species appears, and at even higher concentrations more complicated [AlO_4 Al_{12} $(OH)_{24}$ $(H_2O)_{12}^{7+}$] condensation

polymers can form. In the physiological pH range (5-8) there are usually 4 mononuclear species: $Al(H_2O)_5^{2+}$, $Al(H_2O)_4(OH)_2^{+}$, $Al(H_2O)_3(OH)_3^{0}$ and $Al(H_2O)_2(OH)_4^{-}$ variably present, depending on temperature and hydration time, and as the pH rises even more, Al $(H_2O)_2(OH)_4^{-}$ becomes the exclusive species form (Baes & Mesmer, 1976). Control of hydrolysis products is in a large part dependent upon soil buffering capacity.

Table A3-3-3: Aluminum hydrolysis products at 25°C in 10^{-5}M Al (III)
(Derived from: Baes & Mesmer (1976) In: Haug, 1984)

PH	Aluminum Species	Approximate %
3	$Al(H_2O)_6^{3+}$	95%
	$Al(H_2O)_5^{2+}$	5%
6.5	$Al(H_2O)_3(OH)_3^{0}$	75%
	$Al(H_2O)_4(OH)_2^{+}$	15%
	$Al(H_2O)_2(OH)_4^{-}$	10%
7.0	$Al(H_2O)_3(OH)_3$	70%
	$Al(H_2O)_4(OH)_2^{+}$	5%
	$Al(H_2O)_2(OH)_4^{-}$	20%
10	$Al(H_2O)_2(OH)_4^{-}$	100%

A3-4: Soil Buffering Capacity

Atmospheric gases CO_2, SO_2 and NO_x washed out as acid rain contribute to the acidification of forest soils at an average rate of 7 kmol acid imput per hectar per year in Germany (Ulrich, 1983). Not all forest soils receive the same acidic input however since rainfall and high altitude cloud cover fluctuates, and crowns of variable densities can filter the rain. Not all trees within an ecosystem will receive the same acidification due to variations in genetic type, age, intrinsic health, soil composition, micofauna and microflora activity, abundance and distribution. Calculations of genetic parameters show that phenotypical variation in buffering capacity is governed mainly by genetic factors including differences between families and even clones (Scholz & Reck, 1972).

It is estimated that between 1.6 and 5.3 kmol acid units per hectar per year may actually reach the soils (Ulrich, 1983). However, over the long term, protons will accumulate in

the soil and the buffered acids in the leaves will eventually be transferred via phloem to the roots, and through the apoplast and xylem back to the leaves resulting in a spiral pattern of decreasing pH throughout the arboreal system (Ulrich, 1983). It would be expected that this spiral acidification would continue until the roots and tree approach the pH of the soil, unless they act as a sink for the mobilized toxic ions in which case they may suffer debilitation up to a critical point and then suddenly die.

The vanguards of protection in the subsoil may be the mycorrhizal fungi that control nutrient and ion uptake. If these "gatekeeper fungi" die, then the tree would very quickly go from chronic to acute in their health status. The soil acidification process can in part be counteracted by natural buffer systems, which are dependent upon soil composition, depth, rate and degree of pH change, precipitation patterns, and in another part by natural biomass interactions. At present it is understood that there are several possible soil buffer systems (Figure 3-4-1) which will be briefly described.

A3-4-1: Carbon Buffer System

In the carbon buffer system, at pH 8.6 to 7 calcium carbonate is relatively stable, but as the pH approaches 5, calcium carbonate ($CaCO_3$) is washed out of the soil as calcium hydrogen carbonate ($Ca(HCO_3)_2$) which can dissociate into bicarbonate and free calcium ions (Formula A3-4-1), (Bartels & Knabe, 1990), (Appendix 5-3 to 5-5). Magnesium which exists in the stable $CaCO_3 \cdot MgCO_3$ form in neutral soil can also be leached as $Mg(HCO_3)_2$ and organic anions from the overlying Ahe soil horizon as the pH drops (Hildebrand and Schack-Kirchner, 1990). Both contribute to the sink effect and an overall loss of protons to deeper soil horizons and the ground water.

Formula A3-4-1: Dissociation of Calcium carbonate.

$$CaCO_3 + H_2CO_3 \rightarrow Ca^{+2} + HCO_3^-$$

A3-4-2: Silicate Buffer System

The storage ability of the carbonate and silicate buffer systems above pH 5 is proportional to the clay and humus content of the soil (Ulrich, 1989, p20). Ions such as Na, K, Mg,

and Ca are usually strongly held by the silicate buffered clay micelles, but if the base saturation content is high, and acidification occurs, modest leaching can rid the soil of excess materials (Press & Siever, 1985) or deliver them to the roots. Between pH 6.2 and 5.0, Ca-feldspar and Ca-Al-silicates can be dissociated to release Ca and bicarbonate, leaving Al-kaolinite (Formula A3-4-2), (Bartels & Knabe,1990). It has been estimated that of the 1-7 kmol of protons that are deposited annually throughout Germany, only 0.2 to 2 kmol can be buffered by the silcate system (Ulrich, 1983).

Formula A3-4-2: Dissociation of Feldspar to Kaolinite

$$CaAl_2Si_2O_6 + \text{Ca-Feldspar} + 2H_2CO_3 + H_2O \rightarrow Ca^{+2} + 2HCO_3^- + Al_2Si_2O_5(OH)_4 \text{ Kaolinite}$$

A3-4-3: Cation Exchange Buffer System

In the cation exchange buffer system, progressive acidification can cause the release of Al ions from the silicate buffered clay (Formula A3-4-3), (Bartels & Knabe, 1990). The Aluminumhydroxide cations with their hydration rings can become incorporated between clay micelles forcing Ca, Mg, and K and heavy metal ions to be released and washed away. According to Ulrich (1989), if the pH is above 4.2 and the base saturation is above 15%, there will be intermediate cation storage and increased leaching. At this pH, sensitive plant species will suffer from acid toxicity, with only more tolerant (pine, spruce, fir, larch, beech, and oak) species remaining in the ecosystem (Ulrich, 1989).

Formula A3-4-3: Aluminum Release from Silicate Buffered Clay

$$n[AlOOH + 0.5\,H^+ + \text{Clay} + H_2O] \rightarrow \left[Al(OH)_{2\text{-}5}^{0\text{-}5+}\right]_n + 0.5\,nH_2O$$

A3-4-4: Aluminum Buffer System

As long as the soil is within the cation exchange buffer range (pH 4.0-4.4), a portion of the H_2SO_4 in acid rain will combine with Al-hydroxide and form aluminohydroxosulfate ($AlOHSO_4$) in the soil (Formula A3-4-4a, b,c), (Ulrich, 1989), but as the pH drops due to prolonged dry sulfur deposition (SO_2) (Pfanz et al, 1987) into the aluminum buffer range

(pH 4.2-3.8), the Aluminohydroxysulfates become mobilized releasing reactive Al ions and sulfates (Ulrich et al, 1984; Evers 1985; Fröhlich, 1988; Hildebrand, 1989; Ulrich, 1989, Bartels & Knabe, 1990). Al and Mn stress begins to occur between pH 4.0 and 4.4 and it is estimated that if the pH drops below 4.2, and if the base saturation is less than 5% (or 10% in humus rich soils), then there will be low cation storage (maximal leaching or transfer) irrespective of clay or humus content of the soil (Ulrich, 1989, p.20,21).

Formula A3-4-4a: Aluminum-Sulfate Buffering Formula

$$Al(OH)_3 + H_2SO_4 \rightarrow AlOHSO_4 + 2\,H_2O$$

Formula A3-4-4b: Acidic Destabilization

$$SO_2 + H_2O \rightarrow [\,SO_2 \cdot H_2O\,] \rightarrow H_2SO_4 \rightarrow HSO_4^- + H^+ \rightarrow SO_3^{-2} + 2H^+$$

Formula A3-4-4c: Mobilization of Aluminum ions

$$AlOHSO_4 + H^+ \rightarrow Al^{+3} + SO_4^{2+} + H_2O$$

The ability to form aluminohydroxosulfate in the soil will tie up the aluminumhydroxide and slow down the cation leaching, however acid "pulses" can lead to temporal and spatial variations in forest floor acidification stressing some trees more than others and resulting in selective and variable rhizosphere damage (Ulrich, 1989, Fig.4, p18). In 100 year old Norway spruce forests, the fine root concentrations in various soil depths seems to parallel the time course of acid stress with the highest concentrations of free aluminum ions found in the subsoil at about 60 cm depth (Ulrich, 1989), implying the movement of an acid "front" down through the soil.

A 3-4-5: Iron Buffer System

According to Ulrich (1983), in the iron buffer system, ironoxides are buffered, and contrary to what occurs in the Aluminum system, where there is no color change, the iron binds to the soil creating a dark podsoil. Only with extremely acidic soil (pH < 3.8) does the iron become freed as toxic ions with the lower pH limit of the Fe/Al buffer range

being 2.8 (Ulrich, 1989). From pH 3.8 to 3.0 the Aluminum and Iron buffer ranges overlap, but below pH 3.0 the iron buffer system is exclusively operational releasing trivalent Fe ions and water (Formula A3-4-5), (Bartels & Knabe, 1990).

Formula A3-4-5: Iron Buffer System

$$FeOOH + 3H^{+} \rightarrow Fe^{+3} + 2H_2O$$

Iron is an essential nutrient in small quantities. In *Quercus palustris*, inducing soil acidification can correct Fe and other nutrient deficiencies that can result in the complete elimination of chlorosis (Messenger, 1986). According to Ulrich (1981), forests are normally subject to seasonal acidification pushes alternating with deacidification phases which uncouple and recouple the ion cycles and these phases are controlled by climatic fluctuations but strong changes in climatic or anthropogenic actions can disrupt the cycling. It was determined that humus disintegration and podzolization occurring in a beech (*Fagus sylvatica*) forest in Solling was the direct result of prolonged acid precipitation and not just natural ecosystem variation (Ulrich, 1981). Chemical alteration of the humus layer to form podsoil is an indicator of acid damage.

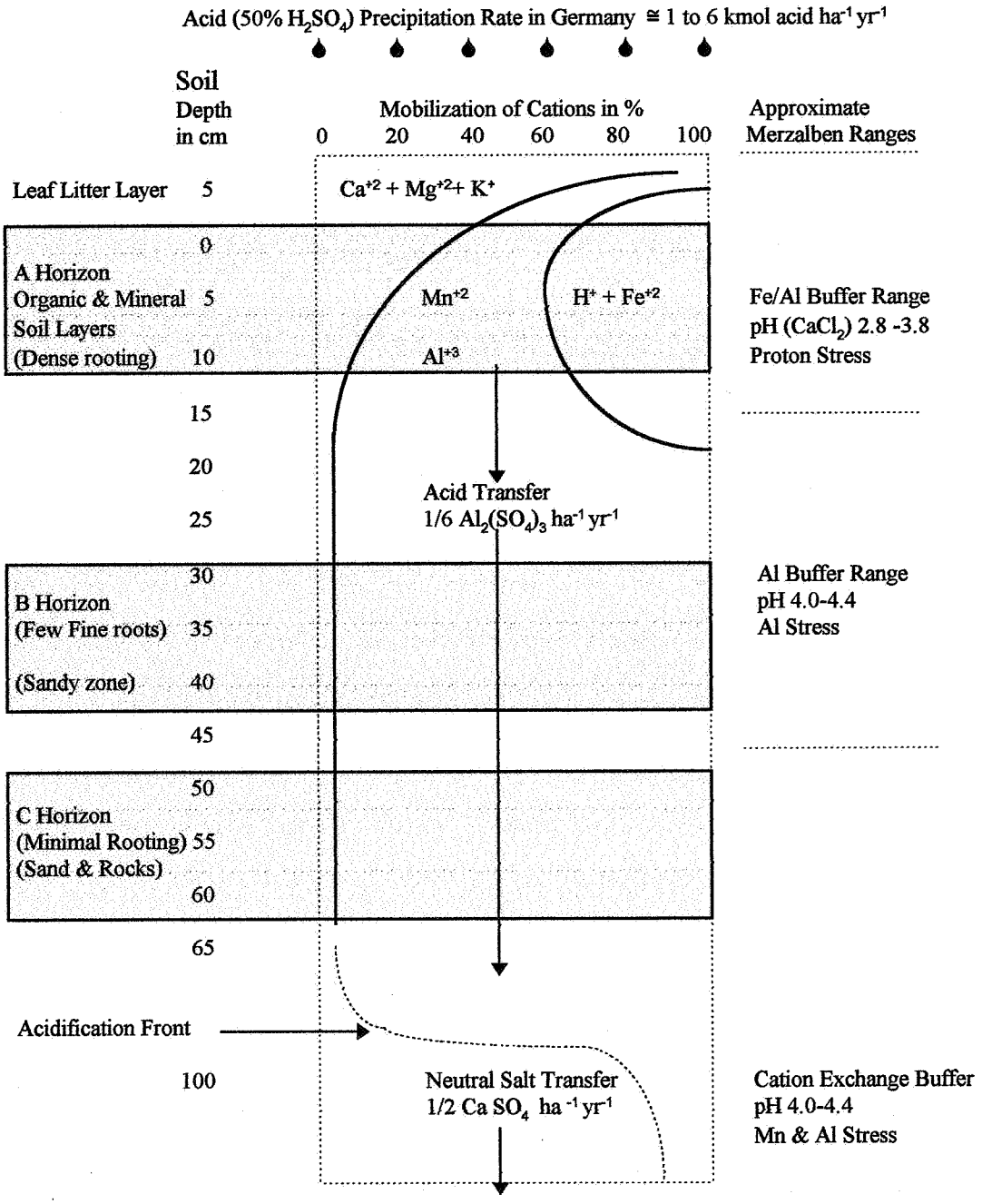
A3-4-6: Soil Buffer Systems Summary

Attempts have been made to mathematically calculate effective ion diffusion coefficients based upon invitro factors such as ion diffusion coefficient in water, impedence factor, nutrient concentration, nutrient quantity participating in the diffusion, and the reciprocal buffering capacity of the soil solution (Junge & Claasen, 1986) with the idea of creating buffer curves and soil depletion profiles. In a living forest ecosystem however application is limited. One can determine how mobilized an ion should be (Fig. 3-4-1) and its leaching rate by direct measurement of the ground water, but not whether it is blocked, sequestered or actively translocated into specific roots of specific plants. In addition, it is difficult to determine if a drop in ion concentration in the soil and ground water is due to mobilization or greater root uptake unless direct insitu comparative measurements are made.

Most damage is reversible as long as buffering capacity is maintained and there is not excessive swinging between the pH buffering ranges. By clinically evaluating the soil pH and changes in the number of free H^+ ions associated with $CaCl_2$ and KCl salts, the status of the soil buffering system can estimated (Ulrich, 1989). Since pH is measured variably in the literature, all three means of estimating pH (H_2O, $CaCl_2$ and KCl) will be used in this study. Another common method of determining rhizosphere stress by direct analysis of soil and root mineral composition. According to Rost-Siebert (1983, 1985), the damage caused by protons and Al ions depends upon the soil solution composition and more specifically upon the Ca concentration since increases in Ca concentrations have been found to reduce the adverse effects of proton (H+) and Al ions. As a result, Ca/Al (and Mg/Al) ratios have been used to characterize stress in spruce and beech forests (Murach & Ulrich, 1988). In spruce and beech trees, a Ca^{+2}/Al^{+3} ratio >1 implies negligible Al stress and <0.3 is associated with apical meristem damage, reduced axial growth and increased root necromass (Ulrich, 1989). The aluminum buffer range is characterized by low Ca/Al molar ratios but can more precisely be estimated by (Ca + Mg + K)/Al ratios (Sverdrup and Warfvinge, 1993). Fine oak root and mycorrhizal, Ca/Al and (Ca + Mg + K)/Al ratios will be compared in this study.

Both soil buffering capacity (pH) and imbalances in the elemental components of the soil have been used in the past to estimate ecosystem elasticity. In a highly elastic system, stability can be maintained for thousands of years unless man or dramatic climatic switches occur, but an ecosystem of low elasticity cannot return to its original vigor, and while spatial and temporal variations in buffering capacities affect a forest, it is generally recognized that nutrients (Ca and Mg along with Nitrates) are washed out as acids and sulfates accumulate in decomposing acidified soils and when not enough humus remains to bind the Al, a toxic concentration of Al will lead to root death (Ulrich, 1983).

Figure 3-4-1: Soil Acidification Front and Ion Mobilization (Ulrich, 1989). Modified from " Depth gradient of soil acidity under the influence of acid deposition" (Ulrich, 1989, p. 21) to indicate soil sampling zones (A, B, C Horizons) used in this study.



A3-5: Merzalben Soil Structure, pH and Mineral Content

A3-5-1: Merzalben Soil Structure

According to Block (1993) the sandstone base of Merzalben is very quartz rich (>80%) and feldspar poor (<13%) with a modest component of clay (<5%) with strong evidence of Aluminum and iron ores (Table A3-5-1a), and according to spectrofluorescent chemical analysis, the soil is very high in SiO_2, low in K_2O and variably low in CaO and MgO (Table A3-5-1b).

Table A3-5-1a: Mineral Composition of Merzalben Soil. Derived from Block (1993) from analysis of samples done by Butz-Braun (1990) using X-ray diffraction techniques.

Mineral	Soil Depth	
	5-10 cm	60-90 cm
Quartz	78-82 %	81-90 %
Alkali-Feldspar	12-13 %	11-13 %
Hornblende	0 %	0 %
Goethite	0 %	0 %
Illite	0-1 %	1-3 %
Smektite	0 %	trace
Cholorite	1 %	1-2 %
Kaolinite	0 %	0 %
Iron & Aluminum	2-3 %	2-3 %
Organics	5-7 %	0 %

Table A3-5-1b: Oxide Composition of Merzalben Soil. Derived from Block (1993) from analysis of samples by Butz-Braun (1990) using spectrofluorescence techniques.

Oxide	Soil Depth	
	5-10 cm	60-90 cm
SiO_2	87.3-90.3 %	89.5-93.3 %
CaO	0.2-0.4 %	0.1-0.3 %
K O	1.7-1.8 %	1.5-1.9 %
MgO	0.3 %	0.2-0.4 %
Na O	0.0-0.1 %	0.0-0.1 %
AlO	**5.2-7.2 %**	**3.4-6.0 %**
Fe O	1.1-1.6 %	0.6-1.6 %
TiO	0.5-0.6 %	0.3-0.4 %

A3-5-2: Merzalben Soil Profile

Under a very thin litter layer, the top soil in Merzalben consists of a very sparse podsoil-brown earth mix with a humus component consisting of deciduous debris from the oak-beech canopy. (From the previous discussion, the presence of podsoil is an indicator of acid stress.) Under the litter layer, the A horizon, darkest because of its high organic content and podsoil complement, varies from 0 to 5 (but up to 30) cm in depth. The B horizon is very sandy with relatively little organic matter and the C horizon (about 60 cm+) is rocky with much sand but little clay in the mix.

According to Press and Siever (1985) it is expected that the A (0-10+ cm) horizon should lack solubilized minerals due to the intense biological activity but the B (mineral) horizon (leaching zone) should have an abundant soluble mineral and iron oxide components. The mineral soil, in the Aluminum buffering range, can be found up to a depth of about 30 cm, overlying a cation exchange buffer zone (Block, 1993). A general soil profile from Block (1993) is given in Table A3-5-1c. In December 1988 an experimental parcel of Merzalben was limed with 6 tons of Dolomite per hectare (Block, 1993).

Table A3-5-2a: Merzalben Soil Profile. Adapted from Block (1993). The numerical ratings are 0 = none to 5 = abundant. The soil types are: Su3 = Brown soil / podsoil / sand, Su2 = Sandy / brown soil, S = Sandy / rocky soil

Horizon	Depth	Soil Type	Humus	Roots
L	2-3.5 cm	Litter	-	-
Of	0-2.0 cm	Litter	-	-
A(e)h	0-6.0 cm	Su3	4	5
Bvh	6-25 cm	Su3	2	4
Bv	25-55 cm	Su2	0	3
Bv-Cv	55-100 cm	Su2	0	2
Cv	80-120 cm	S	0	0

A3-5-3: Merzalben Root Profile

There are no oak roots in the thin litter layer but immediately below it fine roots are abundant especially in the top 10 cm of soil with abundance declining with depth. More overall biomass and fewer subvital roots were found at 0-10 cm depth in the unlimed soil, but root vitality was better in deeper soil in the lime probes sampled in 1995 (Table A3-5-3). We expect some significant variation in the Aluminum content of the fine and finest roots from various soil depths in the unlimed and limed plots.

Table A3-5-3: Merzalben Root Profile. Adapted and modified from Rommel (1998) Tables A-118 and A-119, from random probes containing both Beech and Oak roots. The finest roots (≤1 mm) and fine roots (1-2 mm) are given in kg/ha and only the median values are presented. The Vital (V) roots were determined to be white to light brown and nearly >80% mycorrhizal and the subvital (SV) roots were darker brown and only 10-80 % mycorrhizal.

	Unlimed Forest Soil					Limed Forest Soil			
Soil Depth cm	Finest Roots V	Finest Roots SV	Fine Roots V	Fine Roots SV		Finest Roots V	Finest Roots SV	Fine Roots V	Fine Roots SV
0-10	3044	818	433	205		2513	710	416	421
10-25	2457	1310	662	440		1866	1525	453	809
25-45	1323	1361	362	330		1205	970	446	318
45-65	454	580	230	150		706	862	368	356

A3-5-4: Merzalben Soil Acidity

In 1988, at 0-5 cm depth, the average acidity of the Merzablen region soil was 3.5 and at 10-30 cm depth it was 4.3, with an overall average of 3.9 for the region (BMELF, 1997). The actual pH levels of the soil samples collected will be presented in this study but for all intents and purposes it can be stated here that the pH of the Merzalben forest, 10 years

after liming was only very slightly improved, and that of the unlimed forest showed increased acidification, relative to the 1988 estimates. As will be seen, the soil pH varied with season, depth and relative precipitation but overall, both zones exhibited a temporal decline in pH to within and occasionally below the Al / Fe buffering limits. The Merzalben soil is not as acidic as other forest regions, such as Idar-Oberstein (Block 1993). But, despite relatively low annual input of 0.85 kmol acid ha^{-1} from 1988 to 1992 (Block, 1995), the soil in Merzalben was and still is highly acidified and at risk.

A3-5-5: Merzalben Soil Minerals

The dominant potentially toxic mineral in the Merzalben soil solution is Al, as opposed to Fe or other heavy metals (Block, 1999 pc). A chart of the elemental composition of Merzalben soils (Block, 1993) is given in Table A3-5-5a,b. Elemental analysis of the oak roots was not done. Elemental analysis of oak fine roots over several growing seasons and of selected mycorrhizae will be presented in this study. It will be found that roots and mycorrhizae from the unlimed and limed forest zones differed in their overall ionic composition despite a similarity in basic soil chemical characteristics implying that liming has altered the physiological and ion uptake status of the roots.

Table 3-5-5a: Chemical Characteristics of the Merzalben Soils: Known values adapted from Block (1993, p.19) of the litter layer mineral constituents.

Litter Layer

Ecological Values	pH (KCl)	pH ($CaCl_2$)	C/N	C/P	C / Ca+ Al + Fe
	3.9	4.1	25	407	0.22

Elemental Composition in mg/g									
C	N	P	K	Ca	Mg	Na	Fe	Mn	Al	Si
305	12	0.7	0.9	407	0.9	0.1	5	2.1	5.1	174

Table 3-5-5a: Chemical Characteristics of the Merzalben Soils: Known values adapted from Block (1993, p.19) of the mineral soil constituents. E = cation exchange capacity and BS = base saturation (Na+K+Ca+Mg / E).

Mineral Soil

		Elements			Exchangeable Cations								
Depth	pH	C	N	P	K	Ca	Mg	Na	Fe	Mn	Al	E	BS
cm	$CaCl_2$	%	%	mg/100g	---------- µmol IÄ/g ----------								%
0-5	3.5	5.3	0.3	38	1.7	2.8	1.6	0.5	<0.8	3.6	52.3	66	10
5-10	3.9	2.8	0.2	28	1.1	0.9	0.7	0.3	0.0	1.5	42.7	47	6
10-30	4.3	1.4	0.1	21	0.8	0.4	0.2	0.4	0.0	0.5	26.2	28	6
30-60	4.3	0.5	0.4	16	0.7	<0.4	0.1	<0.4	0.0	<0.4	20.0	21	6

A3-6: Nutrient and Aluminum Uptake

A3-6-1: Biotic pH Control of Nutrient and Aluminum Solubilization

Root tip and fungal tip cells can exude H+ ions as needed to solubilize soil nutrients and induce them to flow along electrochemical gradients ("Proton wires") to the membrane channels where they can be selectively taken into the cells (Haug, 1984). These highly localized and ephemeral chemical gradients created by the tips are essential to nutrient uptake (Raven, 1990). However, in increasingly acidified soils, Al can be released from the soil matrix, and once solubilized can travel the uptake currents to the cell membrane pores and channels where it must be either be blocked, incorporated or translocated. Iwabuchi, Yano & Shimizu (1989) proposed that there may be apical extracellular alkalinization and subapical acidification by rapidly growing algal roots to create circulating currents that will avoid aluminum toxicity at the apex but enhance uptake of nutrient ions subapically. Leisen et al (1990) demonstrated that pH values varied within a very narrow range from the apex (4.7-5.3) to the subapical (4.5-4.7) zones and then rising again with distance from the tip (4.5->5.7) in *Picea abies* demonstrating fine control over pH along the root surface and supporting the "acid growth" theory in the elongation zone (Brummel, 1986). Unfortunately Leisen et al used only non-mycorrhizal spruce roots. Besides fine pH control, complexing with exuded Phosphates may neutralize Al temporarily but possibly at the expense of phosphatase activity (Manning & Goldberg,

1996) which is needed to control the H + antiports. The use of acidic chelators may solve the problem of detoxification and will be discussed shortly.

The fact that plants can sense and alter external pH is extremely important. Proton release and activity of acid and alkaline phosphatases within 2 mm of the root surface are associated with P and K import along the length of the root hairs (Jungk & Claasen, 1986). The rhizosphere pH may be as much as 2 units higher or lower than the pH of the bulk soil with distinct differences in pH along the roots (Marschner et al, 1986). The active extrusion of H^+ ions (via ATP ase membrane antiports) is important for regulation of cytoplasmic (Reid, Smith & Whittington, 1989) and vacuolar pH (Kurkdjian & Guern, 1989) and for various solute transport processes (HCO_3^- use, $2H^+$:Cl^- symport, H^+:Na^+ antiport, and $K^+$and NH_4^+ uniport) (Walker, Smith & McCullough, 1989). Alternatively, mycorrhizal extrusion of OH^- ions may balance uptake, potentially improving Nitrogen nutrition but the observations are controversial (Bown & Smith, 1981). More work needs to be done in this field, but for this research it is sufficient to speculate that some mechanism is probably in place in oak roots and their ectomycorrhizal symbionts that can also control extracellular pH, solubilize nutrients and control their uptake.

A3-6-2: Organic Chelators in the Soil

The pH at which aluminum becomes available can be defined, but the amount of exchangeable metal within the soil is dependent upon the presence of chelators (organic humic acids, galacturonic acids, citrates, and organic phosphates in the soil), ion concentration, and the absorptive and catalytic characteristics of the aluminosilicates and oxides that form the soil matrix (Haug, 1984). According to Haug (1984), some low molecular weight chelators, with aluminum or other metals bound to them, can actually assist in convection and diffusion from the soil to the plant root. The beneficial or toxic effects would then be dependent upon the type of chelate and metal location. Addition of chelating materials (peat, humus) to acidic soil may help to alleviate aluminum toxicity by trapping it in the organic complexes and thus reducing its solubility and mobility gradient to the living roots (Haug, 1984, p. 45-75). It is known that a stable humus accumulation can occur with fungal buildup of complex polymers such as lignin at pH 4.0-4.5, in

conjunction with the silicate, ion exchange and aluminum buffer systems (Ulrich, 1983). However, increased acidification releases Al ions into soil solution almost as fast as the organic complexes become bound resulting in aluminum toxicity symptoms. The simplistic solution to reduce acid-induced aluminum mobility is then to increase the humic components in the soil by the addition of composed materials, rather than exclusively liming. The more complex solution is to consider direct inoculation with acid-resistant fungal chelators. Certain ectomycorrhizal fungi *(Hymenogaster* sp., *Scleroderma* sp. *Pisolithus tinctoris)* can withstand high concentrations of Al, Fe, Cu and Zn better than others (*Cenococcum geophilum* and *Thelephora terrestris*) and therefore may have potential for revegetation schemes in metal contaminated soils (Tam, 1995).

A3-6-3: Fungal and Root Chelators

According to Haug (1984),on the surface of fungal and fine root tip cells, a multitude of potential ligands are present which can form complexes with free soluble aluminum. Depending upon the spatial configuration of the ligand, non-chelating bonds can vary from weak electrostatic to ionic bonds which can be easily dissolved; holding the metals on the dry root surfaces and releasing them when hydrated again. In order to form a true chelate, the ligand must penetrate the secondary hydration ring at two or more points to find a final position within the primary shell in close proximity to the metal ion. This claw-like penetration, coordinated with a release of water molecules will cause stronger bonds to form with the metal cation. The stability of the bond depends upon the geometric configuration of the ligand which will determine if the bonds are weakly coulomic or strongly covalent with a specific metal ion. (Haug, 1984).

The ability of certain mycorrhizal species to "hold" aluminum in their hyphal walls may be related to unidentified chelators released or present in their ultrastructure. The inability of other species to "sequester" aluminum may be due then to a lack of specific chelators. It is not within the scope of this study to identify the chelators but it is important to note that some sort of species-specific chemical modifications are present and some mycorrhizal species can and do " hold " aluminum and prevent its translocation into the roots. It is possible that some of these secreted chelators are organic acids

(Ahonen-Jonnarth et al, 2000) such as oxalic, malate (Notwotny et al, 1998) and citric acids (Landeweert et al, 2001) or internal granular phosphates (Genet et al, 2000). Neumann & Clemens (2001) of the Institute of Plant Biochemistry, Halle (Saale), Germany believe that phytochelatins (PCs) which are small peptides synthesized from glutathione or related thiols by some fungi, plants can be element and species specific and can promote metal hypertolerance bases on evidence that PC - deficient species are metal hypersensitive. At the moment however, nearly all emphasis is being placed on yeast, yeast mutants and bacteria in an effort to elucidate the CAD1 cDNA gene responsible.

A3-6-4: Nutrient and Aluminum Uptake in Mycorrhizal Roots

Nutrient uptake is much more likely to be the function of growth of the root-mycorrhizal complex, than of the mass, surface area or ion-uptake of the root proper (Clarkson, 1985). In order to maintain a flux of P and other ions into the plant, the fine roots and/or the hyphae of the mycorrhizal fungi must continuously extend into undepleted volumes of soil, imposing a carbon cost to initiate, develop and maintain fine roots and the symbiotic mycorrhizae (Cannell & Dewar, 1994). Root exudates (organic carbon, amino acid phytosiderophores, mucilage) may mobilize mineral nutrients directly (chelation) or indirectly by providing the energy substrates for microbial activity in the rhizosphere (Marschner et al, 1986). Bacteria and fungi are 20-50 times more abundant in the rhizosphere than in bulk soil (Rovira, 1979), and it is in this rhizosphere that they act as a source, sink and pathway for the redistribution of P in soil-plant systems (McLaughlin et al, 1986). Most roots adapted to infertile soils have a high uptake capacity for mobile soil nutrients such as nitrate, but low uptake capacities for immobile phosphates when compared to species from fertile soils (Chapin, 1988). Mycorrhizae can compensate for this but are difficult to include in assays of uptake kinetics (Caldwell et al, 1991).

Recent studies have shown *Paxillus involutus* can assist transport of Mg to tree roots, improving host nutrition (Jentschke et al, 2000) and that ^{32}P can be translocated from wood-decomposing fungi to ectomycorrhizae (Lindahl, 1999). In an elegant study done by Sun et al (1999), the mycelium of *Suillus bovinus* slowly absorbed [U-^{14}C] glucose and other tracers from droplets placed on the hydrophobic cords, translocated them to the

peripheral hyphae and the growing tips at a rate of about 10 mm/24 hr and exuded them into fluid drops on the hyphal tips. The exudate compounds were mainly carbohydrates and peptides but acetic acid, oxalic acid and a number of unidentified compounds were also present. Released ions (K, Na, Cl, P, Mg and Ca) were identified by X-ray microanalysis and Calcium oxalate crystals were seen readily under the microscope. The mycelium was shown to reabsorb up to 65% of the ^{14}C compounds in 2 days, with the exception of " unwanted" secondary metabolites (oxalic acid and phenols). With the addition of the respiration inhibitor sodium azide close to the point of application, ^{14}C exudation decreased within 1 hour implying energy is required for active uptake of sugar but for a short period of time translocation can occur by diffusion and acropetal water transport within the cord vessels. Exudation of droplets on the hydrophobic cords may represent an important ecophysiological function, providing an interface for interaction with the hyphal environment and its microorganisms and where the peripheral hyphae can exchange their photosynthetically derived products for nutrients to be used later by the host (Sun et al, 1999).

The health status and diversity of the ectomycorrhizae is of great significance. It was initially assumed that mycorrhizae would be similar in their ability to transfer nutrients and limit Al movement to the fine roots but in the course of the research it was determined that there is incredible diversity in ability amongst the mycorrhizal species emphasizing the need for maintenance, not just of mycorrhizae, but for specific complements of mycorrhizae within the rhizosphere. This is in part accomplished naturally through seasonal diversity and competition (Genet et al, 2000).

Theoretically, aluminum present in an acidified solution with few chelators should remain mobile unless it meets an impregnable barrier. If certain mycorrhizal species lack Al-specific chelators and cannot otherwise halt Aluminum translocation, then the Al should be able to pass freely into the fine absorbent roots where it will then be first influenced by chelators present in the root walls. It will also be shown that there are several barriers to apoplastic translocation present in the root but despite these, some Aluminum can slowly penetrate to the xylem, probably via apoplastic, but possibly also symplastic pathways.

Once in the xylem, although considerable aluminum sequestration occurs in the actual walls, if the xylem fluid is acidified, then solubilization and long distance transport should be able to occur. The toxic ions can then be transported to distal regions where they can affect cellular metabolism. Dyes such as 8 hydroxyquinoline, chrome azurol S, criochrome R (Marczenko, 1981) (Haug, 1984, p. 77) and morin (Bidwell, 1974, Rost-Siebert, 1985) are specific chelators used to photometrically locate trace amounts of Aluminum. Morin will be used in this study.

A3-7: Acid Stress Symptoms

In Germany nearly 1/3 of the forest trees show confirmed damage attributed to acid rain and of these Oak trees rank fifth after Spruce, Pine, Fir and Beech in stress assesements (Federal Ministry for Research & Technology, 1984). Normal, unpolluted rain generally has a pH of about 5.6 due to carbonic acid created from CO_2. The addition of volcanic gases and chlorine and sulfates from ocean spray and anthropogenic sources can the pH of rain well below 5.6 while alkaline dust can raise it above 7 (Cunningham & Saigo, 1995). Fog, snow, mist, low lying clouds and dew can also trap and deposit contaminants, which in addition to dry fall out can account for over half of the acidic deposits on soils especially in high elevation forests bathed in acidic clouds (Cunningham & Saigo, 1995). Sulfate, nitrate and chloride particles can be converted into acids in surface water or when they come in contact with moist tissue surfaces drastically and cumulatively altering the pH (Cunningham & Saigo, 1995).

According to Ulrich (1989), acid stress can be caused by Al ions, protons, Mn ions, Fe ions and some heavy metals which initially probably compete with nutrient cation (Ca, Mg, K) binding sites in the apoplast, symplast, and in biological membranes, and in general acid stress can lead to a reduction in root growth, necrosis, altered nutrient uptake and changes in elemental content (Ca, Mg loss). According to Bartsch (1987) the regeneration ability of spruce roots after a drought period is delayed by a preceding period of acid stress. Root damage begins with the mycorrhizae and fine root tips (Blashke, 1981) and subsequently spreads via penetration of toxic materials and poor metabolic activity as a secondary stressor elsewhere in the root system (Ulrich, 1983). Gross rooting

patterns can be altered, for example, when growing on soils in the Al/Fe buffer range Norway spruce developed only superficial roots within the organic and mineral soils and not the deeper roots commonly characteristic of old spruce stands (Ulrich, 1989). Recognition of root damage can act as an early warning system. According to Ulrich (1989), non-mycorrhizal roots can be generally classified by changes in root growth, biomass and necromass.

- **Damage Class I** : Primary root tip becomes light brown and growth stops. Growth can resume or new lateral roots can form immediately behind the damaged tips when the roots are freed of acid stress. Damage is reversible.
- **Damage Class II:** The primary root tip dies back (meristem abortion), the cortex becomes brown and the lateral roots also show browning and die back.
- **Damage Class III:** The primary and lateral roots are necrotic.

The exact pH at which roots show stress may vary considerably between species and between individuals of the same species, but very generally, roots generally do not grow below pH 3 and most roots are negligibly affected by pH between 4 to 8 if sufficient Ca is available and if excess toxic ions such as Al and Mn are absent (Moore, 1974).

Pennanen et al (1998) used analysis of fungus - and bacterium - specific phospholipid fatty acids in the soils to determine that, when humus pH decreased from 3.83 to 3.65, total microbial biomass decreased as well, but some gram positive acidiophilic bacteria and fungi were not seriously affected. With acidification and phenolic accumulation, bacterial complement declines followed by a concurrent drop in nitrification (Ulrich, 1983). Under more severe field conditions a decrease in mycorrhizal frequency (Blasius et al, 1985), changes in mycorrhizal types (Meyer, 1984), and ultrastructure (Haug et al, 1986) have been used to classify root damage (Ulrich, 1989). But the use of bacteria and mycorrhizae as damage class indicators is limited since knowledge of the "normal" distribution and diversity of is extremely sparse. Changes in mycorrhizal diversity and distribution may act as "red flags" preceding root damage. Certain ectomycorrhizal

species that accumulate aluminum may act as stress indicators allowing more time for appropriate forest remediation.

A3-8: Aluminum Toxicity

A3-8-1: Aluminum Toxicity in Roots

The trivalent form of aluminum is considered to be root toxic (Clarkson, 1965, 1969; Moore, 1974; Ulrich, 1979; Murach, 1983; Rost-Siebert 1983; Blaschke 1985; Alten, 1987; Ulrich and Meyer, 1987, Vogelei and Rothe, 1988; Godbold et al 1988; Jentschke et al, 1991; Hentschel et al, 1993) It is generally assumed that as the pH drops below 5, Al becomes toxic and as the pH drops further, toxicity increases. Aluminum slows root growth, and in association with phosphate concentration changes, results in stubby root tips, lower root mass and eventually necrosis, but the Al can be moderated by gelatinous secretions and binding by mycorrhizae and sequestration in the above ground plant areas often without obvious detrimental effects (Schütt et al, 1992). This scenario is effective only for short-term, low impact acid stresses. What we are currently dealing with however is long-term, high impact acid stress with a prognosis for wide-spread forest loss.

The precise physiological action of aluminum in plant cells is not well understood. Bidwell (1974) stated that aluminum is toxic and will, under natural circumstances, inhibit the growth of plants because it tends to precipitate in plant membranes or around the roots where it can interfere with Fe and Ca uptake. In 1990, Johnson & Wood found that Al could interfere with DNA metabolism in Rhizobium. In roots, the primary site of Al damage seems to occur in the apical meristem (Howell, 1997). Al can accumulate and inhibit root cell division and elongation (Clarkson, 1965; Horst et al, 1982, 1983). Cell elongation is most likely disrupted by displacement of Ca and Mg which are essential for cell wall structure (Ulrich, 1989). In roots treated with Al, not only is Al content high, but Ca is displaced in the cortical walls (Godbold et al 1988, Stienen and Bauch, 1988) inducing fine and lateral (Ulrich, 1983) root damage and reducing vitality (Rost-Siebert, 1983, Lewis, 1989). Aluminum may also "push" calcium out of the middle lamella and in doing so destroy the endodermis (Hüttermann, 1983; Tischner et al, 1983) which as will

be seen here is the primary root barrier to aluminum movement into the stele. In *Picea abies* roots exposed to < pH 2.5 solution the swollen root tip rhizodermis is the first structure damaged and the deeper cells followed with deformities occuring in the middle lamella (Vogelie & Rothe, 1988).

A3-8-2: Aluminum Concentration in Tree Roots

The exact concentration at which aluminum becomes toxic to *Quercus petraea* roots has not been determined, but if other roots can be used as examples, then roots can be sensitive to very low Al concentrations depending upon the species.

In *Triticum aestivum* (wheat) for example, 2 ppm Al in solution is extremely toxic at pH 4.5, but less so at pH 4.0 (Moore, 1974). Since the Al^{+3} form was more prevalent in solution at pH 4 and declined in concentration as the pH rose, Moore concluded that the $AlOH^{+2}$ form present at pH 4.5 was primarily responsible for toxicity symptoms in wheat. It has been estimated that the upper limit of solubilized aluminum in the soil rarely exceeds 4 ppm (~ 140 μM) when the pH is very low and low levels of Ca and P are present (Haug, 1984), but this would be enough to induce toxicity symptoms in many grass roots.

In a study done by Mayer & Heinrichs (1981), fine beech roots from unlimed acidic soils contained 420 mg/kg while the finest roots had 680 mg/kg Al and those from calcareous soils contained 1920 and 7760 mg/kg Al, respectively. Mayer & Heinrichs concluded that the augmentations in the roots were due to reduce root uptake and translocation due to physiological or organic complexing followed by an accumulation at the root surface. They did not consider the mycorrhizae nor determine the damage classes of the trees but did state that both areas were subject to aerosolized acid and heavy metal contamination.

In contrast, the total average aluminum in fine roots from damaged beech growing in pH 3.9 soils exceeded 3800 ppm with a Ca/Al ratio of <1, while in the limed soils at pH 7.3, the roots of damaged beech had only an average of 1600 ppm Al with a Ca/Al ratio of 2.2, and the fine roots of healthy beech in the limed plots contained less than 400 ppm Al and

had a Ca/Al ratio of 9.2.(Flückiger et al, 1984). Flückiger et al (1984) claimed that this puts the aluminum toxicity hypothesis in doubt, but in reality it is more likely a temporal complication. If the aluminum levels in roots of stressed trees are high prior to liming and lower after liming, then one could assume that the aluminum has been mobilized. And so where has it gone? It has either been actively secreted back to the soil against mass flow, or it has gone to the leaves. Since Flückiger et al (1984) found the heavily damaged beech crowns had an overall higher in Al content than usual in their leaves, it might be logical to assume the Al has been translocated from damaged roots to the leaves. The aluminum content of the fine roots and mycorrhizae of unlimed and limed sessile oak trees will be examined.

A3-8-3: Aluminum in Aerial Plant Regions

It is generally accepted that the mineral content of tree stemwood depends upon the chemical composition of the xylem sap and to a limited extent, each tree ring can represent the characteristics of the soil during the year the ring was formed (Lévy et al, 1996). In the study done by Lévy et al (1996), not only were aluminum and nitrogen present in the heartwood of pedunculate oaks of the Armance Forest in France, but they also increased in the 30 year period from 1938 to 1968 corresponding to temporal acidification of the soil. Accumulation in heartwood may be part of a detoxification process but this indicates that Aluminum is definitely translocated.

In many plants the concentration of Al is considerably higher in the roots than it is in the leaves or in seeds (Bollard, 1983). On acidified soils, the Arbuscular Mycorrhizal plants had lower shoot concentrations of Mn, Fe, B, and Al than similar non-mycorrhizal plants (Clark et al, 1999) implying that the mycorrhizae are controlling uptake and/or translocation of potentially toxic minerals.

Aluminum can be translocated to the leaves where it can potentially interact with structural Ca and alter metabolic processes. Low concentrations of Al can be present in pedunculate and sessile oak leaves [0.1-0.15 mg/g (Shütt et al, 1992)] and may even be a required element. Heinsdorf (1996) noted that in poorly nourished trees the Al content of

the oak leaves fell below 0.042 mg/g (42 ppm), while in normal trees it varied between 0.042-0.071 and in luxurious growth it reached a maximum of 0.2 mg/g (200 ppm). Simon and Wild (1998) determined that Al content in penduculate oak leaves in Damage Class I and II were similar (77-79 μg/g DW) and there was a modest but significant difference in Class III (81 μg/g DW) but concluded that the overall concentrations were not high and were more likely the consequence rather than the cause of damage.

Flückiger et al (1984) determined that as the soil pH in the unlimed Rodersdorf forest dropped to < 4.0, Aluminum was translocated from the roots to the leaves of beech, and despite root damage, the leaves showed good vitality despite high aluminum (> >120 ppm) content. At the same time, Flückiger et al (1984) noted that in an area of strong crown and root damage in Chrischona beech forest, with a soil pH >4.0 (and higher $CaCO_3$), the aluminum content of the leaves increased as crown damage increased with the strongest damage occurring when the Al concentration of the leaves exceeded 150 ppm. This indicates that aluminum toxicity is not just a function of soil pH or leaf damage. In comparing the high aluminum content of beech roots (<400-3800 ppm) to that of the leaves (<120 - 150 ppm) it is evident that there is some limiting factor controlling Aluminum translocation. It is likely that interactions between elements such as Ca, Mg, K and P are involved. These interactions will be discussed in Sections F (bound minerals) and G (unbound minerals) of this report. It will be proposed here that the mycorrhizal complements may be determining factors in specific nutrient sequestration and blockage and thus tree survival in untreated and lime-treated, acidic forest soils.

A3-9: Protection from Aluminum Toxicity

A3-9-1: Secretions

In, certain plant species (*Vigna unguiculata* L.) adapted to acidic mineral soils, root exudates such as mucilage may specifically complex to Al^{+3} thus restrict its penetration into meristematic tissue (Marschner et al, 1986). Mycorrhizal species associated with tree roots produce oxalate and malate exudates (Notwotny et al, 1998; Ahonen-Jonnarth et al,

2000; Landeweert et al, 2001) which may also bind aluminum. But these secretions are probably very Ectomycorrhizal species / host species specific. Our understanding of these systems is drastically limited and information on the role of mycorrhizae in particular is practically non-existent.

A3-9-2: Phosphate granules

On the negative side, Aluminum can interfere with phosphate metabolism by causing large accumulations of inorganic P in the roots and by doing so reduce P transport to distal cells (Bidwll, 1974). Free, soluble Al ions show strong preferential bonding to cellular organic phosphate groups, limiting the physiological usefulness of P in cellular energy (ATP) and enzyme systems (Haug, 1984, Manning & Goldberg 1996).

On the positive side, Aluminum is known to elicit exocellular phosphatidylethanolamine (PE) production in *Pseudomonas fluorescens* (Appanna & St. Pierre, 1996). According to their study, the trivalent Al metal was trapped by PE pellets and secreted as an insoluble form in a gelatinous mass from the bacterium, a process which was promoted in the presence of citrate or D-glucose in the growth medium to the point where the cells were eventually totally free of cytoplasmic aluminum! The gelatinous mass when examined by SEM consisted of rounded bodies containing aluminum and long carbon rich fibers. Other ions neutralized by phosphate residues include yttrium (Appanna et al, 1994), lead (Al-Aoukaty et al 1991), indium (Anderson and Appanna, 1993) and gallium (Al-Aoukaty et al, 1992). Polyphosphate granules are present in some fungal cells (Kottke, 1991) and may be possible filter sites for toxins (Thurnau et al, 1993). Metal amelioration for the fungus *Pisolithus tinctoris* involved the secretion of extrahyphal slime in which Cu and Zn formed polyphosphate linkages (Tam, 1995). Phosphate granules were not detected in *Russula acrifolia* (Agerer et al, 1994) but they were detected in *Lactarius subdulcis* and *Lactarius blennius* var viridis growing on *Fagus sylvatica* (Genet et al, 2000). In *Lactarius*, in autumn and early winter, cytoplasmic glycogen and the number of polyphosphate and nitrogeneous granules was high while in late winter intense acid phosphatase activity on the plasmalemma activated the reserves. In summer, acid phosphatase activity declines, and reserves of P and N granules and glycogen are again at

their low point (Genet et al, 2000). Since Al and other toxic ions can be tied up in the polyphosphate granules, the question raised then is: Are the toxic ions released when acid phosphatase activity is high in the winter ?

A3: Summary - A Hypothesis Statement

The selective presence of mycorrhizae over the absorptive root surfaces may be essential to moderate and control ion influx and egress. Fungal chelating sites and proton or mucilage secretions may attract and / or hold ions, as a means of reducing or controlling ion entry and thus protect the cells from potential toxicity in acidified soils. However, the external aluminum chelating sites may also act as sinks and tie up potentially available Phosphate ions, impeding normal ion influx process and perhaps even driving useful ions away as acidification progresses. It is not hard to imagine that Al ions normally held in the soil matrix may become overwhelmingly toxic when either suddenly or chronically solubilized and mobilized directly to the fine mycorrhizal root absorptive surfaces. Selective mycorrhizal presence may protect the roots for a short period of time, but even they may become overwhelmed.

Eventually the Aluminum may be able to penetrate the fine absorbent roots, becoming incorporated into phosphate granules, affecting enzymes, DNA, destroying meristem tissue, or symplastically travel to the xylem from which they can be translocated to sensitive growing areas inducing distal necrosis or at the very minimum interfering with normal cellular processes and toxicity symptoms. Liming may moderate this process.

As acidification continues, excess hydrated ions may be leached, impoverishing the soil, and poisoning the ground water and a spiral cycle of increasing acidification may occur in the roots. In drought, extracellular ions may form strong bonds with phosphates and other essential organic complexes reducing their availability to roots, thus aggravating drought impact. Should the environment become more alkaline, or increasingly dehydrated, aluminum ions, which tend to then be less capable of mobilization will reform oxygen bonds either within the inorganic soil matrix (Haug, 1984), or if in close proximity, within

the plant or fungal cell cytosol, membrane or wall components furthering the potential for damage. Alternation of dry and wet periods may aggravate the problems especially if the wet periods are intensely acidic and the dry periods are prolonged. Since precipitation and drought tend to be unevenly distributed within a forest ecosystem it could be expected that selected trees may be more aggreviously affected than others resulting in seemingly random "sudden oak deaths".

According to the laws of conservation of energy, input must equal export in an open terrestrial ecosystem to maintain stability, a constant cycling and resilience within the ecosystem. Such stability cannot be maintained if climatic input of protons suddenly or continuously exceeds normal tolerance limits. At that point entropy is sure to proceed, soil destabilization, humus decomposition and loss of acid-sensitive species from the microbial, fungal to giant phototrophs, leaving only the most resistant species in the lithosphere. It only makes sense that the species most intricately involved with the acidified soil should be the first affected and first altered. For that reason, soil microfauna and flora complements should be examined as biotic indicators of a sick environment.

Only one small part of this picture will be examined in this thesis. Ectomycorrhizal species will be examined with respect to their abundance and distribution in association with oak roots at various soil depths and at different seasons to shed some light on the types of mycorrhizae present in oak forest soils. A limed forest region will be compared to an unlimed area to determine the effects of changing pH and liming have on mycorrhizal diversity. Representative samples of each mycorrhizal species at the various depths and seasons and soil treatments will be examined to determine if aluminum is present, where it is present and how far into the root it has penetrated. Mycorrhizal species will be classified into groups based upon their ability to sequester Aluminum.

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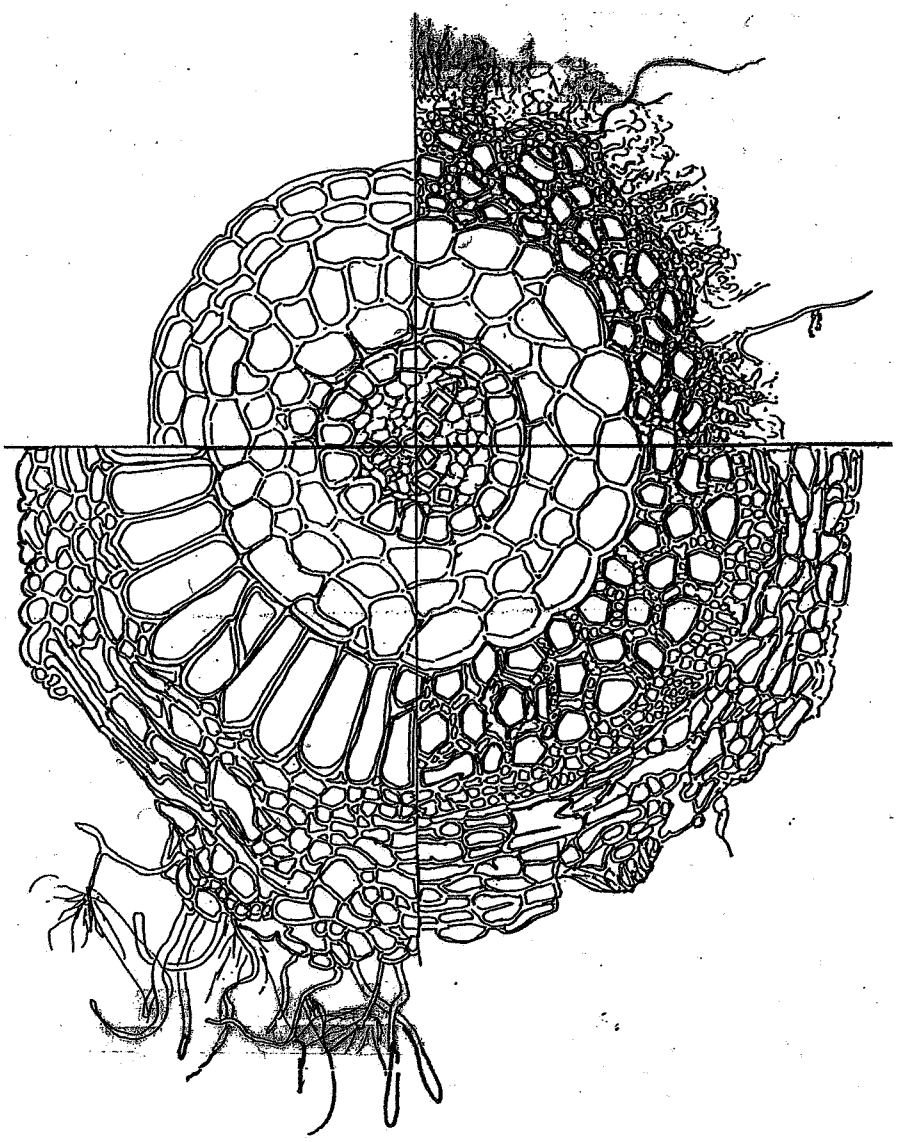
SECTION B

ABUNDANCE AND DISTRIBUTION OF OAK ECTOMYCORRHIZAE



Section B: Abundance and Distribution of Oak Ectomycorrhizae

INDEX

B: Abundance and Distribution of Ectomycorrhizae Associated with Oak from Unlimed and Limed Soils.

B1-1: Abstract

Liming forest soil has a dramatic and long term effect upon the abundance and biodiversity of the symbiotic mycorrhiza and their co-existent fine sessile oak roots. Liming seemed to induce K selection typified by high diversity and low numbers of mycorrhizae, typical for unstable, stressed or changed environments. Whereas, the unlimed forest mycorrhiza had a selective distribution more typical of R selection with low diversity but greater biomass, common in stable unchanging or slowly changing environments. The distribution of dominant ectomycorrhizal species, in 1999 and 2000, was affected by the dynamics of seasonal moisture, pH and the residual effects of the 1989 liming.

B1-2: General Goals

1. To determine the mycorrhizal species associated with *Quercus petraea*
2. To characterize mycorrhizal abundance and diversity in various soil depths.
3. To determine seasonal abundance and diversity and succession trends.
4. To determine how diversity changes in periods of desiccation and hydration.
4. To compare ectomycorrhizal complements in limed and unlimed oak forest plots.

B1-3: Introduction

This section deals with the abundance and diversity of symbiotic mycorrhizae associated with the fine roots of *Quercus petraea* (Matt) LIEBL., at different depths, in unlimed and limed soils of an oak forest. The effects of wet verses dry spring and fall weather will be taken into account when discussing the successional characteristics of specific mycorrhizal species. Section "E" of this report will deal with the precise location and qualitative concentration of Al within mycorrhizal roots relative to all the above factors with specific emphasis upon the effects of liming.

The oak forest region under consideration is located approximately 7.5 kilometers SSE of Trippstadt, in the Rheinland-Pfalz at Merzalben Forest District 04 / 0705, Rheinland - Pfalz (See Sections A1-1-3; A1-2-2). At approximately 523 to 591 meters in elevation, the specific area of study is a mixed forest of primarily *Quercus petraea* (See Section A1) interspersed with common European beech (*Fagus sylvatica* L.), and a minor population of Scott's pine (*Pinus sylvestris* L.). In 1989, a small area of approximately 60 square meters outside the fenced research facility was limed with 6 Tons per hectar of dolomite lime, in an effort to counteract the damaging effects of acid rain (Block, 1999, p.c.) (See Sections A1-4-2; A2; A3-5) and aluminum toxicity (See Section A3).

Heterotrophic mycorrhizae are essential for health and survival of their host species. "The fungus obtains carbohydrates from the tree, and the tree's uptake of essential nutrients is improved by the fungus" (Lewington & Streeter, 1993). Ectomycorrhizae, covering the fine roots of oak, act as an extension of the plant root system, increasing absorption surface area at the tips, extending into the surrounding soil beyond the root's reach and transporting water and liberating critical nutrients, such as P and N, to the root (Kohm & Franklin, 1997). Acid rain, pH 5.5 or lower (Schreiber, 1995), may alter the chemical equilibrium of the soil, resulting in a nutritional imbalance leading to extensive mortality and reduce growth in sensitive forest species (Peterson, 1995).

Some ectomycorrhizae have broad host ranges, while others are restricted to certain tree genera (Molina et al, 1992). *Q. petraea* and its associated mycorrhizae have not been investigated as intensely as *Quercus robur*, (Palfner, 1994; Palfner & Agerer, 1996), *Quercus pubescens* (Zambonelli et al, 1993), *Queurcus ilex* and *Quercus variablilis* (Agerer, 1987-1995) nor, apparently, have the mycorrhizae of the sessile oak been examined for potentially toxic levels of aluminum ions. Very little is known about the ectomycorrhizae of Merzalben (A1-3-6), or of oak forests in general (Section A1-3 and Appendix 1), even though alteration of the normal mycorrhizal complement may act as one of the earliest indicators of pollution stress.

B2: Materials & Methods for Ectomycorrhizal Discrimination

B2-1: Tree Selection

A total of 24 *Quercus petraea* (oaks) were selected, 12 from the limed forest area and 12 from an adjacent region of unlimed forest. Trees of approximately the same age, and state of health, whose root systems were fairly isolated from neighboring beech trees by a minimum distance of 5 feet were selected. The sessile oak trees chosen were between 171 and 198 years of age at the time of sampling (cf: BMELF, 1997). The exact age of each tree was not determined by trunk bore sampling in order to eliminate possible insect infestation damage and reduction in commercial value. Initially the trees were marked with numbered flags in the soil at the base of the trees, but due to the disruptive actions of wild animals, the trees were subsequently marked with large letters on the bark using white spray paint as recommended by the forestry department.

In a survey done in 2002, 22 of the trees selected for this study (11 in each plot) were determined to be in Damage Class 2, but one tree in the unlimed (#11) and one in the limed plot (# 7) had died and both had been removed (Schröck, 2003, p.c.; A1-4, Appendix 3). The health status of the trees in both regions was determined by summer crown light penetration and winter observations of the crown structures. According to a 2003 unpublished report, 88% of the trees in the limed zone had < 25% light penetration, while in contrast only 20% of the trees in the unlimed plot had the ideal (<25%) levels of light penetration (Schröck, 2003, p.c.; A1-4, Appendix 3). The initial conclusion was drawn that the trees in the limed forest were in better overall health in comparison to those in the unlimed forest.

B2-2: Soil Sampling

Sampling was performed following methods developed in the working group of Professor Rothe, Johannes Gutenberg - Universität Mainz. Leaf litter was cleared at a distance of one meter from the base of each tree. In a region where fine roots were evident, usually midway between two large supporting roots, soil samples were extracted. A 10 cm diameter root auger was used to obtain cylinders of soil from 0-10 cm, 30-40 cm and 50-

60 cm depths. Using the standard three dimensional formula for a cylinder [$(\pi d^2 h)/4 = \pi r^2 h$, where d ≅ 9.8 cm inner diameter, r ≅ 4.9 cm inner radius and h ≅ 10 cm height], each cylindrical sample had an average volume of approximately 723.822 cm ≅ 26.9 cm^2. The 0-10 cm sample will be referred to as Horizon A, the 30-40 cm depth sample as Horizon B and the 50-60 cm depth sample will be Horizon C. These depths roughly correspond to the traditional soil horizons of the same names (See Section A3-4 Figure A3-4-1 and Section A3-5 Figure A3-5-2a). The cylindrical soil probes were placed into labelled clear plastic bags and set on ice for transport. The extraction holes were refilled with earth and the leaf litter was replaced.

In 1999 and 2000, soil samples, from 6 trees in each of zones, were taken in the spring and again, from the other 6 trees in each zone, in the fall. Each sampling involved duplicate probe sets. One set was used to: extract and identify the mycorrhizae (Section B), test the soil for moisture and pH (Section C), for fluorescent analysis (Section E) and for bound and unbound chemical analysis (Section F). The other set was used for backup, and general estimations of total root biomass (Section C) and laser analyis (Section D). Specific descriptions of the methodology for each procedure is located at the beginning of the respective sections.

B2-3: Isolating the Roots

Soil samples were refrigerated (4° C) in the dark until cleaned. The probes were freed of adhering soil particles, using techniques modified from those outlined by Schauf (1996), working group Professor Rothe, Johannes Gutenberg - Universität Mainz. The roots were isolated from the soil by gently sieving the loose soil away through a coarse grid. The coarsly sieved soil was resieved through a fine grid to recover lose root fragments. The final soil sample was visually checked to extract stray root pieces. A 100 gram sample of the finely sieved soil from each probe was packaged in plastic ziploc bags, sealed and stored in large sealed plastic containers at 10° C for future pH and moisture analysis.

B2-4: Cleaning the Roots

The extracted roots were placed in a large beaker of ice water for 30 + minutes. The hydrated samples were placed on a fine mesh grid over a basin and the adhering soil particles were gently rinsed away from the fine roots by a stream of cool tap water. The roots and fragment (still with some adhering soil) were stored in a clean beaker of iced distilled water and refrigerated for up to 5 days. Up to this point the duplicate probe sets were handled in the same manner. The probe set that appeared to be the least damaged by the cleaning procedure was used for mycorrhizal analysis.

B2-5: Isolation of Mycorrhizae

Using a dissecting microscope, the mycorrhizal roots were further cleaned of adhering stones and organic debris using fine tip forceps. The mycorrhizal tips were carefully snipped from the fine roots and separated by color (black, brown, yellow, white, other) into individual glass containers of iced water. Some isolates were quickly identified by genus (*Cortinarius, Cenococcum, Xerocomus, Russula*) or even *species (Byssocorticium atrovirens, Paxillus involutus, Laccaria amethystina, Russula ochroleuca*) by their distinctive growth forms, colors and emanating hyphae. When known immediately, similar species were stored together in separate glass containers.

The non-mycorrhizal roots were set aside in iced water to be later segregated by root diameter (< 1mm, 1-5 mm, > 5mm) for subsequent biomass analysis (Section C). The very finest roots (< 1mm) were used for mineral analysis (Section F). Many healthy (turgid) long brown root tips at first glance appear to be non-mycorrhizal when in fact they are covered with a very thin mantle of *Piceirhiza.* As a result, the "non-mycorrhizal" roots isolated by student helpers were re-examined and slightly swollen, long, brown root tips were rescued to be included in the study. Microscopic analysis of tip cross-sections confirmed the presence of *Piceirhiza nigra* (dark brown) or *Piceirhiza chordata* (medium brown) in these samples.

Identifying features tend to change with senescence. Some of these age-induced morphological abberations are known, while most are not. Inevitably the mycorrhizae

isolated from the soil probes were not all precisely the same age. The spring samples contained intermixed tips of fresh new mycorrhizae and forms that probably were overwintering forms. The fall samples also containd fresh new tips and tips that were probably late summer forms. Occasionally "old" mycorrhiza were intermixed with fresh young mycorrhizae on the same fine roots. If the "old" mycorrhizae retained the same basic anatomical details with the exception of browning in damage spots or some die back at the tips of the mycorrhizal trees, they were included in the study. Only fresh young tips were used for the fluorescent studies. Dead tips (whithered, necrotic) were separated out and included with the non-mycorrhizal root (<1 mm) isolates.

B2-6: Mycorrhizal Identification

The mycorrhizae were kept cool and moist throughout the identification process. Using a standard dissecting microscope with a strong point light source for good color and feature discrimination, the isolated mycorrhizae were further segregated. Morphologically similar groupings were identified to genus and species (where possible) using the "Color Atlas of Ectomycorrhizae" (Agerer 1987- 1999). There were 5 isolates which could not be identified even to genus. These tips were not included primarily because they were extremely small and found only once. Ten unidentified species that occurred more than once and could be separated by morphological traits, were included and given pseudonoms. Of these, 2 species ("Unknown Gray" and "Unknown Rosa") that could not be identified but were very frequently found were included in the fluorescence study.

General macroscopic morphological and colormetric features were used as a primary identification tool. Factors such as ramification (branching patterns), color and tones, mantle surface configuration, rhizomorph morphology, emanating hyphae and cystidia, density, soil adhesion, known autofluorescence and overall growth forms were considered as described by Agerer (1985-1999). When appropriate, 15% KOH was used on small isolated samples to determine if pigments would dissolve or if tissues would change colors. This chemical test was employed especially to differentiate between *Cortinarius cinnabarinus* and *Cortinarius armillatus* and occasionally as a confirmation test for *Russula* or *Piceirhiza* species.

B2-7: Mycorrhizal Quantification

When all the tips from one probe had been cleaned, sorted, resorted and identified to satisfactorily isolate the mycorrhizal species, then each species grouping was quantified by counting the tips, including the tips in each branching "treelet". The problem with this data collection was found to be a lack of consistency in the literature for comparative purposes. In some cases an entire "treelet" was considered to be "one" tip and in other cases, the each branch of a treelet was considered to be a tip. As a result, the data was filed but not used in this report. Instead the total gram weight of each grouping was used as the quantitative measure of abundance. Each grouping was gently blotted to remove excess water and weighed to one/thousandth of a gram.

B2-8: Mycorrhizal Macroscopic Documentation

Representative samples of each species were photographed using a high resolution (6.3x to 50x) Leica MZ8 Photo-stereomicroscope using 100 ASA Sensia II Fujifilm for color slides. For the photographic process it was necessary to keep the mycorrhizae completely submerged, with the exception of *Thelephora terrestris* and *Fagirhiza cystidiophora* whose cystidia were more evident when the tips were in the air. The selected samples were placed in a small petri dish, covered with cold tap water and set on top of a piece of black velvet for background contrast. Magnification and duration of exposure were recorded for each. Where appropriate for species verification, photographs are included in the Mycorrhizal Micrographs in the last Appendix.

The isolated mycorrhizae were gently placed in labelled Safe-lock 1.5 ml Eppenndorf caplets containing distilled water, left uncapped for metabolic gas exchange and refrigerated (4° C) until fluorescent analysis. If fluorescence analysis could not be done within 7-14 days the samples in the caplets were sealed and flash frozen in liquid nitrogen and stored at -32° C. Through pretesting procedures (See Section E for Fluorescence testing) it was determined that freezing did not alter fluorescence results.

B2-9: Mycorrhizal Identification Confirmation

Confirmation of the species was made during fluorescent microscopic analysis (Section E), by the examination of the cryossectioned roots. The outer (or inner) mantle patterns, hyphal septation, cystidia distribution and morphology, and rhizomorph ultrastructure were compared to Agerer's descriptions and documented photographically as needed. Some species proved to be difficult to confirm unless both macroscopic and microscopic features were examined. In particular the black mycorrhizae (*Fagirhiza setifera, Fagirhiza spinulosa* and *Cenococcum geophilum*) were problematic, especially if intermixed on a root. Despite the care taken, and consultations made, it is inevitable that some species may have been misidentified. In particular, what is referred to as *Xerocomus chrysenteron* in this report, through genetic analysis of a morphologically identical mycorrhizae with similar fruiting bodies, may actually be what is currently called *Xerocomus pruinatus* in the gene banks (Haese, 2003, p.c), The only assurance that I can give the reader is that within an isolated grouping, all of the mycorrhizae were morphologically identical and identified as accurately as possible given the currently available literature (Appendix 1), minus genetic analysis. Except where the samples were exhausted by analytical procedures, remaining mycorrhizal isolates were retained, frozen.

B2-10: Confirmation via Fruiting Body Collections

Although not all mycorrhizae form fruiting bodies and the numbers present may not be representative of the subsurface cultures, the presence or absence of fruiting bodies can be considered an indicator of presence of the associated mycorrhiza. In fall 2000 and 2001, the forest plots were searched for fruiting bodies. The relative abundance of each species was estimated and ranked on a scale of 0 to 4. Only 1 to 2 representative samples were collected for photographic documentation. These samples were stored on ice until photgraphed and then desiccated at room temperature for 24 hours and then oven dried. The dry specimens were stored in glass jars or ziploc bags at 10°C. The fruiting body abundance was compared to the mycorrhizal abundance. Only 10 of the possible 35 mycorrhizal species that form fruiting bodies were found. An attempt was made to trace 1 fruiting body back to its source mycorrhiza but abandoned due to the complex nature of

the forest floor. Since the fruiting bodies and their potential source structures were displaced by several feet or even meters distance, the activity was considered to be too disruptive. No definitive relationship could be discerned between the presence or absence of the fruiting bodies relative to specific mycorrhizal abundance. Fruiting body presence could only be used as a loose confirmation of possible mycorrhizal species in the soils. Details of the collection, including conclusions, are presented in Appendix 2.

B3: Results

B3-1: Mycorrhizal Diversity Overview

Over 63 known mycorrhizal species (≅ 27 Genuses) were isolated from the fine roots of *Quercus petraea.* The most common 50 species, discussed in Section B, are presented in the literature review which discusses their nomenclature and known ecology (Appendix 1 (80 species)) and again in the fluorescence results (Section E and Appendix 7). Selected samples are presented in Poster 1 and Poster 2 in this section. Raw data sets of mycorrhizal abundance at various soil depths and seasons are presented in Appendix 8A and 8B. Appendix 8A contains Graphs B3-1 to B3-68, one for each species, comparing their individual seasonal growth patterns in the unlimed and the limed soils. Appendix 8B contains the raw data for all 93 isolates found. Isolates were eliminated from this study if there was inadequate sample size, dubious characteristics, or no comparative value. For each of the most common 50 species examined, including unknowns and tentative identifications, notes, photographs, and sketches of the structures used to confirm the species, were produced. These, along with representative fluorescence photographs, are presented, in summary, in the last Appendix: "Oak Mycorrizal Species - Individual Micrographs". Selected isolated samples of the most abundant and versatile mycorrhizae (*Cenococcum geophilum, Quercirhiza fibulocystidiata, Piceirhiza chordata and Lactarius subdulcis)* were used for the seasonal bound and unbound mineral analysis presented in Section F. This report (Section B) focuses on the comparative analysis of mycorrhizal species from unlimed and limed soils with respect to: diversity, abundance, soil depth and season. The goal of this section is to answer the question "How does liming affect mycorrhizal diversity and abundance ?"

Ectomycorrhizae of Fagus sylvatica and Quercus petraea

Photographs from Color Atlas of Ectomycorrhizae, R. Agerer, Ed.

Poster 1

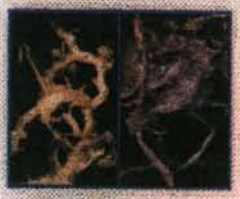

Boletus edulis

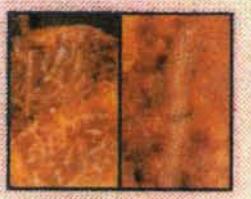

Byssocorticium atrovirens


Cenococcum geophilum


Cortinarius armillatus


Cortinarius bolaris


Cortinarius cinnabarinus


Elaphomyces muricatus


Fagirhiza arachnoidea


Fagirhiza cystidiophora


Fagirhiza fusca


Fagirhiza granulosa


Fagirhiza globulifera


Fagirhiza setifera


Fagirhiza spinulosa


Fagirhiza tubulosa


Fagirhiza vermiculiformis


Geastrum fimbriatum

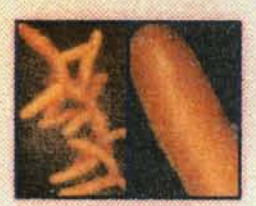

Genea hispidula


Laccaria amethystina

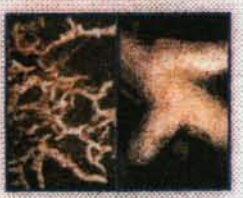

Lactarius acris


Lactarius pallidus


Lactarius rubrocinctus


Lactarius subdulcis


Lactarius vellereus


Paxillus involutus


Piloderma croceum


Russula illota


Russula maireí


Russula ochroleuca


Tomentella ferruginea


Tricholoma acerbum


Tricholoma sciodes


Xerocomus chrysenteron


Xerocomus badius


Xerocomus chrysenteron

Ectomycorrhiza of Quercus petraea

Photographs from Color Atlas of Ectomycorrhizae, R. Agerer, Ed.

Poster 2 - Infrequent species

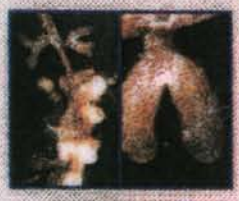
Amphinema byssoides

Boletus cavipes

Cortinarius varicolor

Dermocybe cinnamomea

Genea verrucosa



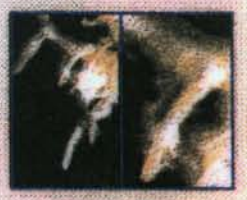
Gomphidius roseus

Hydnum rufescens

Inocybe appendiculata

Inocybe obscurobadia

Lactarius chrysorrheus



Lyophyllum decastes

Phellodon niger

Piceirhiza bicolorata

Piceirhiza chordata

Piceirhiza gelatinosa

Piceirhiza guttata

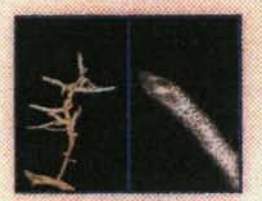
Piceirhiza nigra

Pinirhiza cyaneoviridis

Pinirhiza rufomaculata

Quercirhiza fibulocystidiata

Quercirhiza squamosa



Quercirhiza subbutea

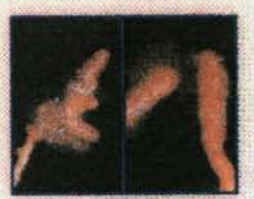
Rumaria subbotrytis

Russula azrifolia

Russula firmula

Russula fuegiana

Tetraberliniaerhiza sp.



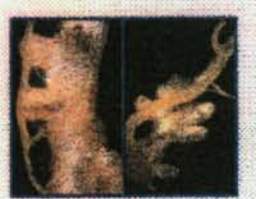
Tuber aestivum

Tuber melanosporum

Tuber mesentericum

Tuber puberulum

Tuber rufum



Xerocomus subtomentosus

Quercus petraea fruit & leaves

Quercus petraea bark & flowers

B3-2: Oak/Beech-Ectomycorrhizal Macroview : Poster 1

Thirty four species that may be found on oak, which are also known to colonize beech roots (*Fagus sylvatica),* are presented in Section B, Poster 1. This includes 5 species (*Cortinarius cinnabarinus, Fagirhiza vermiculiformis, Geastrum fimbratum, Tricholoma acerbum,* and *Tricholoma sciodes*) which were not found in our samplings but were cited in Agerer (1985-1999) as being associated with beech in mixed deciduous forests. All the other species were found on the Merzalben oak roots. After production of the poster, two additional oak/beech-ectomycorrhizal species (*Entoloma sinuatum, Quercirhiza squamosa)* were found both on the Merzalben oak roots and in the literature (Appendix 1). *Quercirhiza squamosa* can be found in Poster 2. Commonality of some ectomycorrhizal species between oak and beech is to be expected due to their close and enduring proximity in forest sylviculture (See Figure A1-1-8).

B3-3: Oak-Ectomycorrhizal Macroview : Poster 2

Poster 2 presents 33 oak mycorrhizae that are not (yet) known to be specifically associated with beech and 16 species not previously known to be associated with oak. Six species included in Poster 2 (*Boletus cavipes, Cortinarius varicolor, Hydnum rufescens, Lyophyllum decastes, Pinirhiza cyaneoviridis* and *Ramaria subbotytris*) were cited in the literature as being oak mycorrhizae (Agerer, 1985-1999), but were not found at Merzalben. The remaining 27 species were present.

According to the literature search (Appendix 1), 11 species in Poster 2 (*Genea verrucosa, Inocybe appendiculata*, Inocybe obscurobadia*, Lactarius chrysorrheus, Quercirhiza fibulocystidiata, Quercirhiza squamosa*, Quercirhiza sublutea, Tuber melanosporum, Tuber mesentericum*, Tuber rufum,* and *Xerocomus subtomentosus*), have been known to be associated with oaks in the literature , but not specifically *Quercus petraea.* Fifteen species are known *Picea abies* - mycorrhizae (*Amphinema byssoides, Dermocybe cinnamomea, Inocybe appendiculata*, Inocybe obscurobadia*, Phellodon niger, Piceirhiza bicolorata, Piceirhirza chordata, Piceirhiza gelatinosa, Picerirhiza guttata, Piceirhiza nigra, Quercirhiza squamosa*, Russula acrifolia, Russula firmula, Tuber mesentericum*, Tuber puberulum).* *Some (4) of these species are known

to be cross-over species between spruce and oak. No spruce trees were present within the selected study sites. However, a small group of (10+) young trees bordered the limed plot in a fenced area and another island group of older spruce trees were 20-30 meters distance from the unlimed area. It is possible that these trees were the source of mycorrhizal species normally associated with *Picea abies,* or conversely, the oaks were a mycorhizal source for the spruce. Regardless, all of the oak-mycorrhizal species listed were successful colonizers with the exception of *Dermocybe* and the *Tuber* species which had very low numbers, as will be shown shortly. The remaining 5 oak-mycorrhizal species [*Gomphidius roseus-Pinus sylvestris, Pinirhiza rufomaculata-Pinus sylvestris, Tuber aestivum-Pinus sylvestris, Russula fuegiana-Nothofagus pumilio* (S. America), *Tetraberliniaerhiza - Tetraberlinia* (Cameroon)] were found to be associated with remote tree species in the literature (Appendix 1), and so may have been incidental introductions (spores carried on hiking shoes...). Of these 5, *Pinirhiza* was the most successful species.

Besides the species depicted in Posters 1 & 2, six other oak-ectomycorrhizal species *(Entoloma sinuatum, Lactarius pornisis, Pisolithus tinctoris, Pseudotomentella tristis* and 2 unknowns) were found and are mentioned in this study, but are not depicted. Subsequent to production of the Posters, 14 other species were found in the literature to be associated with oak as ectomycorrhizae, possible mycorrhizae, or present as fruiting bodies primarily under oak trees (Appendix 1). None of these 14 species were found in Merzalben. Although, fruiting bodies of one species (*Lactarius quietus)* were found (Appendix 2), the ectomycorrhizal forms were not found in the Merzalben probes.

These posters were created for the exclusive purpose of facilitating mycorrhizal identification in the lab. The images were borrowed from the internet, Agerer's Color Atlas, and from photographs produced during the course of this study. The Adope Photoshop presentation was accomplished with the instruction and aid of Jürgen Harf, to whom I owe a debt of gratitude. The posters are presented here to give the reader a quick overview of oak ectomycorrhizae.

B3-4: Mycorrhizal Diversity & Abundance in Unlimed and Limed Soils

In review of the data from both 1999 and 2000 in the spring and fall it was determined that certain species tended to dominant the three soil horizons examined and that these species differed in the limed and unlimed plots. In addition it was determined that seasonal moisture variations and soil depth affected the distribution of ectomycorrhizae in both forest zones.

B3-4A: Dominant Mycorrhizal Species

Table B3-1 lists the top 10 mycorrhizal species at each soil depth, in the combined unlimed and limed forest areas, in both the dry (1999) and wet (2000) years. These species are considered to be the most successful based upon their total weights relative to the other species, expressed as percentages. The single most common species at all depths and in most soil probes was *Cenococcum geophilum.* The other species tended to be more selective and less abundant. In the following verbal descriptions of dominance in the rhizosphere, species are listed in order of abundance, unless otherwise stated.

Tables B3-2 through B 3-5 depict the 10 dominant species for spring and fall 1999 and 2000 in the unlimed and limed soils in each horizon. The discussion will emphasize the disparity between the unlimed and limed zones especially during dry spells. During wet seasons, the diversity and abundance of mycorrhizal species in unlimed and limed zones became more similar. Commonality is indicated by underlining the species names in the discussion. The limed soils typically had more diversity but lower overall gram weights.

The extensive species by species results are pictorally presented in Appendix 8 A: Graphs B3-1 through Graphs B3-68. In these graphs, presented in alphabetical order, the unlimed (N) preceeds the limed (L) and the three depths are represented as horizons A (0-10 cm), B (30-40 cm) and C (50-60 cm). The abundances are all relative and based upon the cumulative gram weights of the collected tips for each season for the 6 trees in each zone sampled. Each season is represented by a color code, in order of occurance. Spring 1999 is purple, Fall 1999 is red, Spring 2000 is yellow and Fall 2000 is blue. If a season is missing it is because the mycorrhizal species was absent (dormant ?). Very little work

has been done on the ecology of mycorrhizae. Next to nothing is known of their seasonal attributes or soil depth preferences. In the course of this study, this data has been preserved to shed some light upon the preferences of mycorrhizal species. Individual growth patterns for each species is discussed in the last appendix " Oak Ectomycorrhizae - Individual Micrographs". The main focus of this study was to determine the selective pressures of soil acidification and aluminum toxicity with respect to soil depth and species distributions.

B3-4B: Dominance at 0-10 cm Depth

Cenococcum geophilum dominated the 0-10 cm depth soil horizons except in three instances: Spring 1999 in the limed soil when it came in 10th in abundance, and fall 1999 and 2000 in the unlimed plot when it came in fifth and second respectively. At 0-10 cm depth mycorrhizal tips were extremely abundant.

During the dry year, in unlimed soils, *Cenococcum geophilum*, *Tuber rufum*, *Tuber melansporum* and *Xerocomus badius* were most common in the spring 1999, at 0-10 cm depth while *Inocybe obscurobadia*, *Quercirhiza squamosa*, *Piceirhiza chordata* and *Russula orcheoleuca* prevailed in the fall 1999. At the same time and depth in the limed soils, *Russula acrifolia*, *Fagirhiza spinulosa* and *Fagirhiza tubulosa* were dominant species in the spring and *Cenococcum geophilum*, *Cortinarius armillatus* and *Cortinarius bolaris* prevailed in the fall. The distribution of dominant mycorrhizal species between the unlimed and limed plots was distinctly different in the dry year (Tables B3-2 and B3-3), however within the plots hierarchy was generally maintained.

During the wet year, in unlimed soils, *Cenococcum geophilum*, *Quercirhiza squamosa* *Xerocomus chrysenteron*, *Lactarius subdulcis* and *Paxillus involutus* were most abundant in the spring 2000, at 0-10 cm depth while *Lactarius subdulcis*, *Cenococcum geophilum* and *Quercirhiza fibulocystidiata* prevailed in the fall 2000. At the same time and depth in limed soils, *Cenococcum geophilum*, *Fagirhiza fusca* and *Fagirhiza tubulosa* were dominant species in the spring and *Cenococcum geophilum*, *Quercirhiza fibulocystidiata* and *Cortinarius armillatus* prevailed in the fall. There was slightly more similarity

between the unlimed and limed plots during the wet year (Tables B3-4 and B3-5), with a better correlation in the fall (4 shared species in the top 10) than the spring (2 shared species in the top 10).

B3-4C: Dominance at 30-40 cm Depth

At 30-40 cm depth, *Cenococcum geophilum* was once again dominant in the unlimed and limed samples except for spring 1999 when it ranked second after *Fagirhiza setifera* for both, and in the very dry fall of 1999 in the limed plot, when it ranked fifth. There was considerably more similarity between the unlimed and limed probes with respect to dominant top ten species distributions as depth increased to 30-40 cm, although the average total biomass declined (Tables B3-3 to B3-5).

In the dry year in the unlimed soils, *Fagirhiza setifera*, *Cenococcum geophilum* and *Elaphomyces muricatus* were most common in the spring 1999, while *Cenococcum geophilum, Fagirhiza granulosa, Genea verucosa* and *Boletus edulis* struggled for life in fall 1999. In the limed soils during the same time period, *Fagirhiza setifera* outcompeted *Cenococcum geophilum* in the spring with *Tuber melanosporum* a distant third, and *Piceirhiza chordata, Paxillus involutus, Tetraberlinearhiza* dominated in the fall. In the spring 1999 the unlimed and limed probes had 5 dominant species in common at 30-40 cm depth (Table B3-2) but during the very dry fall there was more disparity and only 2 dominant species in common (Table B3-3).

In the wet year in unlimed soils, *Cenococcum geophilum Boletus edulis,* and *Quercirhiza squamosa* predominated in the spring 2000, while *Cenococcum geophilum, Piceirhiza chordata, Quercirhiza fibulocystidiata* and *Lactarius subdulcis* were abundant in the fall 2000. In the same time periods, in the limed soils, *Cenococcum geophilum,* Unknown brown & clear, *Elaphomyces muricatus* and *Piceirhiza chordata* were common in spring and *Cenococcum geophilum, Piceirhiza chordata* and *Elaphomyces muricatus* dominated in the fall. In the spring 2000 the unlimed and limed plots shared 6 dominant species (Table B3-4) while in the fall there were only 5 species in common (Table B3-5). Very generally, the unlimed and limed plots were more similar in the fall than the spring and

were more similar in the wet year than the dry year. In addition, similarities in dominance hierarchy was greater at 30-40 cm depth than at 0-10 cm depth.

B3-4D: Dominance at 50-60 cm Depth

Overall, at 50-60 cm depth, *Cenococcum geophilum* was NOT the most abundant species. It was more common in the wet year than in the dry year but in general at this depth, several other mycorrhizal species outcompeted *Cenococcum*. The biomass of mycorrhizal root tips was very low at this depth (Tables B3-3 to B3-5). The unlimed and limed probes were the least similar at this depth.

In the dry year in unlimed soils, *Fagirhiza spinulosa, Piceirhiza nigra* and *Tuber melanosporum* were most common in the spring 1999 and *Inocybe obscurobadia, Pinirhiza rufomaculata* and *Fagirhiza granulosa* were dominant in fall 1999. In limed soils during the same time period, *Fagirhiza fusca, Piceirhiza chordata* and *Fagirhiza setifera* dominated in the spring, and an Unknown, *Piceirhiza chordata, Piceirhiza gutatta* and *Cenococcum geophilum* struggled in the fall 1999. The unlimed and limed probes were the lease similar at this depth with only 2 dominant species shared in the spring, and none in the fall.

In the wet year in unlimed soils, *Quercirhiza squamosa* and *Boletus edulis* out competed *Cenococcum* in the spring 2000, but *Cenococcum* regained its premier ranking followed by *Piceirhiza chordata* and *Lactarius subdulcis* in the fall 2000. In limed soils during the same time period, *Elaphomyces muricatus, Fagirhiza granulosa, Lactarius chryssorheus* dominated *Cenococcum* in the spring and *Elaphomyces,* Unknowns and *Piceirhiza chordata* dominated *Cenococcum* in the fall. The unlimed and limed probes regained similarities in the wet fall with 5 dominant species in common in the spring but only 2 in the fall of 2000. Despite this, the unlimed and limed probes were least similar in species distributions at this depth compared to the overlying horizons.

Table B3-1: Summary of the seasonally dominant (top 10) mycorrhizal species found at various depths (Horizons A, B, C) in the (unlimed and limed) soils of *Quercus petraea* forests, in both the dry (1999) and wet (2000) years, in Merzablen, Germany.

Horizon A (0-10cm depth)			
Spring 1999 / 2000		**Fall 1999 / 2000**	
Cenococcum geophilum	26%	*Lactarius subdulcis*	22%
Quercirhiza squamosa	9%	*Cenococcum geophilum*	21%
Russula acrifolia	7%	Quercirhiza fibulocystidiata	11%
Tuber rufum	5%	*Cortinarius armillatus*	8%
Fagirhiza tubulosa*	4%	*Tomentella ferruginea*	7%
Lactarius subdulcis	4%	Piceirhiza chordata	6%
Piceirhiza chordata	4%	*Russula orcholeuca*	6%
Tuber mesentericum	3%	Fagirhiza fusca	6%
Fagirhiza fusca	3%	Quercirhiza squamosa	5%
Xerocomus chrysenteron	3%	Fagirhiza granulosa	4%
Quercirhiza fibulocystidiata	2%	Unknown gray	4%

Horizon B (30-40cm depth)			
Spring 1999 / 2000		**Fall 1999 / 2000**	
Cenococcum geophilum	27%	*Cenococcum geophilum*	39%
Fagirhiza setifera	14%	Piceirhiza chordata	24%
Boletus edulis	8%	*Elaphomyces muricatus*	4%
Piceirhiza chordata	7%	*Paxillus involutus***	3%
Elaphomyces muricatus	5%	Fagirhiza spinulosa	2%
Quercirhiza squamosa	5%	Unknown- brown & clear	2%
Unknown- brown & clear	5%	Unknown- rosa	2%
Unknown- rosa	5%	*Lactarius acris***	2%
Fagirhiza fusca	4%	*Lactarius subdulcis*	2%
Cortinarius armillatus	2%	Fagirhiza granulosa	1%

Horizon C (30-40cm depth)			
Spring 1999 / 2000		**Fall 1999 / 2000**	
Fagirhiza fusca	16%	Piceirhiza chordata	14%
Quercirhiza squamosa	15%	*Cenococcum geophilum*	14%
Piceirhiza chordata	9%	*Elaphomyces muricatus*	12%
Elaphomyces muricatus	8%	*Inocybe obscurobadia*	9%
Cenococcum geophilum	7%	Pinirhiza rufomaculata	7%
Boletus edulis	6%	Quercirhiza fibulocystidiata	5%
Fagirhiza setifera	5%	*Lactarius subdulcis*	5%
Piceirhiza nigra	5%	Fagirhiza granulosa	4%
Fagirhiza spinulosa	4%	Fagirhiza spinulosa	2%
Unknown-rosa	4%	*Lactarius acris*	2%

*Fagirhiza tubulosa is also known as *Sphaerozone astiolatum*
** *Lactarius acris* was also knowns as *Agaricus acris.*
*** *Paxillus involutus* was also known as *Agaricus involutus*

Table B3-2: Dry Spring 1999. The 10 dominant mycorrhizal species in limed and unlimed soils at various depths given in actual gram weights and relative percentages.

Horizon 0-10 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Russula acrifolia	1.419	31%	*Cenococcum geophilum*	1.336	29%
Fagirhiza spinulosa	0.416	9%	*Tuber melanosporum*	0.830	18%
Fagirhiza tubulosa	0.416	9%	*Tuber rufum*	0.830	18%
Tuber rufum	0.342	8%	*Xerocomus badius*	0.389	9%
Piceirhiza nigra	0.341	7%	Quercirhiza squamosa	0.344	8%
Quercirhiza squamosa	0.302	7%	*Paxillus involutus*	0.329	7%
Tomentella ferruginea	0.271	6%	Piceirhiza chordata	0.192	4%
Piceirhiza chordata	0.161	4%	Fagirhiza spinulosa	0.102	2%
Fagirhiza setifera	0.144	3%	*Phellodon niger*	0.084	2%
Cenococcum geophilum	0.143	3%	*Cenococcum geophilum var.*	0.054	1%

Horizon 30-40 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Fagirhiza setifera	0.314	32%	Fagirhiza setifera	0.568	37%
Cenococcum geophilum	0.236	24%	*Cenococcum geophilum*	0.157	10%
Tuber melanosporum	0.125	13%	*Elaphomyces muricatus*	0.130	8%
Unknowns	0.075	8%	Piceirhiza chordata	0.092	6%
Piceirhiza chordata	0.056	6%	Piceirhiza glutinosa	0.060	4%
Quercirhiza squamosa	0.052	5%	Fagirhiza fusca	0.048	3%
Elaphomyces muricatus	0.040	4%	Piceirhiza nigra	0.045	3%
Laccarius sp.	0.024	2%	*Cortinarius bolaris*	0.040	3%
Fagirhiza fusca	0.015	2%	Tuber B	0.037	2%
Dermocybe cinnamomea	0.009	1%	Fagirhiza granulosa	0.035	2%

Horizon 50-60 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Fagirhiza fusca	0.552	49%	Fagirhiza spinulosa	0.148	23%
Piceirhiza chordata	0.225	20%	Piceirhiza nigra	0.145	22%
Fagirhiza setifera	0.117	10%	*Tuber melanosporum*	0.132	20%
Cenococcum geophilum	0.060	5%	Tuber A	0.082	13%
Elaphomyces muricatus	0.030	3%	Unknown	0.055	9%
Fagirhiza tubulosa	0.025	2%	Fagirhiza setifera	0.033	5%
Unknown	0.024	2%	Fagirhiza granulosa	0.021	3%
Leucangium carthusianum	0.023	2%	Piceirhiza chordata	0.008	1%
Piceirhiza nigra	0.020	2%	*Russula mairei*	0.007	1%
Quercirhiza fibulocystidiata	0.017	2%	*Cortinarius bolaris*	0.005	1%

Table B3-3: Dry Fall 1999. The 10 dominant mycorrhizal species in limed and unlimed soils at various depths given in actual gram weights and relative percentages.

Horizon 0-10 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Cenococcum geophilum	0.299	19%	*Inocybe obscurobadia*	0.311	21%
Cortinarius armillatus	0.286	18%	Quercirhiza squamosa	0.266	18%
Cortinarius bolaris	0.153	9%	Piceirhiza chordata	0.192	13%
Fagirhiza granulosa	0.142	9%	*Russula orcheoleuca*	0.173	12%
Amphinema byssoides	0.138	9%	*Cenococcum geophilum*	0.160	11%
Quercirhiza fibulocystidiata	0.098	6%	*Tuber rufum*	0.150	10%
Genea hispidula	0.063	4%	*Tomentella ferruginea*	0.063	4%
Piceirhiza bicolorata	0.048	3%	*Tuber melanosporum*	0.055	4%
Lactarius subdulcis	0.043	3%	*Paxillus involutus*	0.036	2%
Tuber puberulum	0.042	3%	Fagirhiza granulosa	0.016	1%

Horizon 30-40 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Piceirhiza chordata	0.499	56%	*Cenococcum geophilum*	0.829	77%
Paxillus involutus	0.135	15%	Fagirhiza granulosa	0.075	7%
Tetraberlinaerhiza bicolor	0.078	9%	*Genea verucosa*	0.070	7%
Unknowns	0.047	5%	*Boletus edulis*	0.031	3%
Cenococcum geophilum	0.035	4%	Tuber D	0.022	2%
Piceirhiza guttata	0.030	3%	*Boletus cavipes*	0.014	1%
Russula acrifolia	0.016	2%	Quercirhiza squamosa	0.007	1%
Cortinarius armillatus	0.014	2%	Piceirhiza bicolorata	0.005	1%
Quercirhiza fibulocystidiata	0.011	1%	*Cortinarius varicolor*	0.004	0%
Inocybe obscurobadia	0.010	1%	Quercirhiza fibulocystidiata	0.003	0%

Horizon 50-60 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Unknown	0.074	63%	*Inocybe obscurobadia*	0.262	31%
Piceirhiza chordata	0.023	20%	*Pinirhiza rufomaculata*	0.243	29%
Piceirhiza guttata	0.019	16%	Fagirhiza granulosa	0.098	12%
Cenococcum geophilum	0.001	1%	*Lactarius subdulcis*	0.074	9%
No other species found.			*Pinirhiza cyaneoviridis*	0.043	5%
			Unknown	0.031	4%
			Cortinarius bolaris	0.018	2%
			Fagirhiza setifera	0.017	2%
			Fagirhiza globulifera	0.017	2%
			Tuber melanosporum	0.017	2%

Table B3-4: Wet Spring 2000. The 10 dominant mycorrhizal species in limed and unlimed soils at various depths given in actual gram weights and relative percentages.

Horizon 0-10 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Cencoccum geophilum	1.922	31%	*Cencoccum geophilum*	2.735	31%
Fagirhiza fusca	0.732	12%	Quercirhiza squamosa	1.534	18%
Fagirhiza tubulosa	0.630	10%	*Xerocomus chrysenteron*	0.771	9%
Genea hispidula	0.544	9%	*Lactarius subdulcis*	0.755	9%
Quercirhiza fibulocystidiata	0.518	8%	*Paxillus involutus*	0.770	9%
Unknown- bicolorata	0.463	7%	Piceirhiza chordata	0.294	3%
Russula acrifolia	0.233	4%	Piceirhiza bicolorata	0.271	3%
Piceirhiza chordata	0.217	3%	Picerihiza nigra	0.200	2%
Unknown - brown & clear	0.151	2%	Unknown - black & brown	0.190	2%
Inocybe obscurobadia	0.148	2%	*Byssocorticum atrovirens*	0.187	2%

Horizon 30-40 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Cencoccum geophilum	0.864	42%	*Cencoccum geophilum*	0.672	25%
Unknown - brown & clear	0.316	15%	*Boletus edulis*	0.546	20%
Elaphomyces muricatus	0.225	11%	Quercirhiza squamosa	0.278	10%
Piceirhiza chordata	0.186	9%	Fagirhiza fusca	0.212	8%
Cortinarius armillatus	0.065	3%	Piceirhiza chordata	0.184	7%
Quercirhiza squamosa	0.050	2%	*Cortinarius armillatus*	0.112	4%
Fagirhiza setifera	0.040	2%	Quercirhiza fibulocystidiata	0.092	3%
Unknown rosa	0.036	2%	Fagirhiza setifera	0.088	3%
Unknown bicolorata	0.033	2%	Unknown rosa	0.087	3%
Fagirhiza granulosa	0.033	2%	*Lactarius subdulcis*	0.044	2%

Horizon 50-60 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Elaphomyces muricatus	0.235	41%	Quercirhiza squamosa	0.516	42%
Fagirhiza granulosa	0.094	16%	*Boletus edulis*	0.215	17%
Lactarius chryssorheus	0.065	11%	*Cencoccum geophilum*	0.151	12%
Cencoccum geophilum	0.043	7%	Unknown rosa	0.125	10%
Cortinarius armillatus	0.043	7%	Piceirhiza chordata	0.070	6%
Piceirhiza chordata	0.034	6%	*Lactarius subdulcis*	0.030	2%
Fagirhiza setifera	0.024	4%	Tuber species - sulfur	0.024	2%
Lactarius subdulcis	0.014	2%	*Cortinarius bolaris*	0.018	1%
Quercirhiza fibulocystidiata	0.014	2%	*Cortinarius armillatus*	0.015	1%
Bysoccorticum atrovirens	0.002	1%	Fagirhiza setifera	0.012	1%

Table B3-5: Wet Fall 2000. The 10 dominant mycorrhizal species in limed and unlimed soils at various depths given in actual gram weights and relative percentages.

Horizon 0-10 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Cencoccum geophilum	1.536	21%	*Lactarius subdulcis*	3.202	36%
Quercirhiza fibulocystidiata	0.915	12%	*Cencoccum geophilum*	1.599	18%
Cortinarius armillatus	0.904	12%	Quercirhiza fibulocystidiata	0.849	10%
Russula acrifolia	0.634	8%	*Russula orcheoleuca*	0.639	7%
Genea verrucosa	0.619	8%	*Tomentella ferruginea*	0.650	7%
Tomentella ferruginea	0.607	8%	Piceirhiza chordata	0.429	5%
Pseudotormentella tristis	0.584	8%	Unknown gray	0.415	5%
Fagirhiza granulosa	0.583	8%	Quercirhiza squamosa	0.395	4%
Fagirhiza fusca	0.581	8%	*Xerocomus chrysenteron*	0.374	4%
Lactarius subdulcis	0.512	7%	Fagirhiza fusca	0.341	4%

Horizon 30-40 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Cencoccum geophilum	1.118	40%	*Cencoccum geophilum*	0.339	30%
Piceirhiza chordata	0.716	25%	Piceirhiza chordata	0.205	18%
Elaphomyces muricatus	0.211	7%	Quercirhiza fibulocystidiata	0.177	15%
Unknown brown & clear	0.136	5%	*Lactarius subdulcis*	0.080	7%
Fagirhiza spinulosa	0.099	4%	*Byssocorticum atrovirens*	0.058	5%
Unknowns	0.095	3%	*Paxillus involutus*	0.045	4%
Unknown rosa	0.080	3%	Fagirhiza spinulosa	0.040	3%
Lactarius acris	0.072	3%	*Lactarius acris*	0.040	3%
Fagirhiza setifera	0.070	2%	Unknown rosa	0.034	3%
Russula fueginea	0.034	1%	*Leccinum sacbrum*	0.022	2%

Horizon 50-60 cm					
Limed	**Grams**		**Unlimed**	**Grams**	
Elaphomyces muricatus	0.394	29%	*Cencoccum geophilum*	0.201	24%
Unknowns	0.269	20%	Piceirhiza chordata	0.153	18%
Piceirhiza chordata	0.256	19%	*Lactarius subdulcis*	0.088	10%
Cencoccum geophilum	0.118	9%	Fagirhiza spinulosa	0.07	8%
Quercirhiza squamosa	0.103	8%	*Quercirhiza fibulocystidiata*	0.066	8%
Cenococcum geophilum var.	0.091	7%	*Lactarius acris*	0.064	7%
Lactarius rubrocinctus	0.024	2%	*Lactarius rubrocinctus*	0.036	4%
Fagirhiza setifera	0.020	1%	*Xerocomus submentosus*	0.027	3%
Byssocorticum atrovirens	0.016	1%	*Inocybe obscurobadia*	0.024	3%
Laccaria amethystina	0.014	1%	Piceirhiza bicolorata	0.021	2%

B3-5: Mycorrhizae and Soil Depth.

Mycorrhizal presence was dependent upon a number of mitigating factors. In general although most mycorrhizae were present in the upper soil horizons, the biomass of living mycorrhizae shifted with severe drought. The shifts in biomass and species distributions between the soil horizons in the unlimed and limed samples were not identical but rather characteristically different.

B3-5A: General Biomass Trends

Overall, mycorrhizal biomass distribution varied with depth with approximately 72 % of the mycorrhizal roots being found at 0-10 cm depth, 19 % at 30-40 cm depth and only 9 % at 50-60 cm depth. The comparative mycorrhizal biomass distribution in unlimed and limed soils for each season and depth is provided in Table B3-6. Temporal shifts in biomass distribution with depth were noted in association with seasonal moisture occillations. In the very dry fall 1999, in both the limed, and more dramatically in the unlimed, soils the biomass shifted to the lower horizons. The following moist spring and fall 2000 saw a shift back up into the upper horizons for both zones, with a slightly more rapid recovery rate in the unlimed soils. In the wet fall 2000 the biomass growth was primarily in the upper horizon (0-10 cm depth) for both, indicating either recovery from the drought or opportunistic responses to increased moisture in the soils.

Table B3-6: Total gram weights of mycorrhizal roots and their % distribution with depth.

Depth		Spring 1999 grams		Fall 1999 grams		Spring 2000 grams		Fall 2000 grams	
0-10 cm	Limed	4.551	68%	1.612	62%	6.246	71%	12.382	75%
30-40 cm	Limed	0.989	15%	0.887	34%	2.071	23%	2.817	17%
50-60 cm	Limed	1.122	17%	0.117	4%	0.514	6%	1.352	8%
0-10 cm	Unlimed	4.547	67%	1.489	44%	8.694	69%	12.355	86%
30-40 cm	Unlimed	1.545	23%	1.074	32%	2.677	21%	1.147	8%
50-60 cm	Unlimed	0.646	10%	0.837	25%	1.232	10%	0.855	6%

B3-5B: Soil Depth and Species Commonality

The limed and unlimed probes shared mycorrhizal species but the commonality varied with depth (Table B3-7). In both the unlimed and limed probes, at 0-10 cm depth 77 % of the species were in common, at 30-40 cm depth 72 %, and at 50-60 cm only 46 %. All these differences were significant. The low percentage of common species at 50-60 cm depth may be in part due to extremely low numbers of mycorrhizae normally found at that depth. The high number of common species at 0-10 cm depth was expected since that is the primary rhizosphere growth zone. It was anticpated that species with the same metabolic needs and adaptive responses would be found in the same growing areas and depths, especially since the two plots were in such close proximity sharing the same microclimate, soil types and host species. The 23 - 54 % range difference in identity indicates a strong environmental factor has affected the mycorrhizal diversity and survival.

Table B3-7: Commonality of mycorrhizal species in limed (L) and unlimed (N) probes at each soil depth. The actual number of species isolated each season is provided. See key below.

Depth		Spring 1999	Fall 1999	Spring 20000	Fall 2000	Total	Common	Diff.	Max.	Commonality
0-10 cm	L	32	32	42	50	69	58	11	75	77%
0-10 cm	N	14	24	48	49	64	58	6		
30-40 cm	L	23	14	30	29	53	38	15	53	72%
30-40 cm	N	28	16	37	29	52	38	14		
50-60 cm	L	19	4	17	21	30	22	8	48	46%
50-60 cm	N	16	15	26	27	40	22	18		

Total = Total number of species found.
Common = Number of species common to both the limed and unlimed plots.
Diff. = Number of species unique to either the limed or unlimed probes.
Max. = Maximum number of species found in the combined limed and unlimed probes.
Commonality = % of species in common for both the limed and unlimed probes.

In addition to the disparity in the commonality of species, the dominance hierarchy (relative abundance of each species) between the unlimed and limed plots varied considerably. (Section B3). The dramatic differences in dominance could in part be due to natural variation, but since the hierarchies themselves were relatively consistent it is most likely that the shifts were due to liming and its correlated chemical changes within the biosphere. Lime induces alteration of pH and aluminum content of the mycorrhizae are presented in the next two sections of this report.

B3-5C: Multi-Horizon Species

Some mycorrhizal species were more versitile than others with respect to depth. Very generally, there was 27 to 34 % identity between horizons A/B, A/C or B/C (Table B3-8), but the actual species transiting two horizons was dependent upon whether the samples were from unlimed or limed probes (Appendix 8, Graphs B3-1 to B3-7). In addition, the species varied with season and moisture levels. So it can only be very generally stated that at any given point in time, approximately 1/3 of the mycorrhizal species present in the rhizosphere are capable of existing in an active growing state in more than one horizon. The depth diversity strongly supports the theory that a species must be specifically adapted to selectively survive at a specific depth. But this depth diversity probably also provides the tree with a very nice symbiotic survival insurance policy.

Table B3-8: Commonality of mycorrhizal species between soil horizons A (0-10 cm depth), B (30-40 cm depth) and C (50-60 cm depth) in the limed (L) compared to the unlimed (N) forest zones presented numerically (#) and as percentages (%).

Soil		Spring 1999		Fall 1999		Spring 2000		Fall 2000			Averages
Horizon		#		#		#		#			
A/B	L	14	34%	11	31%	20	38%	21	36%		35%
A/B	N	7	20%	9	29%	21	33%	20	34%		34%
A/C	L	15	42%	4	13%	15	34%	20	39%		32%
A/C	N	5	20%	6	18%	18	32%	21	38%		27%
B/C	L	9	27%	4	29%	9	24%	13	35%		29%
B/C	N	10	29%	6	24%	18	40%	17	44%		34%

Formulae for Commonality of Mycorrhizal Species Between Soil Horizons

% Commonality for A/B horizons = # / [# + (A-#) + (B-#)] x 100
% Commonality for A/C horizons = # / [# + (A-#) + (C-#)] x 100
% Commonality for B/C horizons = # / [# + (B-#) + (C-#)] x 100

# = the number of common species between the two horizons compared (Table B3-8)
A = the number of species at horizon 0-10 cm depth from Table B3-7.
B = the number of species at horizon 30-40 cm depth from Table B3-7.
C = the number of species at horizon 50-60 cm depth from Table B3-7.

Very few species of the species isolated (19 (L) ; 21 (N)) were versitile enough to exist well in all 3 horizons (Table B3-9), and of these only a four species [*Cenococcum geophilum* (L & N), *Fagirhiza granulosa* (N), *Inocybe obscurobadia* (N), and *Lactarius subdulcis* (L & N)}] were present in all 3 horizons during the worst of the drought in fall 1999. Of all the species, only *Cenococcum geophilum* was present at every depth and season in both the unlimed and limed forests. Besides being the most versitile, it was also the most abundant species by gram weight (Section B3-4-1) . *Lactarius subdulcis, Fagirhiza setifera* and *Piceirhiza chordata* were also very versitile and very common but not always amoung the most abundant species present. In contrast, *Fagirhiza granulosa* and *Inocybe obscurobadia* were more numerous only when other species were absent during the dry spell. Most of other multi-depth species were most strongly present in the wet spring 2000 and /or fall 2000 (Table 3-9).

The species listed in Table 3-9 are of interest since they are amoung the mycorhizae most likely to succeed in a dynamic environment. Innoculations of combinations of these species along with some of the dominant species mentioned in Section B3-4 may be useful in forest amelioration. With respect to the ability to sequester aluminum however, some of these species may not be as beneficial, especially if they provide no barrier to aluminum translocation.

Table B3-9: Certain versatile mycorrhizal species were found in all 3 soil horizons during the indicated seasons and forest locations. (L = limed; N = unlimed)

Multi-horizon Species	Spring 1999	Fall 1999	Spring 2000	Fall 2000
Amphinema byssoides			N	L
Boletus edulis			L & N	N
Byssocorticium atrovirens			N	L & N
Cenococcum geophilum	L & N	L & N	L & N	L & N
Cortinarius armillatus			L & N	N
Elaphomyces muricatus				L
Fagirhiza granulosa	L	N	L & N	L & N
Fagirhiza fusca				N
Fagirhiza setifera	L & N		L & N	L & N
Fagirhiza spinulosa				N
Fagirhiza tubulosa	L			
Genea hispidula				N
Inocybe obscurobadia		N		
Lactarius acris			L	
Lactarius pallidus				N
Lactarius subdulcis		L & N	L & N	L & N
Paxillus involutus				N
Piceirhiza chordata	L & N		L & N	L & N
Piceirhiza nigra	L		L	
Quercirhiza fibulocystidiata			L & N	N
Quercirhiza squamosa	L		L & N	L & N
Tomentella ferruginea L				
Tuber melanosporum	N		N	
Tuber puberulum			L	
Unknown rosa			L	L & N
Xerocomus chrysenteron			N	

B3-5D: Soil Depths and Mycorrhizal Biomass Shifts

At 0-10 cm depth, the soil is a sandy humus under a thin litter layer and it supports a good diversity of mycorrhiza. The lowest percentage of mycorrhizal roots at this depth was found in the very dry fall 1999 (Table B3-6). In both the unlimed and limed soils, there was a shift of mycorrhizal biomass to the lower (30-40 cm depth) horizons during the dry spell. With hydration, the following year, the mycorrhizal biomass distribution shifted back up to the 0-10 cm horizon. The biomass shift to the lower horizon did not involve a major shift of species but rather a change in metabolic activity with many species

probably becoming dormant within their rhizosphere zone during the dry spell and recovering with the return of moisture (Appendix 8A). The actual number of species for both the unlimed and limed soils dropped, from a high number (Unlimed: 24, Limed: 32) present at 0-10 cm depth to a lower number (Unlimed: 16, Limed: 14) at 30-40 cm depth during in the dry spell (Table B3-7) and the dominance hierarchies changed (Tables B2-B5). It would be expected that if the mycorrhizae acutally moved, the number of species would increase with depth, but this did not occur. Although it would be possible for the species that can share horizons to physically move to the lower horizons, it is more likely that the shift was metabolic. Most likely, the desiccation-stressed species in the upper soils went into a temporary state of metabolic inactivity or vegetative stasis as reflected by their reduced, and then recovered, biomasses (Appendix 8A).

The biomass shifts were more pronounced in the unlimed than in the limed soils with a much larger biomass shift to the deepest soil horizons in the dry fall 1999, but also with a more elastic recovery in total weight the following spring. (Table B3-6). (The limed probes had less depth displacement in the dry fall 1999, and a slower recovery with respect to total gram weight). By the wet fall 2000, both the unlimed and limed forest regions had similarily recovered with the majority of the mycorrhizal biomass once again in the A horizon.

B3-5E: Species- specific Actual and Delayed Depth Shifts

Several species had strong depth shifts and recoveries to the upper horizon (Appendix 8A). Some of the shifts occured during the drought with certain species simply appearing or becoming more numerous in deeper soils (30-40 cm) during the dry period followed by a shift to the upper soils the following spring. This will be referred to as an "actual" shift. Other species had a "delayed" shift where they disappeared during the drought and tentatively reappeared the following spring in the deeper soils (30-40 cm) but by the next fall were strongly present in the upper soils (0-10 cm) once again, but were then much less evident or absent from the B horizon. Delayed shifts were more common.

It is strongly felt that the mycorrhizae did not actually move between the horizons but rather hyphal strands or spores already present within the rhizosphere simply changed their metabolic states in an adaptive response to microenvironmental changes in moisture. What is of major interest here is the diversity in survival strategies exhibited. An modest attempt will be made in the next report section to determine if a relationship exists between these trends and the aluminum content of individual mycorrhizal species.

In unlimed soils, 11 species exhibited A/B depth shifts (Appendix 8A): *Amphinema byssoides* - delayed (Graph B3-1), *Boletus edulis* - delayed (Graph B3-3), *Cenococcum geophilum* -actual (Graph B3-5), *Cortinarius armillatus* - delayed (Graph B3-8), *Fagirhiza granulosa* - actual (Graph B3-15), *Fagirhiza setifera* - delayed (Graph B3-16), *Genea verrucosa* - actual (Graph B3-21), *Quercirhiza fibulocystidiata* -delayed (Graph B3-43), *Quercirhiza squamosa* - delayed (Graph B3-44), *Tuber mesentericum* - delayed (Graph B3-55) and Unknown - Rosa - delayed (Graph B3-65).

In limed soils, only 8 species had A/B depth shifts and recoveries (Appendix 8A). Most of these were also delayed shifts. The shifts were: *Amphinema byssoides* - delayed (Graph B3-1), *Byssocorticum atrovirens* - delayed (Graph B-4), *Cortinarius armillatus* delayed (GraphB3-8), *Fagirhiza archnoidea* - delayed (Graph B3-11), *Fagirhiza setifera* - delayed (Graph B3-16), *Inocybe obscurbadia* - actual (Graph B3-24), *Piceirhiza chordata* -actual (Graph B3-36), and *Piceirhiza guttata* - actual (Graph B3-39).

Shifts between the C horizons and upper areas were not observed. What is of interest to note is that during the drought, more species with more overall biomass were present in the C horizon of the unlimed probes than the limed (Table B3-3). Regardless of the season, some species tended to be more numerous at 50-60 cm depth than in the upper soils (Appendix 8). These will be refered to as geophilic species while the other ectomycorrhizae will be refered to as rhizophilic species. Despite the fact they are all present at the root tip interfaces some species "prefer" the deeper horizons. The reasons for the preferences are unclear but may include factors such as allotrophic inhibitition of

growth in the upper rhizosphere by more competative mycorrhizal species along with moisture and pH stability of the C horizon and leachate accumulation.

In limed soils, the geophilic species included: *Elaphomyces muricatus* (Graph B3-10), *Fagirhiza fusca* (Graph B3-13), Lactarius acris (Graph B3-26), *Lactarius chrysorrheus* (Graph B3-27), Lactarius rubrocinctus (Graph B3-29), Piceirhiza chordata (Graph B3-36), and *Russula firmula* (Graph B3-49).

In unlimed soils, the geophilic species were: *Fagirhiza granulosa* (Graph B3-14), *Inocybe obscurobadia* (Graph B3-24), Lactarius acris (Graph B3-26), Lactarius rubrocinctus (Graph B3-29), Piceirhiza chordata (Graph B3-36), *Pinirhiza rufomaculata* (Graph B3-41), Tuber unknown A (Graphs B3-58), and *Xerocomus submentosus* (Graph B3-68).

Mycorrhizal species tentatively identified as *Leucangium carthusianum* (L) *Pinirhiza cyneoviridis* (N), and *Russula xerampelina* (N) were isolated only from Horizon C. Similar species were not found in the upper horizons to confirm identification. It is likely that these mycorrhizal tips exhibited poor growth form due to soil depth confounding accurate identification. In contrast *Fagirhiza globulifera* (L & -N), *Lactarius chrysorrheus* (L & N), and *Pinirhiza rufomaculata* (L -N) which were rare, but present at 50-60 cm, were also found in horizon A. Species identification becomes more problematic with depth primarily because of low numbers, isolated tips, poor growth, and mechanical damage. Despite this some species (*Cenococcum geophilum, Byssocorticum atrovirens, Cortinarius armillatus, Cortinarius bolaris, Fagirhiza granulosa, Fagirhiza setifera* and *Piceirhiza chordata*) retained their primary characteristics and were very easy to identify.

B3-5F: Non-competative Dominance Shifts with Drought

A few species opportunistcally filled the gap during the dry spell and became **temporarily*** numerous but often dissappeared the following spring 2000 when the more competative species reappeared (Appendix 8A).

Amoung these species in the limed soils, *Amphinema byssoides* (Appendix 8A: Graph B3-1), *Cortinarius armillatus* (Graph B3-8), *Cortinarius bolaris** (Graph B3-9), *Fagirhiza granulosa* (Graph B3-15), *Lactarius rubrocinctus** (Graph B3-29), *Russula orcholeuca* (Graph B3-51) and *Tuber puberulum* (Graph B3-56) became distinctly more numerous during the dry spell at 0-10 cm depths; while at 30-40 cm depths *Paxillus involutus** (Graph B3-33), *Piceirhiza chordata* (B3-36), *Piceirhiza guttata** (Graph B3-39), *Piceirhiza rufomaculata** (Graph B3-41), and *Tetraberlineaerhiza bicolor** (Graph B3-52) appeared and disappeared the following season.

In the unlimed soils during the dry fall 1999, *Cenococcum geophilulm* (Graph B3-5), *Inocybe obscurobadia** (Graph B3-24), and *Quercirhiza squamosa* (Graph B3-44) were more common; while at 30-40 cm depths *Boletus cavipes** (Graph B3-2), *Cenococcum geophilum* (Graph B3-5), and at 50- 60 cm, Fagirhiza globulifera* (Graph B3-14), and *Inocybe obscurobadia** (Graph B3-24) were ephimerally present.

As can be seen above (by the lack of underlined common species), the unlimed and limed regions were distinctly different. The assumption is being made here that the majority of the species* just mentioned appeared only because the more competative dominant species were dormant and/or the other species competing for root tip infection sites were considerably less numerous. With the return of more favorable conditions most of the ephimeral species* dissappeared.

B3-6: Discussion

The Podsoil horizons present at Merzalben are suitable for the preferential survival of the deep-rooted oak species which predominates the area. At 0 to 10 cm depth, the A horizon is a sandy humus, at 30 to 40 cm depth the B horizon is sandy with a minor component of heavily oxidized humus, and at 50 to 60 cm depth , the soil is a typical C horizon which is sandy with numerous stones and rocks. In a preliminary examination of these aluminosilicate soils, it was found that the uppermost A horizon, under a thin litter layer, supported a good diversity of mycorrhizal species and that the abundance of mycorrhizal species decreased in B and even more dramatically in the C horizons.

In limed soil the diversity was much greater than in unlimed soil (Table B3-7), however contrary to expectations, the visual health and obvious vitality of the individuals was much reduced in limed soil as evidenced by lower accumulated weight for individual species (Appendix 8) and lower total fine mycorrhizal biomass (Tables B3-1 to B3-5). The probes from the unlimed soil, although not as diverse, were larger and healthier looking at every depth. This qualitative observation was supported by a greater biomass for individual species (Appendix 8) and greater or at least equivalent total seasonal biomass (Tables B3-1 to B3-5). Liming seemed to induce K selection. K selection is typified by high diversity and low numbers, typical for unstable, stressed or changing environments (Miller, 1992). Whereas, the unlimed forest mycorrhiza had a selective distribution more typical of R selection. R selection is typified by low diversity but greater biomass, common in stable unchanging environments (Miller, 1992). Abundance, diversity and dominance hierarchies were affected by soil depth, seasonal moisture levels and liming.

The dominance hierarchy of mycorrhizal species varied in the limed and the unlimed forests. In addition to the constant factors of soil composition, depth pressures and elevation, the distribution of dominant species was affected by the dynamics of seasonal moisture, pH and the residual effects of the 1989 liming. Since seasonal changes in dominance hierarchies were found, it is possible that with yearlong sampling, a true succession picture may become apparent. But it is also evident from this study that the seasonal succession in the limed forest would not be identical to that of the untreated forest. Liming has selectively affected mycorrhizal survivability and sensitivity affecting distribution patterns.

At 30-40 cm depth, the soil is sandy with a minor component of heavily oxidized humus. Under favorable conditions, it normally contains fewer mycorrhizae than the humic upper soil. From the commonality calculations (Table B3-8), it was determined that 34% (unlimed) and 35% (limed) of the species were common to both the A (0-10 cm) and B (30-40 cm) horizons. At 0-10 and 30-40 cm depths, it was also noted that the unlimed

and limed species were respectively, 75% and 72% identical (Table B3-7), but the dominance study (Section B3-4) indicated that there were marked differences between the unlimed and limed species with respect to dominance and depth-specific biomass distributions especially during desiccation stress at both the 0-10 and 30-40 cm depths, and that these differences tended to disappear when the soil rehydrated.

Liming has affected not only dominance of the (72-75%) shared and unshared species, but also their responsiveness to depth and desiccation stresses. At 50-60 cm depth where the soil is sandy with numerous rocks and stones, the fewest mycorrhiza tended to be found. Very generally, the number of common species decreased during drought and increased during the subsequent recovery phase with a few exceptions. The commonality of species between the unlimed and limed forests, at 50-60 cm, was very low (46%) (Table B3-7). The number of species common to horizons A/C or B/C was somewhat similar within each zone, but differed between zones (Table B3-8). Even in very deep soils, the long-term effect of liming upon mycorrhizal distribution can be seen.

According to this portion of the study, besides liming, the single largest factor affecting mycorrhizal distribution was moisture, followed by soil depth. In the next section, aluminum content and disparity between the unlimed and limed zones will be examined.

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Section C: Physical Parameters Affecting Growth

INDEX

C1: Moisture Content of Unlimed and Limed Soil Samples

C1-1: Introduction

In Merzalben, during the course of the experimental sampling period, it was noted that the spring of 1999 was relatively dry, but the fall 1999 was extremely dry with a tentative recovery of moisture levels in spring 2000, followed by a full recovery in the fall of 2000 (Section A2-6). The general climatic information for this same time period for western Germany followed a similar trend (Section A2-4-1) (NOAA-NCDC, 1999, 2000, 2001). Soil moisture for the specific forest region under examination (unlimed and limed soils at various soil depths for each of the four seasons) was measured to document the changes.

Soil moisture had a direct effect upon the growth and vitality of roots and their fine mycorrhizal symbionts. In the absence of adequate moisture, mycorrhizal growth and metabolism slowed and with many species entering survival mode. Drought affected some species more adversely than others leading to their total disappearance during the stress period. For most species, the period of dormancy was maintained in the soil for the short period of fall drought followed by an actual or delayed elastic recovery the following year.

Some of the more resistant species were able to produce durable spores (i.e. *Cenococcum geophilum*), somewhat desiccation-resistant mantles (i.e. *Piceirhiza chordata*), hardy rhizomorphs (i.e. *Xerocomus submentosus*), or metabolically shift to the lower soil horizon subspecies where the needed moisture was present (i.e. *Cortinarius armillatus*) (Section B3-5). Elastic recovery from deep soil to the upper soil horizons was species-specific and was either immediate (actual) (i.e. *Piceirhiza*) or delayed (i.e. *Quercirhiza fibulocystidiata*) depending upon the individual adaptive responses (Section B3-5E). Susceptible species become locally extinct (i.e. *Xerocomus badius* (Appendix 8: Graphs B3-66)) or severely reduced in numbers (i.e. Tuber melanosporum (Appendix 8: Graph B3-54 and *Tuber rufum* (Graph B3-57)) with no spontaneous recovery.

C1-2: Material & Methods

Soil samples were obtained using a 10 cm diameter metal root auger. The sample sites were located at one meter distance from the base of each oak tree, mid way between two

major root extensions. The 10 x 10 cm core samples were from horizons A, B and C, at 0-10 cm, 30-40 cm and 50-60 cm depths respectively. The probes were packaged individually and immediately into plastic bags and stored on ice. Once back in the lab, the roots were removed from the soil for the mycorrhizal study. Approximately 100 grams of a coarsely sieved (4 mm sieve) soil sample was retained and packaged in small ziploc plastic bags and double bagged in larger plastic bags to provide a moisture barrier and then stored at 10^0 C in a cool room.

From the original soil probes, either 75 gram (0-10 cm depth), or 60 gram (30-40 and 50-60 cm depth) samples were accurately weighed to 4 decimal places and placed in labeled glass containers which were also weighed. The soil samples were dried for 3 days in a 60^0 C drying chamber (Lab oven, Memmert GmbH, Schwabach, Germany). Alternatively, the soil samples could have been room air dried and weighed every 12 hours until two standard equal readings were obtained (Carter, 1986) but this method is highly dependent upon relative humidity and so less reliable and more time consuming than slow drying in a control chamber and so was not used.

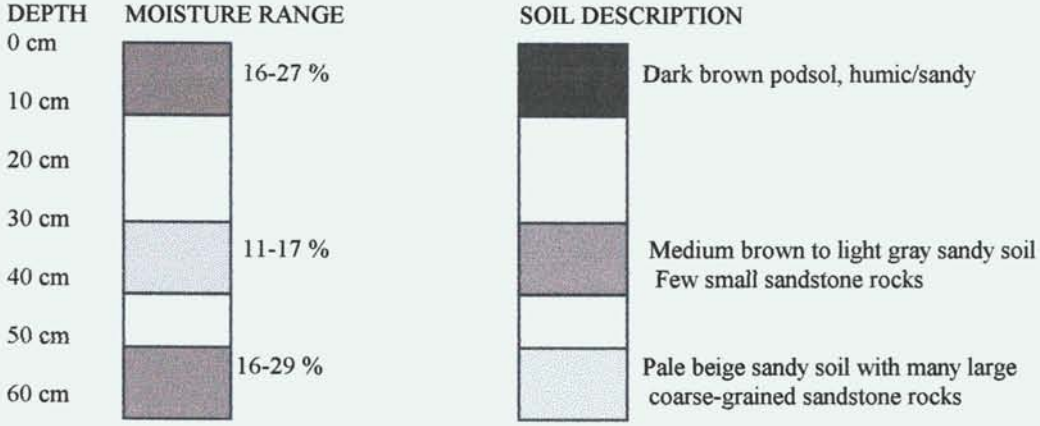
Immediately upon removal from the desiccation chamber, the glass and soil samples were weighed and the moisture loss recorded. The relative percentage of initial water content for each sample was calculated. Average moisture content was compared for various soil depths, various seasons and various soil treatments. Statistical analysis of the samples was completed to verify the significance of the differences in moisture content using the standard student t-test.

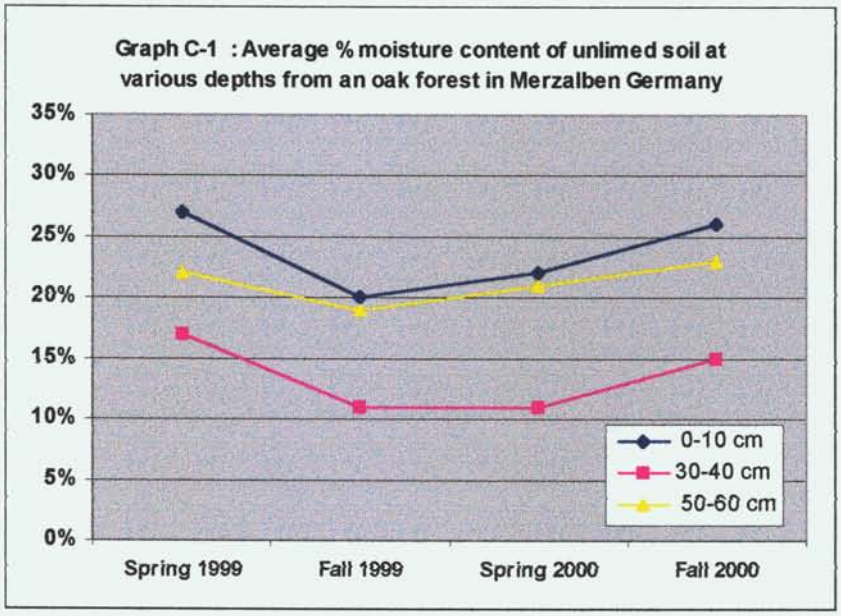
C1-3: Results

Actual seasonal variations in moisture content of the soil samples mirrored the general observations made during on-site sampling. On the average, for all depths, spring 1999 was fairly moist (16-29% moisture) but Fall 1999 (11-20% moisture) was very dry. Spring 2000 (11-22% moisture) was only slightly better than the previous fall but not as moist as the previous spring. Fall 2000 (15-27% moisture) continued the improved moisture trend, almost returning to "normal" (Graphs C1 - C3, Appendix 9A). Appendix 9A contains the

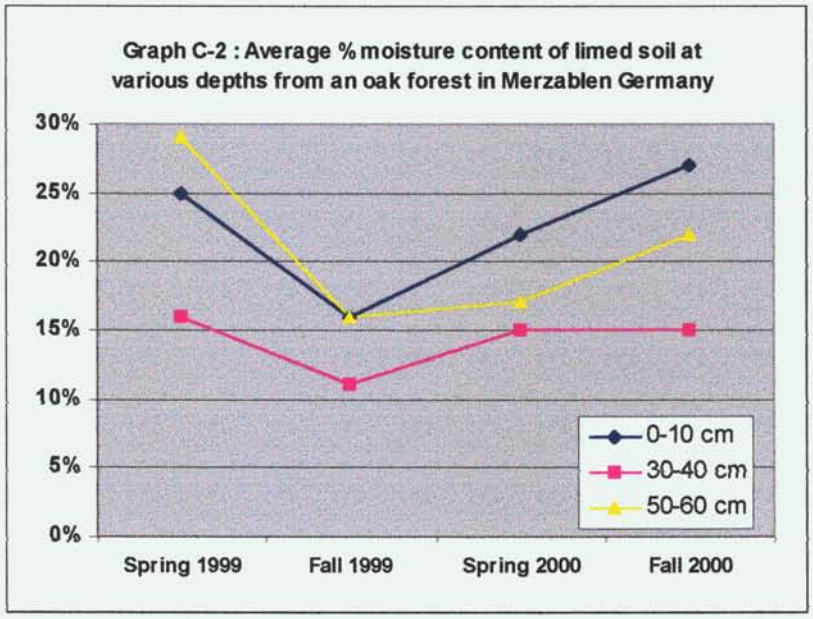
raw data sets used to create Graphs C1 and C2 plus the statistical analysis tables. Moisture retention varied with soil composition and depth. Horizon A and C had similar moisture while horizon B was significantly less hydrated in both the unlimed and limed soils.

Diagram C1-3: Soil Depth, General Moisture Content, Soil Type





Unlimed	Spring 1999	Fall 1999	Spring 2000	Fall 2000
0-10 cm	27%	20%	22%	26%
30-40 cm	17%	11%	11%	15%
50-60 cm	22%	19%	21%	23%



Limed	Spring 1999	Fall 1999	Spring 2000	Fall 2000
0-10 cm	25%	16%	22%	27%
30-40 cm	16%	11%	15%	15%
50-60 cm	29%	16%	17%	22%

C1-4: Discussion

C1-4A: Soil Depth and Moisture Content

The A and C horizons had significantly more water content than the B horizons (30-40 cm) sandwiched between them, with one exception (dry Fall 2000, limed soil only) (Appendix 9) . The surface soil receiving the initial water input had fairly good retention ability while the lower, B, horizon formed a leaching zone. The lowest horizon, C, may form the upper reaches of the forest water table. With respect to the mycorrhizal complement (Section B), the 0-10 cm depth, probes had many fine roots and abundant recognizable mycorrhizae, while the 30-40 samples were rather poorly populated which may be related to the lowered fluid retention of this horizon. The 50-60 cm depth probes had the fewest mycorrhizae, but had sufficient water. Lowered mycorrhizal abundance at this depth was therefore not related to the abundance of water but rather affected by other geophysical factors. These factors most likely included: soil pressure, nutrient deficiencies accumulation of leached surface waste products and toxic Al ions.

C1-4B: Limed vs. Unlimed Soil Moisture Content

It is interesting to note that there was no significant difference between the unlimed (Graph C1) and limed (Graph C2) soil treatments with respect to their relative moisture content for 0-10 cm depth regardless of the season (Appendix: Table C1-9). This despite a registered 4% lower average moisture content in fall 1999 in the limed soil samples.

At 30-40 cm depth there was also no significant difference between the unlimed and limed soils with respect to their moisture contents for Spring 1999, Fall 1999 and Fall 2000 (Appendix 9: Table C1-9). In spring 2000, the limed soil samples had an average of 4% more moisture (Graphs C1-C2) than the unlimed, and this difference was significant.

The soil samples were collected from 0-10 cm depth and then again at 30-40 cm depth. The interim gap of 10-30 cm was not examined. It is possible that from fall 1999 to spring 2000, this interim could have contained the "transition" water that would account for the average 4% difference observed between fall and spring in the limed soils. Whether this implies a liming effect upon the soil with drought is unclear but possible.

At 50-60 cm depth there were significant differences between the unlimed (Graph C1) and limed soils (Graph C2) for all seasons except fall 2000 when the moisture differences were negligible. During spring 1999, the limed soil had an average of 7% more moisture than unlimed soil at the same depth, but had 3-4% less moisture than the unlimed soils the following very dry fall (1999) and moist spring (2000). If there is a liming effect upon moisture retention then it can be said that liming the upper soils adversely affects the moisture retention of deeper soils, especially during drought, but conversely improves moisture retention during wet seasons! The dichotomy of the effects may be related to the leachates, but that is the subject of another study.

C1-5: Conclusions

With respect to the effective comparison of mycorrhizal numbers and diversity, at 0-10 and 30-40 cm depths, moisture content can be generally ignored as a complicating factor in analysis since both plots were similar. For 50-60 cm depth, the significant differences in moisture content between the forest zones must be taken into account in determining

the depth effect of liming and Al upon the mycorrhizal complement. Liming was associated with improved moisture retention during wet seasons but adversely affected moisture retention during the desiccation event, but only in the C horizon. Significantly lower soil moisture can only partly account for the lower mycorrhizal diversity and abundance found in the limed zones at this depth during the dry spell. Other geophysical factors come into play which severely reduce mycorrhizal numbers. When the moisture levels returned to their normal highs (16-29% of soil weight, depending upon season), the mycorrhizal numbers were still very very minimal at 50-60 cm depth.

At 0-10 cm the moisture content of the soils was similarly high (16-27% of soil weight, depending upon the season) in both zones. Because of this, and the similarity in elevation, soil types, tree distribution, and general climate, any species differences can be attributed directly to the general effects of liming, minus normal random variation due to precise probe locations.

In the B horizon, moisture was lower in both zones (11-17% of soil weight, depending upon the season). The 30-40 cm depth leaching zone may form a hydration barrier to hyphal extension, especially during wet seasons, which would partly account for the lower mycorrhizal complements found as depth increases in both forest zones. During dry spells, the deeper and drier, B horizon may be important for spore and hyphal reserves of select xero-tolerant subspecies. The fact that more species shifted during the dry fall to the B horizon in unlimed soils cannot however be simply attributed to the moisture content of the soils since both the limed and unlimed zones were essentially similar in this respect. It can be concluded then that the forest zones differences in species shifts between horizons A and B, both during and after the short term drought, was directly related to the long-term residual effects of liming.

C2: pH of Unlimed and Limed Soil Samples

C2-1: Introduction

The toxicity of aluminum (Al/Ca >1) appears concomittantly with proton toxicity (H/Ca >1) (Rost-Siebert, 1983; Vogelei & Rothe, 1988, 1993; Schaedle et al, 1989). On one hand, liming of forests has been recommended to alleviate possible Al- and proton effects on trees (Ulrich, 1982, 1983). On the other hand, in Norway spruce, the damage caused by acid can be intensified by aluminum treatments (Vogelei & Rothe, 1988), especially the Al^{3+} forms (Rost-Siebert, 1983). It is known that Al^{3+} becomes freely solubilized in acidic soils below a pH of five (Section A3-3). Once freed into solution Al and nutrients can travel hydrogen ion uptake currents to the soil-ectomycorrhizal root interface where they can potentially be translocated across the membranes into the cytosol (Section A3-6). This portion of the "acid growth" theory allows for nutrient uptake in the elongation zone of fine roots and, in part, explains the intricate relationship between H^+ and Al ions.

The action of soil buffer systems (Section A3-4), mycorrhizal membrane chelators, selective uniports, cytoplasmic sequestration, and antiports may control entry of potentially toxic aluminum ions (Section A3-6). Very generally, it is assumed that if sufficient calcium is present, root damage will be negligible even down to pH 4 but at pH 3 damage and death will be extensive (Moore, 1974) (Section A3-7). Conversely stated, one of the major causes of root dieback is probably the progressive accumulation of toxic aluminum ions at pH levels below 4.5, especially if the soil is low in Calcium and / or Phosphate (Section A3-8). The exact pH at which damage occurs is largely unknown may be highly dependent upon the physiological abilities of individual mycorrhizal species. This thesis assumption will be discussed briefly here and in Section F. *The primary focus of the current section is to determine the actual pH values of the collected soil probes.*

In 1988, the average acidity of soil in the Merzalben region is pH 3.9 (See Section A3-5-4). In 1993 the pH ($CaCl_2$) of the soil at 0-5 cm depth was pH 3.5, at 5-10 cm the soil was pH 3.9, and from 10-60 cm depths it was 4.3 pH (Section A3-5-5). According to these facts, the surface soils in Merzalben were definitely within the range of pH for

aluminum to be solubilized. The 1993 report did not differentiate between unlimed and limed forest zones. This report will also focus on the difference in pH between the unlimed and limed forest regions in the 1999 and 2000 study period.

There are several methods to determine how acidic an area is. The first is by direct measurement of the pH of the ambient or runoff water. This method estimates the hydrogen ion loss from the soil matrix. According to the Trippstadt Forestry department, the runoff from the limed soil plot is acidic, but less acidic than that of the unlimed forest (Block, 1999 p.c.). This would imply that, in the limed zone, hydrogen ions are either not being released or are being retained or more heavily metabolized in the rhizosphere zone. The second is by direct measurement of the pH of the soil. This second method should portray the pH of the interactive biogeochemical rhizosphere. This should shed some light on the availability of aluminum to the mycorrhizae in direct contact with soil micelles. If the soil pH is more acidic than the runoff water, then we can assume that hydrogen ions are being held within the physiological realm of the root-soil interaction sphere. The third method calculates acidity responses by determining the acid neutralization capacity (ANC) of soil or roots by using base cation (Ca, Mg, K, Na) mineral analysis (Fox et al, 1989). The direct soil pH and ANC results and will be discussed in Sections G & H (Bound & Unbound mineral analysis).

C2-2: Materials & Methods

C2-2A: Soil Preparation

In the spring and fall of 1999 and 2000, sessile oaks from the limed and unlimed plots in Merzablen high elevation forest were selected and numbered. A total of 24 trees were examined, 12 from each test area. Of these, six trees were sampled on each plot in the spring and the other six in the fall in 1999. The procedure was repeated the following year. An area about 1 meter from the base of each tree was cleared of litter. In a region between two large primary roots where fine roots were evident, soil root auger (9.9 x 10 cm) samples were taken from 0 to 10 cm, 30 to 40 cm and 50 to 60 cm depths. Each core sample was placed in separate plastic bags, labeled and stored on ice for transportation back to the lab where they were refrigerated at 4 degrees centigrade overnight. The next

day the mycorrhizal and fine roots were carefully removed by hand (Dähne et al, 1995) and the fresh soil was sieved (4 mm plastic sieve) to retain a 100 gram sample (Mettler P1200 Weigh Scale, Mettler Waagen GmbH, 63 Giessen 2, Switzerland), which was placed into a labeled plastic Ziploc bag and stored in the dark at 10°C until analyzed. The soil samples were double bagged to preserve moisture. Precisely weighed soil samples were oven-dried in glass beakers for a period of 3 days at 60 degrees centigrade in a Memmert drying chamber (Memmert GmbH, Schwabach, Germany) and then reweighed The difference between the fresh and dry weights was used to calculate the percentage moisture content of the soil (Section C1). After determining the moisture content, oven-dried soil samples were passed through a 1 mm stainless steel sieve to improve soil surface area, in preparation for pH testing (Butzke, 1969).

C2-2B: Preliminary Testing Procedures

In preliminary testing of extra soil samples, ten (100 mg) samples were air-dried and weighed every 12 hours until two standard readings were achieved (Carter, 1986). The same number of soil samples were oven dried at 60 degrees centigrade for a number of days until at least two standard readings were achieved over 12 hours. The pH readings (Section C2-2C) of sieved soil samples was recorded before (moist) and after (dry) oven or air desiccation to determine if the mode of drying the sample would have an adverse effect on the pH readings. Three modes of reading pH ($CaCl_2$, H_2O and KCl) were tested (See C2-2C) for procedures.

The results of the preliminary tests are presented in Appendix 9B. The air drying method recommended in the literature proved to be highly dependent on the relative humidity, more time consuming and less consistent than slow drying in a standardized desiccation chamber for 3 days at 60 degrees centigrade. Both modes of desiccation produced nearly identical pH readings. Moist soil was difficult to sieve (1 mm diameter), lost weight due to evaporation and gave higher and more variable pH readings. Since oven-dried samples were easier to accurately measure and more consistent in their pH readings than the moist soil, this technique was chosen as a standard parameter.

C2-2C: pH Testing

Three methods of determining pH using H_2O, $CaCl_2$ (Meiwes et al, 1984) and KCl (AFZ, 1983; Meiwes et al, 1984) were used for ease of comparison to literature sources. To standardize the testing procedures, a sample:solution ratio of 1:2.5 (Canada Soil Survey, 1978; Carter, 1985) was used. In each case, after combining the soil with the appropriate reagents, the solutions were thoroughly mixed using a magnetic stirrer for one hour (Haese, Arbeitsgruppe Rothe, p.c. 2001) to equilibrate the temperature of the electrodes, solutions and soil. At the end of the hour, the pH value was obtained using a standard pH meter (Knick Digital 646) with glass electrodes washed and stored in the appropriate solutions between readings to assure consistency. Readings were recorded when the values remained unchanged for a minimum of 30 seconds.

Method 1: pH H2O. Of the three methods of determining pH, the most direct method, pH H_2O, measures the *actual* acidity. For this method, 10 grams of sieved fine (dry) earth was suspended in 25 ml of distilled water. After being thoroughly mixed using a magnetic stirrer for one hour, the pH value was obtained.

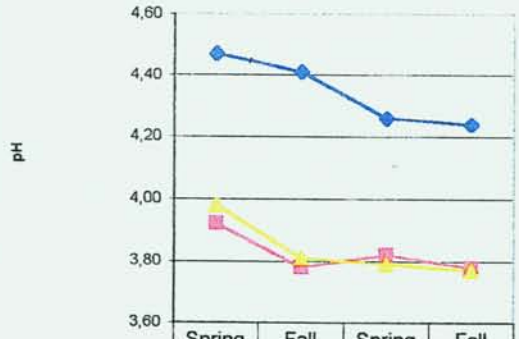
Method 2: pH CaCl2. The second method, pH $CaCl_2$, measures *total* acidity. About 10 grams of sieved (dry) soil was added to 25 ml of 10 mmol/liter Ca Cl_2 (alkaline) solution. After mixing for one hour with a magnetic stirrer, the pH values were obtained.

Method 3: pH KCl. For the third method, pH KCl, was used. Ten grams of dry soil was mixed with 25 ml of 0.1 mol/l KCl solution. Three mol/l KCl was diluted with distilled water to a ratio of 1:30 to create the acidic 0.1 mol/l KCl solution (AFZ, 1983). After 60 minutes of mixing with a magnetic stirrer, the *total* pH of the suspension was determined.

C2-3: Results

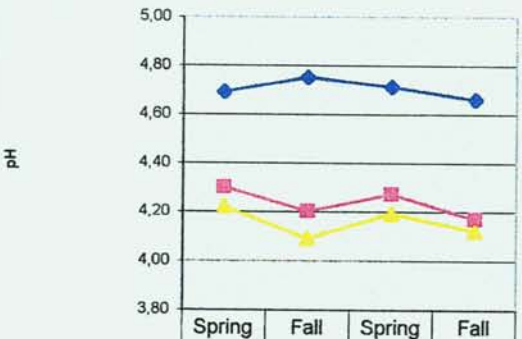
The raw data sets for the pretests and tests are presented in Appendix 9B. Graphs C2-1 through C2-6 show the average pH readings for the limed and unlimed probes for spring 1999, fall 1999, spring 2000 and fall 2000 for all three of the pH test procedures.

Graph C2-1: : Average pH values for soil samples from 0-10 cm depth in Limed soil of an Oak Forest



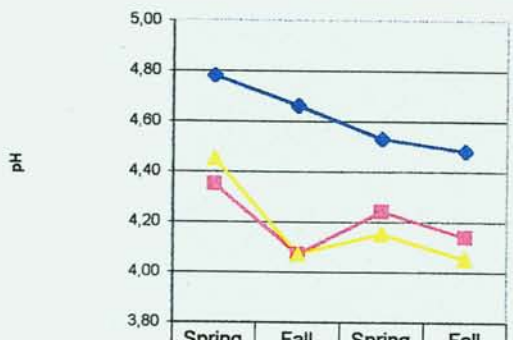
	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	4,47	4,41	4,26	4,24
pH CaCl2	3,92	3,78	3,82	3,78
pH KCl	3,98	3,81	3,79	3,77

Graph C2-2: Average pH values for soil samples from 30-40 :m depth in Limed soil of an Oak Forest



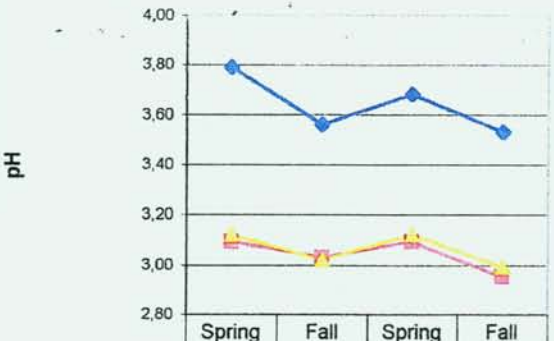
	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	4,69	4,75	4,71	4,66
pH CaCl2	4,30	4,20	4,27	4,17
pH KCl	4,22	4,09	4,19	4,12

Graph C2-3: Average pH values for soil samples from 50-60 cm depth in Limed soil of an Oak Forest



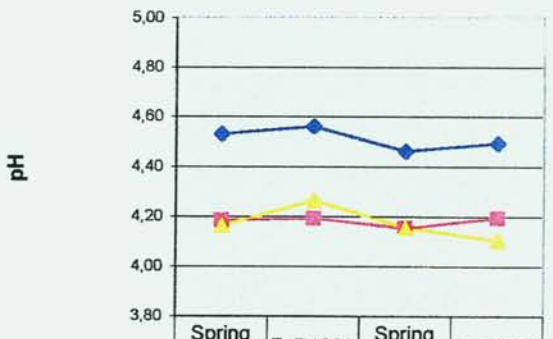
	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	4,78	4,66	4,53	4,48
pH CaCl2	4,35	4,07	4,24	4,14
pH KCl	4,45	4,07	4,15	4,05

Graph C2-4: : Average pH values for soil samples from 0-10 cm depth in Unlimed soil of an Oak Forest



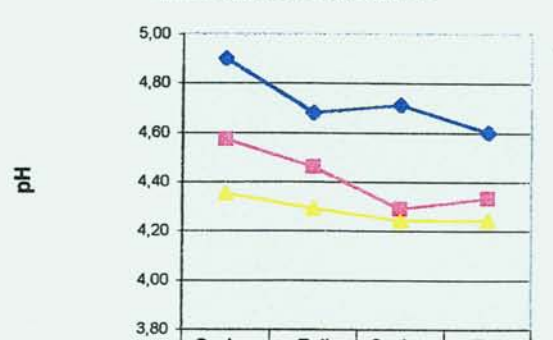
	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	3,79	3,56	3,68	3,53
pH CaCl2	3,09	3,03	3,09	2,95
pH KCl	3,12	3,02	3,12	2,99

Graph C2-5: Average pH values for soil samples from 30-40 cm depth in unlimed soil of an Oak Forest



	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	4,53	4,56	4,46	4,49
pH CaCl2	4,18	4,19	4,15	4,19
pH KCl	4,16	4,26	4,15	4,10

Graph C2-6: Average pH values for soil samples from 50-60 cm depth in Unlimed soil of an Oak forest



	Spring 1999	Fall 1999	Spring 2000	Fall 2000
pH H2O	4,90	4,68	4,71	4,60
pH CaCl2	4,57	4,46	4,29	4,33
pH KCl	4,35	4,29	4,24	4,24

C2-4: Discussion

C2-4A: Reliability of the pH Tests

According to Meiwes et al (1984), inaccuracy in pH readings can become evident if the electrodes, solutions and the soils are not at the same temperature, which is especially true if the soil is very cold and the probe and solutions are warm. A mixing time of one hour at room temperature seemed to provide good results with pH readings stabilizing in under 3 minutes for most $CaCl_2$ and KCl tests with the acidic KCl providing the most rapid and consistent readings. The pH H_2O readings generally took longer (4-5+ minutes) to stabilize and were more variable. Readings were recorded when the pH remained unchanged for a minimum of 30 seconds.

In the preliminary tests (Appendix 9B), the pH (H_2O) tests were found to be the most variable in range (+0.07 to -2.08) when compared to the pH $CaCl_2$ (-0.06 to -0.75) and pH KCl (-0.07 to -0.81) test methods. The pH (H_2O) may be the preferred method for field work due to its simplicity, but pH values obtained from fresh soil in as little as 5 to 10 minutes after a good suspension with either $CaCl_2$ or KCl, give more rapid and consistent readings. The water pH method also gave the highest pH readings, averaging 1 to 2 pH increments above the $CaCl_2$ and KCl values. The pH $CaCl_2$ readings averaged 0.03 (to maximum of 0.1) pH increments above the KCl values. Although the $CaCl_2$ readings tended to be less acidic than KCl readings, the seasonal fluctuations were nearly identical (Graphs C2-1 to C2-6). The water pH method, though not very stable, was a good indicator of momentary pH fluctuations, while the $CaCl_2$ and KCl methods gave more reliable estimates of potential total pH due to H^+ ion release. The pH (H_2O) tests are used to measure the *actual pH values* while both pH $CaCl_2$ and pH KCl tests are used to determine *potential acidity* due to the extra release of H^+ ions into solution (Evers & Shöpper, 1988; Rothe, 2004, pc).

Overall, the pH H_20 values were significantly and consistently higher than the measured $CaCl_2$ and KCl values by a factor range of 0.30 to 0.59 pH units with an average

difference of 0.44 units. Between H_2O and $CaCl_2$ the average difference was 0.52 units and between H_2O and KCl the average difference was 0.46 units which was found not to be a significant difference. The $CaCl_2$ and KCl values were very similar in most cases with a factor range of –0.01 to 0.13 and an average non-significant difference of 0.043 units. The values for all three tests tended to follow the same general patterns, rising or falling in seasonal fluctuations dependent upon depth and liming treatment. The results of the preliminary tests supported the observation that "$CaCl_2$ (was) a very stable reagent providing consistent results over time and a variety of concentrations (up to 100 fold increase)" (Hiatt, 1967). KCl was considered to be less stable with its physiological pH changing with concentration from a neutral substance at 10^{-5} KCl, to alkaline at 10^{-4} and 10^{-3}, to acidic at 10^{-2} (Pitman, 1970). At the concentration used here, the 10^{-1} KCl pH tests proved to be very reliable.

Soil under pH KCl of 3.02 is considered to be strongly acidic (Evers & Schöpper, 1988). Using a 0.1 mol/l KCl solution with air dried soil, pH readings were obtained to establish soil health criteria (AFZ, 1983). According to the AFZ (1983), an average humic soil pH KCl reading above 3.35 was found in 47% of the "healthy" stands of mixed spruce, white fir, pine and beech, while a pH of 3.06 was associated with 33% of the "weakened" stands, and a pH of 2.94 or less was present in 20% of the "very damaged" stands. Using the standards just mentioned, the unlimed forest region was in the "weakened" to the "very damaged" categories, while the limed forest was "healthy".

Dry soil consistently averaged nearly a full pH (-0.96) unit lower than wet soil in the pH (H_2O) test, but only 1/2 a pH (-0.41) unit lower in the pH ($CaCl_2$) test, and only 1/3 of a pH (-0.29) unit lower in the pH (KCl) test (Appendix 9B). For the purposes of this study it needs to be noted that within a living, hydrated, microecosystem the actual pH of the soil is most likely slightly more alkaline than the readings presented here. If we consider the hydrated pH values then it is quite possible that trees with adequate moisture in the humic soils may have a higher general soil pH and so not be quite so stressed as drier

trees in the same vicinity. Despite this site to site variation, the actual pH readings were all well within the range of aluminum solubilization.

C2-4B: Aluminum Solubility & pH

According to the results of the soil analysis (Graphs C2-1 to C2-6) all the soil samples tested registered below pH 5 confirming that the mycorrhizal roots were exposed in all the collected probes to soil solutions probably containing free aluminum ions. The most acidic soils (pH 2.95 - 3.79) were found at 0-10 cm depth in the unlimed forest zone (Graph C2-4) and so theoretically these roots should have had the greatest aluminum ion exposure. The next most acidic soil samples (pH 3.77 - 4.47) were found at 0-10 cm depth in the limed forest zone (Graph C2-1). Theoretically then, this zone should have had the second highest free aluminum ion content. Seasonal fluctuations in pH in both zones (Graphs C2-1 and C2-4) were comparable and both zones exhibited temporal decline in pH values over the two year period.

With increasing depth, the soil pH became more alkaline in both the unlimed (Graphs C2-5 and C2-6) and limed (Graphs C2-3 and C2-4) forests, so theoretically should have had less aluminum freely available. The pH fluctuations between the unlimed and limed zones at 30-40 and 50-60 cm depths were not identical, but they did exhibit a temporal decline in pH values over the two year period. A temporal decline in pH implies greater availability of solubilized aluminum. The actual Al contents of the mycorrhizae will be qualitatively (Section F) and quantitatively (Sections G and H) demonstrated.

C2-4C: Temporal Trends in Acidification

In 1988, at 0-5 cm depth, the average acidity of soil in the Merzalben region was 3.5, and at 10-30 cm depth it was 4.3, with an overall average of 3.9 for the region (BMELF, 1997) (See Section A3-5-4). In 1993 the pH ($CaCl_2$) the soil at 0-5 cm depth was pH 3.5, at 5-10 cm the soil was pH 3.9, and from 10-60 cm depths it was 4.3 pH, while the litter layer varied from pH (KCl) 3.9 to pH ($CaCl_2$) 4.1 (Block, 1993; Section A3-5-5). From 1999 to 2000, the average pH ($CaCl_2$) acidity of the soil at 0-10 cm depth was 3.83 in the limed area but only 3.03 in the unlimed zone. Combining the B and C horizons, the average

pH($CaCl_2$) was 4.3 (unlimed forest) and 4.2 (limed forest) in the same time period. Over the last 12 years there has been a very slow temporal acidification trend which was more evident in the unlimed forest. Liming slowed acidification in the upper horizon, but a slightly more pronounced acidification front moved down into the lower horizons.

C2-4D: Seasonal pH Variations

In the limed forest, at all depths the pH ($CaCl_2$) values dropped in the fall and rose in the spring (Graphs C2-1, C2-2, C2-3). In the unlimed forest, the lower pH ($CaCl_2$) values dropped in the fall and rose in the spring at 0-10 cm depth (Graphs C2-4), but the seasonal trends were reversed or lost in the lower horizons (Graphs C2-5, C2-6). Liming induced a modified response to seasonal acidification at all soil horizons.

C2-4E: Liming Effects upon pH values

The greatest effect upon pH with liming occured at the 0-10 cm horizon where the pH was ameliorated by a factor of approximately 0.73+ units. Although the pH readings at 30-40 cm depth were significantly different, the difference was due to opposing seasonal variations rather than a great spread in pH values. At this intermediate horizon, the limed probes averaged only 0.08+ pH units higher than the unlimed. At 50-60 cm depth, an acid front was very evident in the limed soils with the limed soils actually becoming more acidic than the unlimed by a factor of approximately 0.14- units. The differences in pH values were significant at all depths. Liming definitely affected the pH. According to Hildebrand (1999), liming should improve the Ca/Al ratios in the Ah an Bv horizons (the C horizon was not examined). By converse logic then, the C horizon of the limed zones should have more freed Al than the unlimed soil, based upon the pH values.

What will be interesting to note in the next two sections of this report is the enhanced uptake of aluminum in the roots from the A and B horizons of the limed forest. In face of the fact of the lowered free aluminum content in the runoff water found in tests performed by the Trippstadt forestry department (Block, 1999 pc), and supported by the observations of other research (Hildebrand, 1999), we have a dilemma. The runoff water is being used as a measure of "successful" amelioration of freed aluminum ions. The assumption being

that the ions were being held in the soil and not freed to the soil solutions. The reality being, as will be shown shortly, that the freed ions are probably being effectively moved into the rhizosphere and into the roots due to the presence of calcium "carriers" and proton "wires" which more than adequately accounts for their absence in the runoff waters. Aluminum has been shown to accumulate in limed roots of *Picea abies* (Vogelei & Rothe, 1988, 1993; Nowotny et al, 1998).

C2-4F: pH & Mycorrhizal Diversity

From Section B of this report, it was noted that liming affected not only mycorrhizal diversity and dominance hierarchy but also the abundance of various mycorrhizal species at different soil depths. In general, the limed oak roots had more mycorrhizal diversity but less overall individual species abundance (K selection) while the unlimed roots had lower ectomycorrhizal diversity but more overall abundance of each represented species (R selection) (Sections B; H4-2F). From the pH analysis, the limed (pH H_2O range 4.24 to 4.76) and unlimed (pH H_2O range 3.53 to 4.9) plots varied significantly in pH at all depths. The measure of unbound (Section G) and bound (Section H) Calcium and Aluminum contents, and species-specific Al uptake (Section F) will be discussed separately. Initially it was difficult to say with any certainty, which had the greatest effect upon oak-mycorrhizal diversity in the *limed plot,* liming shock, the increased availability of Calcium, the definitive rise in pH, moisture changes, or the altered accumulation of ions. As will be shown in Section F (Chart F17-1) all of these factors are interactive. Beyond that, each oak-mycorrhizal species is independent in its responsiveness and uptake of specific ions (Sections B, E, F, G, H). For now we will deal only with general mycorrhizal trends which do tend to indicate that pH is a prime factor controlling mycorrhizal diversity and abundance.

In studies of the effects of pH upon mycorrhizal growth and survival, it was determined that a change in pH affects diversity, morphology and survival of the mycorrhizae and the biomass of seedling root systems, but the effects are not consistent from species to species or from host to host (Literature review by Singh, 2000). The majority of the studies to date deal with immature trees and seedlings of host species other than the mature oaks

represented in this study. Despite this, the findings have relevance in that they tend to indicate intricate host-mycorrhizal relationship. Short term exposure of neutro basic pH substrates to acids (pH adjusted to: 7.5, 6.0, 4.5 and 3.0) enhanced ectomycorrhizal formation in some cases *[Pinus-Pisolithus* (Honrubia & Diaz, 1996), *Eucalyptus-Laccaria laccata*-Isolate A (Thomson et al, 1996)], but did not necessarily increase plant growth. In other cases, total ectomycorrhizal infection was not altered by pH 5.5 or 7.5, but the (*Quercus palustris)* seedling biomass was augmented in acidic soils (Hauer & Dawson, 1996). In yet other cases, the ectomycorrhiza (except for *Paxillus involutus)* showed similar changes in infection potential in response to pH changes, irrespective of whether lime or ash had been added to *Pinus sylvestris* soils (Erland & Soderstrom, 1991). This last example implies that variation in pH was more significant than Calcium upon general mycorrhizal metabolism. In the case of *Paxillus involutus*, labeled Ca uptake was enhanced at pH 4, but at higher pH levels, the Ca uptake did not differ from non-mycorrhizal plants (Andersson et al, 1996). In this report we will see later that the uptake of Calcium was strongly influenced by pH effects on specific mycorrhizal species rather than by pH alone (Sections G & H).

We do know that lower pH values, lower Ca and higher Aluminum concentrations can negatively impact both non-mycorrhizal (Vogelei & Rothe, 1988) and mycorrhizal (Vogelei & Rothe, 1993) Norway Spruce roots in unlimed soils. The fine roots of Norway spruce accumulated large amounts of Al irrespective of their mycorrhizal state if exposed to acid nutrient solutions or an acidified soil of pH 3.5 (Vogelei & Rothe, 1993).

In a study by Nowotny, Schwanz and Rothe (1998), soil acidification stimulated the activities of three enzymes (glucosephosphate isomerase, pyruvate kinase and phosphogluconate dehydrogenase) by a factor of 1.5. The stimulatory effect was limited to mycorrhizal roots from the humus with no effect on mycorrhizal roots from the upper mineral (0-5 cm) soil. Liming and compensatory liming had no influence on the enzyme activities although short term seasonal cycling was apparent. Within the same study, mycorrhizal roots from the humus had approximately ten times more citrate and two times more malate than mycorrhizal roots from the upper soil. In this instance however, acid

treatment decreased and liming increased both citrate and malate contents in the mycorrhizae from the humic soil but had no effect on the mycorrhizae from the mineral soil. It is probable that pH had a direct influence on the metabolic activities described, but only as moderated by the mycorrhizal species involved. Of the various explanations for the discrepancies between the soil regions, the one that stands out is a dependency upon the types of fungi which participate in the ectomycorrhizal symbioses. *Amphinema byssoides*, *Tylospora* species and *Piceirhiza nigra* are more frequent in humus than in the upper mineral horizon whereas the fungi *Elaphomyces* species and *Russula orchroleuca* are more frequent in the upper mineral soil than in humus (Brand and Taylor, 1991, p.c. In: Nowotny, Schwanz & Rothe, 1998).

Higher pH values are also known to impact mycorrhizal roots. From a study of Norway spruce (*Picea abies*) by Nowotny et al (1998), after liming there was a shift in fine roots to the humus layer as well as an increase in mass of the mycorrhizal roots in both the humus and the upper mineral soil (0-5 cm) with both attributed to an increase in pH to 4.5 rather than alleviation of Al toxicity. The quantity of "free" Al in the mycorrhizal roots of the humus were 1/2 those of the upper mineral soil, however despite the fact the upper mineral soil mycorrhizae contained considerable quantities of Al it did not seem to be toxic. The aluminum may not be toxic at higher pH values because it is immobilized by chelators (Sections A3-6-2, A3-6-3, A3-9). Nowotny et al (1998) suggested that the aluminum may be tied up in polyphosphate granules present in the hyphae of some ectomycorrhizae such as *Paxillus involutus* (Turnau et al, 1993) and *Laccaria bicolor* (Martin et al, 1994). Once past a mycorrhizal barrier, the Al may be immobilized in the cortical walls where it is know to strongly bind (Vogelei and Rothe, 1993) and should it enter the root cell cytoplasm, because of the neutral pH value of the cytosol it may complex with proteins (Aniol, 1984; Rengel, 1992), silic acid (Exley et al, 1994) or phospates (Section A3-9-2). In concert with all the probable explanations of why the aluminum was not toxic, the distribution of ectomycorrhizal species is more than likely to play a major role. "Under liming (of the Höglwald spruce forest) the fungi *Amphinema byssoides*, *Piceirhiza nigra* and *Tuber puberulum* appeared and made about 50% of all mycorrhizal fungi in the humus (Brand and Taylor, 1991, p.c.). This effect can be

attributed to a preference of these fungi for high pH values (or higher Ca-values (cf. Agerer et al., 1986) and a better availability of organic nitrogen compounds (cf. Dähne et al, 1995) " (Nowotny et al, 1998). The actual mechanism of detoxification is not known but as will be shown, aluminum sequestration in oak-mycorrhizae is related to both the pH of the soil and the specific fungal species that inhabit the rhizosphere (Sections G & H).

Clearly, we are dealing with a very complex picture of individual species-specific responses and interdependent host-species interactions in response to environmental stimuli. What can be stated is that pH is a critical contributing factor in mycorrhizal growth, survival and diversity.

C2-5: Conclusions

Both $CaCl_2$ and KCl pH tests gave similar, reproducible, results with the only qualification being that the actual pH values in hydrated soils in the natural environment are probably 0.5 to 0.3 pH units higher, respectively. The Merzalben forest soils have become more acidic since 1988 with the effects being most pronounced in the unlimed area used in this study. While temporal acidification is more evident in the upper horizons of the unlimed forest, an acidification front is more evident in the deep soils of the limed forest. The pH values fluctuated seasonally, with the fall values tending to be slightly lower than the spring in the upper soils of both zones. In deeper soils, these same seasonal fluctuations were evident in the limed soils but not the unlimed. All the soils were within the pH range needed for Al solubilization.

C3: Root Biomass Comparison of Unlimed and Limed Soils

C3-1: Introduction

Fine roots along with their mycorrhizal partners are essential for nutrient and water absorption (Section A1-3). The relative abundance of fine roots in comparison to the larger secondary roots, and total root biomass in different soil horizons, can be indicators of health. These methods of health determination are infrequently used however since it is expected that removal of roots can potentially harm the trees. Instead measurement of non-invasive growth factors such as crown dieback, leaf yellowing, light penetration in

summer, crown shape in winter and fruiting strength are generally used (Section A4). According to the analysis of these later factors, the trees in the Merzalben forest (Appendix 3), the majority of the oak trees in both the limed and unlimed zones were in damage class 2. Evaluation of the light penetration of the crowns in 2003 indicated that the trees of the limed region were in better overall health in comparison to those in the unlimed zone (Section A1-4-2).

Since root samples were removed to obtain the mycorrhizal tips, it seemed prudent to evaluate the relative and total root biomass in the unlimed and limed probes. It will be shown that abundance of fine and larger roots varied with season, depth, drought and with the liming treatments. During drought root biomass declined in the upper soils, but some mycorrhizae and larger roots exhibited a vertical shift to the moister, lower horizons.

C3-2: Materials & Methods

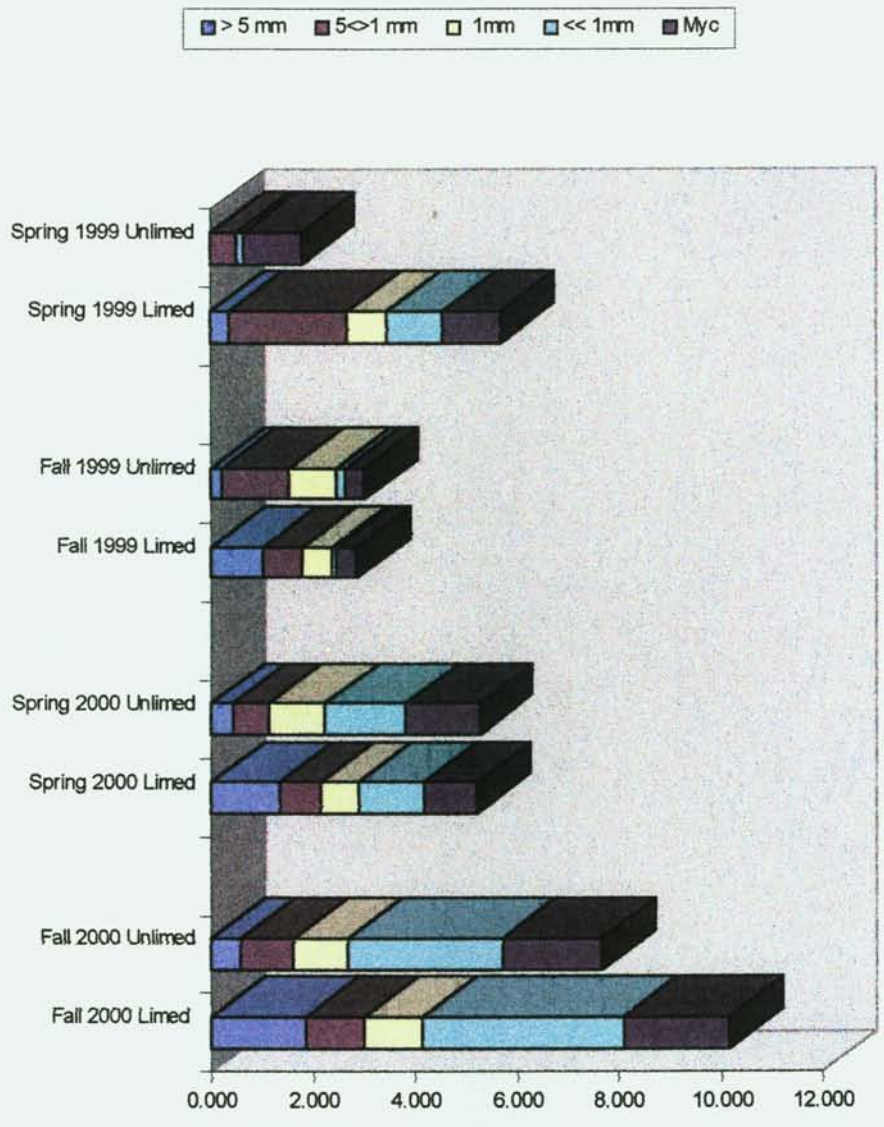
See Sections B2-1 to B2-4 for tree selection, soil sampling, root isolation and cleaning procedures. After cleaning, the mycorrhizae were removed (See Section B2-5) and the remaining roots were sorted by diameter and classified as very fine or finest (<< 1 mm), fine (1 mm), large (5< > 1 mm), and very large (> 5 mm) (Vogelei & Rothe, 1991). The wet roots were spread out and allowed to air dry for 2 hours. The remaining soil debris was shaken or gently brushed away. The roots were weighed to obtain a "fresh" weight and then allowed to completely air dry in the dark, and were stored in ziploc bags at room temperature in the dark. The finest and fine ($\leq$ 1 mm diameter) roots were used for mineral analysis. Information concerning the mycorrhizal components was added to the data after the mycorrhizal root tips were sorted and identified (See Sections B2-5,6,7).

C3-3: Results

Estimations of limed and unlimed biomass abundance per square hectare (Tables 9C-1 to 9C-3) for various soil depths and seasons and significant differences are presented in Appendix 9C. Raw data (Tables 9C-4 to 9C-7) are also in Appendix 9C. Formulas for statistical analysis are presented in Appendix 9D. Comparisons of the unlimed and limed probes containing roots of the various diameters are averaged and presented in Graph C3-

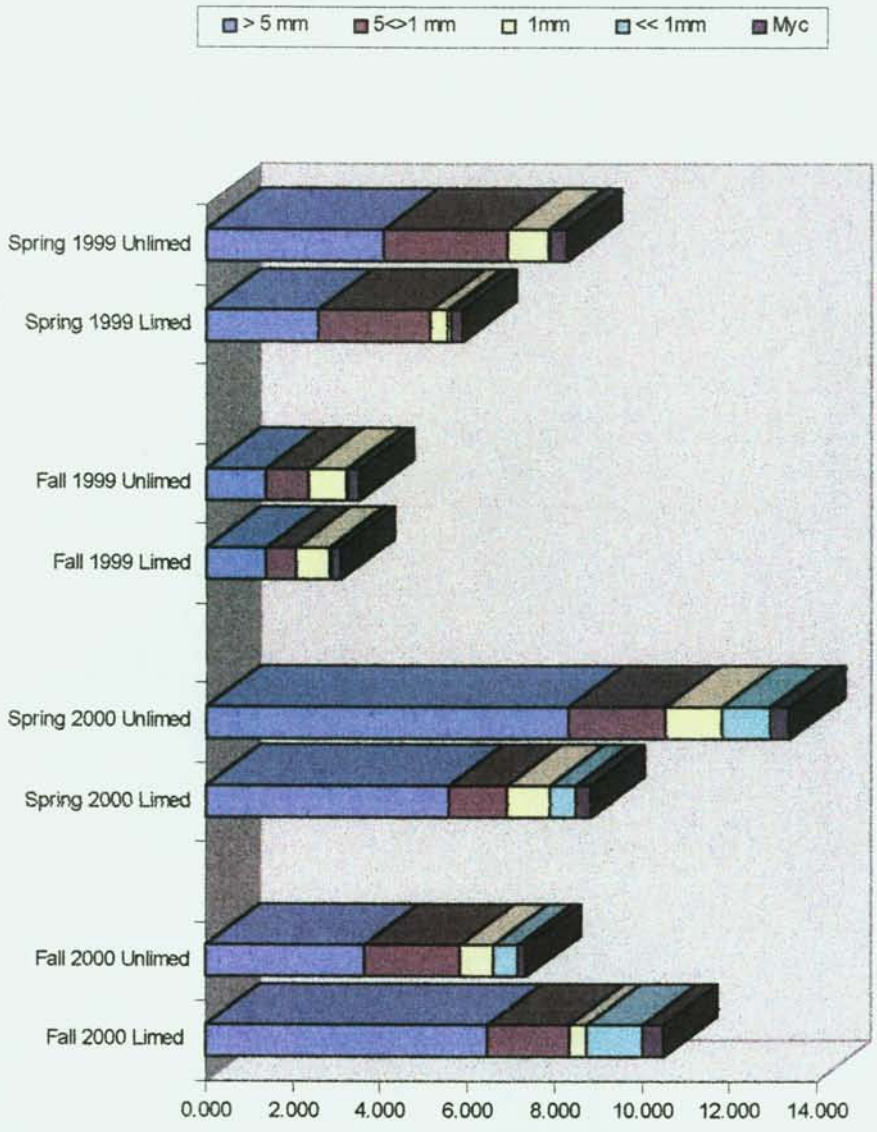
1 (0-10 cm depth), Graph C3-2 (30-40 cm depth) and Graph C3-3 (50-60 cm depth) for all four seasons. The bar graphs are additive but not cumulative. For example, in fall 2000, at 0-10 cm depth, the limed probes, on the average, contained approximately 2 grams of very large (> 5 mm diameter) roots, 1 gram of large (5< > 1 mm) roots, 1 gram of fine (1 mm), 4 grams of very fine (<< 1 mm) roots, and 2 grams of mycorrhizal (Myc) tips for an average grand total of approximately 10 grams of root per 10 x 10 cm cylinder of extracted soil. A total of 126 probes were analyzed (63 from the unlimed and 63 from the limed forest). One third of the probes were from horizon A, 1/3 from horizon B and 1/3 from horizon C. A more accurate representation could have been made if more probes were used since the standard deviation in a few cases was large, where some probes had no roots of a specific diameter, while due to the random sampling, others had many. Despite this, the final graphs presented are a fairly accurate representation of the distribution of roots in the unlimed and limed zones at various seasons and soil depths.

Graph C3-1: Comparison of unlimed and limed roots of various diameters from very large (>5 mm) to large (5<>1 mm) to fine (1 mm) to very fine (< 1 mm) plus finest mycorrhizal tips (Myc) at 0-10 cm depth over 4 seasons.



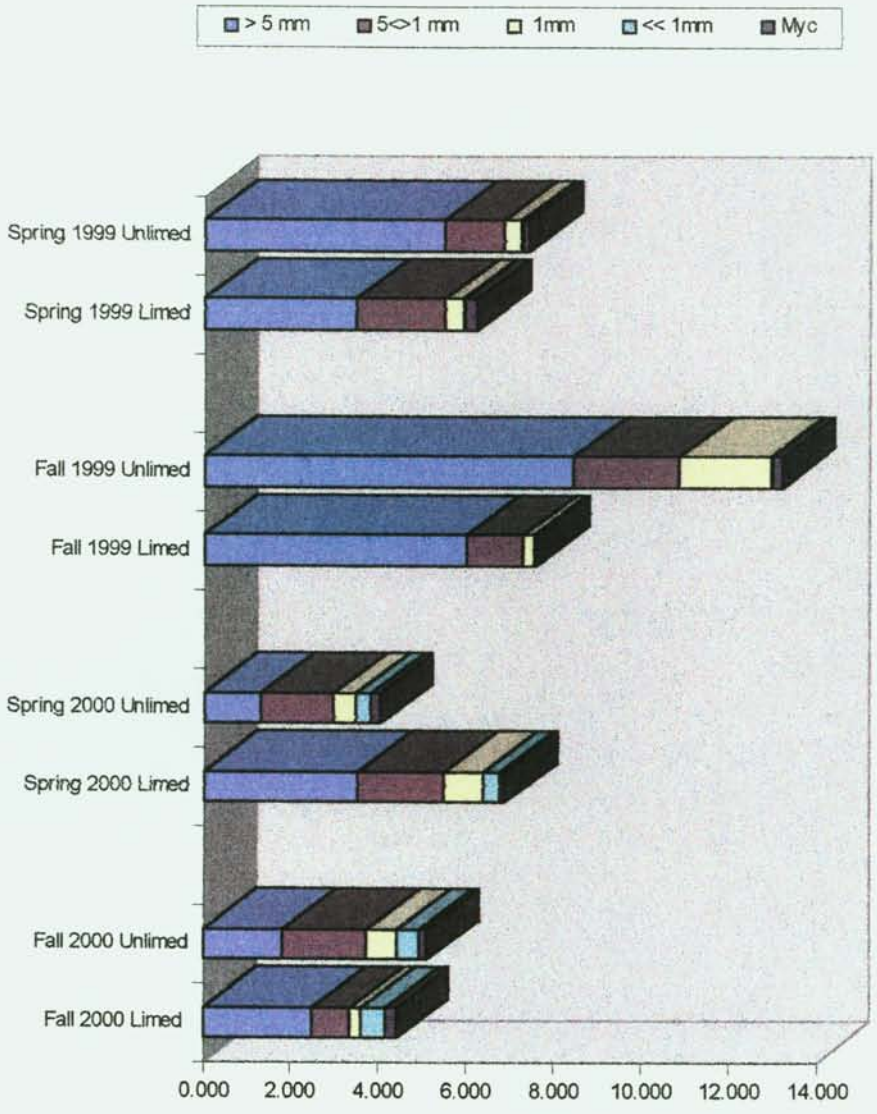
Weight in grams averaged from collected probes. Each root type is independent rather than cummulative in weight.

Graph C3-2: Comparison of unlimed and limed roots of various diameters from very large (<5 mm) to large (5<>1 mm) to fine (1 mm) to very fine (<1 mm) plus finest mycorrhizal tips (Myc) at 30-40 cm depth over 4 seasons.



Weight in grams averaged from collected probes. Each root type is independent rather than cummulative in weight.

Graph C3-3: Comparison of unlimed and limed roots of various diameters from very large (>5 mm) to large (5<>1 mm) to fine (1 mm) to very fine (<1 mm) plus finest mycorrhizal tips (Myc) at 50-60 cm depth over 4 seasons.



Weight in grams averaged from collected probes. Each root type is independent rather than cummulative in weight.

D3-4: Discussion

From the statistical analysis it was determined that the TOTAL biomass of the unlimed and limed probes was significantly different for all depths and seasons except at 0-10 cm (fall 1999, spring 2000), 30-40 cm (fall 1999) and 50-60 cm depth (spring 1999 and fall 2000). Total biomass was the least during the drought and greatest in the recovery period for both forest zones. In the unlimed plot, the greatest biomass was in the deeper horizons, especially during the drought. In contrast, in the limed plot, the greatest biomass was in the upper most horizons, except during the drought when it declined abruptly.

D3-4A: Largest (> 5mm) Roots

With respect to the largest roots (> 5 mm) (Graphs C3-1 to C3-3, purple bars), there was a significant difference between the unlimed and limed probes except for fall 1999 at 30-40 cm depth. In both the zones, during the dry fall 1999, the largest roots were found at 50-60 cm, but with the return of moisture the following spring and fall, the roots at 30-40 cm had the largest average diameters. It is known that during a dry period carbon allocation shifts from the leaves to lower stem and roots for growth or storage and that many oak species, including *Quercus petraea*, are morphologically and physiologically adapted to difficult sites (Dickson & Tomlinson, 1996). It is believed that tap roots, penetrating 3 to 5 meters plus, confir drought resistance (avoidance) due to their access to the deep ground water (Dickson & Tomlinson, 1996). In confirmation, during the fall 1999 drought, biomass is shifted, not just to the roots in general, but rather to the deeper, larger roots. This temporal physiological biomass shift was associated with a corrresponding shift in mycorrhizal infection, especially in the unlimed soil (Section B).

D3-4B: Large (5< >1mm) Roots

With respect to the large (5< > 1 mm) roots (Graphs C3-1 to C3-3, red bars), there were significant differences between the unlimed and limed probes except for 0-10 cm (fall 2000), 30-40 cm (spring 1999, fall 1999, fall 2000), and 50-60 cm (spring 2000). In both plots, the occurrence of large roots was most similar at the 30-40 cm horizon.

C3-4C: Fine (1mm) Roots

The fine root (1 mm) biomass (Graphs C3-1 to C3-4, yellow bars) was significantly different between the unlimed and limed plots except for: 0-10 cm depth (fall 2000), 30-40 cm depth (fall 1999 and spring 2000), and 50-60 cm depth (spring 1999). It is generally known that the abundance of fine roots drops with soil depth especially in acidic soils (Heinz-Werner et al, 2000). As expected, the fine roots were least abundant at 50-60 cm depth. At 30-40 cm depth the fine roots were the most abundant during and right after the drought. At 0-10 cm depth the fine roots were very common. The limed probes had some biomass loss during the drought. The unlimed probes had very few fine roots before the drought but did have fine roots during and after the drought, roughly equivalent in abundance to the limed probes. Fine roots were less abundant in deeper soils, except during drought. Recovery after drought in both zones was strong.

C3-4D: Very Finest (<< 1mm) Roots

The very finest (<< 1 mm) roots (Graphs C3-1 to C3-3, blue bars), were the most variable in the 0-10 cm depth horizon. The limed probes had the most very fine roots in spring 1999 and again in fall 2000, but in between, during, and immediately after the drought, the unlimed and limed probes were very similar at 0-10 cm depth. In deeper soils there was no significant difference between the unlimed and limed probes. In all cases the very finest roots were absent, or nearly so, in the dry spring and fall 1999 and minimally present in the moist spring and fall 2000. It can be stated that the very finest roots prefer the humic soils and that very fine roots were more common in limed soils with good moisture levels. This corresponds to the observation by Nowotny et al (1998) that after liming Norway spruce, there was a shift of fine roots to the humus layer.

C3-4E: Mycorrhizae

The mycorrhizal components of the roots were most abundant at 0-10 cm depth and declined in abundance with depth (Graphs C3-1 to C3-3, dark purple bars). In the lower horizons, the abundance of mycorrhizal tips was low but relatively constant, and in the uppermost horizon, it was much more variable. General biomass trends for mycorrhizae (Section B3-5A, Table B3-6) indicated that mycorrhizal tips were reduced in abundance at

0-10 cm depth during the dry fall 1999 and subsequently recovered the following spring and fall. The subsequent recovery was more elastic in the unlimed probes. By fall 2000 both the unlimed and limed probes had roughly the same complement of mycorrhizae with respect to gram weight, but the limed probes had more total root biomass due to a much enhanced fine root recovery.

According to the literature review by Singh (2001), moist humus supports the highest level of mycorrhizae except during drought when moister decaying wood becomes the most active site and, in addition, during drought root dormancy increases, elongation rates of parent roots declines, and fewer growing mycorrhizae are present. Furthermore, it has been observed that a decline in root biomass, vitality of fine roots and a reduction in the number of mycorrhizal apexes has been associated with reduced nutrient transport and tree decline (Literature review in Causin et al, 1996). Thomas & Hartmann (1996) found distinct reductions in fine root biomass and increased dead fine roots only in severely damaged pedunculate oak trees. They determined that root decay was not a primary factor but more likely the result of complex oak decline.

According to Causin et al (1996) there was a significant decrease in the proportion of mycorrhizae between healthy and declining 50-55 year old common oak (*Quercus robur* L.) trees, but among the already damaged trees, they found no correspondence between the probability of finding vital mycorrhizae and decline intensity. Most mycorrrhizal morphotypes were found to be distributed homogeneously in the different decline classes but a few morphotypes were especially present in the declining plants. It was determined that the occurrence of a high proportion of vital mycorrhizae could allow even the most damaged trees to produce a denser crown than the previous year.

Morte et al (2000) observed that although drought stress did not affect the amount of mycorrhizal colonization in herbaceous plants, the survival rate of mycorrhizal plants was higher than that of non-mycorrhizal plants. It was suggested that in stress tolerant mycorrhizal species, (i.e. *Suillus bovinus*) specialized hydrophobic (ho) strands may morph into hydrophilic (hi) water seeking strands altering the function of the

extramatrical hyphae to suit the microenvironment and improve survival (Unestam & Sun, 1995). Dense ho cords form when no suitable substrates are available and exude droplets at substrate interfaces, but when water is present the strands fan out into a (hi) mycelial mat. Hydrophilic species (i.e. *Cenococcum geophilum, Thelephora terrestris, Laccaria laccata*) can produce short hi strands but not the ho strands and so are limited to wicking water in moist soils (Unestam & Sun, 1995). It is most likely not the mass of mycorrhizae that determines survival but rather the combination of adaptive species.

Although this present study did not compare different decline classes, distinct variations in root biomass, mycorrhizal abundance and composition, were directly correlated to drought dieback. Decline and recovery in both unlimed and limed soils are both definitely correlated to variations in the quantities of mycorrhizae and fine roots. Beyond this, there are also shifts in biomass (Section C) and mycorrhizae (Section B) to lower horizons during periods of stress. In these depth shifts, roots growing in limed and unlimed soils differ in the strength and timing of the responses. Allowing for a lack of significant difference in the moisture content of the soils, the only other variable factors were the pH, lime and corresponding diversity in mycorrhizal dominance and abundance. Since the mycorrhizae form the most important component of the soil-root nutrient interface, it is most likely that they are the primary controlling influence for root function and survival.

C3-5: Conclusions

If it can be stated that root biomass is representative of overall tree health, then during the drought, trees in both the unlimed and limed zones suffered stress, characterized by distinctly lower root biomass in the upper soils. However, it must also be stated that the unlimed soils had more fine roots, a stronger shift to the lower horizons during the same time frame, and a faster recovery of fine roots after the dry spell. This implies that the unlimed roots were more responsive to microclimatic shifts. When adequate moisture returned to the soils in the fall of 2000, the limed roots exhibited a delayed but very strong recovery, exceeding that of the unlimed roots. Liming then can be considered a stabilizing feature inducing slower responses to environmental stimuli. Liming may be detrimental during drought, but highly beneficial during periods of adequate moisture.

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Section D: Laser Analysis of Heavy Metals and Radioactive Quantification

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Section D: Laser Analysis of Heavy Metals and Radioactive Quantification

D1: Introduction

One of the premises for studying Oak mycorrhizae was to determine if Aluminum was present in the symbiotic fungi and the associated roots. To this end fluorescence microscopy provided precise localization of aluminum within the mycorrhizae and root but it was felt that a confirmation test was required. Some preliminary testing was done to determine if Plasma Quod Laser (Inductively Coupled Plasma or ICP) analysis could be used to support the fluorescence research. It was thought that with a very fine laser beam it might be possible to determine if Al or other minerals could be precisely detected with the idea of pinpointing the Al within a root cross-section and the mycorrhizal sheath. As it turned out, the prepared specimens were not able to be selectively analyzed point by point. The organic material could not withstand the targeted energy source. However, some interesting results were obtained concerning the presence of radioactive particles, in addition to the confirmation of the presence (but not the amount) of Aluminum in the samples of *Cenococcum geophilum* which were tested. In addition to Al, the presence of Mg, Ca, Ar, Sr, St, Ba, Th, Hg, Pb and U were confirmed. Aluminum may not be the only culprit associated with oak decline. In light of this data, fresh mycorrhizae and fruiting bodies gathered from Merzalben were tested for the presence of radioactivity to determine if sufficient amounts of radioactive materials were present to be easily detected, quantified and correlated. Indeed using a standard Geiger counter, the fruiting bodies emitted radiation in excess of the background levels but the mycorrhizae did not despite the fact that known fission byproducts were present.

D2: Materials & Methods for ICP Analysis

D2-1: General Tissue Preparation

Fresh *Cenococcum geophilum* tips, which had been collected from 0-10 cm depth from unlimed and limed soils in the fall of 1999, were cleaned and identified according to the procedures outlined earlier. Within 72 hours of collection, mycorrhizal tips were excised and placed in plastic Safe-Lock 1.5 ml Eppendorf tubes. As recommended for ICP

analysis, no glass equipment was used. For the preliminary tests, a portion of the tips were cross sectioned into 5 mm long root cylinders and another portion were cut length wise into 5 mm long segments and mounted on plastic films. Normally 0.5 to 1.0 grams of material are needed for laser analysis (Kritsotakisk, Department of Geology, Johannes Gutenberg University, Mainz 1999, p.c.) but each tip used weighed about 0.001 to 0.002 grams. In addition, from both the unlimed and limed zones, three samples of 0.01 grams of *Cenococcum geophilum* mycorrhizal roots were prepared for extraction. These tips were air dried and ground to a fine powder using a ceramic mortar and pestle and the powder was placed into an eppendorf container.

D2-2: Whole Mycorrhizal Root Tip Mounting

The fresh cross and long root sections were affixed using Pattex plastic (Butylacetate + Acetone - Henkel KGaA, Dusseldorf) to firm 22 x 22 x 1mm plastic squares. The long sections were placed so that the cut interior was exposed face up, and the cross sections were placed so that the cut at 5mm from the tip was upright and exposed. The prepared sections were placed into plastic petri plates, covered to permit air circulation and allowed to air dry.

D2-3: Mineral Extraction

Using the following suggested parameters (Dr. Kritsotakisk,1999, p.c.), the ground material was further dried at 80 °C for 24 hours, in the eppendorf caplets. Normally for geological mineral analysis 5-10 grams of material is extracted in 20-25 ml of 2% HNO_3 super pure but since our sample sizes were so small, the extraction volumes were reduced. The dried ground material was extracted by adding 1 ml of 2% HNO_3 super pure to 0.5 grams of dry material and heating to 60°C for 2 to 3 hours. The Nitric acid (65% HNO_3, Merck) was diluted with triple distilled water (HPLC Grade Chromatography LiChrosolv, Merck) to obtain the final 2% solution. According to the Merck labels, 65% HNO_3 super pure may contain up to 0.005 ppm Al and the triple distilled water may contain up to 0.002 mg/l and so diluted, a 2% solution, may contain approximately 0.00207 ppm

(0.00015 ppm + 0.001938 ppm) of aluminum which was felt would not interfere with the much stronger readings expected from the root preparations (Kristotakisk, 1999, p.c.). The extract was filtered using plastic millipore filters (Filter Nr. 595, Schleicher & Schull, D-3354 Dassel, W. Germany) to obtain a clear supernatant which was stored in clean capped eppendorf caplets at room temperature.

D2-4: Plasma Quad Device

According to Ahmad et al (2001, p.126-144), Inductively Coupled Plasma (ICP) sources operate on the principle of the passage of a high voltage current through an electrically hot ionized region of an inert gas (argon) stream. "The nebulized (fine droplets in a compressed gas stream) sample is carried along a quartz tube to the head of a torch by the argon gas. Ionisation is initiated by a spark from a Tesla coil and the closed-circle motion of electrons and ions is sustained by applying a strong oscillating magnetic field from a water-cooled induction coil." Normally the samples (10-15 mm^3) which can be dissolved in a solvent or are fine powders are introduced into the ICP devices by the nebulisation process. In this experiment, whole dry organic root sections (not powders) were used in addition to the liquid (2 % HNO_3) extractions.

The limits of detection by ICP devices varies with the device and for different elements. "Absorption spectroscopy of elements based on electrothermal atomization provides the best sensitivities for the detection of aluminum (0.005 ng ml^{-1}), lead (0.002 ng ml^{-1}),and cadmium (0.002 ng ml^{-1})" but quantitative determination of over 60 metals or metalloid elements can be made depending upon sample presentation, and the production of accurate calibration curves (Ahmad et al, 2001, p. 132-3). The experimental goals were to be able to detect aluminum in very small samples (individual roots) and to establish a calibration curve for measuring aluminum content for comparative purposes.

Computer analysis of the wavelength dependence of the absorption and emission of electromagnetic radiation data produces a characteristic atomic spectrum for atoms and ions of elements. The atomic absorption/emission lines produced in hot environments

often form a symmetrical spread in wavelength or wave numbers about a peak value which is identifiable for each element as a set of spectral lines (also known as the natural linewidth) occurring with specific raw counts per second (Kritsotakisk, 2000, p.c.). Despite high resolution spectroscopy, these lines can be adversely affected by Doppler broadening (atomic motion at high temperatures), Pressure broadening (Atomic collisions), and Excitation state lifetime, and Maxwell-Boltzman distributions (Velocity distributions) (Ahmad et al, 2001, p. 127). As a result, for each type of sampling made a precise calibration scale must be established prior to comparative analysis. Interpretation of the spectral information and identification of the elements was made, with grateful appreciation, by Dr. Kritsotakisk (Department of Geology, Johannes Gutenberg University, Mainz, 2000).

D3: Materials & Methods for Radioactive Quantification

As a result of the data obtained from the ICP spectral analysis an alternative non-destructive method for estimation of the presence of radioactive isotopes was examined. On Oct 17, 2001 all the mushroom fruiting bodies collected from Merzalben from both the unlimed and limed oak forests were examined. All the samples were identified to species, where possible, prior to testing. In addition, random samples of fresh, whole mycorhizal roots of *Cenococcum geophilum* from both the unlimed and limed zones were tested. These roots were washed free of all adhering soil and 10 cm long mycorrhizal sections were removed and placed in plastic petri dishes. In addition, a variety of common objects in the lab were randomly chosen to determine back ground radiation.

Samples were placed on a selected counter top and a Standard Geiger counter, supplied with an electric current, was placed approximately 2-3 cm above the samples. Readings were taken until a 30 second mean value was established. The minimum, maxim and mean were recorded. According to Dr. Beutelmann (Department of Biology, Johannes Gutenberg University, Mainz, 2001, p.c.), the average indoor background radiation detected by this piece of equipment hovers around 10-12 becquerel units. (One becquerel

unit equals one emission per second.) The pulse counting rate is considered to be proportional to the energy of incident radiation (Ahmad et al, 2001).

D4: Results

D4-1: Laser Analysis of Mineral Extraction

According to Dr. Kritsotakisk (2000, pc), the prepared extractions were sufficient to give a reading confirming Al presence above the minute quantity possibly present in the 2% HNO_3, but the actual quantity of aluminum could be not be determined with confidence for comparative purposes . Clearly larger sample sizes would be needed to create the calibration curves necessary for quantitative analysis. This effectively eliminated the possibility of a root to root extraction analysis which was the desired outcome. As a result, traditional mineral analysis techniques, which were also very time and cost effective, were used for the root mineral quantifications and replicated fluorescence techniques were used for qualitative analysis.

D4-2: Laser Analysis of Prepared Mycorrhizal Tips

Electrothermal vaporization of the prepared whole roots resulted not in a point to point nebulization as hoped but rather a large area burn which encompassed the majority of the fine root diameter (1-3 mm) and did not allow for distinction between the mycorrhizal sheath and the root proper. Production of a finer argon stream would solve this problem, but was not possible with the equipment available. Laser destruction of the whole tiny root segments which were to be the comparative controls eliminated the possibility of creating a calibration system. In view of the calibration problem, it was decided that Al, Mg, Ca and Pb, which were the minerals of the most interest, would be tested for using traditional mineral analysis.

The nebulisation of the mycorrhizal samples did however lead to some interesting results. A sample of the atomic spectra for atoms with masses from 0 to 240 amu which were present in the *Cenococcum geophilum* dry root sections are given in Graph D-1. A clean example of the natural linewidth of aluminum is given in Graph D-2. First, abundant

Aluminum was clearly present in the limed roots. This supported the fluorescence observations of chemical reactions using the dye Morin which colormetrically indicated the presence of aluminum. Unfortunately since the calibration curves could not be established, the relative concentrations of Aluminum in unlimed and limed roots could not be quantified with confidence.

Second, the physiologically important minerals Mg and Ca were precisely identified as being present. In addition, radionuclides (Uranium (U), Thorium (Tr), Strontium (Sr) and Barium (Br)) were also present, which corresponds to previous research identifying this area as a post-Chernobyl (April 26, 1986) hot spot (Block, 1993). The heavy metals mercury (Hg) and lead (Pb) were also among the identified elements. Since research had already been done with the fruiting bodies of various mushroom species in Merzablen to detect Cesium (Cs 137 & Cs 134) and potassium (K40) (Block, 1993), the interest here was to see if individual mycorrhizae would provide enough material for simple Geiger counter testing, relative to the fruiting bodies.

D4-3: Radioactivity

The background radiation of the counter top had a range of 8-20 and a mean of 13, while room air averaged a mean of 12 becquerel units per second during the random 48 hour period of the testing . This was slightly higher than the 10-12 units expected.

The readings for *Cenococcum geophilum* mycorrhizae never exceeded the background radiation levels. The 10 mycorrhizal samples tested each had a range of 9 to 14 units with a mean of 12. In otherwords, when placed upon a countertop for Geiger counter analysis, the extracted 100± mg samples of mycorrhizal root tips could not be differentiated from the table top. The mycorrhizal roots were either too small to affect the Geiger counter or contained no radioactive elements. Since we know from the ICP analysis that certain radioactive components were indeed present, we can assume the lack of a definite response may have been most likely due to sample size. Either way since the

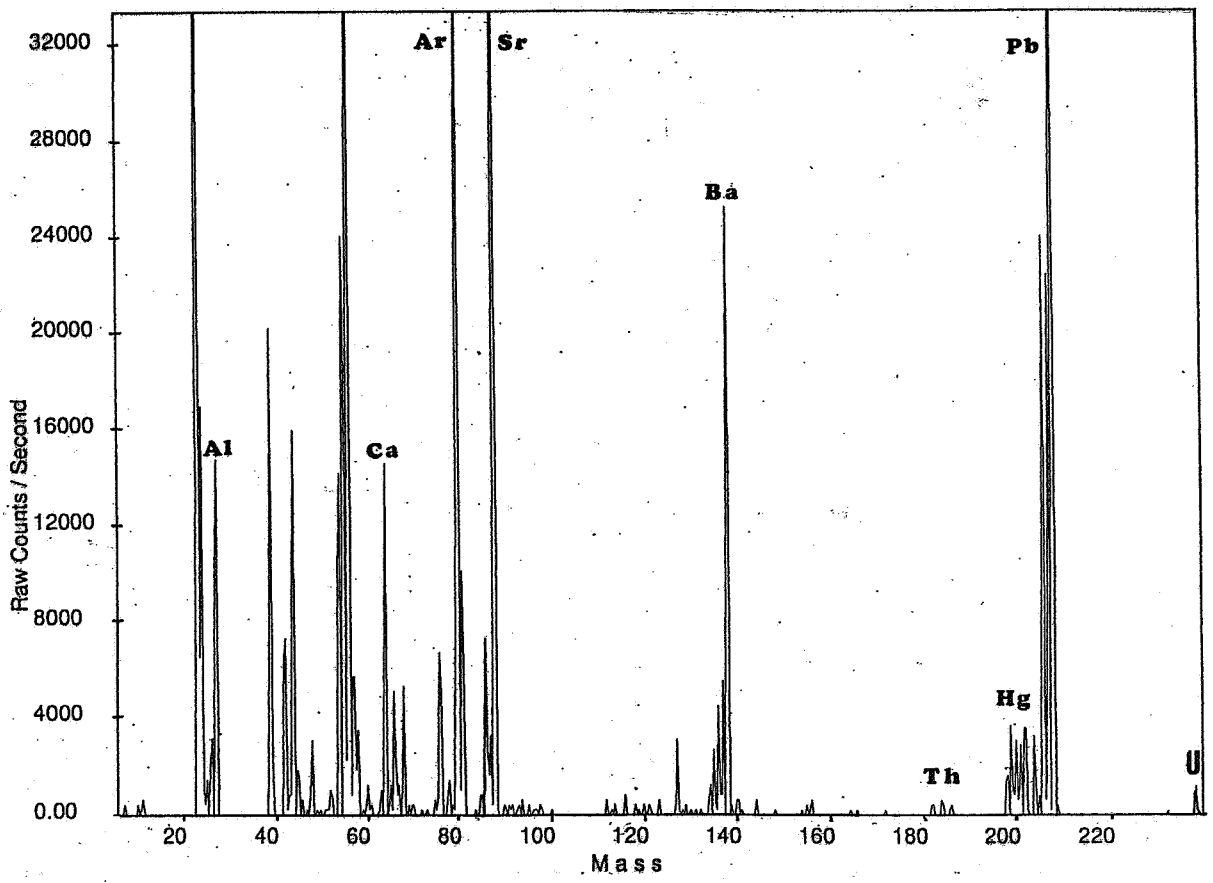
emissions, if any, were indistinguishable from back ground radiation this was not a useful procedure. On the other hand, the fruiting bodies did produce readable scintillations.

From the unlimed oak forest, 38 fruiting bodies of various species were tested (Table D1). The average minimum reading was 10.32, the average maximum reading was 18.89 and the mean was 14.05. The alarm was set off only once by *Ticholoma fulvum* with a rate of 25 emissions per second. Of the 27 mushroom fruiting bodies collected from the limed forest that were tested, the average minimum reading was 10.07, the average maximum was 18.78 and the average mean was 13.98 (Table D2) The alarm was set off only once by *Stereum rugosum* with a rate of 25 emissions per second. There was no significant difference in the overall readings of the fruiting bodies isolated from the unlimed and limed forest.

Only one acorn of *Quercus petraea* was examined along with the other random objects collected (Table D4-3-3) The acorn had a range of 10 to 15 and a mean of 12 Bq units which was well within the normal background radiation levels. Of the other items examined, the forest collection had an average range of 9.92 to 18 and an average mean of 12.92 which was slightly below that of the fungal fruiting bodies. Of the fomites examined, the average range was 9.6 to 16.6 with a mean of 13 units . Of the people examined, all of whom were right handed, the average range for their left hands was 9.6 to 15.2 with a mean of 12.4 and for their right hands was 10.8 to 18 with a mean of 14.2. Since we all worked in the forest, and handled soil and fungi, it may not have been a fluke that our radiation levels were similar to the fungi.

From this very basic testing it might be assumed that the incidence of radiation from the mycorrhizae is negligible and that of the fruiting bodies is higher than the normal background radiation. This implies a modest concentration of scintillating minerals in the mature fungal matrix.

Graph D-1 : ICP Atomic spectra for elements present in a dry root crossection of the ectomycorrhiza *Cenococcum geophilum* which was collected from 0-10 cm depth LIMED soil in a region of oak forest in Merzablen Germany in the spring of 1999. Included is the Argon laser signature (Ar). The test was originally done to determine if Aluminum (Al) was present, which it is, but there was also strong indiction of heavy metals (Pb, Hg) and radioisotopes (Th, Ba, Sr, U).



Graph D-2 : Detail of the calibrated ICP spectral lines and peaks for the elements Aluminum (Al) with an atomic mass of approximately 27 amu (actual = 26.9815 amd) and Magnesium (Mg) with an atomic mass of approximately 26 amu (actual = 24.312). Al and Mg were present in a dry root crossection of the ectomycorrhiza *Cenococcum geophilum* which was collected from 0-10 cm depth LIMED soil in a region of oak forest in Merzablen Germany in the spring of 1999. Fluorescence data indicated that Al was absent from the mycorrhiza but strongly present in the root proper. The ICP process was unable to distinguish between the tissues.

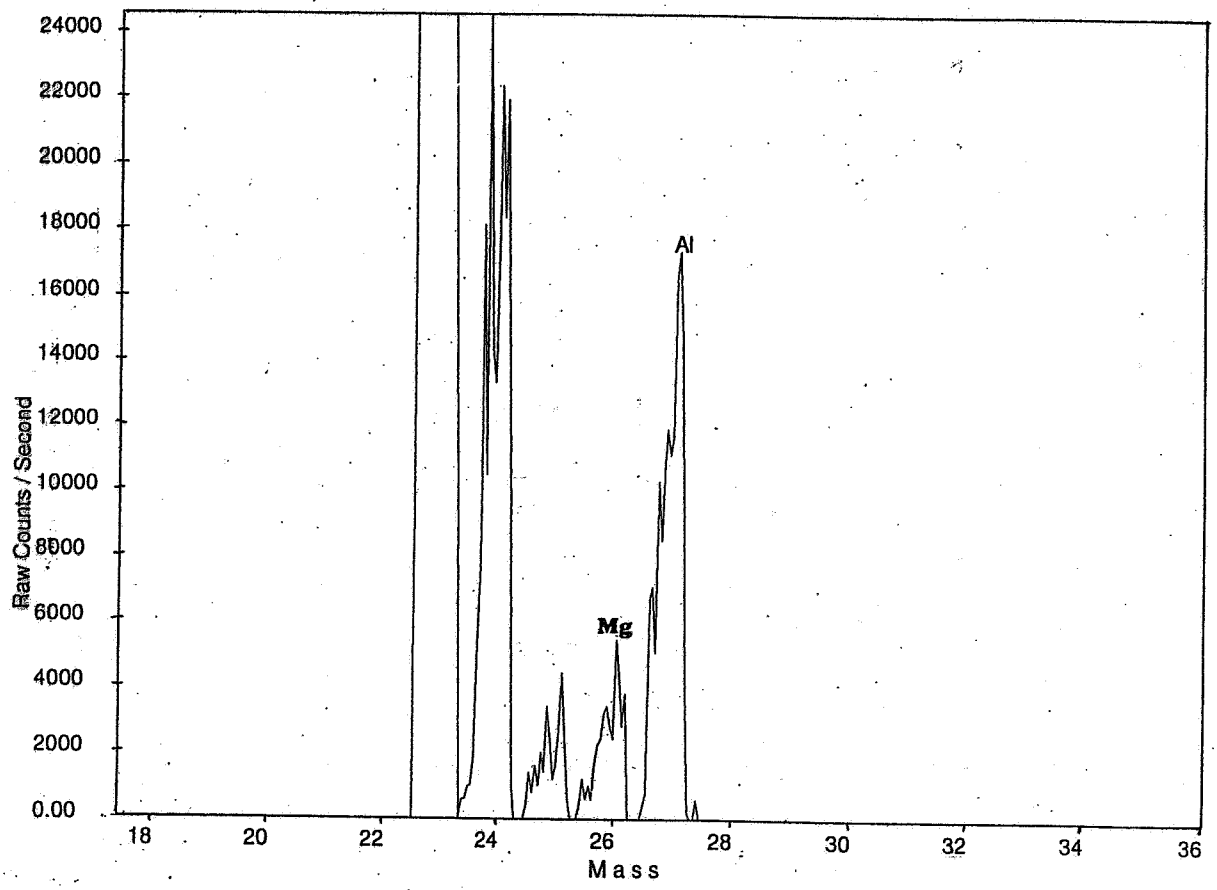


Table D-1 : Radioactivity of mushrooms collected October 17, 2001 from the UNLIMED region of an oak forest in Merzalben, Germany. Min.= lowest reading, Max. = highest reading, Mean = Bq count held over 30 seconds, (M) = Forms Mycorrhizae.

Fungal Name	Notes	Min.	Max.	Mean
Amanita citrina	young (M)	10	18	16
Amanita citrina	older (M)	10	20	15
Amanita citrina	oldest (M)	11	21	14
Amanita eliae	(M)	11	18	14
Amanita pantherina	very large (M)	8	18	14
Amanita rubescens	(M)	10	20	12
Armillaria ostoyae	single	11	17	15
Armillaria ostoyae	group	10	20	14
Armillaria ostoyae	malformed	11	18	16
Boletus erythropus	(M)	11	18	14
Boletus impolitus	(M)	10	20	14
Callistorporium olivascens		11	18	14
Conocybe subovalis		10	20	15
Cortinarius palustris		11	18	12
Cortinarius pholideus		9	18	13
Hypholoma fasiculare	Group A	11	18	14
Hypholoma fasiculare	Group B	10	18	12
Hypholoma fasiculare	Group C	11	20	13
Laccaria amethystina	(M)	12	20	14
Laccaria tortilis		11	20	16
Lactarius quietus		11	20	14
Leucocortinarius bulbiger		10	18	13
Macrolepiota procera		10	18	13
Melanoleuca cognata var.		10	22	15
Mycena pura		10	22	14
Paxillus involutus	(M)	10	20	14
Paxillus atromentosus	(M)	10	19	14
Pluteus diettrichii		10	16	12
Psathyrella hydrophila var.		8	18	14
Psathyrella spadicea		9	17	12
Psathyrella spadicea		10	20	15
Psathyrella species		12	18	16
Russula albonirga var.		11	19	15
Russula ochroleuca	(M)	10	18	14
Tricholoma fulvum		11	17	13
Tricholoma fulvum	ALARM	10	25	17
Tyromyces albellus		10	15	14
Unknown (very large)		11	18	12
Total		392	718	534
Average		**10.32**	**18.89**	**14.05**

Table D-2 : Radioactivity of mushrooms collected October 17, 2001 from the LIMED region of an oak forest in Merzalben, Germany. Min.= lowest reading, Max. = highest reading, Mean = Bq count held over 30 seconds. (M) = Forms Mycorrhizae.

Fungal Name	**Notes**	**Min.**	**Max.**	**Mean**
Armillaria ostoyae	malformed	9	18	13
Armillaria ostoyae	normal 1	10	18	14
Armillaria ostoyae	normal 2	9	18	14
Clintocybe graminocola	in moss	10	20	15
Collybia erythropus	in moss	10	14	12
Hypholoma capnoides		11	17	12
Hypholoma fasiculare	young	10	25	15
Hypholoma fasiculare	older	8	20	15
Hypholoma faiculare	older	10	18	14
Hypochnicium vellereum	on wood	10	19	14
Laccaria amethystina	(M)	12	18	15
Laccaria bicolor	small	9	18	12
Lachnellula subtilissima		10	16	13
Macrolepiota mastoidea	sample 1	10	20	14
Macrolepiota mastoidea	sample 2	10	20	14
Mycena filopes		11	18	14
Mycena lactea	very small	11	16	14
Mycena leucogala		10	16	14
Mycena speirea	very fine	8	17	14
Pysathyrella spadicea	on bark	12	20	15
Russula kromboholzii	large	10	21	15
Russula kromboholzii	small	10	21	13.5
Stereum rugosum	ALARM	10	25	14
Unknown	cap	8	18	14
Xylaria hypoxylon	on wood	10	18	14
Xylaria hypoxylon	Sample 2	12	20	15
Xylaria hypoxylon	Sample 3	12	18	15
Total		272	507	377.5
Average		**10.07**	**18.87**	**13.98**

Table D-3 : Radioactivity of objects, randomly collected on Oct. 17, 2001, including fungi from the LIMED Forest in Merzalben and the Uni-Mainz Laboratory. Min.= lowest reading, Max. = highest reading, Mean = Bq count held over 30 seconds.

Forest Collection

Fungal / Item Name	**Notes**	**Min.**	**Max.**	**Mean**
Aleurodiscus amorphus	on wood	11	20	13
Daedalea quercina	Bracket fungi	10	10	14
Gloeophyllum oderatum	Bracket fungi	11	15	13
Grifola umbellata	on twigs	11	19	13
Hypochnicium vellereum	mushroom	10	18	13
Merulius tremellosus	blasseform 1	10	18	13
Merulius tremellosus	blasseform 2	8	16	13
Lichen A		10	21	12
Lichen B		8	21	12
Lichen C		8	16	11
Quercus petraea acorn		10	15	12
Soil & white hyphae	wood destroyer	9	20	14
Total		119	216	155
Average		**9.92**	**18**	**12.92**

Lab Collection

Item Name	**Notes**	**Min.**	**Max.**	**Mean**
Room air		8	14	12
Fomites:				
Counter top		8	20	13
Glass slides in a box		10	16	13
Cup of cold tea		11	14	13
Average		**9.6**	**16.6**	**13**
Humans:				
Left Hand-Subject 1	Female	12	16	14
Left Hand-Subject 2	Male	10	14	12
Left Hand-Subject 3	Female	8	14	12
Left Hand-Subject 4	Male	9	16	12
Left Hand-Subject 5	Female	9	16	12
Average		**9.6**	**15.2**	**12.4**
Right Hand-Subject 1		12	20	17
Right Hand-Subject 2		12	18	14
Right Hand-Subject 3		10	20	14
Right Hand-Subject 4		10	18	14
Right Hand-Subject 5		10	14	12
Average		**10.8**	**18**	**14.2**

Table D-4: Comparison of temporal changes in radioactivity of Merzalben fungal fruiting bodies collected during the time span 1987 to 1990 and similar species collected in 2001. Cs 134 and Cs 137 readings of dry mass were measured in Bq/kg (Block, 1993), while the general radioactivity was calibrated in Bq by averaging the direct readings of fresh fruiting bodies using a standard geiger counter.

Fungus	Year	Cs 134 Bq/kg	Cs 137	General Radioactivity
Amanita rubescens	1987	375	127	
	1989	103	<5	
	2001			12
Boletus erythopus	1990	330	42	
	2001			14
Hypholoma fasciculare	1988	1024	520	
	2001			13
Paxillus involutus	1986	5748	2946	
	2001			14
Russula atropurpurea	1989	2469	919	
	2001			15
Lab Background	2001			13

D5: Discussion

D5-1: Laser Analysis

The use of ICP Sources to analyze chemical content has the distinct advantage of not requiring one to chose which chemicals to test for. On the other hand, only a positive or negative presence and a vague indication of concentration based on peak strength was possible. In addition, when using the whole dried organic samples, the laser beam could not be selected to analyze from point to point, but rather burned large regions resulting in the destruction of the sample. Calibrating the system to produced comparative results was abandoned primarily due to the destruction of the control test samples. The computer analysis allowed identification of the elements but not the concentrations nor localizations, so no useful comparative interpretations could be made. What was still needed was a way to confirm the aluminum depositions in discrete areas within the mycorrhizal sheath and root regions as determined by fluorescence microscopy. It was decided to increase the number of replicates in each sampling whenever possible to confirm the basic observed fluorescence patterns (Section F). An alternate promising procedure involving ESEM (Environmental Scanning Electron Microscope) was also considered but eliminated due to time / cost / availability constraints. The ESEM has distinct possibilities for non-destructively locating and confirming heavy metals in a root cross-section and is highly recommended for future examination.

D5-2: Radioactivity

According to Ahmad et al (2001), traces of radioactive material are widely distributed in the earth's crust with approximately 45 occurring naturally such as uranium and thorium and radon which occur naturally in rocks, with radon accounting for a large proportion of the back ground radiation. Of these, using ICP technology, Uranium and Thorium were identified as being present in the common successful mycorrhiza *Cenococcum geophilum.*

Of the man-made radionuclides produced and released into the upper atmosphere, many rapidly return to the ground as scattered dry fallout, or as rain drop nuclei in precipitation, forming hotspots. Some important fission byproducts include: Cesium 137, Strontium 90, Iodine 131 (Ahmad et al, 2001), Cesium 134 and Potassium 40 (Block, 1993). Of

these, Strontium was positively identified in the ectomycorrhiza *Cenococcum geophilum* by ICP spectral analysis in addition to Uranium and Barium (Graphs D1 & D2). Since this was a very limited mycorrhizal sampling, the presence of the other radionuclides should be considered as distinctly possible, especially since Cs 137, Cs 134 and K 40 were previous localized in 30 species of fungi in Merzalben from 1986 to 1991 (Block, 1993).

Despite the positive presence of radionuclides in the mycorrhizae by ICP methods, a traditional Geiger counter did not detect emissions from the mycorrhizae in excess of background radiation levels. This would imply that insignificant levels of radionuclides were present. The fruiting bodies however, even in very small pieces, did have emissions exceeding the background radiation readings. Which in turn implies that the radionuclides were moved to and were more concentrated in the larger above ground organic structures.

In comparing hot spots, considerable variation in the presence of radioactive nucleotides can occur, probably dependent upon point by point contact of the nucleotide fall out with the soil microflora, rather than the species. For example, in 1986, the Cesium 134 and Cesium 137 levels for *Paxillus involutus* and *Xerocomus chrysenteron* fruiting bodies from two hotspot [Berlin-Wannsee (Gans, 1987) and Merzalben (Block, 1993, p.71)] were recorded. In Berlin-Wannsee park forest the Bq/kg readings were low [*Paxillus*: Cs 134: 13, Cs 137: 29 and *Xerocomus*: Cs 134: 23, Cs 137: 58] relative to the higher concentrations in the Merzalben samples [*Paxillus*: Cs 134: 5748, Cs 137: 2946 and *Xerocomus:* Cs 134: 2351, Cs 137: 1162].

Of the Merzalben fungal fruiting bodies examined by Block (1993) which contained fission byproducts, the following species were amongst those collected in 2001 and retested for radioactivity: *Amanita rubescens, Boletus erythropus, Hypholoma fasiculare, Paxillus involutus, Russula kromboholzii = Russula atropurpurea.* While these fungi had high readings in their samplings in the early part of this decade (1986-1991), they had low readings in the samples collected in 2001 (Table D4). Only *Hypholoma fasiculare* was common to both the unlimed (Table D1) and limed (Table D2) forest in this comparative sampling. In the unlimed plot *Hypholoma* had a mean of 13 units while the

limed samples had a mean of 14.6 units in 2001. Insufficient data was available to determine if this was a significant difference for this particular species. However cumulative results for all the species tested showed that there was no significant difference between the unlimed (Table D1) and limed (Table D2) zones in 2001.

D6: Conclusions

One of the directives of this study was to find potentially host-toxic elements. Another was to determine how the chemicals differentiated in the unlimed and limed plots. The toxicity of aluminum has been previously discussed (Section A2). Using Laser-quod technology, Aluminum was detected in *Cenococcum geophilum* tips but the mantle and the root proper could not be distinguished, as hoped, to confirm the observation of intense Aluminum-Morin fluorescence in the root cortex and its absence in the mantle.

The slow, long-term toxicity of radionuclides is assumed, but beyond the scope of this study. As a result of the examination of the radioactivity of the mushroom fruiting bodies it was determined that although the radioactive materials were probably translocated in the fungi from the subsurface mycorrhizae to the fruiting bodies, there was no significant difference in radiation between the fruiting bodies from the unlimed and limed plots.

D: References

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Kritzotakisk 2000 & 2001. Department of Geology, Johannes Gutenberg University Mainz, Personal communications

Section E: Fluorescence Studies

INDEX

E1: FLUORESCENCE MICROSCOPE FILTER & FIXATION COMBINATIONS

E1: INTRODUCTION

Preliminary testing was needed to establish the most ideal experimental parameters since it was expected that the longevity of excised mycorrhizal root tips in vitro would be severely limited. Despite hopes that the tips could be fixed in formaldehyde for long term storage, it was determined that the Morin-aluminum colorimetric responses could be altered in the presence of formalyn solutions. Embedding medium was also found to be unsuitable due to its sticky nature. Samples stored in distilled water whether kept fresh or flash frozen maintained their colorimetric responses to Morin. The most ideal fluorescent filter combinations were determined to be LRF 1 (lowest intensity), SRF 2 (UG1 green excitation filter) and CSF c (no barrier filter).

E2: PURPOSE:

1. To determine the reactivity of the Morin dye to aluminum.
2. To determine the best filter combinations to demonstrate Morin dye complexes.
3. To determine if formaldehyde fixing affects sectioning or fluorescence.
4. To determine if freezing affects sectioning or fluorescence.
5. To determine if mycorrhizae and Beech root tissues vary in their cell wall autofluorescence.

E3: MATERIALS AND METHODS:

Mycorrhizal roots were collected from a pure Beech (*Fagus sylvaticus*) wood stand located in a protected, unlimed forest area near Presburg, Germany. Fine root samples were extracted from 0.5 to 1.0 meter from the base of mature Beech trees at a depth of 5-10 cm. Under a dissecting microscope, fresh, healthy, mycorrhizal tips from the fine Beech tree roots were carefully cleaned using fine forceps and water and placed individually into 1.5 ml safe-lock eppendorf caplets (Netheler-Hinz GmbH, Hamburg, Germany). The mycorrhizal species selected for testing was *Fagirhiza setifera* because of abundance and easy of identification using Agerer (1987). Once the method was worked out, *Fagirhiza tubulosa* tips were also examined to see if there was a potential difference between mycorhizal species.

Two sets of eppendorf caplets containing the prepared mycorrhizae were labeled and each was

half filled with one of the following solutions: A = tap water , B = 1.75 % formaldehyde in distilled water, C = 3.5 % formaldehyde in distilled water D = 7.0% formaldehyde in distilled water, E = 10.5 % formaldehyde in distilled water, F = Einbettmedium für Gefrierschnitte [Leica 0201-08926] [embedding medium], G = distilled water. One set was refrigerated at 4^0 C while the other was flash frozen in liquid nitrogen and stored at -20^0C. The samples were stored for a period of 3 to 8 weeks.

A Leitz freezing microtome (Leitz Kryomat 1703) with a cold knife (B for soft tissues) was used for thin sectioning (Fiedler, 1989). The stage was pre-cooled for 15 minutes to -32.5 ^{0}C. Slightly cooler (-35 ^{0}C) dial temperatures proved to be better during the especially hot summer days for maintenance of a consistent mounting platform temperature (below -27^0 C) The knife was kept cold by periodic refreezing so tissue sections from 1 to 50 µm thick could be cleanly cut.

For these trials, each mycorrhizal tip was placed onto the precooled stage and covered with a drop of Leica 0201-08926 embedding medium and /or water, allowed to freeze, and cut into 20-25 µm thick sections. The sticky embedding medium was used sparingly. The tips were oriented either upright for cross-sectioning from the tip to progressively older regions or lengthwise for long-sectioning. Subsequent experimentation showed that the roots could be mounted on the cold stage in just a drop of distilled water or pH 6 buffer for cleaner [less sticky] sectioning and better staining. A single drop was allowed to freeze on the stage, the mycorrhizal tip was gently placed on top and a second drop of medium was added to cover the root tip. This raised the tip a few millimeters for more complete sectioning.

The cut sections were collected from the knife using a fine-haired paint brush and transferred to a drop of pH 6 buffer solution on clean, new slide and covered with a new glass coverslip. The sections were viewed immediately using a Leitz Ortholux 2 epifluorescent microscope to determine autofluorescence using all possible filter combinations (Key Chart B1). The same sections were then stained with a 0.01% Morin solution by allowing it to diffuse under the coverslip and across the sections which were then viewed again using all possible filter combinations (Tables B1 to B12). Aluminum was assumed to be present if, in the presence of

Morin, a yellow color change occured (Ross-Siebert, 1985; Vogelei & Rothe, 1993).

To test the responsiveness of Morin to aluminum, a drop of Morin dye solution on a clean slide was examined under all possible filter combinations to determine its natural autofluorescence and again in combination with 0.01% Al^{+3} (aluminum oxide in pH 6 buffer) to determine its fluorescence in the presence of reactive aluminum. The work room was darkened to prevent premature breakdown of the Morin dye and for better microscopic viewing.

E3-1: FLUORESCENCE MICROSCOPE

A Leitz Wetzlar Ortholux 2, trinocular Fluorescence microscope (Leica Mikrosysteme Vertrieb GmbH, D-64625 Bensheim) with NPL Fluotar objective lenses (16x, 25x, 40x and 100x) and an OSRAM HBO 50 W/AC-L1, 39-45V/50W Leica Wetzlar short arc mercury vapor lamp was used. To control the electrical flow to the mercury vapor lamp a Leitz Regel RED4x Nr.9 – 68427 DO transformer was employed. The high intensity ultraviolet light produced was filtered via three independently operated systems which will be referred to as the large rotary filter (LRF), small rotary filter (SRF) and the color sliding filter (CSF). The approximate wavelengths for UV-A,B,C excitation (100-380 nm) and visible light range (380 to 780 nm) were obtained from Leitgeb (1990, p. 194). The most useful excitation range is known to be 361-435 nm for other types of histochemical testing (Wilson & Peterson, 1983).

- The large rotary filter (LRF) system provides 5 levels to control the intensity of the ultraviolet (UV) light as it emerges from the lamp, with 5 being the most painfully intense.
- The small rotary filter (SRF) through which the light passes next provides excitation to produce specific wavelengths of light of which only certain refracted / reflected components are visible :

Excitation light #1: (UV-C: 100-280 nm → Visible components fluoresce violet blue to blue)

Excitation light #2: (UG1 light consisting of UV-B light: 280-315 and UV-A light : 315-380 nm → Visible components fluoresce blue green to green),

Excitation light #3: (UG2 350-450 nm →Visible components fluoresce green yellow to yellow)

Excitation light #4: (UR1 450-510 nm → Visible components fluoresce orange red to red)

The last two have a KP (kinetic potential) of 490 (350-510 nm range) and so are expected to be less useful but still within a possible range for Al-Morin fluorescence.

- The color sliding filter (CSF) provides four barrier filters (#1 = clear, #2 = green, #3 = yellow and #4 = blue) to block slower, shorter waves. The filters had a TK of 400/K 400 which blocks waves longer than 400 nm or a TK of 510/K510 which blocks waves longer than 510 nm. Since yellow light has a wavelength of 650 nm it is expected that color sliding filter #1 which is actually clear with no wavelength barrier will be the most useful in the Morin-aluminum reactions.

Thin tissue sections containing chemicals which are excited at UV-A, UV-B or UV-C wavelengths will autofluoresce releasing visible light from the blue to the infrared range (380-780 nm). The energy of a quanta of light (E) is inversely related to its wavelength (λ) which basically means that short length UV waves are high energy and when energy is lost, slower light quanta with longer wavelengths will be released. UV light at 350 nm has an energy level of about 80 kcal/mole (or 80 kcal/einstein where 1 mole quantum = 1 einstein = Avogadro's number = 6.02×10^{23} quanta particles of light) and as energy is lost, visible light is formed (Bidwell, 1974). Blue light at 450 nm contains 70 kcal/einstein, yellow light at 550 nm has 60 kcal/mole and red light at 660 nm contains 50 kcal/mole quantum (Bidwell, 1974).

The UV light energy can cause excitation of particular molecular bonds, which can split the bonds or induce vibrations or oscillations which release (reflect) the unused energy in the form of slower visible light. Morin dye can attach to normally non-fluorescent ions such as aluminum and when excited, will release light in the yellow range (450-550-650 nm) with colors ranging from bright green (450 nm) to green to green-yellow to yellow-green to yellow (550 nm) to bright yellow. Intensity and quality of the yellow light was assumed to be directly related to the effective relative abundance of excitable Al-Morin binding sites (Ross-Siebert, 1985).

The microscope was adapted for photography using a Leitz-Wetzlar 22 mm adapter base along with a Hama T2 camera adapter for the Leica DRP Nr. 677597 (Leitz-Wetzlar GmbH) with a

Hama 5331 flexible remote shutter control device. Duration of exposure was determined using a Profisystem light meter (Profisix, Gossen Germany). Color photography was accomplished using 135 mm, 100 / 21^0 ASA Sensia II Fujichrome color slide film. A special non-fluorescent steroid based immersion oil, 70-80% Di (TCD-Methylol)-adipat, (Leica Microsystems Wetzlar GmbH) was used to improve visibility and reduce defraction of the energetic UV light when using the 100x objective.

E3-2: REAGENT PREPARATIONS

E3-2A: Morin Dye:

The Morin dihydrat dye ($C_{15}H_{10}O_7.2H_2O$ = 3,4,7,2',4'-Pentahydroxyflavonol) which is known to bond to aluminum-organic complexes (Vogelei & Rothe, 1993) was obtained from Merck, (stock number 507K1798, Art. 6098, C.I.Nr.75660, S.Nr.1366, gmw 338.27 g/mol.). The 0.01% Morin staining solution was derived from a 0.1% stock solution which was kept refrigerated, in the dark for up to 2 weeks. The Staining solution was composed of 1 ml of v/v stock solution (0.1% w/v Morin in 96 % v/v ethanol) + 9 ml pH 6 phosphate buffer solution (100 $mM.l^{-1}$ pH 6) (Rost-Siebert, 1995).

E3-2B: Aluminum Solution:

Aluminum oxide from Merck (Art. 1090, Al^{+3}) was prepared by mixing 0.0014 g with 1 ml of pH 6 phosphate buffer to create a 0.14 % w/v solution.

E3-2C: Phosphate Buffer:

The phosphate buffer was prepared from dry chemical stock, 500 ml at a time. To create a pH 6 buffer, 2.3637 grams of di-sodium hydrogen phosphate dodecahydrate salt [$Na_2HPO_4.12H_2O$, gmw 358.14] from Merck GmbH and 5.9888 grams of sodium dihydrogen phosphate monohydrate acid [$NaH_2PO_4.H_2O$, gmw 137.99] also obtained from Merck, were placed into a flask which was filled to the 500 ml mark with distilled water. The mixture was agitated using a magnetic stirrer until completely dissolved and the solution was then stored, refrigerated, in a dark glass container between uses. The pH of the staining solution was important for improving Morin-aluminum complexing.

E3-2D: Formalyn Solutions:

The formaldehyde (1.75 %, 3.5 %, 7.0 %, 10.5 %) solutions were prepared using formalyn from Roth GmbH [CH_2O, gmw 30.03, Art. 7398.1] and distilled water. All solutions were refrigerated when not in use in brown (UV resistant) glass containers for up to 2 weeks and then replaced with fresh solutions.

E4: RESULTS KEY CHART : Key for Tables E1-E12

FILTERS

LRF = Large Rotary Filter with 5 numbered filters
SRF = Small Rotary Filter with 4 numbered filters (Excitation filters)
CSF = Color Sliding Filter with clear, green, yellow and blue Barrier filter discs

LRF #1 - Allows the least amount of UV light to pass but does not change the wave lengths.
LRF #2 - Allows more light to pass than #1, but may have a reddish tint
LRF #3 - Allows more light to pass than #2, but may have a yellowish tint
LRF #4 - Allows more light to pass than #3, too bright, may damage retina
LRF #5 - Allows the most light to pass, painfully bright

SRF #1 - blue	CSF #1 clear
SRF #2 - green	CSF #2 green
SRF #3 - yellow	CSF #3 yellow
SRF #4 - red	CSF #4 blue

TREATMENTS

A = Autofluorescence of Beach tree mycorrhizae (fresh & frozen) *
A+M = Fluorescence of Beach tree mycorrhizae stained with Morin (fresh & frozen)
F = Autofluorescence of Formalyn treated mycorrhizae (all concentrations) **
F+M = Fluorescence of Formalyn treated mycorrhizae stained with Morin (all concentrations)

RS = ROOT SECTIONS

x = xylem
c = cortex
m = mycorrhizal sheath

FLUORESCENCE COLOR KEY

W = white
B = blue
G = green
R = red
P = purple
Y = yellow
C = clear
\+ = brightness
\- = faintness
X = too bright to use

* It was determined that both the fresh and frozen tissues in every instance for tap or distilled water had the same autofluorescence and the same responses to Morin, and so they are for simplicity put together.
** The concentration of the formalyn did not alter the autofluoresence or Morin responses, so the formalyn results are amalgamated, however those responses were not the same as was found in the fresh and frozen water samples.

TABLE E1 : ROTARY FILTER COMBINATIONS = LRF # 1 + SRF # 1(BLUE) RATING: 5

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	B+	B++	B++	B++	NO
	c	YB	W	W	W	NO
	m	-	P	P	P	NO
G	x	B+	B++	B+	B++	MAYBE
	c	B-	B	B-	B	NO
	m	-	-	**R**	**R**	**MAYBE**
Y	x	Y	Y	Y	Y	MAYBE
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	**R**	**R**	**MAYBE**
B	x	B	B	W	W	NO
	c	YB	YB	Y	Y	MAYBE
	m	-	-	P	P	NO

TABLE E2 : ROTARY FILTER COMBINATIONS = LRF # 2 + SRF # 1 (BLUE) RATING: 0

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	P-	P-	P-	P-	NO
	c	P-	P-	P-	P-	NO
	m	P-	P-	P-	P-	NO
G	x	P+	P+	P+	P+	NO
	c	P-	P-	P-	P-	NO
	m	P-	P-	P-	P-	NO
Y	x	Y	Y	Y	Y	NO
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	-	-	NO
B	x	P-	P-	P-	P-	NO
	c	P-	P-	P-	P-	NO
	m	P-	P-	P-	P-	NO

TABLE E3 :ROTARY FILTER COMBINATIONS = LRF # 3 + SRF # 1 (BLUE) RATING : 0

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	B++	B++	B++	B++	NO
	c	B++	B++	B++	B++	NO
	m	B++	B++	B++	B++	NO
G	x	B++	B++	B++	B++	NO
	c	B++	B++	B++	B++	NO
	m	B++	B++	B++	B++	NO
Y	x	Y	Y	Y	Y	NO
	c	RY	RY	RY	RY	NO
	m	R-	R-	R+	R+	NO
B	x	B++	B++	B++	B++	NO
	c	B++	B++	B++	B++	NO
	m	B++	B++	B++	B++	NO

TABLE E4 : ROTARY FILTER COMBINATIONS = LRF #1+ SRF #2 (GREEN) RATING : 11

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	G	G	YG+	YG+	MAYBE
	c	G-	G-	YG	YG	MAYBE
	m	-	-	**R**	**R**	**MAYBE**
G	x	G	G	BG+	BG+	MAYBE
	c	G-	G-	B+	B+	MAYBE
	m	-	-	**R**	**R**	**MAYBE**
Y	x	Y	Y	Y	Y	MAYBE
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	**R**	**R**	**MAYBE**
B	x	B	B	YG	YG	MAYBE
	c	B-	B-	Y-	Y-	MAYBE
	m	-	-	**R**	**R**	**MAYBE**

TABLE E5 : ROTARY FILTER COMBINATIONS = LRF #2 + SRF #2 (GREEN) RATING : 2

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	YG	YG	Y	YG	NO
	c	Y-	Y-	Y-	YG	NO
	m	-	-	P-	P-	NO
G	x	GY	GY	Y	YG	MAYBE
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	P-	P-	NO
Y	x	Y+	Y+	Y	Y	NO
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	-	-	NO
B	x	YG	YG	Y	YG	NO
	c	GY-	Y-	Y-	Y-	MAYBE
	m	-	-	P-	P-	NO

TABLE E6 : ROTARY FILTER COMBINATIONS = LRF #3 + SRF #2 (GREEN) RATING : 0

CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	X	X	X	X	NO
	c	X	X	X	X	NO
	m	X	X	X	X	NO
G	x	X	X	X	X	NO
	c	X	X	X	X	NO
	m	X	X	X	X	NO
Y	x	Y	Y	Y	Y	NO
	c	RY	RY	Y-	Y-	NO
	m	R	R	R	R	NO
B	x	X	X	X	X	NO
	c	X	X	X	X	NO
	m	X	X	X	X	NO

TABLE E7 : ROTARY FILTER COMBINATIONS = LRF #1+ SRF #3 (YELLOW						
CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	YG	YG	Y+	Y+	MAYBE
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	**R**	**R**	**NO**
G	x	YG	YG	Y+	Y+	MAYBE
	c	Y-	Y-	Y-	Y-	NO
	m	-	-	**R**	**R**	**NO**
Y	x	BY	BY	Y	Y	NO
	c	Y-R	Y-R	Y-	Y-	MAYBE
	m	-	-	**R**	**R**	**NO**
B	x	YG	YG	Y	Y	MAYBE
	c	-	-	Y-	Y-	MAYBE
	m	-	-	R-	R-	NO

RATING: 5

TABLE E8 : ROTARY FILTER COMBINATIONS = LRF #2 + SRF #3 (YELLOW						
CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	YG	YG	Y	Y	MAYBE
	c	Y-	Y-	YR	YR	NO
	m	-	-	-	-	NO
G	x	YG	YG	Y	Y	MAYBE
	c	YG-	YG-	Y-	Y-	MAYBE
	m	-	-	-	-	NO
Y	x	Y+	Y+	Y	Y	NO
	c	YR-	YR-	Y-	Y-	MAYBE
	m	-	-	-	-	NO
B	x	YG	YG	Y	Y	MAYBE
	c	YG-	YG-	Y-	Y-	MAYBE
	m	-	-	-	-	NO

RATING : 6

TABLE E9 : ROTARY FILTER COMBINATIONS = LRF #3 + SRF #3 (YELLOW						
CSF	RS	A	F	A + M	F + M	COMMENTS
C	x	Y	Y	Y	Y	NO
	c	YR	YR	YR	YR	NO
	m	R	R	R	R	NO
G	x	Y	Y	Y	Y	NO
	c	YR	YR	YR	YR	NO
	m	R	R	R	R	NO
Y	x	Y	Y	Y	Y	NO
	c	YR	YR	YR	YR	NO
	m	R	R	R	R	NO
B	x	Y	Y	Y	Y	NO
	c	YR	YR	YR	YR	NO
	m	R	R	R	R	NO

RATING : 0

TABLE E10 : ROTARY FILTER COMBINATIONS = LRF #1 + SRF #4 (RED) RATING : 0

CSF	RS	A	F	A+M	F+M	COMMENTS
C	x	R	R	R+	R+	NO
	c	-	-	R	R	NO
	m	-	-	R	R	NO
G	x	R	R	R+	R+	NO
	c	-	-	R	R	NO
	m	-	-	R	R	NO
Y	x	R	R	R+	R-	NO
	c	-	-	R	R-	NO
	m	-	-	R	R	NO
B	x	-	-	R-	R-	NO
	c	-	-	R-	R-	NO
	m	-	-	R-	R-	NO

TABLE E11 : ROTARY FILTER COMBINATIONS LRF #2 + SRF #4 (RED) RATING : 0

CSF	RS	A	F	A+M	F+M	COMMENTS
C	x	R	R	R+	R+	NO
	c	-	-	R	R	NO
	m	-	-	R	R	NO
G	x	R	R	R+	R+	NO
	c	-	-	R	R	NO
	m	-	-	R	R	NO
Y	x	R	R	R+	R-	NO
	c	-	-	R	R-	NO
	m	-	-	R	R	NO
B	x	-	-	-	-	NO
	c	-	-	-	-	NO
	m	-	-	-	-	NO

TABLE E12 : ROTARY FILTER COMBINATIONS = LRF #3 + SRF #4 (RED) RATING : 0

CSF	RS	A	F	A+M	F+M	COMMENTS
C	x	R+	R+	R+	R+	NO
	c	R	R	R	R	NO
	m	R	R	R	R	NO
G	x	R+	R+	R+	R+	NO
	c	R	R	R	R	NO
	m	R	R	R	R	NO
Y	x	R+	R+	R+	R+	NO
	c	R	R	R	R	NO
	m	R	R	R	R	NO
B	x	R+	R+	R+	R+	NO
	c	R	R	R	R	NO
	m	R	R	R	R	NO

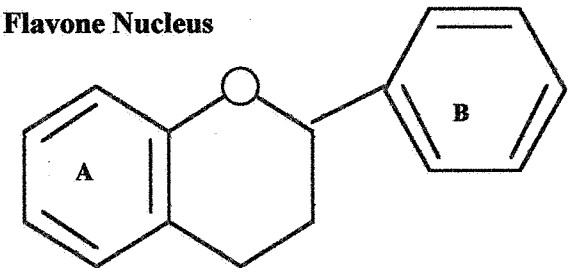
E5: OBSERVATIONS & DISCUSSION

In order to determine the best possible filter combinations, all three filter (LRF, SRF and CSF) possibilities available on the Leitz fluorescent microscope were variously tested using, aluminum solutions, fresh, frozen and fixed tissues and Morin staining.

E5-1: MORIN-ALUMINUM REACTIVITY

It was determined that the pale yellowish Morin dye alone is non-fluorescent. When using the red filters, it was difficult to determine if it contributed to the evident fluorescence since the red filters generally made the whole field red possibly due to reflections from the water and glass coverslip. The clear aluminum solution was also independently non-fluorescent. When Morin and aluminum solutions were mixed, a definite yellow fluorescence was visible especially evident using the LRF #1 and SRF #2 and the CSF c or CSF g. The basic test was repeated with a newly purchased Morin stock chemical. Morin and aluminum definitely can complex and form a yellowish fluorescence. The ability of Morin ($C_{15}H_{10}O_7.2H_2O$ = Pentahydroxyflavone) to complex with aluminum may be in part due to its aromatic flavone ring nucleus (Figure B5-1), (Bidwell, 1974, p. 221).

Figure E5-1: Flavone Nucleus



E5-2: FILTERS ?

The various filter combinations (LRF, SRF, CSF) were rated according to whether they showed any possible aluminum-Morin reactions (MAYBE) or not (NO) and good contrast to natural autofluorescence (Tables B1-B12). Filter combinations were excluded where the natural autofluorescence was yellow since there was not enough contrast between the natural and the Morin-treated coloration. The best filter combinations were deemed to be where the natural autofluorescence was blue and the dye complexing was yellow. The best rated combination

(giving the most definitive responses with a rating of 11) was filter LRF #1 and SRF #2 (Green) (Table B4). Of the CSF filters, in order of clarity, the clear, green and blue were all very good, giving definitive color changes when Morin was added. The yellow and red CSF barrier filters did not provide suitable color contrast.

With respect to the other filters, they were of only limited use. LRF #2 gave a few obvious fluorescence changes with SRF 2 and 3 but not enough to be usable. LRF #3 was useful for autofluorescence only. LRF #4 and #5 were far too (painfully) bright to be of any use and were not included in the tables. Small rotary filters SRF #3 and SRF #4 provided some autofluorescence information with SRF #3 showing differential autofluorescence in the green, yellow and red range while SRF #4 illuminated only in the red range.

It was decided that for the filter combinations, all the samples before and after Morin staining would be examined using LRF # 1, SRF #2, and CSF clear with CSF green or CSF blue used only if they gave greater definition of the localization of the aluminum-Morin reaction for photographic purposes.

E5-3: FRESH, FROZEN or FIXED TISSUES ?

It was determined that the tap and distilled water samples, whether fresh or frozen gave the same autofluorescent (A) and Morin responses (A + M) and so were for simplification, grouped together in the tables B1-B12. Probes stored in distilled water and the 7.0 and 10.5 % formaldehyde solutions produced the thinnest, cleanest sections with greater tissue integrity than either tap water or the lower formaldehyde concentrations. Fresh probes cyosectioned much more cleanly than the frozen probes from storage, although freezing did not seem to affect the location or intensity of the Morin staining reactions.

The formalyn samples were grouped together since differences in the concentrations of formalyn (1.75 to 10.5 %), whether fresh or frozen did not alter the autofluorescence reactions (F) or the reactions to Morin (F + M) , although these reactions were not always the same as with the samples stored in water (Tables B1-B12). The formaldehyde (F) solution had some small effects, producing variations in autofluorescence color (Table B5), but most other differences

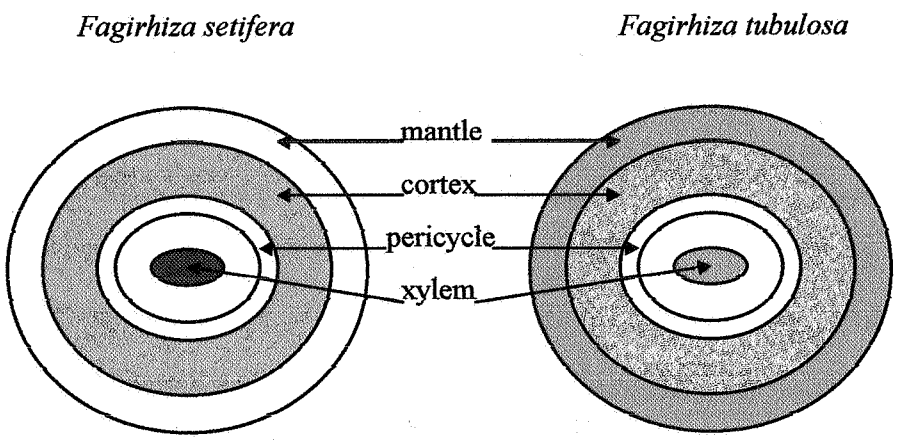
were generally limited to a somewhat brighter autofluorescence (Table B1). Formalyn had a small effect on the Morin staining response (F + M) when compared to the water samples (A + M) resulting in some color variations (Table B5) or fluorescence loss (Tables B10 & B11).

It was further determined that the samples stored in embedding fluid were unusable due to the thick sticky fluid making the sections difficult to remove from the knife and transfer to the slide, as well as interfering with diffusion of the stain, and preventing contact of the stain with the sections.

E5-4: DIFFERENTIAL FUNGAL-ROOT FLUORESCENCE

For these trials only the xylem (x), cortex (c) and mantle (m) were examined and recorded. The phloem, pericycle, endodermis did not change in fluorescence in the presence of Morin and hypodermis, and epidermis were not included because they mimicked the mantle. It was definitely possible to differentiate the tissues on the basis of their autofluoresence (Tables B1, B4, B5, B7, B8, B9, B10, B11) and also their (yellow) reactions to Morin (Tables B1, B4, B5, B7, B8, B10, B11). In order to see if there was a potential difference between mycorrhizal species, a second ectomycorrhiza, *Fagirhiza tubulosa*, was examined using the parameters established. As luck would have it, the two species differed consistently in their autofluorescence (Figure B5-4).

Figure E5-4: *Fagirhiza setifera* and *Fagirhiza tubulosa* cross-sections. A diagram comparing the natural autofluorescent areas (uncolored), and the regions reactive to Morin. Intensity of the gray area corresponds to stronger Morin-complexing.



E5-5: ALUMINUM CHARACTERIZATION BY FLUORESCENCE

The sensitive and non-destructive nature of fluorescence techniques are ideally suited to studies of intact fresh tissues (Wilson & Peterson, 1983, McGown & Levinson, 1999). These differential color reactions are assumed to be relative to the cell wall chemical composition (Wilson & Peterson, 1983). Metal aggregates can fluoresce in different spectral regions depending upon the type of ligand and as a result it is extremely important to carefully choose the excitation and emission wavelengths (McGown & Levinson, 1999).

Through the use of steady-state fluorescence anisotropy the size and shape of cation-organic aggregates can be determined and in the case of aluminum it was established that the longer excitation wavelengths were representative of smaller ligand particle complexes (McGown & Levinson, 1999). This would imply that, depending upon the filter combinations, fluorescence in the Green -Yellow (GY) range for example would be due to Aluminum complexing with larger ligand particles and than when fluorescent in the Yellow-Green (YG) or Yellow (Y) range. Despite of the fact we do not know what the exact Al-ligands combinations are, variations in yellow Morin reaction tints will be recorded in this study.

It is interesting to note that lignin-rich xylem which autofluoresces blue (Wilson & Peterson, 1983), in the filter combinations chosen for this study frequently becomes bright yellow in the presence of Morin; while the endodermis and pericycle which are suberized and also autofluoresce blue (Wilson & Peterson, 1983) are non-fluorescent in the presence of Morin. Also of interest was the fact that fluorescence intensity of the cortical walls varied often with gradations in intensity from weak to strong with increased proximity to the pericycle barrier. McGown & Levinson (1999) determined quenching of Al fluorescence was due to a decrease in organic (humic) concentration associated with a non-fluorescent precipitation of the added excess Al ions. Since variations in the intensity of Al-Morin fluorescence were observed in *Fagirhiza* roots, for this study the color intensities will be recorded as low (-) or high (+, ++) with the assumption that a lack of reaction implies the absence of Al, a quenching of the intensity means less Al is present and a strong bright reaction implies high Al concentrations.

E5-6: MYCORRHIZAL AUTOFLUORESCENCE

According to Agerer (1986) autofluorescence has been recorded for various mycorrhizal species (Trappe 1967, Zak 1971, 1973) but it is rarely used to characterize relationships of fungi despite some striking differences. The known autofluorescence of various mycorrhizae have been noted in Appendix 1, but as a cautionary note, some of the samples may have been fixed in FAA (Brand 1991) prior to fluorescence examination as a standard practice according to Agerer et al (1994). Formaldehyde treatments can occasionally alter normal autofluorescence (Table B1 and B5). In addition most of the fluorescence information involves host species other than oak and so may not be representative for *Quercus petraea.*

E6: CONCLUSIONS

It was concluded that cryosectioning and fluorescent analysis of aluminum complexing in fresh roots was a rapid way of determining aluminum absence, presence, approximate abundance and probably ligand variations within the cell walls of ectomycorrhizal root tips. It was established that for the experimental procedures to follow, fresh probes stored in distilled water only would be used, and that, only if necessary, probes stored in distilled water and flash frozen could be employed. Formalyn fixed probes would not be used because of the possible risk (although small) of interference with the Morin staining reaction. The dye Morin did complex with aluminum and differential staining was clearly evident in the root tissues using the described pH 6 buffer and Morin dye solutions. For consistency in analysis with the Morin dye, a pH 6 buffer solution will be used for cryosectioning instead of distilled water or the sticky embedding medium. For the filter combinations, all the samples before and after Morin staining would be examined using LRF # 1, SRF #2, and CSF clear. CSF green or CSF blue would be used only if they gave greater definition of the localization of the aluminum-Morin reaction for photographic purposes.

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E2: ALUMINUM CONTENT OF MYCORRHIZAE USING FLUORESCENCE

E 2-1A: Introduction

There is evidence that aluminum, which is normally part of the argilaceous minerals is soluabilized in an acid soil and freed to ground water in a potentially toxic form Al^{3+} (Cunningham & Saigo, 1995; See Section A3, A3-8). When the soil pH becomes acidic (below pH 5), aluminum can precipitate phosphate within the correspondingly acidic plant tissues, preventing P translocation, leading to phosphate deficiency symptoms and signs of aluminum toxicity (Bollard, 1983). According to Bollard's (1983) summary of early work, low levels of aluminum can be stimulatory for some species (p.697) and may even be an essential nutrient for other plants (p.699) but slightly higher concentrations Al has distinctly toxic effects causing damage to root caps and meristems and similar restricted growth in plant tops with mottling and necrosis of leaves (p. 697). It is known that some Pine species can contain 1,000 to 2,000 ppm (of aluminum) in their foliage with little damage, and deposits of aluminum succinates, oxalates and citrates are known to occur as storage forms in the heart woods of some tree species (Bollard, 1983), but very little is known of the levels of tolerance in tree roots (See Section A3-8-2). When the mineral soil drops below pH 4.5, freed aluminum (Rost-Siebert 1985) may enter at certain mycorrhizal tips (to be studied) and may be stored or slowly translocated to aerial regions via the xylem (See Section A3-8-3). In the roots, excessive aluminum may bind to meristem cell DNA, thus interfere with cellular division, preventing elongation of primary and secondary roots and shoots, but adaptation and recovery are possible (Horst, Wagner & Marschner, 1983). In forest soils, an aluminum buffering range exists between pH 4.2 and 3.8 (Evers, 1985; Frohlich, 1985; Hildebrand, 1989; Ulrich & Meyer, 1987, Notwotny et al, 1998) which may moderate aluminum uptake by the roots. Despite this, damage and forest decline has been associated with aluminum toxicity (Godbold et al, 1988).

The presence of aluminum within the ectomycorrhizal tips of *Picea abies* and *Fagus sylvatica* was noticed by Rost-Siebert (1985) and documented again using fluorescent

aluminum localization in Norway spruce (*Picea abies*), (Voglelei and Rothe, 1988, 1993). Bound and free aluminum was detected in fresh mycorrhizal Norway spruce (*Picea abies*) root crossections using the fluorescent chemical marker Morin (Nowotny, Schwanz & Rothe, 1998b). The autofluorescence and the aluminum content of mycorrhizae associated with oak roots is virtually unknown. In preliminary testing on sections of fresh mycorrhizal root tips from *Quercus petraea* stained with Morin, aluminum was clearly detected bound to the certain walls of some mycorrhizal cells. This thesis specifically targets the acid stressed roots of *Quercus petraea* isolated from limed and unlimed regions of a high elevation forest in the Merzalben region of Rheinland-Palatinate with the primary goals of determining where aluminum localizes in the mycorrhizal-root complex and how it is affected by liming.

E2-1B: Autofluorescence of Mycorrhizal species in literature - A Comparison

According to Agerer (1986) autofluorescence has been recorded for various mycorrhizal species (Trappe, 1967; Zak 1971, 1973; Moser, 1978) but it is rarely used to characterize relationships of fungi despite some striking differences. The natural autofluorescences for various mycorrhizal species, which have been recorded by previous researchers, are presented in Appendix 12 for comparative purposes. These autofluorescent comparisons will be used a secondary means of species verification with the following disclaimers: (1) The autofluorescence of a mycorrhizal species may vary with its respective alternate host and not necessarily be representative for *Quercus petraea.* (2) Some of the samples may have been fixed in FAA prior to fluorescence examination as a standard practice according to Agerer et al (1994) and formaldehyde treatments can occasionally alter normal autofluorescence (See Section E1-5-3). (3) Autofluorescence can vary in a single species dependent upon a variety of known or unknown environmental factors including, but not limited to: pH, desiccation stress, liming and soil depth.

Previous examples autofluorescence work were rare but include:

1. Dermocybe cinnamomea / Picea abies Autofluorescence: UV 340-380 nm - bluish Mantle and Rhizomorphs, some hyphae distinctly copper orange tint in lactic acid, Blue -

450-490 nm- yellowish green Mantle and Rhizomorphs, some hyphae orange-green with no distinct differences in lactic acid (Agerer, 1987). Not found in Merzalben.

2. Dermocybe sanguinea / Picea abies Autofluorescence: UV 340-380 nm - bluish-green Mantle and Rhizomorphs; Blue 450-490 nm - greenish (Agerer, 1987). Not found.

3. Gomphidius glutinosus / Picea abies Autofluorescence: UV 340-380 nm -slightly blue Mantle; Blue filter 450-490 nm - slightly yellow (Agerer, 1991). Not found.

3. Hydrum rufescens / Picea abies Autofluorescence: UV 340-380 nm -slightly **yellow** whole mycorrhiza (White light: light ochre mantle with small oily drops inside hyphae, oily exudates, crystal-like grain exudates) (Agerer et al, 1996). *Hydrum rufescens / Quercus petraea* (Appendices 7-18 and 12-18) (UV 280-380 nm) was found to have a non-fluorescent to blue outer mantle and the Hartig net only was faint **yellow** (Figure 12-18A).

*4. Laccaria amethystina / Fagus sylvatica A*utofluorescence: UV 340-380 nm- mantle and hyphae light **blue**, Blue filter 450-490 nm - mantle and hyphae **greenish** orange (Brand & Agerer, 1986). *Laccaria amethystina / Quercus petraea* (Appendices 7-20 and 12-20) (UV 280-380 nm) was found to have a tri-layer mantle with a dull layer frequently sandwiched between two bright **blue** layers but the mantle was yellowish in the spring and **greener** in the fall in unlimed soils (Figure 12-20C) with the reverse trend in limed soils. The Hartig net was faint orange-blue in the spring to light orange-blue in the fall in both soils.

*5. Lactarius subdulcis / Fagus sylvatica A*utofluorescence: UV 340-380 nm- creamy white whole mycorrhiza, Blue filter 450-490 nm - **greenish** orange to greenish yellow (Brand & Agerer, 1986). *Lactarius subdulcis / Quercus petraea* (Appendices 7-25 & 12-25) (UV 280-380 nm) had various tints of **green** with the younger cells often being

yellowish-green and the older cells (further from the tip, or in the fall) being duller green (Figures 12-25C, 12-25E, 12-25 G), all with a dull ochre Hartig net.

6. Lactarius vellereus / Fagus sylvatica Autofluorescence: UV 340-380 nm- outer mantle: bright light blue, middle: olive-yellow, inner: faint blue; Blue filter 450-490 nm - outer & inner mantle: greenish yellow, middle layer somewhat more greenish orange (Brand & Agerer, 1986). Not found.

7. Russula acrifolia / Picea abies Autofluorescence: UV -340-380 nm-greenish blue cystidia & inner mantle more intensive than middle parts and **Blue** - 450-490 nm - **yellow** (Zonation like above) (Agerer et al, 1994). *Russula acrifolia / Quercus petraea* (Appendices 7-38 and 12-38) (UV 280-380 nm) mantle and cystidia fluoresced **yellow**-green in spring and faint green in fall while the Hartig net cells were faint orange in unlimed soil. In limed soil, the primary difference was that the Hartig net cells were non-fluorescent (Figure 12-38B).

8. Xerocomus chrysenteron / Fagus sylvatica L. Autofluorescence: UV 340-380 nm - mantle - none, rhizomorphs pale **ochre** with pale blue central hyphae (Brand, 1989). *Xerocomus chrysenteron / Quercus petraea* (Appendices 7-47 and 12-47) (UV 280-380 nm) the outer mantle usually fluoresced very faint yellow but the rhizomorphs varied from blue to ochre to yellow (unlimed soil) and blue to green to yellow (limed soil). The Hartig net cells were usually pale orange-yellow (**ochre**) (limed soil) to some immature regions being non-fluorescent (unlimed soil) (Figures 12-47C, 47E, 47F).

As can be seen by these few comparisons that wall composition, as determined by fluorescence examination, can be very similar (highlighted words) but may also be highly host dependent with fine or distinctive variations. Besides differing host species, the variations have been linked, in this study, to moisture, season, soil depth and liming.

E2-2: Materials & Methods

For a complete description of the following materials and methods see Sections B and E. In brief summary, 24 trees were selected, 12 from an unlimed region and 12 from a limed region of a high elevation *Quercus petraea* (sessile oak) forest. Samples were collected from the base of the trees from three soil horizons (A, B, C) from which mycorrhizae were isolated, identified and quantified. Fifty of the mycorrhizal species (≈ 584 samples of 5 to 10 tips each) were examined for their aluminum content. Fresh samples of each species, from all soil depths, and for each season, present were characterized from both the limed and unlimed forest zones.

The following technique was modified from Vogelei & Rothe (1988, 1993) and Nowotny et al (1988a, 1988b). The isolated mycorrhizae (See Section B2) were stored in distilled water in eppendorf caplets and used immediately or flash frozen in liquid nitrogen and stored at -20 °C and thawed just enough to separate the tips. Using a Leitz freezing microtome (Kryomat 1703), selected mycorrhizal tips from each sample were placed onto a pre-frozen drop of buffer, on a pre-cooled stage (-32.5 °C), and covered with additional pH 6 buffer. Once frozen into position, the tips were cross- or long- sectioned (20-25 µm thickness) using a (type B) cold knife. Sectioning was done to obtain samples from < 1 mm and > 5 mm from each tip. The cut sections were rescued from the knife edge using a fine-haired paint brush and transferred to a drop of pH 6 buffer solution on a clean, new slide and covered with a coverslip. The sections were then viewed immediately using a Leitz Wetzlar Ortholux 2, trinocular fluorescence microscope with LRF#1, SRF#2 and CSF clear filter combinations (See Section E1-3). The normal autofluorescence of each section was determined and photographed or recorded. A 0.01% Morin staining solution [1 ml of 0.1% w/v Morin in 96% v/v ethanol stock diluted with 9 ml (100 mM.l $^{-1}$ pH 6) phosphate buffer] which complexes with aluminum to produce an intense yellow fluorescence was introduced and allowed to diffuse under the coverslip. Aluminum was assumed to be present if an intense yellow fluorescence developed in contrast to the normal autofluorescence of the cell walls. Presence of aluminum was confirmed using

laser analysis (See Section D) and quantified via bound and unbound mineral analysis of the fine roots and mycorrhizae (See Section F).

The fluorescence microscope, besides being used to detect aluminum, was also be used to confirm the original species identification. Ultramicroscopic details of the mantle, cystidia and rhizomorph structures were located and photographed.

The primary goals of the fluorescence examination were to:

1. Determine if aluminum was present in oak ectomycorrhizae.
2. Determine where the Al accumulated.
3. Determine if the mycorrhizae blocked entry of Al into the root.
4. Determine if Al accumulation differed with distance from the tip.
5. Determine if Al patterns varied with soil depth, moisture or pH.
6. Determine if Al patterns varied in the limed soils.

E2-3: RESULTS

E2-3A: General Results

The results of the histochemical tests are presented in Appendix 7 where the fifty species (including 2 unknown morphotypes) of mycorrhiza are presented in alphabetical order (and numbered). Sections were made of the mycorrhizal tips for each season (S = spring, or F = fall) and for each soil depth (0-10, 30-40 or 50-60 cm) at which they were found in the isolated probes from the limed and / or unlimed forest regions. If data is absent, that is because comparative mycorrhizal samples from both the limed and unlimed forests were not present at that particular depth or season. The data sets include the initial natural autofluorescence (A) plus the color changes, if any, when Morin (M) was introduced. Color changes for each type of cell wall (xylem, phloem, pericycle, endodermis, cortex, [(hypodermis, epidermis) = Hartig net], fungal mantle, emanating hyphae, rhizomorphs, and cystidia) are presented, unless that feature was absent in a particular fungus. In addition, where the mycorrhizal tips were long enough, the roots were sectioned to

demonstrate the potential difference between regions at < 1 mm from the tip and > 5 mm from the tip. A key to the summary charts is presented at the beginning of Appendix 7. Prose descriptions for each species along with photographic documentation and diagrams are presented in Appendix 12.

E2-3B: Root Autofluorescence

The walls of the oak root tips (from both unlimed and limed zones) primarily autofluoresced blue. The xylem was nearly always blue with insignificant variations (2.7% were non-fluorescent, very faint blue-yellow or blue-green). The phloem was non-fluorescent in 92-96% of the samples with some samples having very, very faint orange fluorescence (L = 8.7%; N = 4.1%). Orange tones were common in dead or damaged roots. (Dead roots were not used in the mycorrhizal study since it was assumed they would no longer have any control over aluminum sequestration which is for all intents an purposes considered to be an active physiological process). The phloem color anomalies (orange tones) could not be associated with any other external factor and will be considered natural random aging variations. The pericycle and endodermis primarily fluoresced blue and if the fluorescence did vary it tended to stray to the blue-green end of the spectrum (L = 7%; N = 8.7%) or was non-fluorescent (L = 0.3%; N = 4.1%) especially at < 1 mm from the tips. The cortical walls also primarily fluoresced blue with slightly greater variance from green-blue (L = 3.5% ; N = 5.9%) to yellow-blue (L = 4.9%; N = 5.9%) to the orange (L = 0.8%; N = 2 %) end of the range. The variance from blue in the cortex was more frequently noted in the root regions > 5 mm from the tip. For all intents and purposes, the autofluorescence of the fungal Hartig net and that of the epidermis and hypodermis were identical due to the intimate nature of the symbiosis. A basic blue autofluorescence was evident in areas where the Hartig net was absent or poorly developed. The fluorescence of the hypodermal-epidermal-Hartig net walls was entirely species specific. For all intents and purposes, the general basic autofluorescence typical of the living root proper, with the exception of the non-fluorescent phloem, will be considered to be faint to bright blue. The blue being most likely due to the cellulose wall matrix (Wilson & Peterson, 1983).

E2-3C: Fungal Autofluorescence

While some fungal species are non-fluorescent (lacking UV excitable chemical groups), other species are fluorescent (contain UV excitable chemical groups). The natural fluorescence patterns for a given species was found to vary with proximity to the root tip, soil depth, season and with the liming treatment, but to be very consistent from root tip to root tip within a given probe. What is apparent from these fluorescence variations is that the mycorrhizae are highly dynamic, but controlled, in their physiological responses to their microenvironments. Some species have the ability to alter the chemical composition of their hyphal cell walls in specific regions within the fungal structure so that the cystidia, rhizomorphs, outer, middle and inner mantles and the outer and inner Hartig net may fluoresce differently but in a manner consistent for a given species. In some cases the fungi may also alter the fluorescence (chemical composition) of its host's cells. The best example of chemical alteration of the root cells is in the Hartig net, where in most cases observed, the root epidermal and / or hypodermal cell walls take on the characteristic fluorescence of the invading hyphae while the cortical cells (which are not penetrated) do not. In a few species, even the cortical (non-invaded) cells are fluorescently altered from their normal blue tones to green or yellow. From the numerous roots examined it seems that orange tones in the root cell walls are associated with root damage, invasion defense or aging response. The fluorescence of the Hartig net regions did not necessarily match that of the inner, outer mantle regions or other emanating fungal structures but was consistent from root to root within a given species for any particular season, depth or soil treatment. In a converse manner then the host cells may also alter the chemical fluorescent structure of the fungal cell walls which are in intimate proximity in the Hartig net with subtle color changes which differ from the surrounding mantle cells. All of these responses are very species specific and are presented on a species by species basis in Appendix 12.

Many fungi which appeared black in natural light were also non-fluorescent (black) in UV light (*Cenococcum geophilum, Quercirhiza squamosa, Fagirhiza fusca, Fagirhiza setifera, Fagirhiza spinulosa).* On occasion the non-fluorescence was not 100% consistent. If the Hartig net was non-fluorescent, in rare instances it had a very faint

orange (*Cenococcum geophilum, in older regions)* or yellow (*Fagirhiza setifera)* tone. In contrast, some mycorrhizal mantles which were black in natural light, entirely fluoresced yellow (*Piceirhiza bicolor, Piceirhiza nigra)* or green (*Phellodon niger)* in UV. These variations were very characteristic and so could be used to differentiate some of the black ectomycorrhizae.

Rarely was the reverse true. If a mycorrhizal mantle was brown, or other light shades in natural light, it usually had some fluorescence. Only in rare cases (*Unknown Rosa, Hydnum rufescens)* was a colored mycorrhizae non-fluorescent. *Byssocorticium atrovirens* which had a steel blue mantle, either autofluoresced blue or was non-fluorescent in regions. *Genea verrucosa* had a yellowish outer mantle but non-fluorescent inner mantle. These variations allowed for species differentiation.

Some of the mycorrhizae that appear white, light or cream color fluoresced blue (*Amphinema byssoides, Boletinus cavipes, Lactarius acris*), yellow-green (*Boletus edulis, Inocybe appendiculata, Laccaria amethystina),* green *(Fagirhiza arachnoidea, Lactarius subdulcis, Russula acrifolia, Russula fuegiana),* yellowish (*Lactarius chrysorrheus, Lactarius rubrocinctus, Russula ochroleuca, Xerocomus chrysenteron)* or even faint orange (*Fagirhiza tubulosa, Paxillus involutus, Russula mairei, Xerocomus submentosus*)

Some were highly variable with changes in fluorescence from spot to spot even in a single tip which could not be attributed to aging, damage or any other factor (*Cortinarius bolaris*). And some were very variable, but the changes could be associated with distance from tip, soil depth or other factors (*Lactarius pallidus, Quercirhiza fibulocystidiata, Quercirhiza sublutea*).

Brown, or darker toned, mycorrhizal mantles also differentially fluoresced: blue-green (*Pseudotomentella tristis*), green (*Fagirhiza granulosa),* yellow-green (*Piceirhiza chordata, Piceirhiza guttata, Tuber mesentericum*), yellow (*Piceirhiza gelatinosa, Piceirhiza glutinosa, Tuber melanosporum, Tuber puberulum*) or orange (*Genea hispidula, Tuber rufum*).

The preceding statements must be construed as general trends for the outer mantle cells only and are presented here to demonstrate that mycorrhizal species can be differentiated or confirmed based by their autofluorescence. It must be noted that the Hartig net and inner mantle cell layers were frequently distinctly, but differentially, colored. The nuances in autofluorescence were known to vary with liming, depth and season but tended to form patterns very characteristic for each species (Appendix 12).

E2-3D: Goal 1. Determine if Al was present in oak ectomycorrhize.

Using the test reagent Morin, a yellow autofluorescence was considered to be a positive sign of aluminum presence. The sensitivity of the dye for aluminum was pretested (Section E1) and the presence of Al in select samples of ectomycorrhizal roots was confirmed using Plasma quod laser analysis (Section D) and by ICP mineral analysis (Section F). Aluminum was found in oak ectomycorrhizae and the host roots and could be differentiated by location within the cryosectioned samples (Appendix 7 & 12).

E2-3E: Goal 2: Determine where the Al accumulated.

The nature of the Morin tests was sensitive enough that bound Al could be qualitatively localized very specifically as yellow or green specks or deposits, outside the fungal mantle and as yellow deposits within the fungal and host cell walls, or in the cytoplasm if this latter fluid was not lost during sectioning. The presence of bound and unbound Al and other mineral content was quantitatively determined by mineral analysis (Section F). What could not be determined precisely using fluorescence was whether the Al was in the pectinaceous middle lamella, although the technique was sensitive enough to see relative differences in concentration in adjacent cell walls. It was possible also to determine relative concentrations of Al proceeding from the outer mantle to the xylem by the intensity and speed of the Morin-Aluminum response in individual cell walls. Possible locations for Al deposition are presented in Diagrams E2-3E1 and E2-3E2. Precise locations are given for each species in Appendix 7 and photographic evidence is presented in Appendix 12. The following discussion will be of general trends.

Diagram E2-3E1: Possible aluminum deposition sites in mantle cells (Cx) based upon fluorescent examination. (Actual locations varied with fungal species).

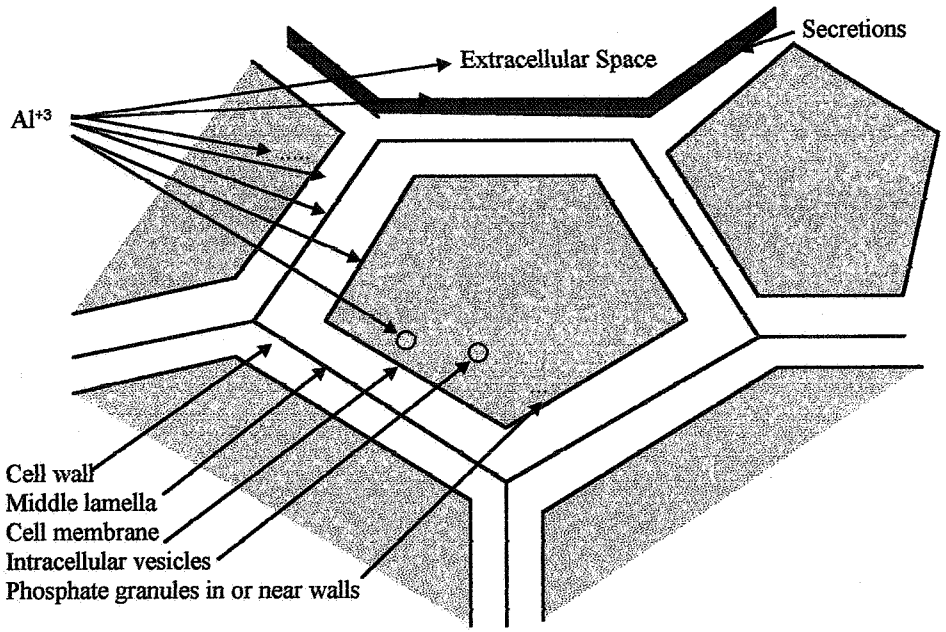
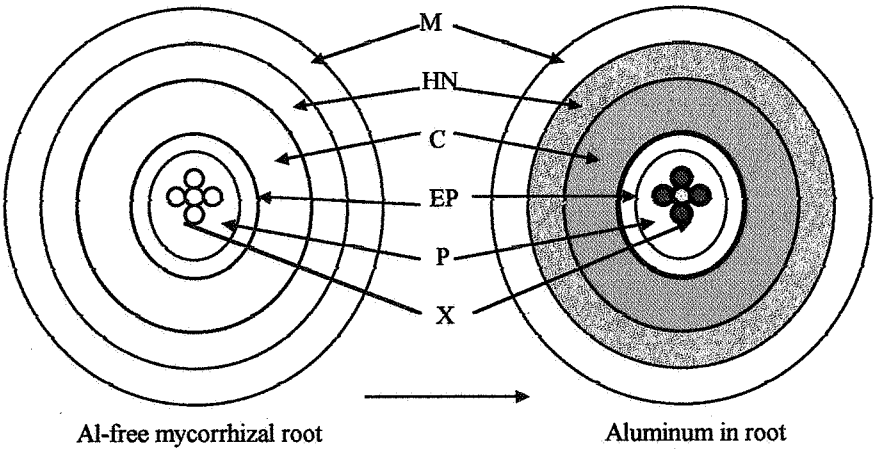


Diagram E2-3E2: Usual locations of aluminum deposition within the fine roots (Cx) from fluorescence examinations. Mycorrhizal mantle (M), Hartig net (HN), Cortex (C), Endodermis and Pericycle (EP), Phloem region (P) and Xylem (X). Darker color indicates regions of greatest aluminum accumulations commonly seen in the cell walls of *Quercus petraea* fine mycorrhizal roots, when the mycorrhizae failed to sequester the Al.



As shown in Diagram E2-3E1 there are many possible sites for aluminum deposition in and around the fungal sheath. With fluorescent analysis specks of Al were visible external to the mantle, but Al-phosphate granules could not be specifically localized inside the cells, nor could middle lamellar depositions be segregated from the cell walls. Despite this, aluminum deposits could be localized by region and from cell to cell and concentration could be related to the intensity and speed of the reactions.

The primary site of control of aluminum was in the fungal sheath (To be discussed later). The endodermis was the second major barrier to aluminum movement (Diagram E2-3E2). Once past the mantle and Hartig net, the intensity of Al concentrations frequently increased from the external to internal cortical regions with the most intense deposits occurring in the cortical walls next to the endodermal ring - in a fashion similar to water behind a dam (-i.e.- Appendix 12- Figures 12-1C, 3D, 5N, 6C, 8B, 10C, 11C, 18B, 25H, 26F, 27B, 27D, 33D, 37B, 44E, 45B).

The only obvious routes past this barrier had to be either via: symplasm (entering the cell cytoplasm and traveling via plasmodesma), growing points (meristem) or at wound sites. Aluminum did in some fashion breach the endodermal barrier because it was very evidently present in the ringed portions of the xylem walls in the great majority of roots sectioned. The rings of the xylem may be the ultimate sequestration point in the fine roots before entering the flow gradient of water to the leaves. Aluminum not held here, after saturation has been reached, may move quickly up to the stem, especially if the xylem sap is rapidly taking in water.

Hydration rings can form around Al (See Section A3-3) when the roots are well saturated with acidified water, preventing the Al from attaching to potential bonding sites along the way. From other studies it is known that Al can accumulate in heart wood, and leaves (See Section A3-8-3), which directly indicates movement with xylem flow with potential sequestration in aerial regions distal from the roots.

In addition to the bound form (sequestration) seen in the fluorescence studies, Al was definitely present in the unbound form (Section F). The mineral tests for unbound Al (Section F) could not determine if the free form was apoplastic or symplastic, but definite apoplastic routes and barriers could be determined in the fluorescent sections. The unbound Al most likely translocated intercellularily along mass flow diffusion gradients towards the xylem. If it passed ectomycorrhizal sequestration points and then somehow breached the Hartig net and pericyclic barriers and then entered the stele it could be drawn directly to the xylem.

If the motion of the ions was not controlled by mass flow, then Al should have also accumulated in the phloem as soon as it entered the stele. The conflicting directional flow of the phloem most likely physically prohibited Al approach and accumulation. It is entirely possible however that the phloem walls contain specific non-fluorescent chemicals (potentially similar to those found in the non-fluorescent *Cenococcum geophilum* walls) which prohibited uptake. In the few cases where Al was found in phloem walls, it was present in the cytoplasm. Logically then, the only way Al could have reached this point was either by : 1. Traveling upwards via the xylem tubes to the leaves and back via the phloem flow or 2. The phloem was damaged.

Since phloem flow slows in winter, and during drought, and there was no obvious accumulations during these time periods of slow ion translocation, the living phloem must contain blocking chemicals within their wall ultrastructure. When alive and undamaged, these non-fluorescent blocking chemicals of the phloem differ from the bright blue pericycle and endodermal wall Al-blocking constituents. When aged, dying or damaged, the phloem occasionally took on orangish autofluorescence. On occasion Al was present in the phloem and the xylem (Figure 12-3C, 12-3D). In these cases, the roots were considered to be very unhealthy.

Besides increasing nutrient uptake, the mycorhiza may selectively block Al, protecting the root from the side effects due to sequestration of toxins.

E2-3F: Goal 3: Determine if the mycorrhizae blocked entry of Al into the root.

E2-3F-1: Extracellular Aluminum

Aluminum was externally present as green (weak) or yellow (strong) specks in the outer mantle areas in the fungal secretions, but only in **deeper moister soils,** limed (K) or unlimed (N) in 2000: [*Piceirhiza guttata* (N), Xerocomus chrysenteron (K) spring 2000, 30-40 cm; *Xerocomus submentosus* (K), 0-10 cm (an exception)] [*Byssocorticum atrovirens* (N), *Cenococcum geophilum* (K), *Fagirhiza granulosa* (N), *Unknown Rosa* (K), fall 2000, 30-40 cm depth] [*Lactarius rubrocinctus* (N & K) *Lactarius subdulcis* (K), *Fagirhiza granulosa* (N & K), *Tomentella ferruginea* (K) fall 2000 at 50-60 cm depth] (Data from Appendix 7 & 12). These specks (Appendix 12 - Figures 12-4D, 5H, 11C, 13A, 13B, 26F, 27C, 36C, 36D, 50B) were not seen in the dry 1999 seasons. Because of dehydration, Al may have formed tight external connections which were indistinguishable from the truly incorporated Al in the outer mantle regions. ESEM (Environmental SEM) technology may provide better resolution. The external specks did not affect mantle structure in any apparent way and so are probably not toxic. What this does indicate is that in moist, deep soil, many mycorrhiza are able to precipitate Al outside the mantle.

E2-3F-2: Intramural Mantle Aluminum

The presence of aluminum within the walls of the fungus varied with depth, season, soil moisture and liming treatments and most importantly, species by species. In the root, both the cortical and xylem cell walls were very competent at Al sequestration, often having intense accumulations, however, the endodermis, pericycle and phloem cell walls were nearly always devoid of any Al residue. Some fungi produced effective barriers to root Al at certain times of the year and when they did so, the Al content of the cortical and xylem cell walls declined. If there was no or little evident barrier or sequestration in the symbiotic fungus, then Al content of the cortical and subsequently, the xylem, both increased. Those species which were present both spring and fall, that tended to produce seasonal barriers at 0-10 cm depth are listed in Figure E2-3F-2A.

Figure E2-3F-2A: Ectomycorrhiza that form seasonal barriers to aluminum translocation at 0-10 cm depth in mantles (or only in the Hartig net*).

LIMED SOIL

Spring : *Boletus edulis, Piceirhiza bicolor, Piceirhiza chordata, Quercirhiza squamosa**

Fall: *Amphinema byssoides, Fagirhiza fusca*, Fagirhiza setifera*, Fagirhiza spinulosa*, Genea hispidula*, Inocybe obscurobadia, Laccaria amethystina, Paxillus involutus, Quercirhiza sublutea, Russula acrifolia, Tuber melanosporum*

Spring and fall: *Fagirhiza granulosa, Piceirhiza nigra*

Variable Barriers: *Cenococcum geophilum*, Lactarius subdulcis, , Quercirhiza fibulocystidiata, Tuber rufum, Xerocomus chrysenteron*

UNLIMED SOIL

Spring: *Lactarius subdulcis, Piceirhiza bicolor, Piceirhiza chordata, Quercirhiza squamosa*, Quercirhiza sublutea, Russula acrifolia, Tuber rufum*

Fall: *Boletus edulis, Cortinarius armillatus, Fagirhiza fusca*, Fagirhiza setifera*, Fagirhiza spinulosa*, Fagirhiza tubulosa*, Genea hispidula, Laccaria amethystina, Paxillus involutus, Piceirhiza nigra, Quercirhiza fibulocystidiata, Xerocomus chrysenteron*

Spring and Fall: *Fagirhiza granulosa*

Variable Barriers: *Cenococcum geophilum *, Lactarius subdulcis, Tuber melanosporum*

E2-3F-3: Aluminum in the Hartig Net

Liming altered seasonal barriers to Al uptake in several species (Figure E2-3F-2A). In some species (*-i.e.- Cenococcum geophilum, Fagirhiza sp., Lactarius subdulcis*) the seasonal Al barriers were in the Hartig net* instead of the mantle. Where the mantle failed to sequester Al, the Hartig net often had significant accumulation (Appendix 12 - Figures 12-5K, 5L, 13D, 20F (unlimed), 24B, 35D, 50B, 50D). In other cases, the Hartig net did not sequester Al, but rather provided a physical barrier to apoplastic transport, often resulting in enhanced mantle accumulation and lowered cortical content, but no appreciable increase within the Hartig net area. Very generally, the Al content of the Hartig net was

considerably reduced when Al content in the mantle was strong (Appendix Figures 12-1B, 4D, 8D, 10B, 20H (limed), 21D, 32B, 33G, 42C, 44E).

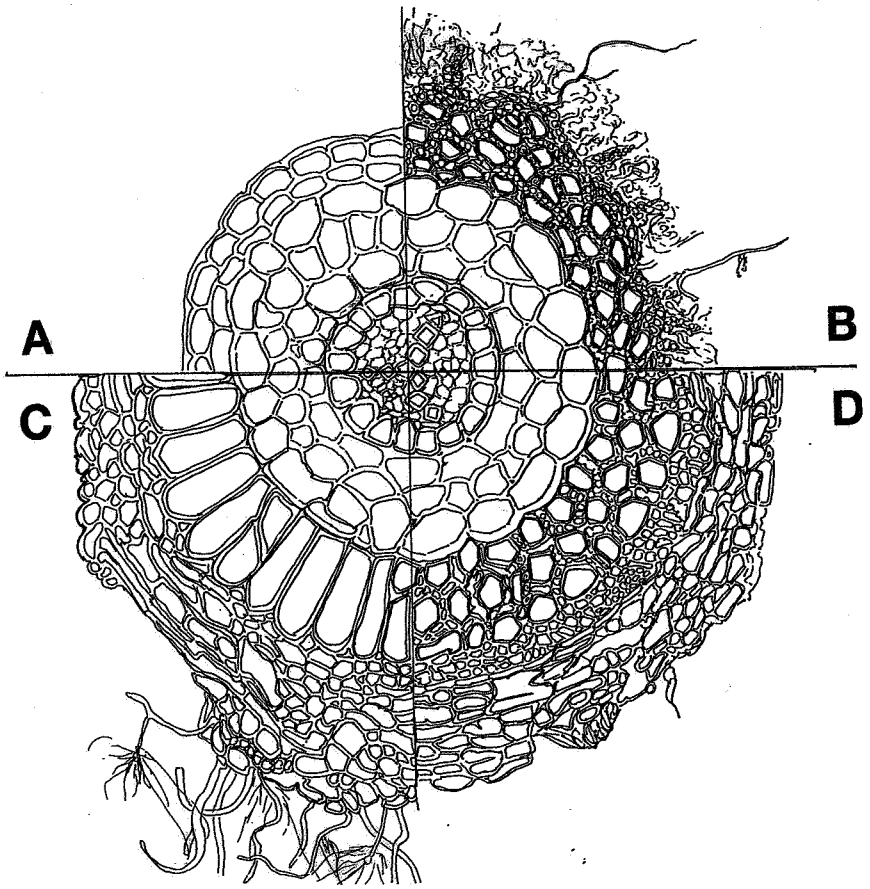
E2-3F-4: General Fungal Morphology and Aluminum Sequestration

The ability to sequester Al was not related to the thickness of the outer mantle, but the morphological appearance of the Hartig net may have an effect on ion movement (Figure E2-3F-4A and E2-3F-4B). The strongest control of Al was related to physiological and / or chemical differences within the walls at various points in the fungal architecture rather than the mantle architecture itself (Appendices 7 & 12).

E2-3F-4A: Pale Ectomycorrhizae

Three morphologically distinct pale ectomycorrhizae were compared (Figure E2-3F-4A). *Cortinarius armillatus, Amphinema byssoides* and *Cortinarius bolaris*. *Cortinarius armillatus* had very tiny hyphal strands, densely invading the 2-3 outer root layers to form a thick Hartig net, but a thin outer mantle with numerous long, very fine emanating hyphae. In natural light, this ectomycorrhiza appears white but in UV it usually fluoresced variably from blue to green to orange depending upon location in the soil (Appendices 7-6 and 12-6, Figures 12-6A, 6B, 6C). In unlimed soil, the mantle was usually Al-free, but not in limed soils. Upon aging, or with soil depth *Cortinarius armillatus* lost the ability to block Al uptake into the cortex. In contrast, *Cortinarius bolaris* had the same type of penetration and Hartig net formation, but larger hyphal strands and a much thicker outer mantle, lacking emanating hyphae. It had even greater variability in autofluorescence (Appendices 7-7, 12-7) but overall, much less Al content. Reduced Al presence could be associated with greener or orange autofluorescences but not Hartig net morphology, which did not change. The third mycorrhiza, *Amphinema byssoides* typically had a distinctly different Hartig net consisting of one layer of radially elongated cells. In between the cells, the hyphal strands were usually singular, also running radially. The mantle was intermediate between *Cortinarius bolaris* (very thick, large hyphae) and *Cortinarius armillatus* (with regions of very fine long emanating hyphal strands). The mantle and Hartig net which were non-fluorescent to bluish, did not accumulate aluminum but allow it to pass into the cortex where it strongly accumulated.

Figure E2-3F-4A: Comparative cross-sectional morphology of pale ectomycorrhizae. In most ectomycorrhizal roots, the cortex and stele were unmodified, however the epidermal and hypodermal (outer two) cell layers were frequently physically altered by fungal hyphal penetration. These physical nuances could not be related to altered ability to sequester aluminum. A = *Quercus petraea* non-mycorrhizal root, B = *Cortinarius armillatus*, C = *Amphinema byssoides*, D = *Cortinarius bolaris.*

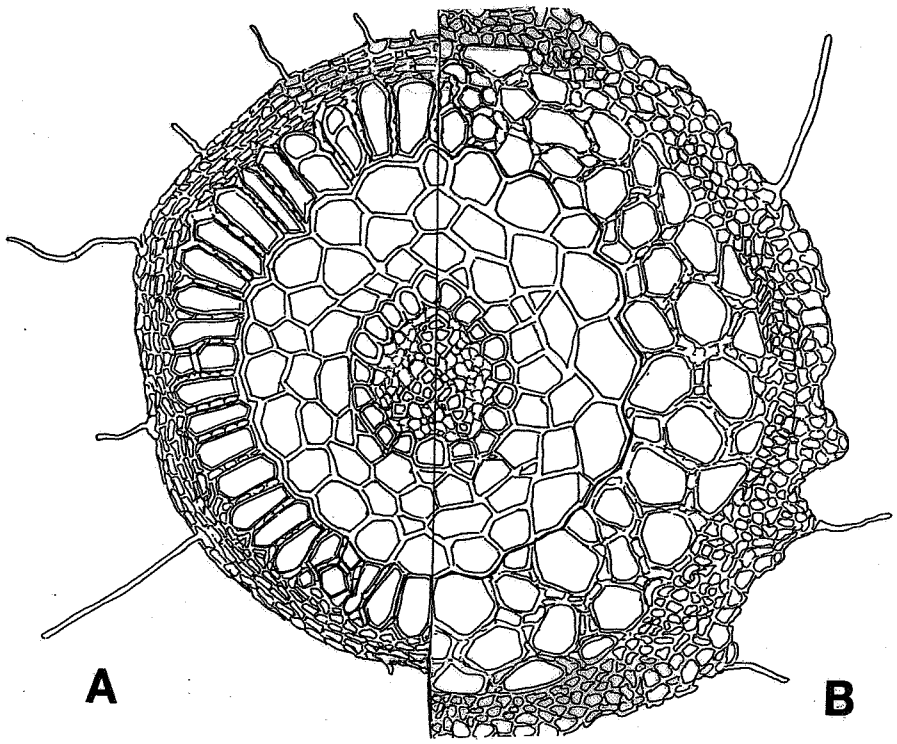


ER-3F-4B: Dark Ectomycorrhizae

Cenococcum geophilum had a dense non-fluorescent mantle and Hartig net which was composed of elongated exodermal cells with tight hyphal connections (Figure E2-3F-4B), but despite this the Aluminum passed almost freely and accumulated intensely in the cortical cell walls. Only on occasion, did the Hartig net accumulate tiny amounts of Al (Appendices 7-5 and 12-5). *Fagirhiza setifera* (also a black ectomycorrhiza), was more variable then *Cenococcum* in autofluorescence, sometimes having faint yellow regions in

the normally non-fluorescent mantle and emanating hyphae. Despite looser and more irregular hyphal connections, *Fagirhiza setifera* variably contained Al in the mantle, emanating hyphae and Hartig net and tended to accumulate qualitatively less Al in the cortex then *Cenococcum*, but was much more dependent upon variations in pH and liming. The ability to control or accumulate Al is not related the black macroscopic coloration, but may be related to slowing apoplastic movement into the roots via adsorption or incorporation along long tortuous intercellular routes rather than along the radially elongated Hartig net channels. It is also possible that less common mycorrhizae such as *Fagirhiza* may illicit more effective invasion response cortical barriers.

Figure E2-3F-4B: Comparative morphology of dark ectomycorrhizae. *Cenococcum geophilum* (A) was black in white light with a thick dense mantle with many radiating black hyphae and a tight Hartig net surrounding elongated exodermal cells. *Fagirhiza setifera* (B) was also black in white light but the hyphae were more irregular and looser in their penetration and less definitive in alteration of epidermal and hypodermal morphology producing a more tortuous pathway for apoplastic flow.



E2-3F-5: Mycorrhizal Control of Aluminum Movement

Each mycorrhizal species was unique in its ability to control aluminum adhesion, sequestration or mobility in its own fungal walls and into its host roots. Some of the control can be attributed to morphological structure (elongated Hartig net cells) but the primary control was in biochemical make up of the fungal walls and the living physiological action of the fungi and its ability to alter the chemical composition of its walls under variable natural conditions. The dynamic abilities of individual mycorrhizal species is presented in detail in Appendices 7 & 12. Summaries of the data are presented in Figure E2-3F-5A (Mycorrhiza that seldom contain Al), Figure E2-3F-5B (Mycorrhiza that usually contain Al). What was most evident from the mycorrhizal studies was that liming definitely affected the ability of certain species to sequester Al. There were fewer mycorrhizal species from the limed zones (26) than the unlimed (31) that did not sequester (or rarely contained) Al in their mantles. Sixteen of these mycorrhizal species were the same for both forest zones, but there were subtle differences in the uptake of Al in 8 of those species (Figure E2-3F-5A). The most common observation was that the limed mycorrhizal roots contained more Al than the unlimed. This was later confirmed by the mineral analysis (Section F).

Of the 21 species from the limed, and 17 from the unlimed plot, that always or nearly always had Al in their mantles, 12 were common to both areas (Figure E2-3F-5B). Of the common species, 6 had very similar Al uptake. Of the remainder, the limed species (4) tended to have more Al present in the associated cortex than the unlimed (2). Once again, the Al content of the cortex of the limed roots tended to be higher than that of the unlimed but the differences were often less distinct. What is of the greatest importance to note was that if the mantle contained Al in abundance, the cortical cell walls were less pronounced and conversely, if the Al content of the mantle was low, the cortical content was usually higher. This last observation is not evident from Figures E2-3F-5A and E2-3F-5B but it is evident in the more specific fluorescence results in Appendices 7 & 12.

Figure E2-3F-5A: Mycorrhiza that seldom contain Aluminum. Table A : Aluminum generally absent (A) from mantle (M) but variably present (V), usually present (P) or absent (A) in the Hartig Net (HN) or cortex (C). Table B: Aluminum absent or rarely present. Occasionally autofluorescence interference made it difficult to determine if Al was present (?) and sometimes Al was present only as tiny specks (*) outside the mantle. Mycorrhizal species with similar physiological responses are highlighted.

A: ALUMINUM GENERALLY ABSENT FROM MANTLE

Limed	M	HN	C	Unlimed	M	HN	C
Amphinema byssoides	A	P	P	*Byssocorticium atrovirens*	A*	V	V
Cenococcum geophilum	**A**	**V**	**V**	*Boletus cavipes*	A	A	P
Fagirhiza arachnoidea	A	A	V	***Cenococcum geophilum***	**A**	**V**	**V**
Fagirhiza fusca	A	V	P	*Cortinarius armillatus*	A	V	V
Fagirhiza setifera	A	A	P	*Elaphomyces muricatus*	A	A	V
Genea hispidula	A	A	V	**Fagirihiza arachnoidea**	A	V	V
Phellodon niger	A	P	P	**Fagirhiza fusca**	A	A	V
Piceirhiza glutinosa	A	A	V	Fagirhiza spinulosa	A	A	V
Piceirhiza guttata	**A**	**A**	**P**	*Laccaria amethystina*	?	?	V
Pseudotomentella tristis	A	A	P	*Lactarius acris*	A	A	V
Quercirhiza squamosa	**A***	**V**	**P**	*Lactarius chrysorrheus*	?	?	P
Russula fuegiana	**A**	**A**	**V**	*Lactarius rubrocinctus*	?	?	P
Unknown Rosa	**A**	**P**	**P**	**Piceirhiza guttata**	**A**	**A**	**P**
				Quercirhiza squamosa	**A***	**V**	**P**
				Russula fuegiana	**A**	**A**	**V**
				Russula mairei	A	A	V
				Tomentella ferruginea	A	A	P
				Unknown Rosa	**A**	**P**	**P**

B: ALUMINUM ABSENT OR VARIABLY PRESENT IN MANTLE

Limed	M	HN	C	Unlimed	M	HN	C
Cortinarius armillatus	V	A	P	*Amphinema byssoides*	V	V	V
Cortinarius bolaris	V	A*	V	**Fagirhiza granulosa**	**V**	**V**	**V**
Fagirhiza granulosa	**V**	**V**	**V**	Fagirhiza setifera	V	A*	V
Fagirhiza spinulosa	V	A	V	Fagirhiza tubulosa	V	V	V
Genea verrucosa	V	A	P	*Genea hispidula*	V	A	V
Lactarius pallidus	**V**	**P**	**V**	***Genea verrucosa***	V	A	V
Paxillus involutus	V	V	P	***Lactarius pallidus***	**V**	**P**	**V**
Piceirhiza bicolorata	V	A	P	***Paxillus involutus***	V	V	V
Quercirhiza fibulocystidiata	V	V	P	**Piceirhiza bicolor**	V	V	V
Quercirhiza sublutea	**V**	**V**	**V**	**Quercirhiza fibulocystidiata**	V	?	V
Russula acrifolia	**V**	**A**	**V**	**Quercirhiza sublutea**	**V**	**V**	**V**
Tomentella ferruginea	V	V	P	***Russula acrifolia***	**V**	**A**	**V**
Xerocomus chrysenteron	V	A	P	***Xerocomus chrysenteron***	V	A	V

Figure E2-3F-5B: Mycorrhiza that usually contain Aluminum. Table A : Aluminum generally present (P) in mantle (M) but variably present (V), usually present (P) or absent (A) in the Hartig Net (HN) or cortex (C). Table B: Aluminum absent or rarely present. Occasionally autofluorescence interference made it difficult to determine if Al was present (?) and sometimes Al was present only as tiny specks (*) outside the mantle.

A: ALUMINUM PRESENT IN MANTLE

Limed	M	HN	C
Elaphomyces muricatus	P	A	P
Fagirhiza cystidiophora	**P**	**A***	**P**
Inocybe appendiculata	**P**	**V**	**V**
Inocybe obscurobadia	P	A*	P
Laccaria amethystina	P	A*	P
Lactarius acris	P	A*	P
Lactarius chrysorrheus	P	P	V
Lactarius rubrocinctus	P	?	P
Piceirhiza chordata	P	V	P
Piceirhiza gelatinosa	P	?	?
Russula ochroleuca	P	?	P
Tuber melanosporum	P	V	V
Tuber messentericum	**P**	**A**	**P**
Unknown gray	P	A	P
Xerocomus submentosus	P	A	V

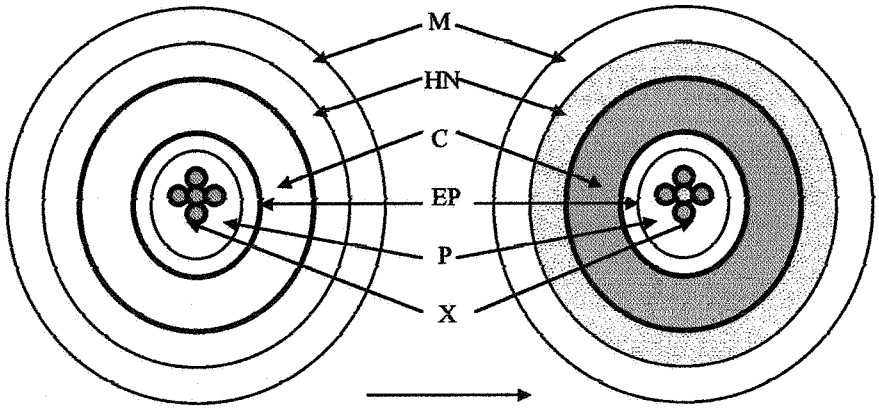
Unlimed	M	HN	C
Fagirhiza cystidiophora	**P**	**A***	**P**
Inocybe appendiculata	**P**	**V**	**V**
Inocybe obscurobadia	P	A*	A
Piceirhiza nigra	P	A	?
Piceirhiza gelatinosa	P	P	P
Pseudotomentella tristis	P	?	P
Russula ochroleuca	P	?	V
Tuber messentericum	**P**	**A**	**P**
Unknown Gray	P	A	V
Xerocomus submentosus	P	A	A

B: ALUMINUM PRESENT OR RARELY ABSENT IN MANTLE

Limed	M	HN	C
Boletus edulis	**V**	**A**	**V**
Lactarius subdulcis	**V**	**V**	**V**
Piceirhiza nigra	**V**	**V**	**V**
Russula mairei	V	A	P
Tuber puberulum	V	P	P
Tuber rufum	V	A	V

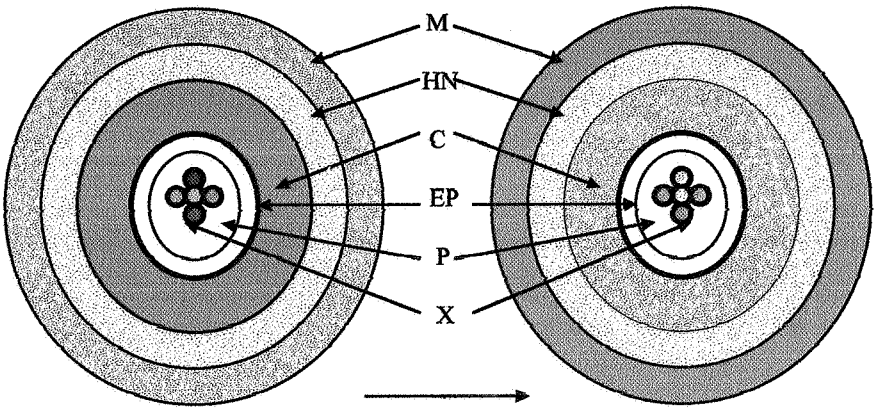
Unlimed	M	HN	C
Boletus edulis	**V**	**A**	**V**
Lactarius subdulcis	**V**	**V**	**V**
Piceirhiza chordata	V	V	P
Piceirhiza glutinosa	V	V	P
Piceirhiza nigra	V	A	V
Tuber melanosporum	V	V	V
Tuber rufum	V	A	P

Figure E2-3F-6A: Mycorrhizal Classification. This is a preliminary attempt to classify mycorrhizal species based upon their abilities to sequester aluminum. Typical Al accumulation is indicated by the intensity of the gray shading. Mantle (M), Hartig Net (HN), Cortex (C) Endodermis & Pericycle (EP), Phloem (P) Xylem (X).



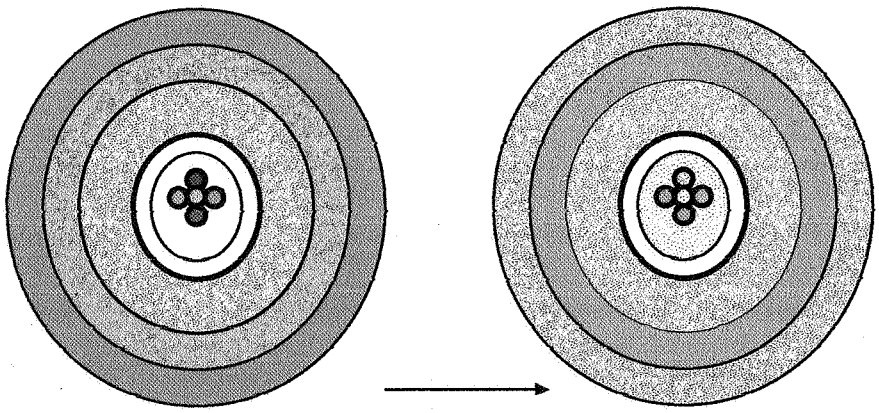
Class 1A: Al-free mycorrhizal root

Class 1B: Mycorrhizal mantle "traps" Al



Class 2A: Mantle-some Al

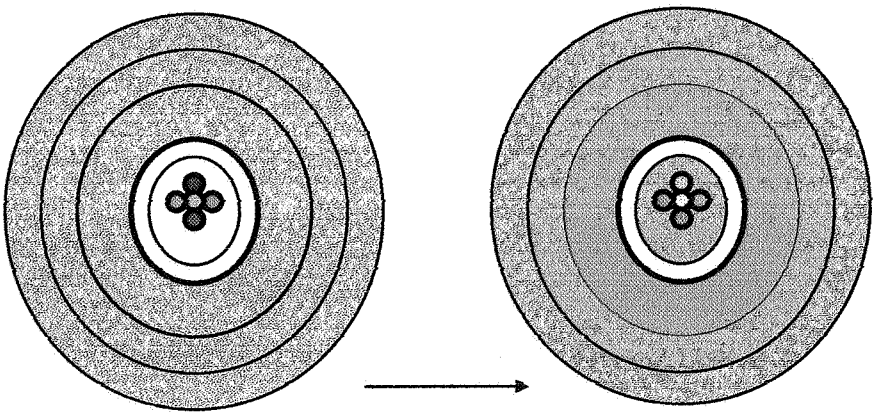
Class 2B: Mantle-abundant Al



Class 3A: Hartig net-some Al

Class 3B: Hartig net-abundant Al

Figure E2-3F-6: Mycorrhizal Classification Continued:



Class 4A: Al Evenly distributed Endangered Root

Class 4B: Heavy Al content, even in Phloem Wounded or Dying root

E2-3F-6: Classification of Mycorrhizal Control of Aluminum Movement

In the tentative classification system shown in Figure E2-3F-6A, six general forms of Al accumulation are depicted based upon the sequestration in the mantle and / or Hartig net of the fungi. In general, it was observed that if aluminum made it past the fungal barrier it accumulated in the cortical cells up to the endodermal barrier and if it made it past the endodermal (outer ring) or peridermal (inner ring) of the steele it was aggressively taken up into the rings of the xylem tubes, but not the phloem. Once past the mycorrhizal barrier, accumulation in the root proper was independent and followed consistent patterns with accumulation occuring along the diffusion pathway in competent root cell walls.

CLASS 1A: These ectomycorrhizae do not sequester Al unless severely damaged or dead. Some may act as barriers to apoplastic flow due to tight intercellular connections but have no apparent symplastic pathway for Al. Class 1A can change to Class 1B.

CLASS 1B: These ectomycorrhizae do not sequester Al unless severely damaged or dead. Once the Al is in the cortex however, especially in older regions > 5 mm from the tip, backwards diffusion (out of the root) is hampered by the fungal sheath.

CLASS 2A: These ectomycorrhizae weakly sequester Al within their mantle walls. The Hartig net is usually Al-free or low in content, but the cortical cells generally contain abundant Al. Class 2A can change to Class 2B.

CLASS 2B: These ectomycorrhizae strongly sequester Al within their mantle walls. The Hartig net can be Al-free, low in content or high in content, but generally, the more Al content the fungal cells contain, the less is evident in the cortex. Class 2B can revert back to Class 2A under certain circumstances.

CLASS 3A: These ectomycorrhizae weakly sequester Al within the Hartig net. The mantle may be Al-free or low in content, but is often higher in content. Generally, the cortical cells are lower in content then the Hartig net. Class 3A can revert back to Class 2B or forward to Class 3B.

CLASS 3B: These ectomycorrhizae strongly sequester Al within the Hartig net. The mantle may be Al-free or higher in content, but it is often lower in content. Generally, the cortical cells are much lower than the Hartig net in content. Class 3B can revert back to Class 3A or may change to 4A.

CLASS 4A: These ectomycorrhizae have mantles and Hartig nets with the same approximate accumulations of Al. The cortical cells can be Al-free, low in content, the same or higher in content. Generally the cortex is similar or higher. Class 4A can change to 4B. It may be possible to revert back to Class 3, but not likely.

CLASS 4B: These ectomycorrhizae have abundant Al throughout. In some cases even the endodermal outer wall contained Al, and on rare occasions the phloem did as well. These roots often showed signs of physical abrasions and damage in the areas of heaviest Al accumulations.

The primary difficulty with this classification system is that the mycorrhizae are highly dynamic and can alter their physiology to microenvironmental conditions. The most transition occurs between Classes 2 and 3. A Class 1 mycorrhiza usually does not change to a Class 2, or the other way, Class 2 to Class 1, but both may taken on some characteristics of Class 3 (Hartig net metabolic changes) or Class 4 (dying). Figure E2-3F-6B lists a few examples of the various classes.

Figure E2-3F-6B: Examples of Mycorrhizal Classes. A few examples for each class are presented here. The photographs are provided in Appendix 12.

CLASS	SPECIES	Photographs
1A	*Cenococcum geophilum*	12-5J
1A	*Lactarius acris*	12-21B
1B	*Cenococcum geophilum*	12-5L, 5H
1B	Fagirhiza fusca	12-11C
1B	*Hydrum rufescens*	12-18B
2A	Fagirhiza granulosa	12-12E, 12F
2B	Fagirhiza granulosa	12-12D
2B	*Russula ochroleuca*	12- 41A
3A	*Lactarius acris*	12-21D
3A	Fagirhiza setifera	12-13B
3A	Piceirhiza guttata	12-32B
3B	Fagirhiza setifera	12-13B
3B	*Lactarius rubrocinctus*	12-24B
4A	*Lactarius subdulcis*	12-25F, 25H
4A	Quercirhiza sublutea	12-37B, 37C
4B	Piceirhiza chordata	12-29D, 29F
4B	*Tuber rufum*	12-45D

E2-3G: Goal 4. Determine if Al accumulation differed with distance from the tip.

The fungal barriers, in many cases were incomplete (thin, poorly formed), especially near the growing tips and allowed massive Al penetration to the meristem (-i.e.- Appendix 12 - *Cenococcum geophilum*, Figures 12-5F, 12-5G, and *Fagirhiza setifera*, Figure 12-13A, *Lactarius subdulcis* Figure 12-25F, *Tuber rufum* Figure 12-45C, D). In these cases, where the fungal mantle and their root endodermis were both complete just beyond the tip, Al penetration to the xylem declined abruptly. In other species, also with incomplete mantle coverage, Al was absent from the meristem (-i.e.- Appendix 12 - *Byssocorticium atrovirens*, Figure 12-4B; *Laccaria amethystina*, Figure 12-20E). Meristem zones of other species were not examined, but it can be assumed that at least in some cases, Aluminum access to the xylem can occur in the meristem region.

Most of the sections examined were taken from 1 and 5 mm from the tip. At regions < 1 mm from the tip the ectomycorrhizae architecture was well developed. In regions > 5 mm from the tip, the mantle was mature. Frequently the two areas were identical in their autofluorescences and Morin reactions in fresh spring or moist fall roots. Limed roots often differed from unlimed roots. Sometimes the "mature" mantle retained the basic structure or sometimes the cells had collapsed, but often the mature mantle was biochemically different in the fall, or in deeper, dryer or limed soils. The accumulation of greenish or orange fluorescing chemicals in these areas was common and was considered a sign of aging, along with collapsing architecture. These chemicals usually reduced Aluminum sequestration but also prevented its movement into the root in these older areas. In regions of the roots distal from the tip, Aluminum could access the cortex in bifucations (Piceirhiza chordata - Appendix 12 - Figure 12-29E, F, G, H).

Comparisons between roots from < 1 and > 5 mm were made for all species examined and are presented in Appendix 7 and 12. Photographic records for *Cenococcum geophilum* show various stages of development from the apex (meristem) (Appendix 12 - Figures 12-5E, 5F, 5G) to < 1 mm from the tip in unlimed soils (Figures 12-5I, 5J) and limed soils

(Figures 12-5K, 5L). Several other species also have representative photos of proximal and distal cross-sections showing mantle structure and Morin reactions. [*Elaphomyces muricatus* - Figures 12-8A-D; Fagirhiza granulosa - Figures 12-12D-F; Lactarius acris - Figures 12-21B-D; Lactarius subdulcis - Figures 12-25C-H; Paxillus involutus - Figures 12- 26C-F; Piceirhiza chordata - Figures 12-29A-D; Piceirhiza nigra - Figures 12-33A-G; Pseudotomentella tristis - Figures 12-34A-E; Quercirhiza squamosa - Figures 12-36B-F; Tuber rufum - Figures 12-45A-D; Unknown Rosa - Figures 12-50A-D)

Autofluorescence, which gave some indication of the biochemical nature of the mycorrhizae could be used to predict Al sequestration ability for some species. Fungal walls with a moderate to bright blue to yellow autofluorescence were often more capable of sequestration than those of the same species which developed orange or green autofluorescence in areas distal from the tip. In many cases, a dull orange or green autoflurescence was directly associated with damaged, mature, aging or deep roots and better sequestration and lower Al translocation to the root. Just by counting the number of species presented in Figure E2-3F-2A that sequester Al, it is evident that mature mycorrhizae have enhanced ability to block Al in the fall. From the cryosectioning at < 1 and > 5 mm from the tips (Appendix 7), Al is best excluded in regions distal to the growing tip. This can be translated as: Older or more mature mycorrhiza tend to block Al better than growing tips, but their true nature is species dependent and dynamic.

E2-3H: Goal 5. Determine if Al varied with soil depth, moisture or pH.

Aluminum content did vary with soil depth, moisture and pH. Each species was specific in its responses to these microenvironmental fluctuations. These qualitative changes are discussed in great detail for each species in Appendices 7 and 12.

From the fluorescence study, it was determined that in general, the whole roots growing in unlimed soil at 0-10 cm depth had the least aluminum content, while the roots growing in limed soil at 0-10 cm depth had more intense Al deposition. In deeper soils and with aging, the mycorrhizae tended to lose their ability to control Al sequestration or

translocation, but this was not universal. Al accumulation in the extracted roots was greater as depth increased. Less Al accumulated in roots from acidic soil than the limed soils possibly due to increased mobility of the ions in acids. During the very dry fall, very few mycorrhizae were extracted but those that were had a far greater aluminum content than ectomycorrhizae of the same species that were extracted in the very wet fall. Quantitative changes that support these observations will be presented in Section F (Bound and Unbound Mineral Analysis).

E2-3I: Goal 6. Determine if aluminum patterns varied with liming.

Throughout the study, in numerous samples, it was found that the limed ectomycorrhizal roots exhibited the strongest Morin reactions implying the greatest Al depositions (Appendix 7 & 12). For example: In unlimed acidic soil, even though *Cenococcum geophilum* did not incorporate Al (Appendix 12-5I, 5J), no Al was found in many roots sampled. In contrast, in limed soils at the same depth, *Cenococcum* still did not incorporate Al, but it was abundantly present in the cortex (Appendix 12-5K, 5L). Both pH and liming played roles in Al translocation in this particular species.

Besides having greater Al accumulation, the limed ectomycorrhizal roots frequently had strikingly different sequestration patterns. While some species were either Class 1(i.e.- Fagirhiza arachnoidea, Appendices 7-9 and 12-9), or Class 2 species (Inocybe appendiculata Appendices 7-19 and 12-19) in both limed and unlimed soils, some were variable. For example: (1) *Elaphomyces muricatus* (Appendices 7-8 and 12-8). Where lime was present, this species sequestered Al (Class 2) and in the unlimed soils it did not (Class 1) at all depths, including at 50-60 cm, where the pH was greater than in the limed soils. Liming, not pH, altered the metabolic action of this species. (2) Fagirhiza setifera (Appendices 7-13 & 12-13) had Al deposition patterns that were almost mirror images of each other - if Al was strongly present in one group at a certain depth and location, in the other, it was weakly present to absent. In this species liming tended to reduce sequestration (Class 2A) while the unlimed samples were Class 2B. The intense diversity in lime induced reactions is discussed in detail for each species in Appendix 12.

E2-4: Summary

The dilemma faced was that the trees from the limed plot exhibited better crown growth and higher root Al content than the trees from the unlimed zone which had 25% more light penetration due to leaf loss and lower root aluminum content. The initial assumption was that the liming improved growth due to blockage of aluminum access to limed roots, but contrary to expectations, the limed roots had intense Al deposits.

If the limed roots had strong Al content then it would naturally be assumed that there should be indications of Al toxicity in the leaves..... unless the Al was being preferentially held in the limed roots, and not being translocated. In contrast, if the Al was not held in the unlimed roots then it could be translocated to the leaves causing toxicity damage. The only logical explanation is that liming affects root metabolism, promoting growth and nutrient uptake and Al sequestration. Because the *Cenococcum,* for example, does not sequester Al but promotes nutrient transfer with its hundreds of emanating hyphae, this mycorrhiza is abundant and in good health, but the corresponding root then receives abundant Al (along with nutrients) which it must neutralize or translocate.

In acidified soil, Al is hydrated and mobilized with the water and can move along proton channels to the roots. As long as it remains acidified, it can continue moving and in this way the acidified aluminohydrates would then be free to translocate past the ectomycorrhizae, past the cortex and eventually into the xylem, where also because of the acid nature of the bathing solution it could also move up the xylem with minimal sequestration along the way. Al^{+3} ions would be free to pass via mass flow directly to the leaves where they could interfere with photosynthesis, cellular metabolism and cell division inducing leaf mortality and crown thinning. Only those mycorrhizae capable of changing the pH in their immediate vicinity would be capable of capturing Al ions and sequestering them. The unfortunate side effect of Al accumulation would be the potential mortality of the poisoned ectomycorrhiza. As a result in the unlimed forest, only those species which were not capable of capturing the Al would survive the selection process.

This might account for the extremely low diversity (and great abundance of surviving species) found in the unlimed zone. These survivors would have low affinity for Al allowing it to pass unhindered or with minimal hindrance into the roots. The genetic drift would be reinforced each year as soil acidification increased.

Liming was shown repeatedly to increase Al deposition in the roots. The amount of Al available to the fine roots was to an extent controlled by the large diversity of ectomycorrhizal species, but once in the cortex, the competence of the cortical walls determined whether sequestration was possible or not. The competence of the cortical walls may have been the same in the unlimed and limed roots, but the pH of the bathing solution may have ultimately determined whether Al was available (unhydrated enough) to attach to the binding sites available. If you reduce the total translocated Al, due to ectomycorrhizal or root sequestration the leaves should show less damage. The enhanced sequestration of Al in the limed roots may be a significant contributing factor.

The downside of this process is that the mycorrhizae and the roots that hold the Al will then be subject to its toxic effects. As roots slowly die and mass upflow declines, the Al should temporarily remain in the rhizosphere, incorporated into newly active mycorrhizal roots with some translocation to aerial region. As long as the bathing fluid remains within the buffering range, the Al may move only slowly, being sequestered along the aerial route. If however a severe drought or winter damage occurs, the Al which may have been tightly incorporated into the root walls may be suddenly released with the return of mass flow in the spring or after the dry spell, especially if the ectomycorrhiza are inactive or the transport fluid is acidic. A temporal (multi-year) accumulation or a sudden massive influx of Al may have an acute effect resulting in sudden tree mortality, several months after a threshold stressor event. The result would be "sudden oak death".

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SECTION F: BOUND MINERALS

INDEX

Section F: Bound Minerals

F1: INTRODUCTION

A small region of oak forest (Section A1) was limed in 1989 with the intent of improving Mg and Ca uptake and with the hope that enhanced Calcium would reduce Aluminum toxicity and so improve the health status of the trees (Block, 1999) (Section A, Appendix 3). Since Aluminum may not be the only heavy metal culprit in tree mortality (Appendix 6), other ions were also analyzed (Section D). With increasingly acidified soil it is possible that some essential ions may not be as easily translocated into the roots while potentially toxic ions may be more readily taken in (Sections A3 & E). A selection of macro (Ca, Mg, K, Na, P, S), micronutrients (Mn, Zn, Fe) and non-essential potentially toxic (Al, Cd, Pb), elements were examined to determine their bound (Section F) and unbound (Section G) concentrations in fine roots and four common (Section B) ectomycorrhizae (*Cenococcum geophilum, Lactarius subdulcis, Quercirhiza fibulocystidiata* and Piceirhiza chordata) of *Quercus petraea* taken from unlimed and limed soils in spring and fall of a dry year (1999) and a wet year (2000) (Section A2 & C1). It was determined from mineral analysis that the bound content of aluminum was highest in roots from the limed soils.

F1-1: Mineral Selection

At this point in time, the few accounts of mycorrhizal mineral content available concentrate upon enhanced uptake of essential nutrients and very few deal with controlled entry of potentially toxic minerals (Sections A1-2, A1-3, A3) Even rarer are accounts of the specific roles of oak ectomycorrhizae (Section A1-3). Much more information is available concerning above-ground fungal forms and their associated subsurface hyphae (Appendices 1 & 2 & 6) and other forest stress factors (Appendix 4). The following is a brief introduction to the minerals generally associated with fungi and plant roots.

According to a review done by Jellison et al (1997), patterns of comparative accumulation of the macronutrients Mg and K in brown rot fungi are contradictory and difficult to discern, however in general, Ca and Mg tend to be retained while K tends to be

leached despite apparently high fungal requirements. Ca plays a major role in cellular metabolism acting as a secondary growth signaler (Jellison et al, 1997) and is specifically involved in cytoskeleton formation (Brockerhoff & Davis, 1992) and as a major component of cell walls and the middle lamella of plant cell walls (Devlin, 1975). Loss of calcium has been associated with increased membrane permeability (Devlin, 1975). Mg can act as an essential cofactor for cell wall synthesis, ATP utilization, DNA repair but even so it is considered to be of secondary importance when compared to potassium (Griffin, 1994). Phosphate is a well known macronutrient. Besides ATP activity, polyphosphates within the fungal cytosol act as primary mineral binding sites (Doonan et al, 1979, White & Gadd, 1986,). Sulfur in *small quantities* is an essential plant nutrient, important in the structure of amino acids (cysteine, cystine and methionine) and protein structure (Bidwell, 1974), but excessive deposition has been shown to provide a stimulus to mobilize aluminum into soil solutions and that long term acidity due to sulfur deposition is also believed to affect nutrient cycling by leaching ions such as Ca, Mg and K (Ulrich, 1989). In organic solutions, Ca^{++} and Mg^{++} act as base exchangers as do Na^{+} and K^{++} (Long et al, 1970). Although Na is not considered a serious macronutrient, it will be included in this examination because of its well known base exchange relationship to K, within the soil solution. But it must be noted that at the plant membrane, K^{+} influx and H^{+} efflux is the most common exchange mechanism (Devlin, 1975). Oak mycorrhizae will be examined for five macronutrients (Ca, Mg, K, P, S) plus Na.

The trace metals required by fungi include manganese, zinc, iron, copper, molybdenum, and nickel (Gadd, 1993). Trace Elements such as Mn, Zn and Cu are essential for all cells but generally difficult to obtain and so all cells including fungi have specific mechanisms for uptake and transport of these ions (Winkelman and Winge, 1994). In fungi, the uptake systems are energy-dependent, specific and of high affinity (Kosman, 1994). Iron is of particular interest because it is known to be solubilized in acidified soils. From research done by Ulrich et al (1989) the Fe acid buffer range is well known but little information is available concerning the interactions of the other ions and oak mycorrhizae. Of these minerals, the micronutrients S, Mn, Zn, and Fe were selected for study.

Non-essential metals commonly found in fungi include cadmium, lead, chromium, mercury and silver (Gadd, 1993). Fungi must be able to repel or sequester rare metals from the soil, but toxic levels of the essential and non-essential metals may accumulate in heavily contaminated areas. Since metals can be potent growth inhibitors of microorganisms, it is not surprising to find reduced fungal diversity in contaminated sites (Zibilske & Wagner, 1982) with a shift towards metal-tolerant species. Shifts in mycorrhizal diversity and abundance were definitely evident in the unlimed and limed forest zones examined (Section B). The limed forest soils had a much greater diversity, but each individual species was poorly represented in a weight to weight comparison. The unlimed forest soils had a marked lower diversity but the individual species that survived the selection pressures were well represented. It was expected that Al would be high in the unlimed roots and low in the limed, but the reverse was true according to the fluorescence examinations (Section E). Aluminum was positively identified as being present in the mycorrhizal root tips (Section D & E) but was especially abundant in the limed probes (Section E)! According to the laser analysis radioactive minerals (Sr, Ba, Th and U) and heavy metals (Hg and Pb) were present (Section D). Iron was minimally present, but since it is important in acidified soils approaching the iron buffer range (Sections A3-4, A3-5 and C), it was included in this study. Cd was chosen as a negative control. It should be (it was) absent or in extremely low concentrations.

Aluminum is generally considered to be a non-essential, but often tolerated element when in low concentrations (Haug, 1984). What was not clearly understood was exactly how much Al was present in bound and unbound forms and which of the other potentially toxic elements were present in the oak roots. The other problem that needed to be solved was to confirm the fluorescence results (Section E). It was logical to assume that a more intense or more rapid Morin reaction indicated a greater concentration of aluminum but concrete evidence was needed to confirm this supposition. The quantitative mineral analysis, as will be seen, gave that confirmation.

F1-2: Mineral Binding

Metals solubilized from the soil (Section 3-1) can bind to a wide variety of ligands (Section 3-5). Within fungal cell walls, metals can bind to various functional groups including phosphate, hydroxyl, carboxyl, sulfhydryl and amines (Section 3-5). When these functional groups are part of enzymes this metal binding may result in blockage of the functional group, displacement of essential metals, conformational changes, denaturation of the enzyme, or interference with membrane transport (Gadd & White, 1989). Fungi have two basic mechanisms to preclude metal interference: extracellular avoidance (Class 1) and intracellular sequestration (Classes 2, 3, 4) (Section E & Appendix 6). Extracellular avoidance may involve more than external precipitation. Some mycorrhizal species (Class 1) may actually promote translocation past the hyphae along apoplastic (proton) channels directly into the roots and thus avoid the potential toxic effects associated with sequestration (Section E2-3F).

Externally, the hyphal sheath may contain special selective functional groups to preferentially bind the toxic metals preventing access to the cell wall and plasma membrane (Sutter et al, 1983). Secretions of oxalic acid (Murphy & Levy, 1983), and melanins (Daniel & Nilsson, 1989), which can contain phenolic, carboxylic and hydroxyl functional groups (McDougal & Blanchette, 1996) may be located external to or within the wall to immobilize the metals. Metal-laced halos (gel sheaths) or externally bound metals may be somewhat protective of the fungi preventing macrophage attack or by providing competitive advantages to mineral tolerant species (Appendix 6).

Lack of an external defense or an overwhelming of the protective shield would require an internal backup system in order for a fungus to survive in a contaminated area. Minerals can be bound in the pectin rich regions within cell walls (Ridolfi et Al, 1996) and in lignified tissues (Orgeas & Bonin, 1996). Once a metal or other mineral has breached the external barriers, the fungi may be able to sequester ions by binding them to orthophosphates within vacuoles (White & Gadd, 1986, Gadd, 1993). These polyphosphates appear as visible granules in micrographs when they are bound to metals

(Doonan et al, 1979). Some similar preferential accumulation of metals in fruiting bodies may also occur (Zabowski et al, 1990). Besides vacuoles, other membrane bound organelles, although of lower affinity, such as the fungal mitochondria (Carafoli et al, 1970) and endoplasmic reticulum (Morales & Ruiz-Herrara, 1989, Belde et al, 1993, Jellison et al, 1997) can store minerals.

Energy-dependent transport of divalent cations (Ca, Mg, Mn, Zn...) and elemental phosphate across the tonoplast channels into (and out of ?) vacuoles plays an important role in regulating the cytoplasmic (unbound) concentration, sequestering potentially toxic ions, and thus allowing normal, controlled, activation of essential metabolic processes (Gadd, 1993). The free cytoplasmic and symplastic content of ions will be discussed in Section G: Enigma of Unbound Minerals.

Ions held in place or stored may be temporarily unavailable, semi-permanently or permanently sequestered. When physiological activities create a chemical sink, some of these ions may be made functionally available. The effectiveness of toxic mineral exclusion, storage and / or release may be dependent upon the Calcium content of the roots and the specific abilities of the mycorrhizal species present. The primary goal of this particular section is to review bound mineral content of combined fine mycorrhizal root tips and to determine if there were major differences between the unlimed and limed forest regions. The secondary goal of the review is to determine how changing microenvironmental factors affected mineral presence.

F1-3: Original Hypothesis

Due to the general healthier appearance of the tree crowns in the limed forest (the unlimed forest had 25% more light penetration) it was initially suspected that liming may have reduced aluminum toxicity and possibly also total root Al content. The original hypothesis set out to confirm this suspicion. In contrast to what was expected, the mycorrhizal roots in the limed forest were actually frequently higher in bound Al content than in the unlimed zone. This was surprising. Analysis of the unbound Al content

provided another surprise and an a possible explanation for lower tree mortality in the limed forest (See Section G: Enigma of Unbound Minerals).

F1-4: Revised Hypothesis

Liming improves sequestration of potentially toxic aluminum and certain other minerals within ectomycorrhizal roots. Specific ion binding simultaneously depends upon the mycorrhizal species, moisture content of the soil, pH, seasonal oscillations and soil depth.

F2: Materials & Methods

F2-1: Root Isolation

After sample collection (Section B), a separate set of oak roots from 0-10, 30-40 and 50-60 cm depths from both the unlimed and limed forest plots were stored undisturbed in their loosely closed plastic bags in a refrigerated chamber for up to 5 days. Within that time period the roots were extracted from the soil by sieving, followed by gentle rinsing in cold tap water to remove adhering soil particles and stones. The cleaned roots were placed in open beakers, covered with iced tap water and refrigerated at least overnight. Prior to segregation, the roots were removed from the beaker to a fresh container. The settled soil was discarded and the transferred roots were well rinsed with cold water to remove the remaining hydrated soil particles. The roots from each probe were separated into very fine mycorrhizal tips (root diameter < 1 mm), larger roots (>1 mm diameter) and very large roots (>5 mm diameter). These samples were blotted dry, weighed to obtain their fresh weights, set in separate open glass containers, and placed in a 60 °C drying chamber for 12 hours and reweighed to obtain their dry weights. The dried root sections were stored in sealed plastic Ziplock bags at room temperature. Only the very fine mycorrhizal root tips were used for mineral analysis. The larger roots were used for biomass comparisons. Subsequent to the fluorescence analysis, the unlimed and limed mycorrhizal tips from the four most abundant species (*Cenococcum geophilum, Lactarius subdulcis, Quercirhiza fibulocystidiata, Piceirhiza chordata*), from the various depths and seasons, were further cleaned to remove any possible foreign contaminants (soil or non-mycorrhizal root fragments). The tips were then dried in the manner described above.

F2-2: Root Preparation

The very fine (< 1mm) mycorrhizal rootlets were separately ground up with a porcelain mortar and pestle with a small quantity of liquid nitrogen. Upon evaporation of the nitrogen, the roots were ground to a fine powder. Exactly 100 mg of root powder was placed in new plastic 2 ml Safe-lock Ependorf caplets, reweighed using Sartorius precision measurement scale, labeled, sealed and stored at room temperature in sealed plastic sleeves. A second set of caplets, also with 100 mg of root powder, was prepared for the unbound mineral analysis (Section G).

F2-3: Mycorrhizal Preparation

Following fluorescence examination, the mycorrhizal tips were flash frozen in their caplets using liquid nitrogen and stored at -35^0 C. The species which might provide the required 100 mg for mineral analysis were thawed and further cleaned to remove any extraneous debris. The mycorrhizae were freed of all non-mycorrhizal root portions. Due to the extremely small sampling size of the individual mycorrhizal species, the tips from several probes (from the same depth, season and soil treatment (unlimed or limed), but different trees) were combined. These single species samples were air dried at room temperature (22-25^0 C) for 3 days in new eppendorf caplets. The resulting material was ground using a small quantity of liquid nitrogen and transfered to a new ependorf caplet of a known weight to obtain the required 100 mg. Extra sample was retained in a separate caplet. There was not enough sample to run an analysis of unbound minerals so only the bound minerals were evaluated. In some cases, less than 100 mg was collected for bound mineral analysis. In those cases, the final mineral concentrations were extrapolated. All the results are presented in mg/kg in the tables and graphs.

Due to the nature of certain mycorrhizal species, despite thorough cleaning, some tiny specks of material may have remained on the mantle surfaces. For example, ***Cenococcum geophilum, Quercirhiza fibulocystidiata*** and *Lactarius acris* all tend to have strongly adhering debris. To a lesser extent, *Quercirhiza squamosa, Fagirhiza fusca* and *Genea hispidula* which all have long loose hairs may retain a tiny amount of adhering sand but

are usually easily swept clean. Some species such as ***Lactarus subdulcis*** and ***Picerhiza chordata*** are glabrous and free of any adhesion. Only the highlighted species were used for mineral analysis. Although some of the mineral readings may be slightly higher (estimated << 1 %) for *Cenococcum* and *Quercirhiza* due to the adhesions, the results will be considered normal since this is relevant feature of their architecture.

F2-4: Mineral Analysis

The prepared dried mycorrhizal root, and later the individual mycorrhizal samples, were delivered to the Forstliche Versuchsanstalt (FVA) Rheinland-Pfalz, Trippstadt and through them the probes were analyzed by Landwirtschftliche Untersuchungs und Forschungsanstalt (LUFA), (LUFA, Obere Langgasse 40, 67346 Speyer, Hd Herr Wies). The dried materials were reweighed in a teflon container and extracted with Nitrous acid for 4 hours at 170^0 C in a sealed pressurized chamber. The extraction was injected in 50 ml increments and analyzed using ICP-OES (Wies, 2002, pc). The mg/kg concentrations of Ca, Al, Mg, K, Na, Mn, S, Zn, Fe, Cd and Pb and g % P were determined for each fine root probe. *Cenococcum geophilum*, the most common mycorrhiza, was analyzed for Ca, Al, Fe and K. *Quercirhiza fibulocystidiata, Piceirhiza chordata* and *Lactarius subdulcis* were analysed for Calcium and Aluminum. Unlimed and limed probes were compared.

F3: RESULTS

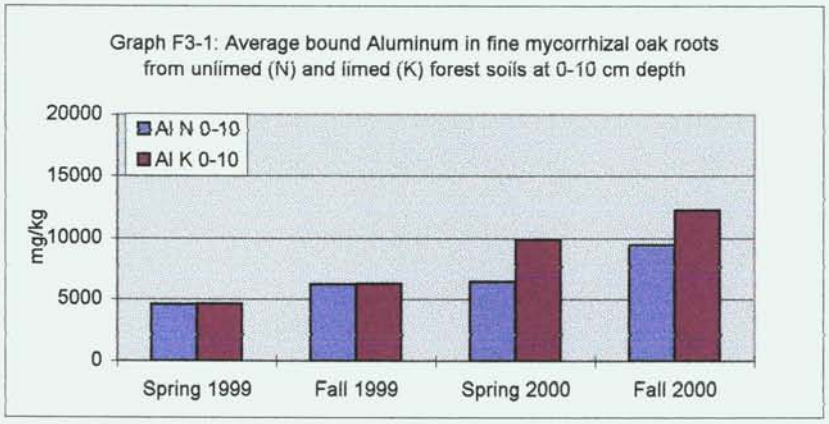
The mg/kg concentrations of Ca, Al, Mg, K, Na, Mn, S, Zn, Fe, Cd, Pb and g % P were determined for each probe by LUFA, Speyer. Appendix 10A presents the original data sets from unlimed [Tables 10A-1 (0-10 cm), 10A-3 (30-40 cm), 10A-5 (50-60 cm)] and limed fine roots [Tables 10A-2 (0-10 cm), 10A-4 (30-40 cm) 10A-6 (50-60 cm)]. Statistical analyses of the data are presented in Appendices 10B, 10C and 10D. In Appendix 10B, the significant differences between unlimed and limed probes for the dry year 1999 compared to the wet year 2000, for each depth [0-10 cm (Tables 10B-1 & 10B-2), 30-40 cm (Tables 10B-3 & 10B-4) and 50-60 cm (Tables 10B-4 and 10B-6)] are presented. In Appendix 10C, unlimed and limed roots are statistically compared with respect to soil depth [0-10 to 30-40 cm, 0-10 to 50-60 and 30-40 to 50-60 cm depth,

respectively] for each season [spring 1999 (Table 10C-1), fall 1999 (Table 10C-2), spring 2000 (Table 10C-3), fall 2000 (Table 10C04)]. In addition, the bound Ca / mineral ratios were compared (Appendix 10D) to determine if there was any overall statistical difference between the unlimed and limed probes for each depth [0-10 cm (Tables 10D1-10D4), 30-40 cm (Tables 10D5-10D9), 50-60 cm (Tables 10D10-10D12). For this portion of the report, individual minerals were graphed to show their average abundance in unlimed (N) and limed (L) soils at 0-10, 30-40 and 50-60 cm depths for spring and fall of 1999 and 2000. (**Graphs F3:1-36** : Al: F3:1-3: **Ca**: F3:4-6, **Mg**: F3:7-9 **K**: F3:1-12 **Na**: F3:13-15, **Fe:** F3:16-18, **Mn:** F3:19-21, **P:** F3:22-24, **S:** F3:25-27, **Zn:** F3:28-30, **Cd:** F3:31-33, **Pb:** F3:34-36). The seasonal significant differences between the unlimed and limed samples were determined (Appendix 10B) and recorded below each graph as percentage confidence units. All the minerals (except P) were graphed together to create an **overview** differentiated on the basis of depth and seasons **(Graphs F3:37-42).**

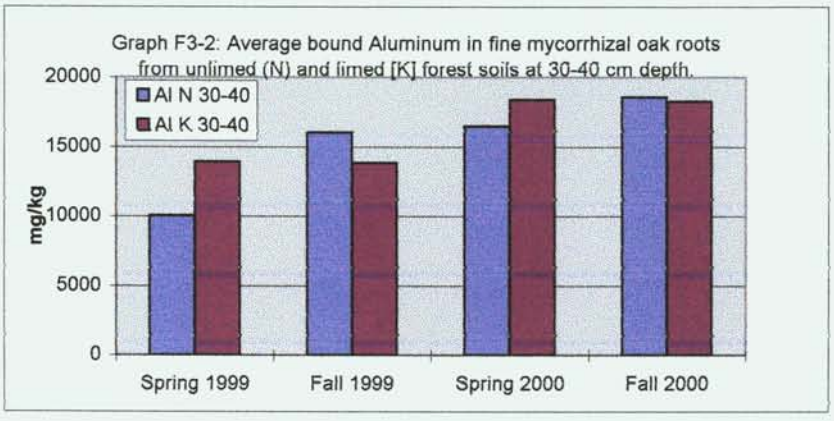
The four isolated ectomycorrhizal species [*Cenococcum geophilum, Quercirhiza fibulocystidiata, Piceirhiza chordata* and *Lactarius subdulcis*] were compared for calcium (Graphs F3:43-46) and aluminum (Graphs F3:47-50) content at 0-10 cm depth in spring and fall 2000. In addition, a comparative mineral analysis (Ca, Al, Fe, K) of ***Cenococcum geophilum*** tips from various depths from unlimed (Graph F3:51) and limed (Graphs F3-52) soils was possible. The Ca:Al content of the ectomycorrhizae ***Piceirhiza chordata*** was compared from various soil depths from limed probes(Graph F3: 53) only since there was insufficient material from the unlimed samples.

Previously established parameters including [moisture (Sections A1-2, A3-3, A3-4 & C1, Appendix 9A) acidification (Sections A1-2, A1-5, & C2, Appendix 9B), ectomycorrhizal abundance (Section B & Appendix 8 & 12), heavy metal (Section D), and aluminum localization (Section E, Appendices 7 & 12)] will be discussed. Overall, the mineral contents of unlimed and limed roots will be compared and contrasted, with particular emphasis upon the discovery of an augmented presence of bound Al within limed roots.

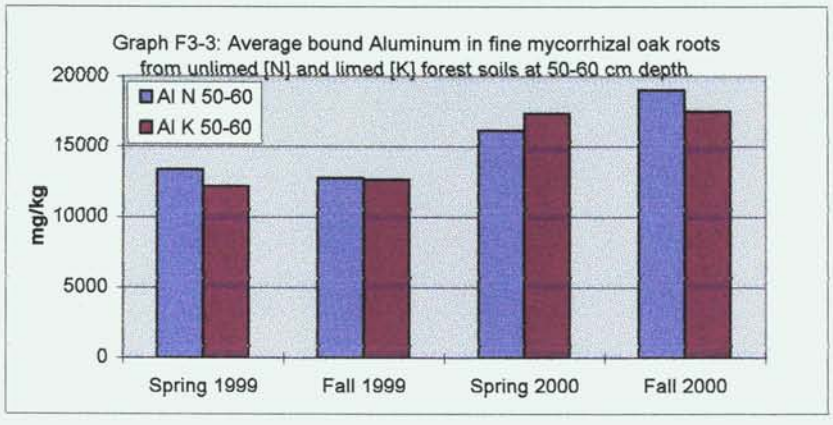
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples. NS = No significant difference.



NS NS >95% >80%

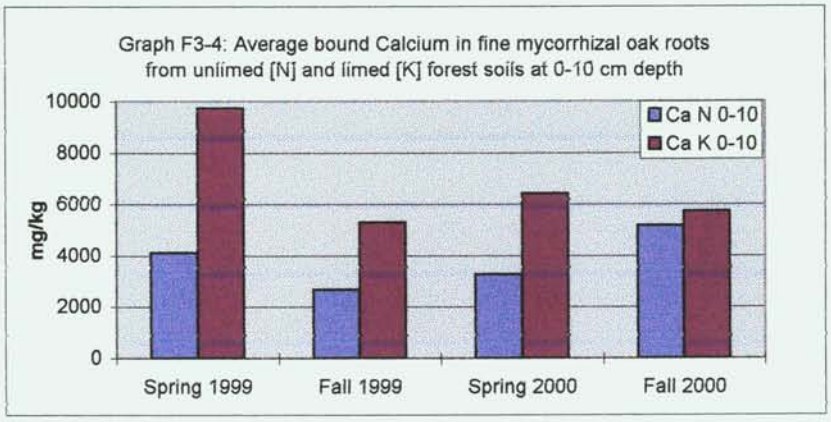


>90% >50% >50% NS

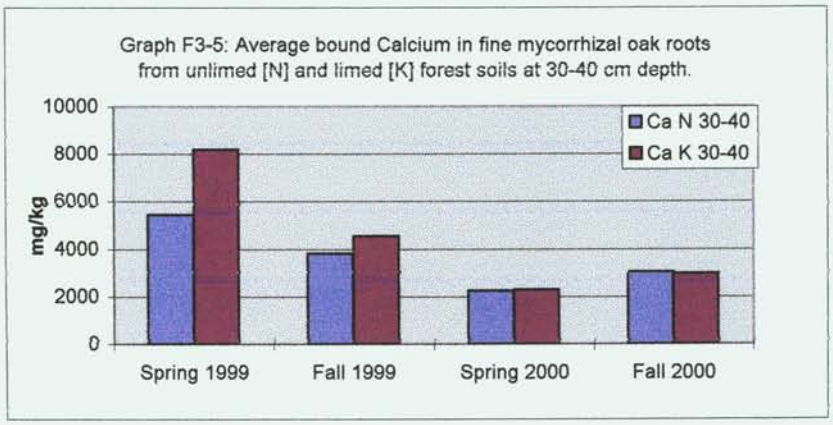


>60% NS NS NS

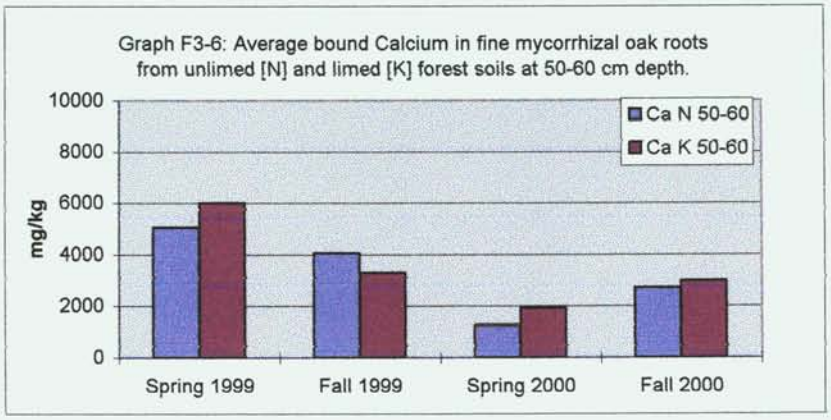
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples. NS = No significant difference.



>99%	>95%	>99%	>95%

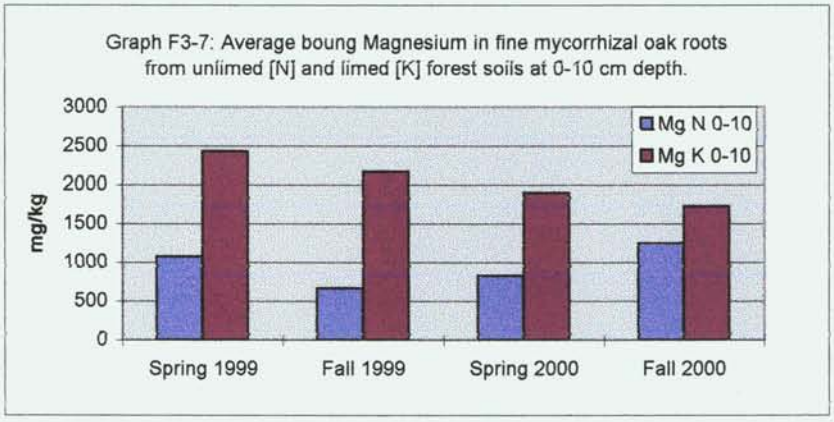


>95%	NS	NS	NS



>80%	>60%	>60%	NS

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples. NS = No significant difference.



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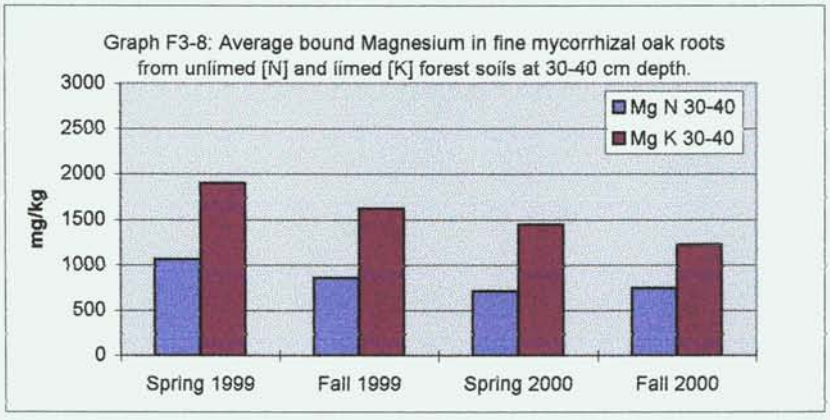
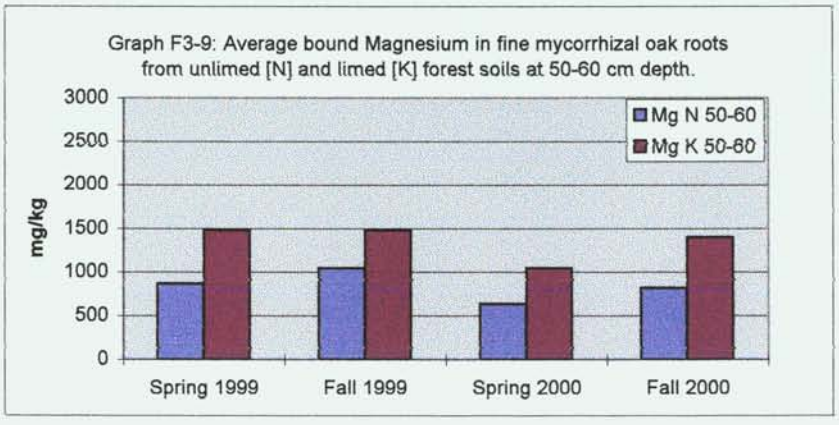
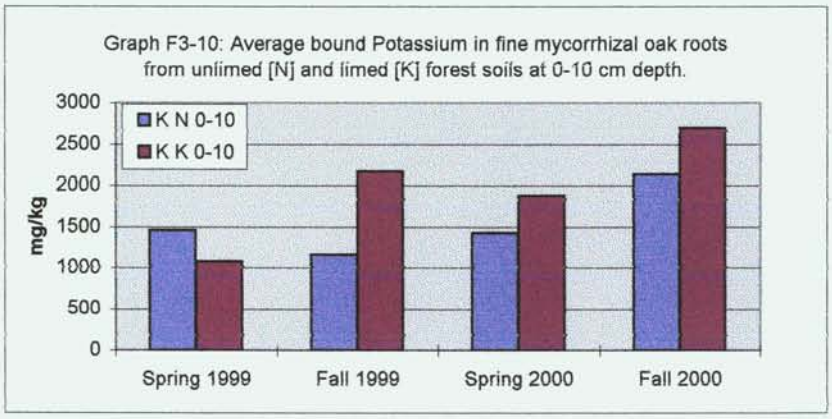


>95% >99% >99% >99%



>99% >80% 98% >99%

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples. NS = No significant difference.



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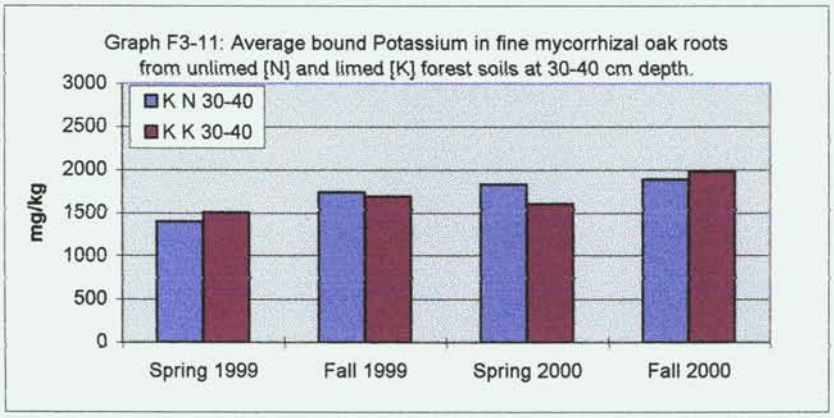
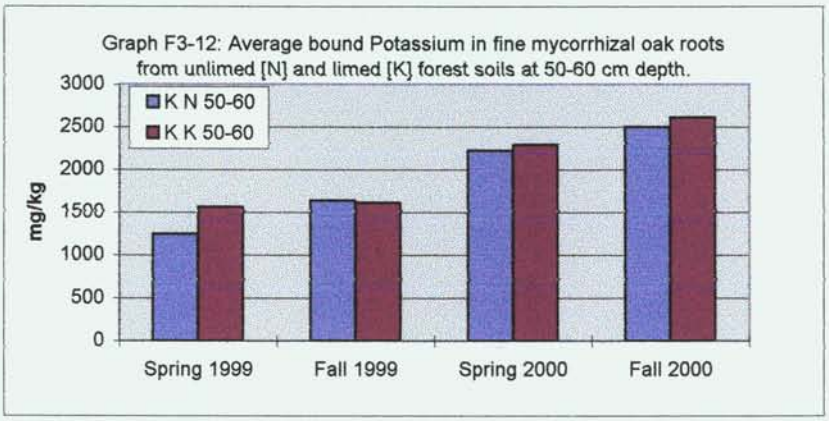
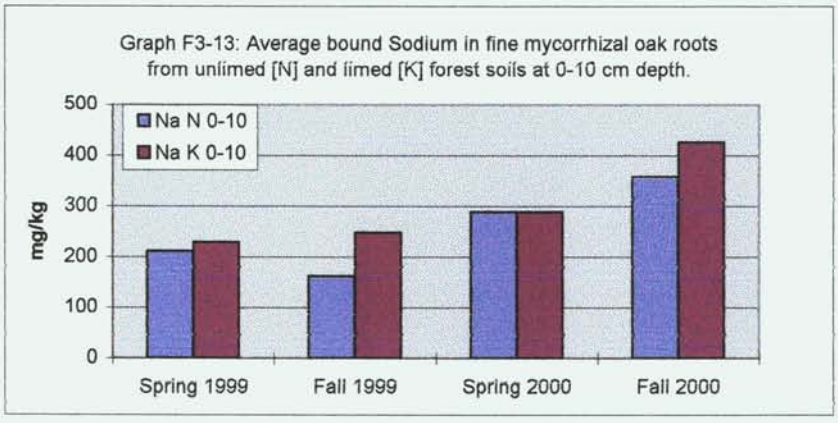


NS NS >70% NS

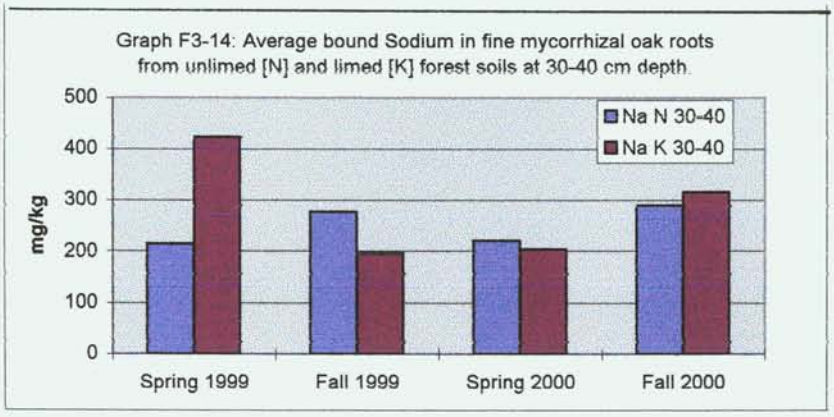


>70% NS NS NS

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples. NS = No significant difference.



NS >50% NS >70%



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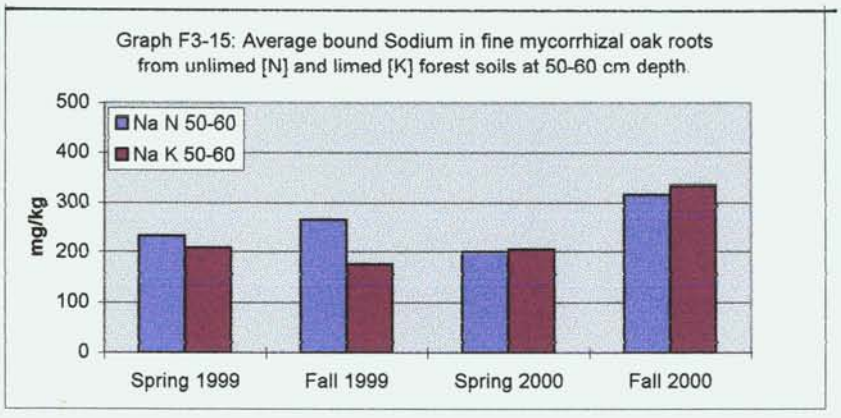
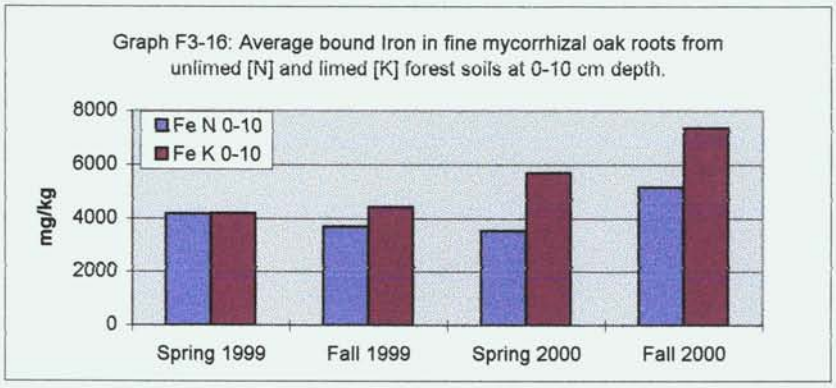
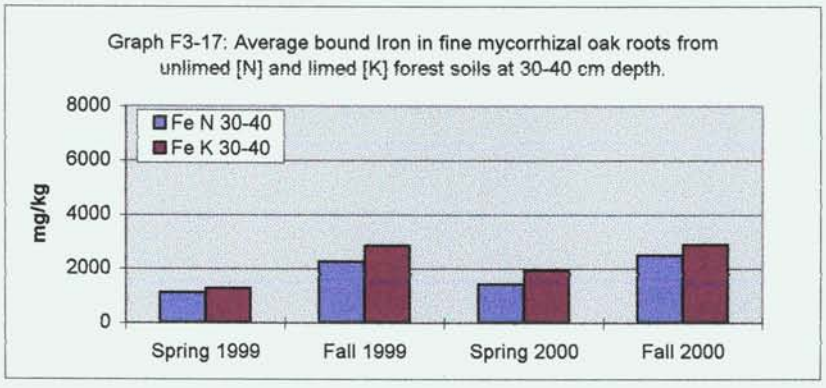


NS >60% NS NS

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS = No Significant Difference



NS >60% >98% >80%



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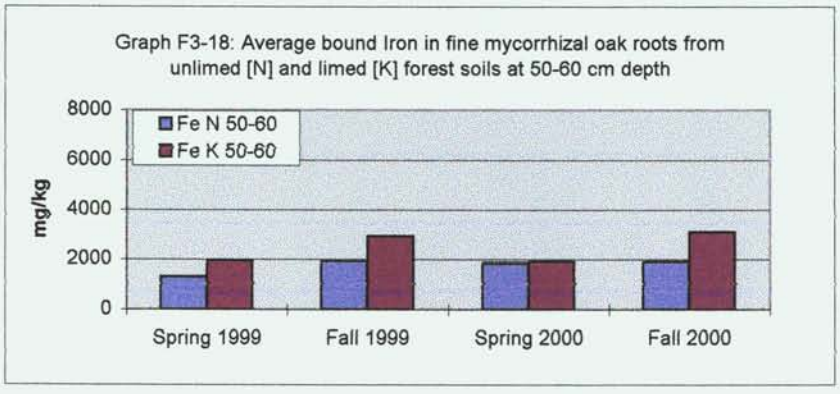

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Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
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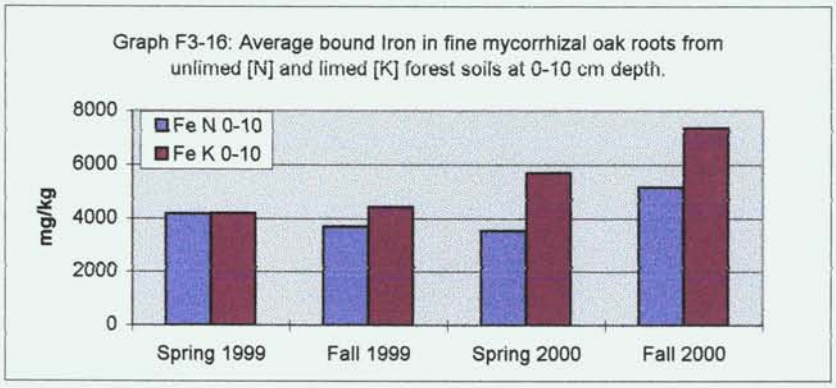
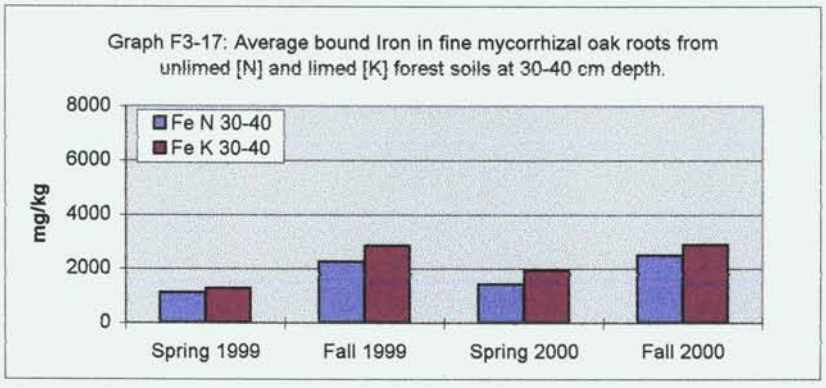
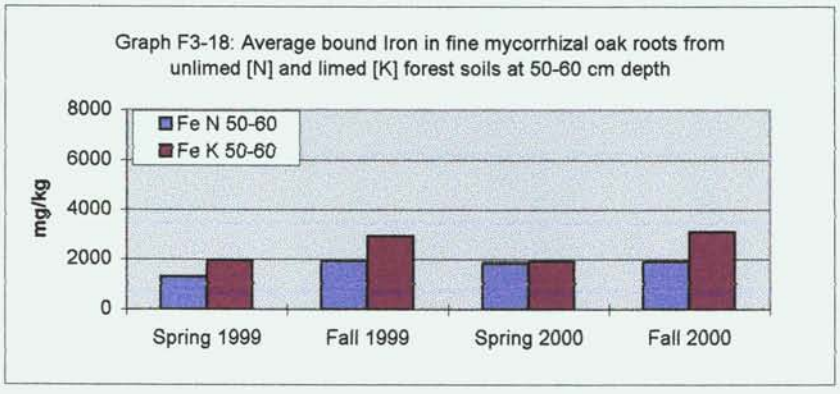


NS >60% >98% >80%

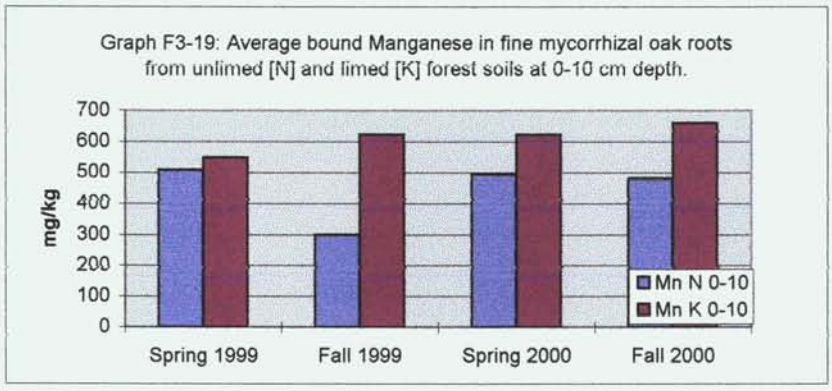


NS >70% >80% NS

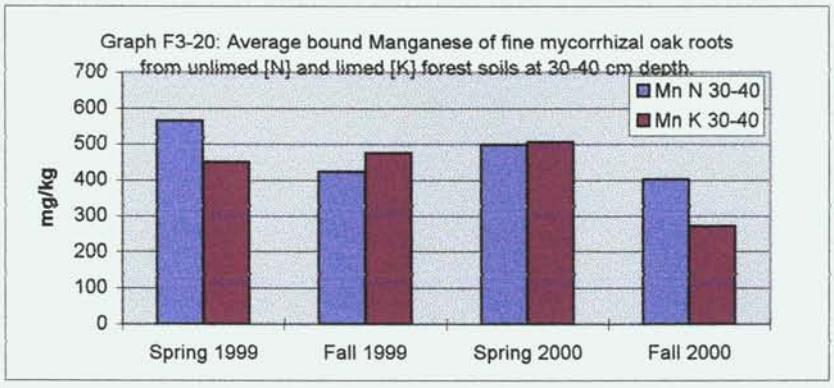


>70% >70% NS >90%

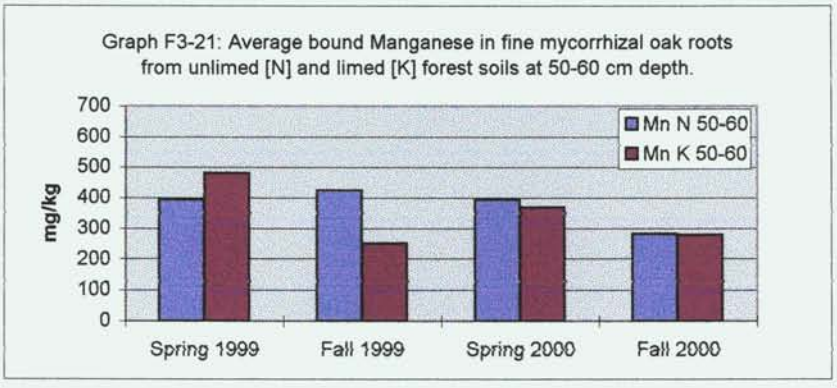
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS = No Significant Difference



NS >80% >50% NS



>50% NS NS >60%



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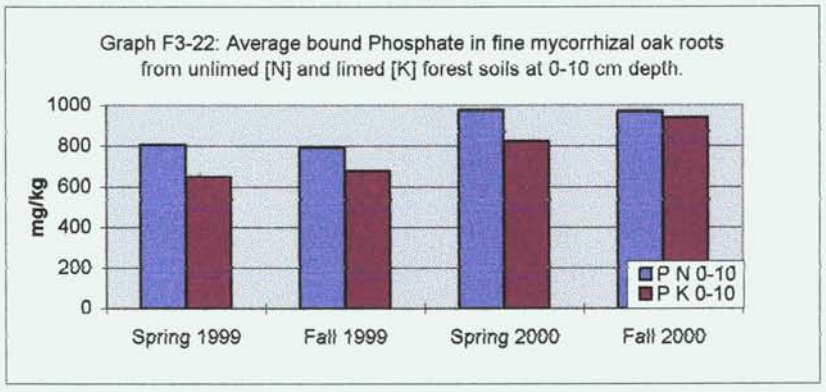
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS = No Significant Difference



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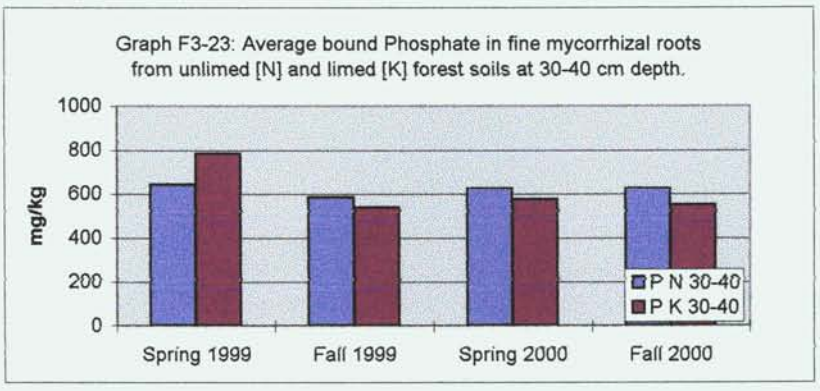

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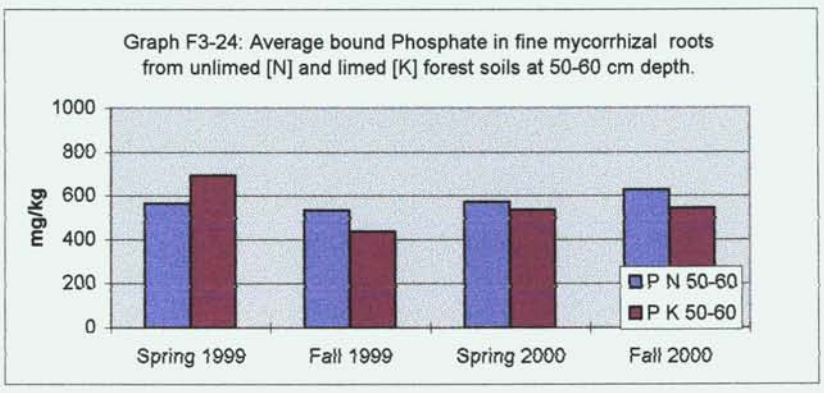
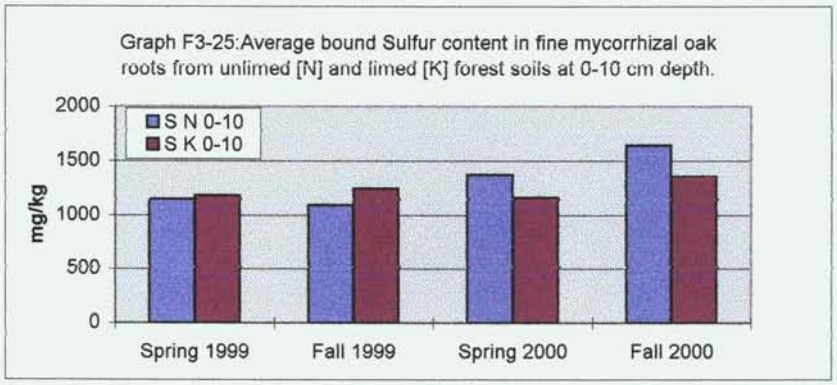
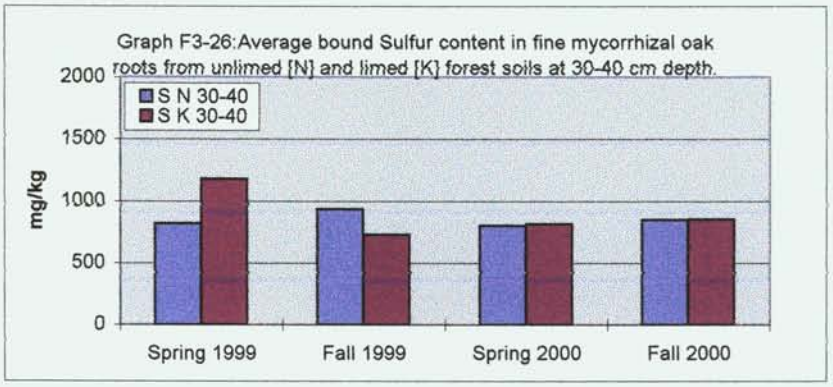


>90% >50% >50% >95%

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS =No Significant Difference.



NS >70% >95% >98%



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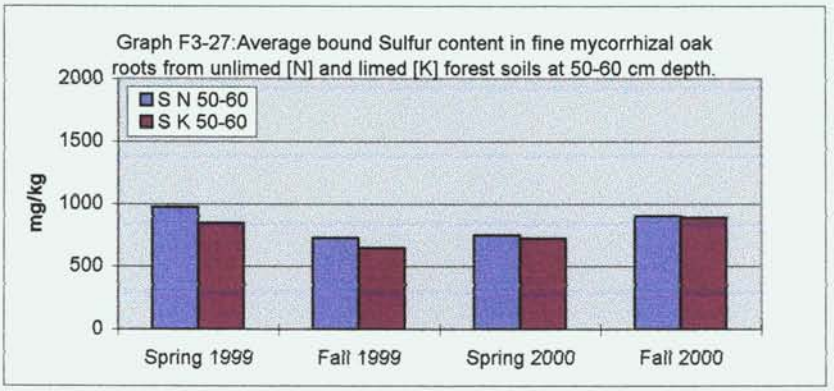
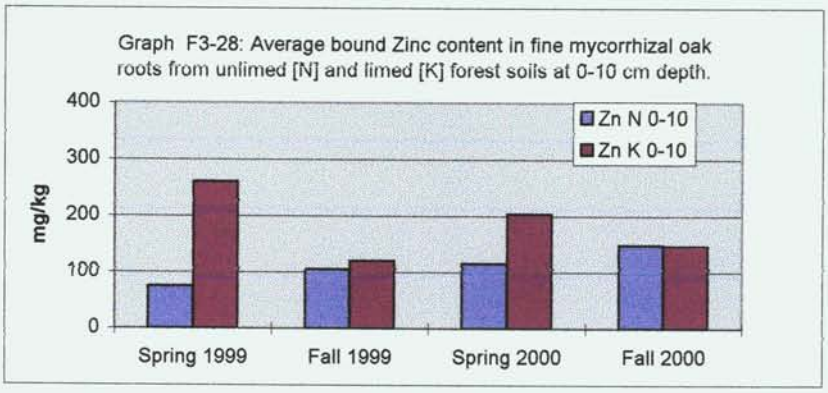


>80% NS NS NS

Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS =No Significant Difference.



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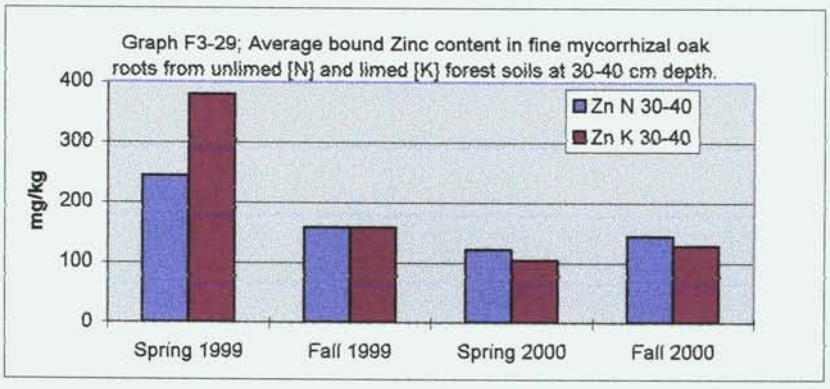

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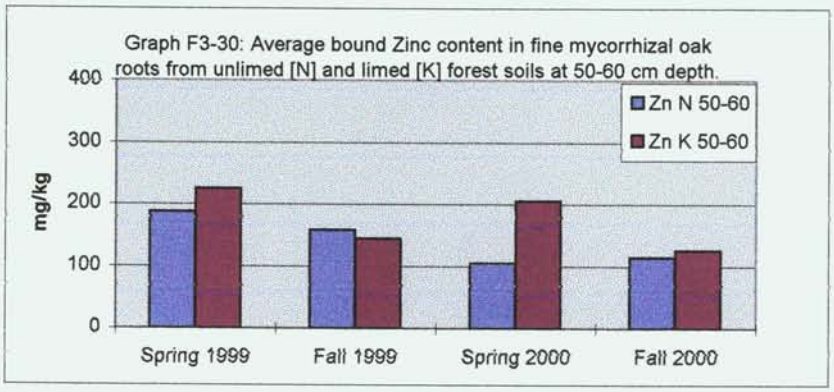
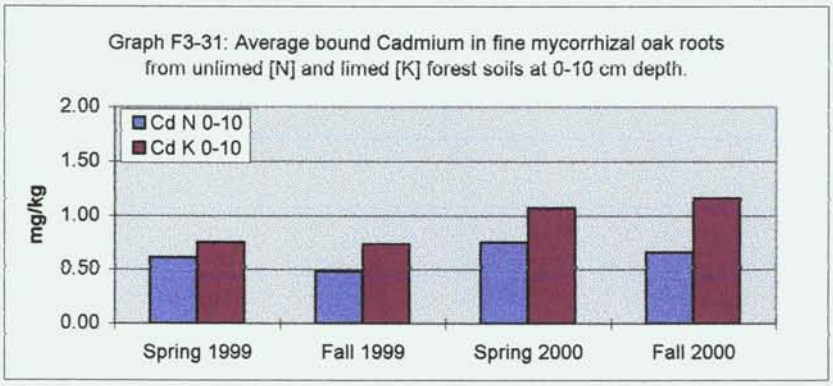
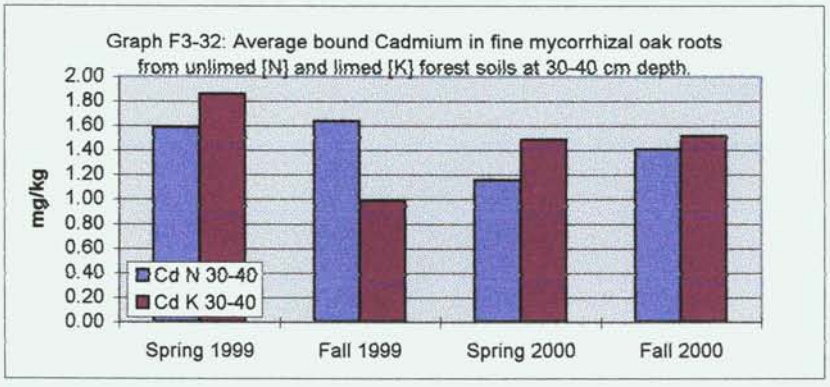


NS NS >70% NS

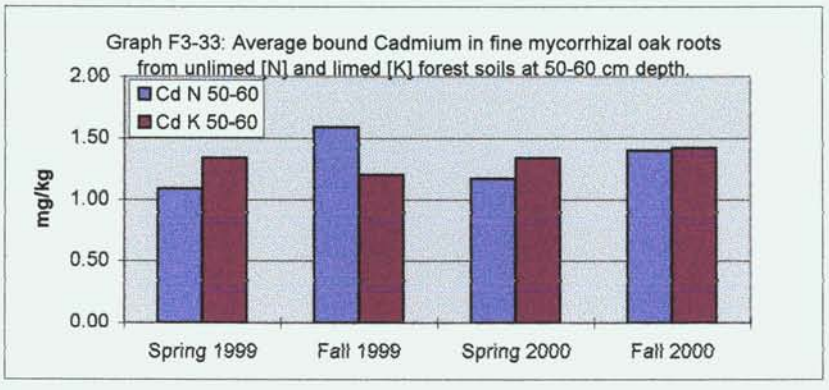
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS =No Significant Difference.



>50% >95% >80% >90%

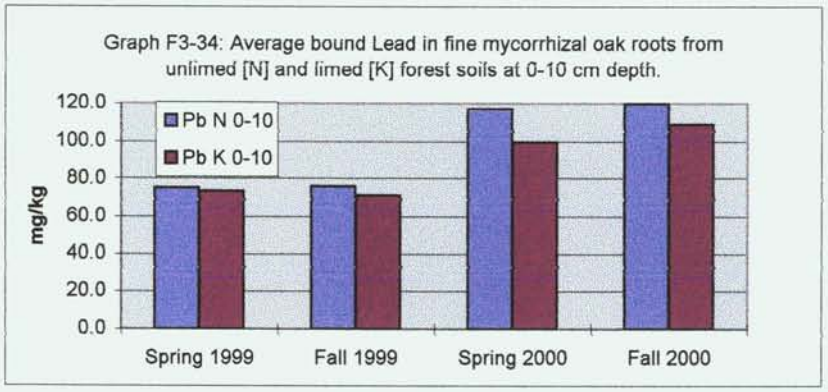


NS >95% >80% NS

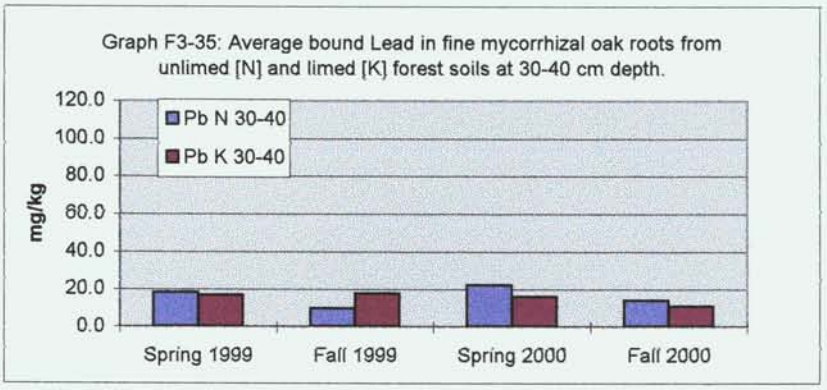


NS NS >50% NS

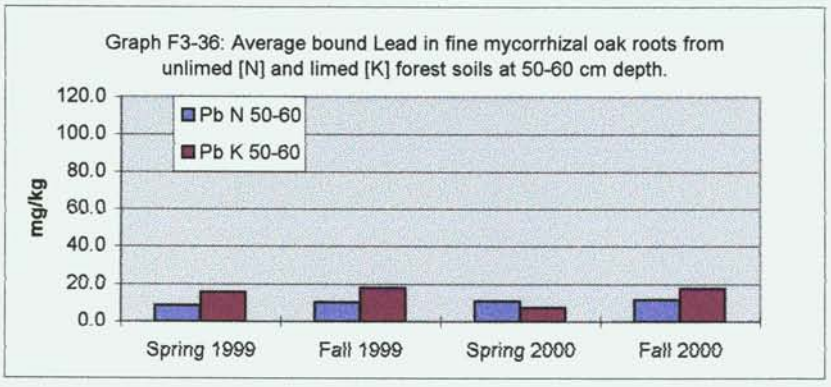
Analysis of bound root minerals with % Significant Difference between unlimed and limed samples.
NS =No Significant Difference.



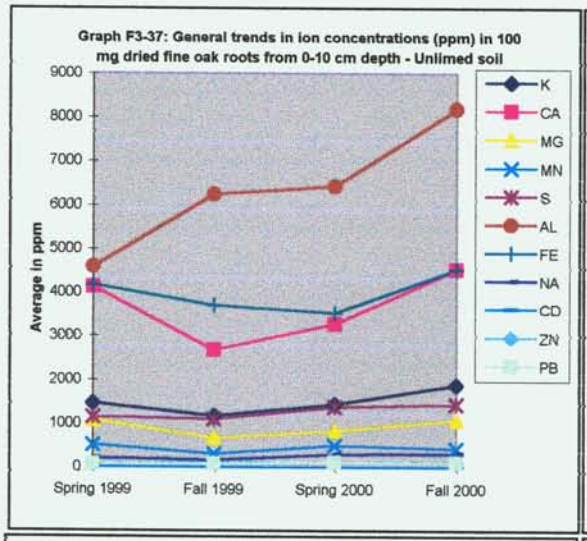
NS >50% >80% >50%

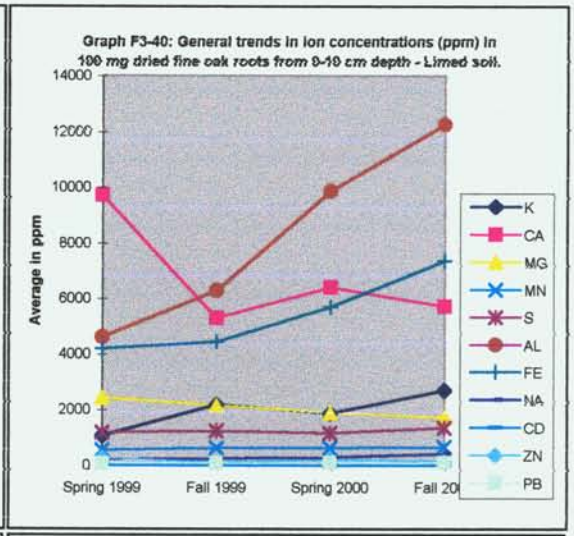


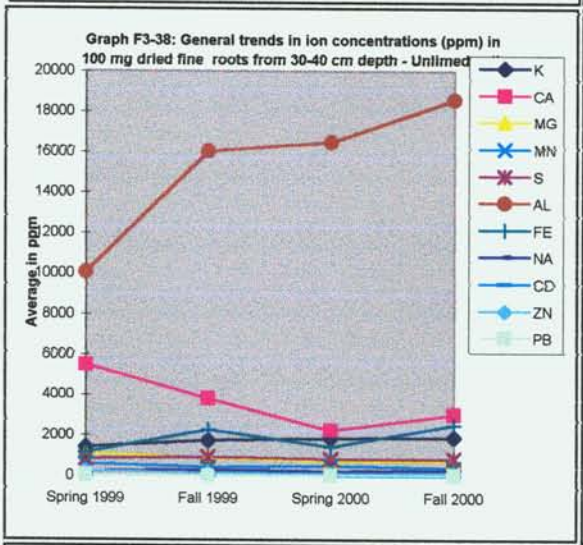
NS >95% NS NS

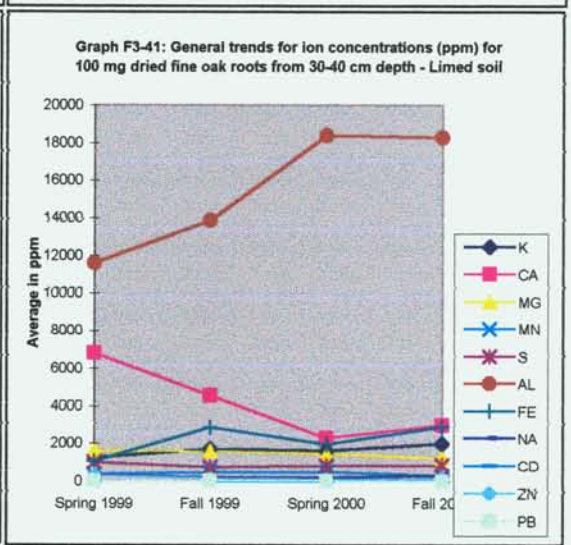


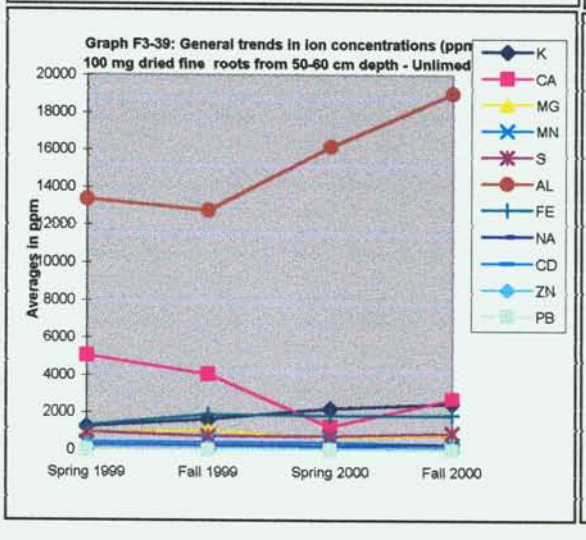
>70% >90% >80% NS

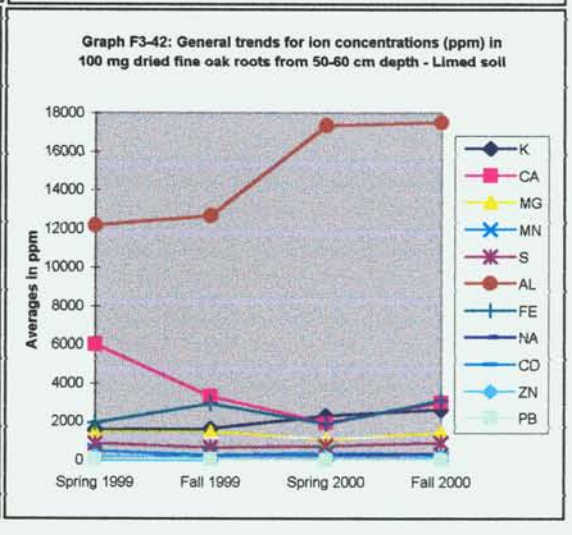

Graph F3-37: General trends in ion concentrations (ppm) in 100 mg dried fine oak roots from 0-10 cm depth - Unlimed soil
Average in ppm
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Spring 1999
Fall 1999
Spring 2000
Fall 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB


Graph F3-40: General trends in ion concentrations (ppm) in 100 mg dried fine oak roots from 0-10 cm depth - Limed soil.
Average in ppm
14000
12000
10000
8000
6000
4000
2000
0
Spring 1999
Fall 1999
Spring 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB

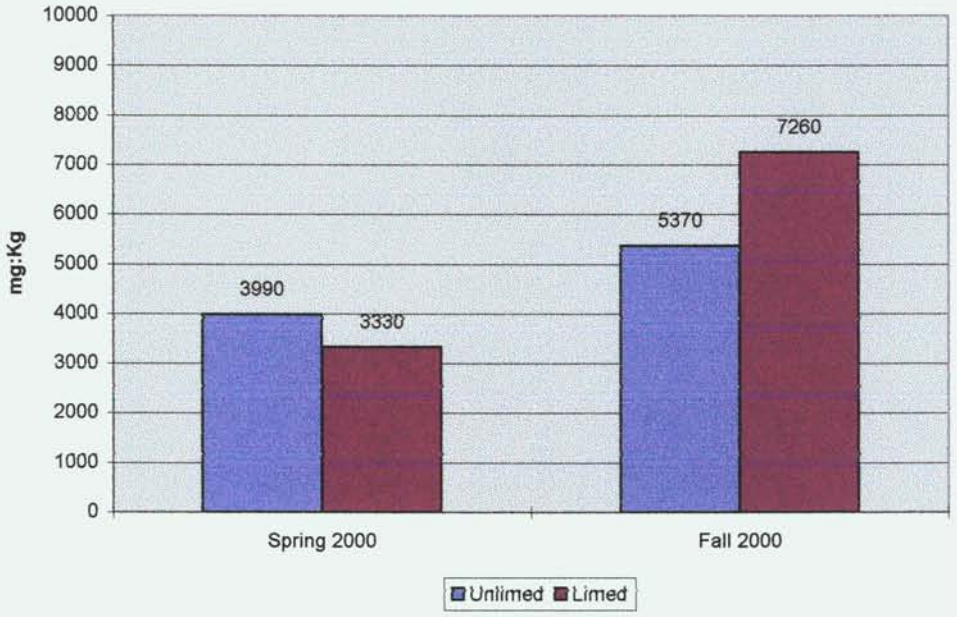

Graph F3-38: General trends in ion concentrations (ppm) in 100 mg dried fine roots from 30-40 cm depth - Unlimed
Average in ppm
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Spring 1999
Fall 1999
Spring 2000
Fall 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB


Graph F3-41: General trends for ion concentrations (ppm) for 100 mg dried fine oak roots from 30-40 cm depth - Limed soil
Average in ppm
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Spring 1999
Fall 1999
Spring 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB

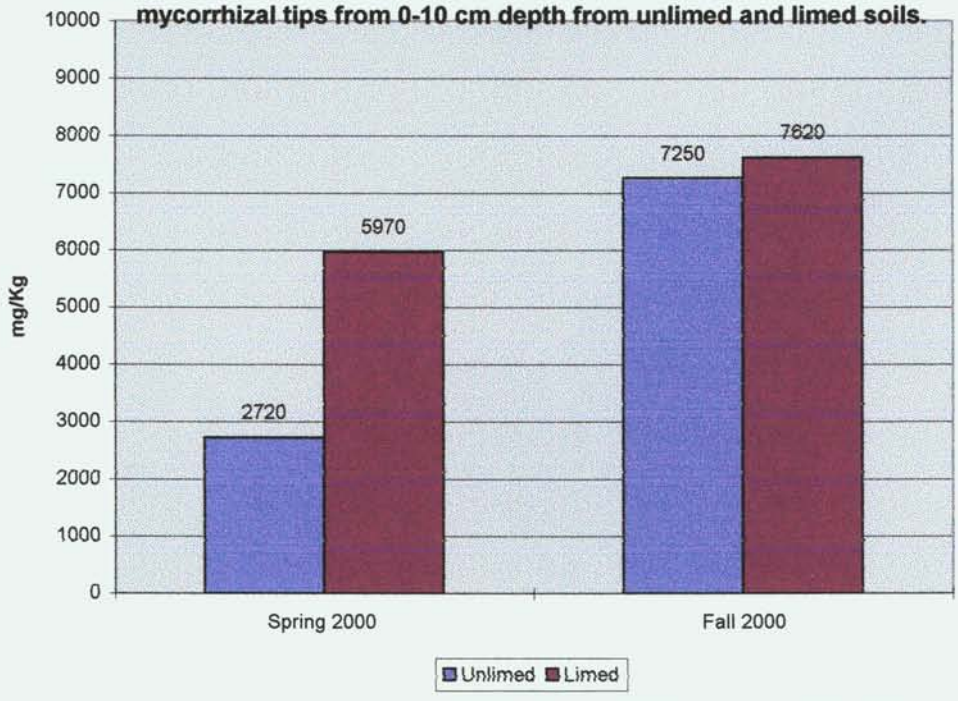

Graph F3-39: General trends in ion concentrations (ppm
100 mg dried fine roots from 50-60 cm depth - Unlimed
Averages in ppm
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Spring 1999
Fall 1999
Spring 2000
Fall 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB


Graph F3-42: General trends for ion concentrations (ppm) in 100 mg dried fine oak roots from 50-60 cm depth - Limed soil
Averages in ppm
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Spring 1999
Fall 1999
Spring 2000
Fall 2000
K
CA
MG
MN
S
AL
FE
NA
CD
ZN
PB

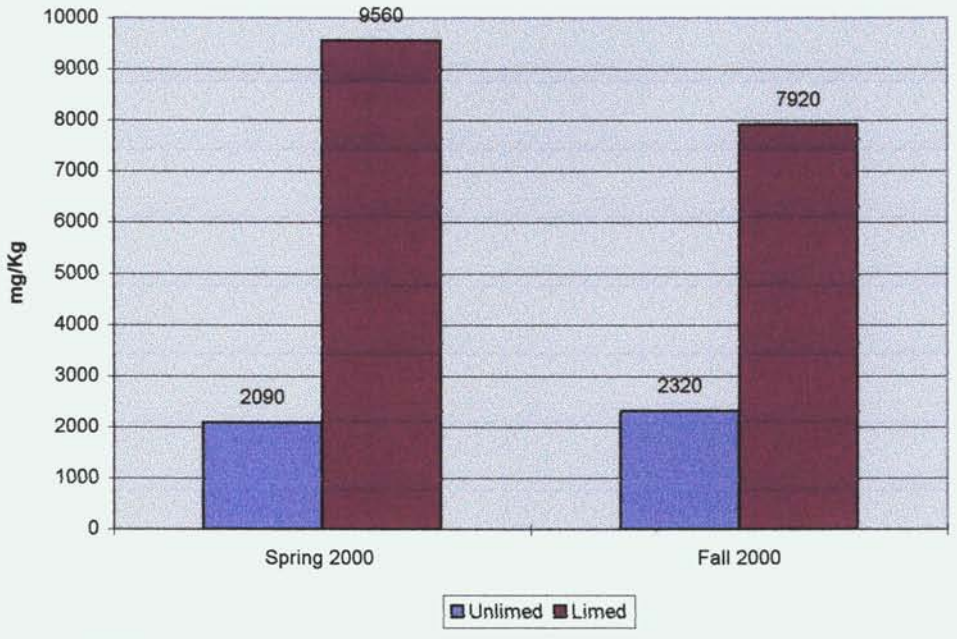
Graph F3-43: Calcium content of *Cenococcum geophilum / Quercus petraea* mycorrhizal tips from 0-10 cm depth in unlimed and limed soils.



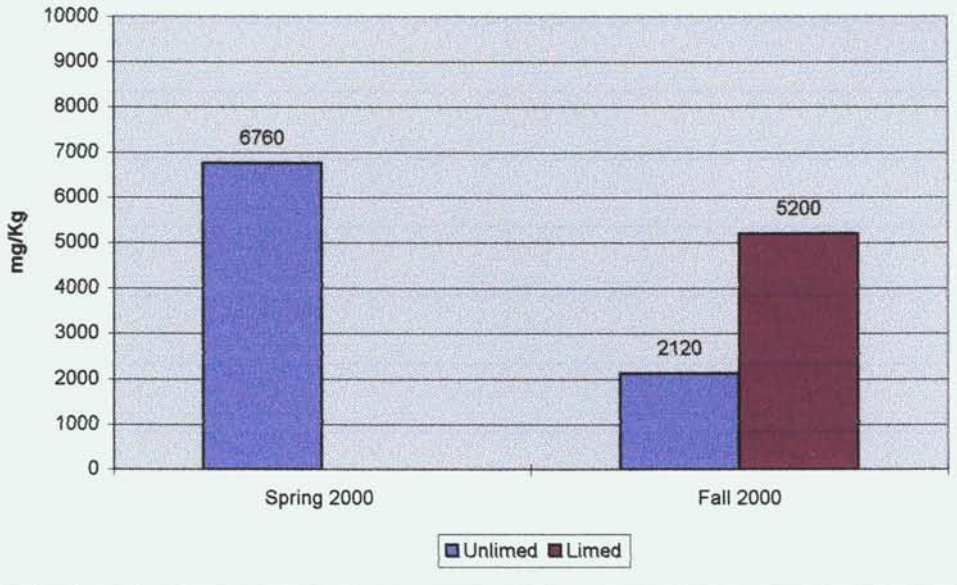
Graph F3-44: Calcium content of *Lactarius subdulcis / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



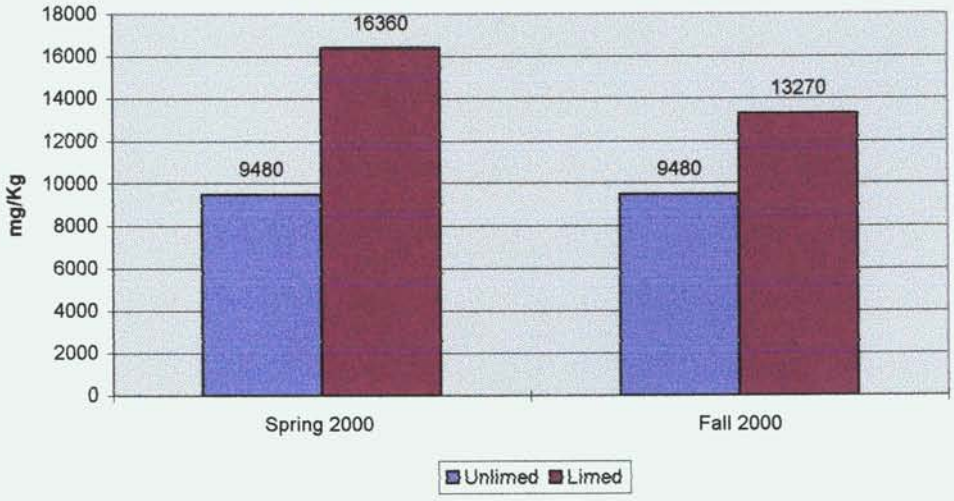
Graph F3-45: Calcium content of *Piceirhiza chordata / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



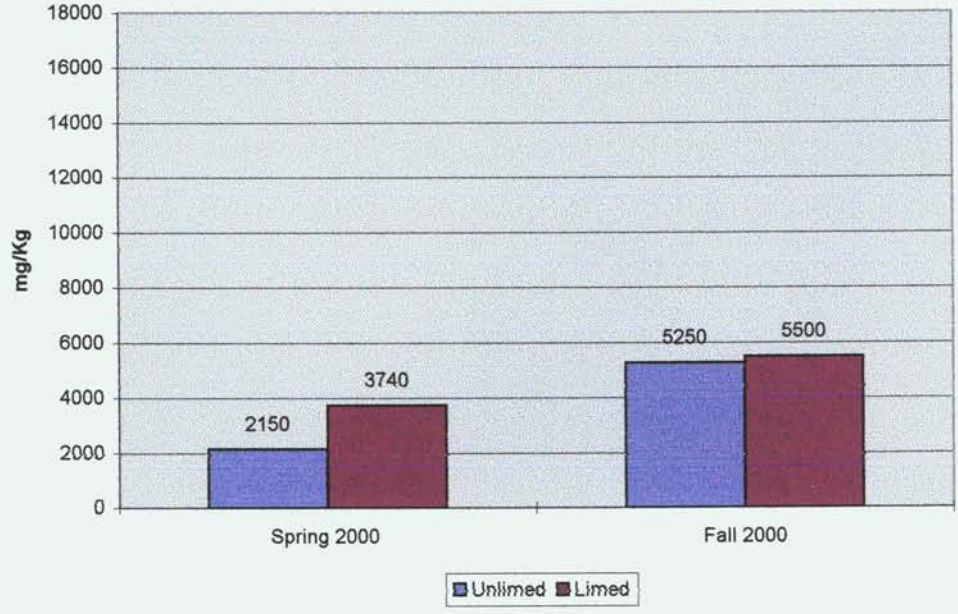
Graph F3-46: Calcium content of *Quercirhiza fibulocystidiata / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



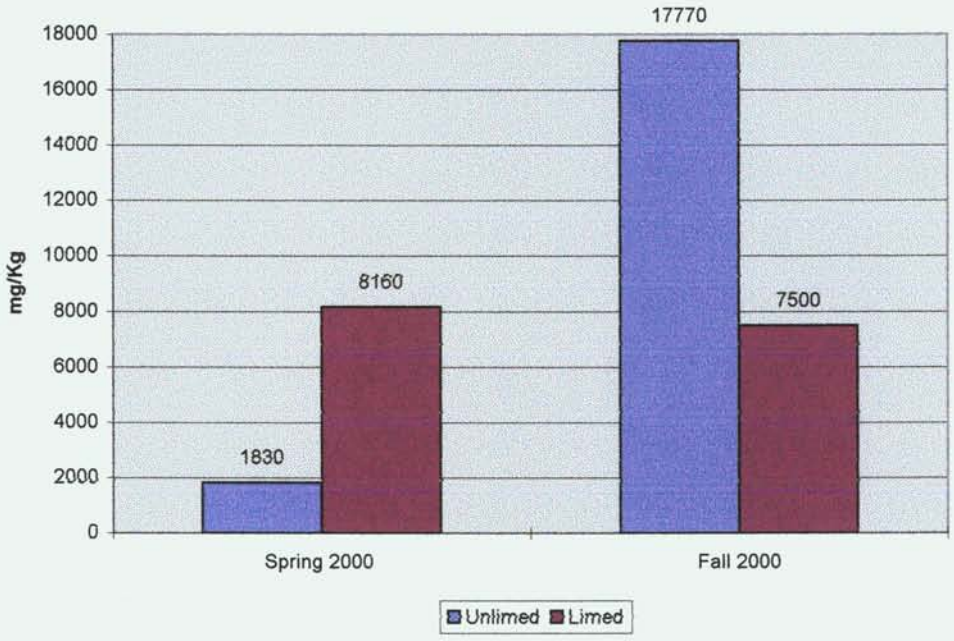
Graph F3-47: Aluminum content of *Cenococcum geophilum / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



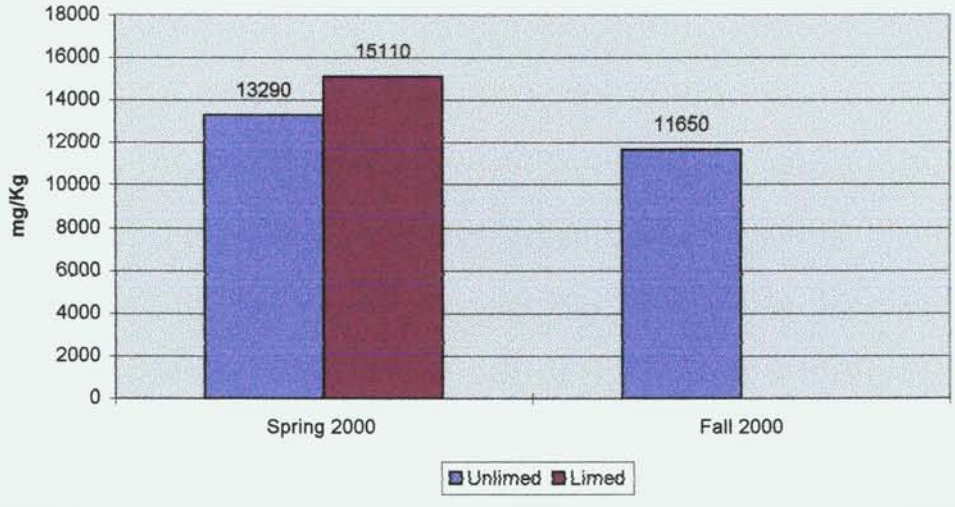
Graph F3-48: Aluminum content of *Lactarius subdulcis / Quercus petraea* mycorrhiza tips from 0-10 cm from unlimed and limed soils.



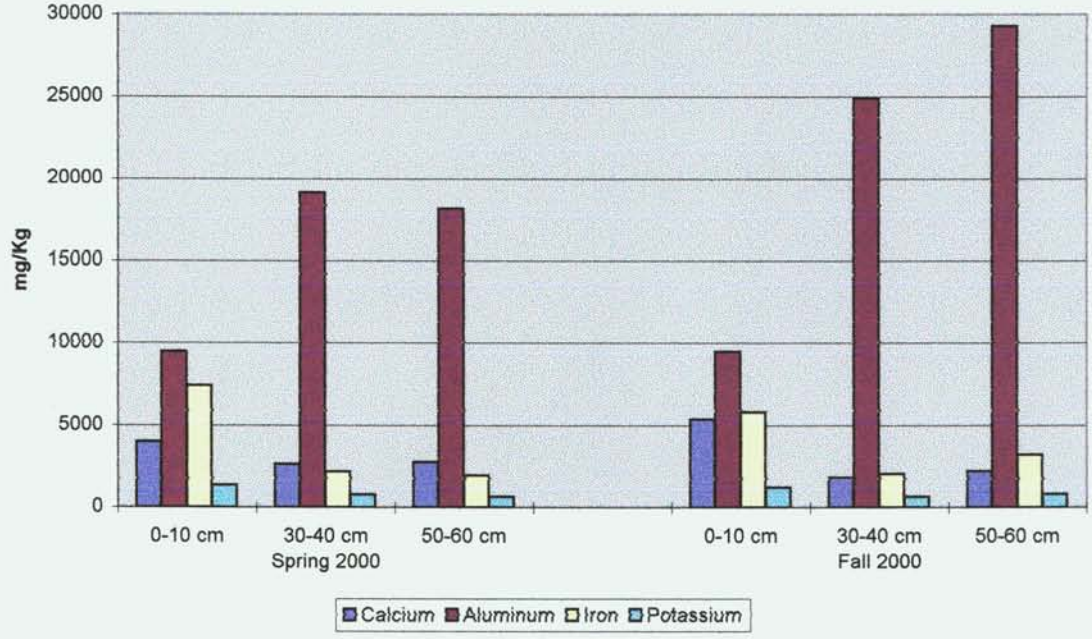
Graph F3-49: Aluminum content of *Piceirhiza chordata / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



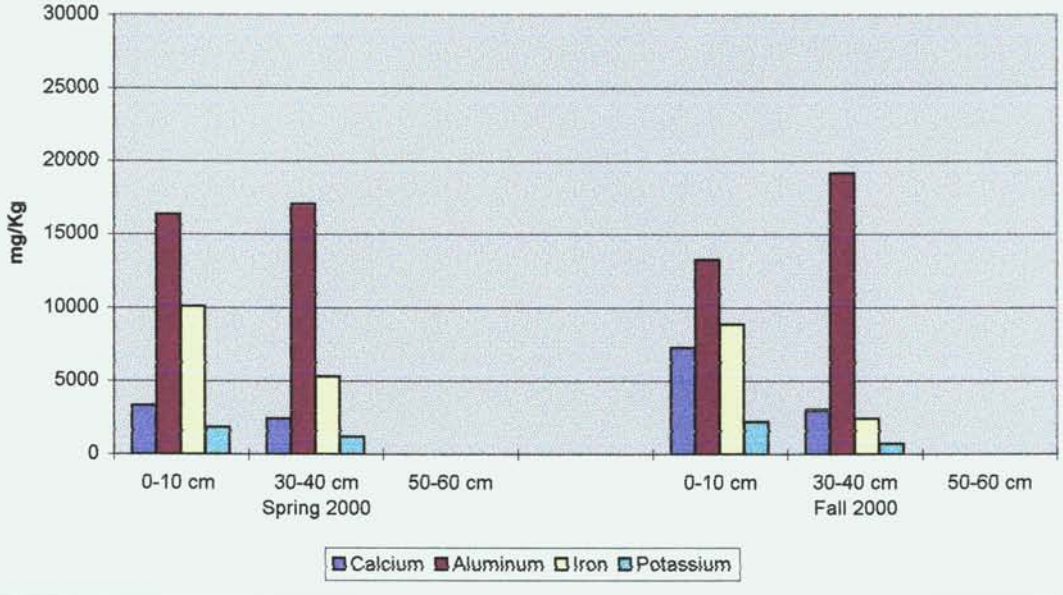
Graph F3-50: Aluminum content of *Quercirhiza fibulocystidata / Quercus petraea* mycorrhizal tips from 0-10 cm depth from unlimed and limed soils.



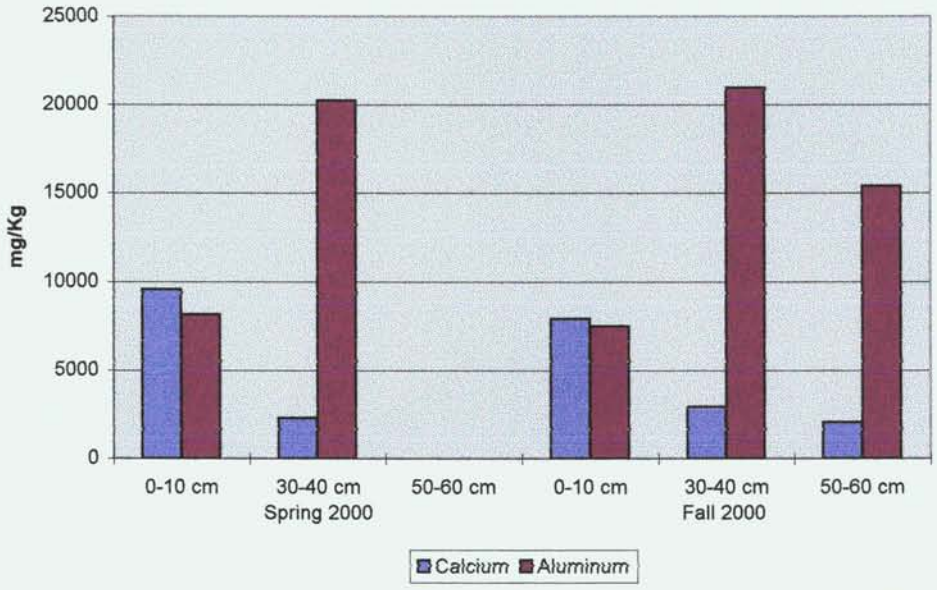
Graph F3-51: Mineral content of *Cenococcum geophilum / Quercus petraea* mycorrhizal tips from various depths from unlimed soil.



Graph F3-52: Mineral content of *Cenococcum geophilum / Quercus petraea* mycorrhizal tips from various depths from limed soils. (No samples from 50-60 cm depth were available).



Graph F3-53: Calcium and Aluminum content of *Piceirhiza chordata / Quercus petraea* mycorrhizal tips from various soil depths in limed soil only (No data was available for 50-60 cm depth in spring 2000).



F4: INTERPRETATION OF RESULTS

Seasonal cycles known to exist in living mycorrhizal roots (Rothe & Vogelei, 1991; Nowotny et al, 1998) may affect mineral uptake, sequestration and deposition. The role of mycorrhizae and liming was definitely complicated by natural variations in soil moisture and pH at various depths and seasons. The purpose of this section is to determine the patterns of moisture and pH and their relationships (if any) to the mineral concentrations within the isolated roots. Once these are known, then the effects of mycorrhizae and liming upon mineral content can be evaluated.

The moisture content at various soil depths was described in detail in the Section C1. Discussions of the moisture parameters here will include: seasonal oscillation pattern (F4-1A), seasonal accumulation (F4-1B), and moisture and soil depth (F4-1C) with respect to bound minerals. The pH at various soil depths was described in detail in the Section C2. Discussions of acidification in this section will include temporal acidification trends (F4-2A), empirical pH (F4-2B) and the effects of liming (F4-2C) The diversity , abundance and distribution of oak ectomycorrhizae was described in detail in Section B. Discussions of mycorrhizal effects on bound mineral presence will include: mycorrhizal abundance relative to soil depth (F4-3A), season (F4-3B), moisture, pH effects and liming (F4-3C).

Since the bound minerals examined were affected by the same environmental parameters, these factors will be considered first. A summary of these parameters and a potential rational explanation (or prediction) of the variations in the mineral concentrations will be presented first (Section F4), followed by a discussion of the actual mineral results with respect to liming and mycorrhizal abundance (Section F5). Due to the complexity of the natural systems, each mineral will be discussed in terms of their (1) Moisture Responses (2) Acidification Responses and (3) Mycorrhizal Responses with a (4) Summary.

In the "Bound Minerals General Conclusion" a chart is provided ranking the factors that seemed to have the greatest influence upon the bound mineral content in the mycorrhizal roots isolated from the humic soil (horizon A) in both unlimed and limed forest. In a broad general summary the ions Al, Mn, Zn, Cd, Pb tended to be heavily controlled

(accumulated) by mycorrhizae, while Ca, Mg, S were most heavily affected (accumulated) by liming. The minerals K and Na were primarily controlled by soil moisture, while P and Fe were most directly affected by soil pH with mycorrhizae and / or liming playing secondary roles. This is not to say that secondary effects cannot override the primary controls, but rather to show general trends in the dynamic flexibility of the natural system.

F4-1. Moisture Parameters - Background Information

F4-1A. Seasonal Oscillation Patterns

In brief summary, both plots experienced similar changes in water content consistent with the climatic variance. From spring 1999 to fall 1999, the average soil moisture content fell, followed by a gradual rise in the wet spring 2000 and fall 2000 (Section C1: Graphs C-1, C-2). There were no statistical differences between the unlimed and limed plots in this respect for the upper two soil horizons where the primary mycorrhizal growth occurs, but the lowest (50-60 cm depth) moisture levels were affected by liming especially in the dry spring 1999 (Section C1). If the bound and / or unbound mineral components of the root were directly affected by soil moisture, there should be a direct and nearly identical correlation between the contents of the minerals and the seasonal moisture levels in both plots. Conversely if moisture was not a controlling feature, the accumulation of a specific mineral should bare no obvious correlation. It was determined that some minerals (K, Na) varied more directly with moisture than others, but all minerals were in some way affected by moisture (Section F5). For these minerals, the seasonal oscillating patterns and mineral accumulation at A (0-10 cm), B (30-40 cm) but not C (50-60 cm) horizons should be similar unless the minerals were affected by liming, pH and / or mycorrhizal distribution.

F4-1B. Seasonal Accumulations

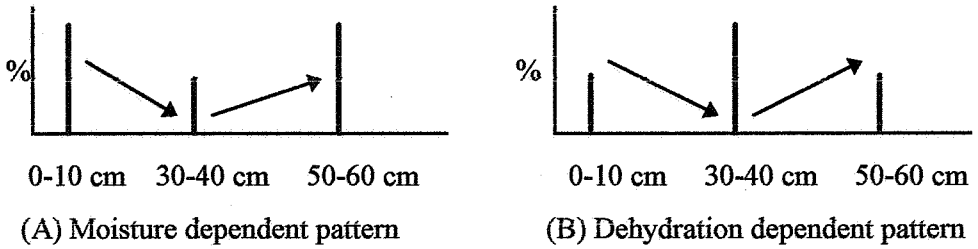
If a mineral requires hydration, it should translocate best to and into the roots during wet seasons, unless excessive leaching occurs. In the spring, as the xylem one-way flow increases, the mass mineral flow may increase unbound mineral concentrations, but total bound root content may as a result be artificially low. Conversely, in the fall, as soils dehydrate and /or xylem flow declines, a mineral may become trapped within the root

resulting in an apparent increase in bound, and decrease in unbound mineral content, unless it is actively secreted by the root mycorrhizae. (1) If there is a „balance" in the mycorrhizal activity, then the uptake and sequestration may follow the expected hydrological and climatic pattern with augmentation of the bound mineral in the fall or during dry periods. (2) These general assumptions will not apply if there is aggressive preferential mobilization, sequestration or blocking of mineral transport by selective mycorrhizae or interference due to the effects of liming.

F4-1C. Moisture & Soil Depth

In addition to seasonal moisture changes and their concurrent oscillation patterns, the total moisture content at various soil depths needed to be reviewed. At 0-10 cm (humic soil) and 50-60 cm (rocky-sandy soil) depths the moisture contents were not significantly different except in the spring of 1999 when the unlimed soil had (2%) more water in the 0-10 cm depth and the limed plot had (7%) more water in the 50-60 cm deep soil. The upper horizon retained water probably due to the absorptive nature of humus and root biomass while the lower horizon held water possibly due to the sandy-clay-rock mix or the possible presence of a solid sandstone base raising the water table. At 30-40 cm depth where the soil was primarily sand, the water content was consistently and significantly lower (11-17 %) than either the upper humus soil (16-27 %) or lower sandy-rocky soil horizons (16-29 %). This is consistent with rapid sandy soil leaching. At 30-40 cm, both the unlimed and limed plots were not significantly different except for the faster spring 2000 recovery in the limed soil (4% more water). The differences between the moisture 0-10 and 50-60 cm depths can be then contrasted to the drier 30-40 cm levels. If uptake of nutrients was affected by soil depth and moisture content, then two possible opposing tends may occur depending upon the mineral involved: (1) the mineral will be higher in concentration in both 0-10 cm and 50-60 cm depths and lower in 30-40 cm depth producing a "moisture dependent pattern" (Figure F4-1C-A), or (2) the mineral will be lower in concentration at 0-10 and 50-60 cm and higher at 30-40 cm depth producing a "moisture independent pattern" (Figure F4-1C-B). (3) Where no obvious pattern is evident, or where the 0-10 and 50-60 cm depth accumulations differ we may assume that the roots are less affected by moisture and more by liming, pH, or mycorrhizae.

Figure F4-1C: Patterns of bound mineral accumulation (indicated by lines) based upon moisture gradients (indicated by arrows) at various soil depths. If a mineral is dependent upon moisture to accumulate, it should increase as the moisture content rises (A) but if a mineral becomes bound with dehydration it will follow pattern (B).



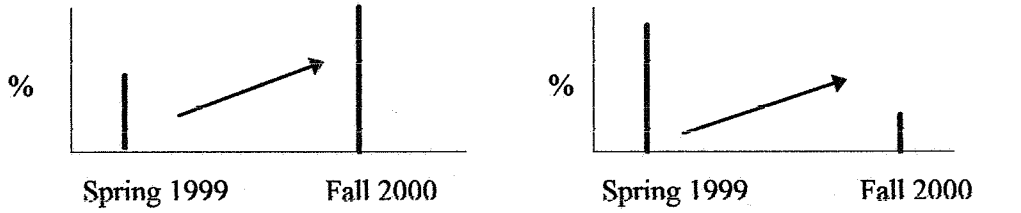
(A) Moisture dependent pattern (B) Dehydration dependent pattern

F4-2. Acidification Parameters

F4-2A. Temporal Acidification

Over the last 12 years there has been a slow progressive acidification (Section C). Liming has slowed the acidification in the upper horizon but with a slightly more pronounced acidification front moving down into the lower horizons (Graphs C2-1 to C2-3). Averaging the three pH test procedures (pH H_2O, pH KCl, pH $CaCl_2$) used, at 0-10 cm depth the soil acidified considerably (Unlimed: from 3.33-0.167 units to 3.17; Limed: from 4.12-0.183 units to 3.94) and while a pH drop at 30-40 cm occurred (Unlimed 4.3-0.03 units to 4.27; Limed 4.4-0.087 units to 4.32), it was not dramatic nor significant. The most significant temporal pH drop from 1999 to 2000 occurred at the 50-60 cm depth (Unlimed 4.6-0.217 to 4.39; Limed 4.53-0.303 units to 4.22). In all cases, the pH was much less than 5, well within the Al buffering range and entering the Fe buffering range (Section A3-4). If the content of bound minerals is altered by long term acidification then the relative amounts should reflect the increasing temporal acidification. (1) A rise in bound minerals may occur if the mineral is made more mobile in acidic soil. (2) A drop in bound minerals should occur if the mineral is made less mobile in acid soil. (3) No obvious pattern may be evident if other factors are more prominent in their effects. (Figure F4-2A)

Figure F4-2A: Potential mineral accumulation (indicated by lines) related to temporal acidification (indicated by arrows). If a mineral is mobilized and accumulated in acidifying soils, its concentration should increase over time (A) but if it is not mobilized, or mobilized but not bound, its concentration should decline (B).



(A) More binding with acidification (B) Less binding with acidification

F4-2B. Empirical pH

There were significant differences in pH in between the unlimed (N) and limed (L) plots at various soil depths. At 0-10 cm horizon, the unlimed soil was very acidic with the average pH H2O ranging from 3.53 to 3.79, while the limed soil ranged from 4.24 to 4.47. At 30-40 cm depth, the ranges narrowed and were closer in relative value (N: 4.49 to 4.53 and L:4.66 to 4.69). At 50-60 cm depth, the ranges widened and interestingly, unlimed soil was often more alkaline at this depth than the limed soil (N: 4.6 to 4.9 and L: 4.48 to 4.78). In light of these factors, if uptake and binding of minerals is dependent upon the empirical pH values, then the mineral contents should either vary (1) directly with the most acid soils at 0-10 cm depth having the highest mineral content or (2) indirectly with the most alkaline soil at 50-60 cm depth having the highest mineral content or (3) with no obvious pattern if pH is not a primary control factor.

F4-2C. Liming

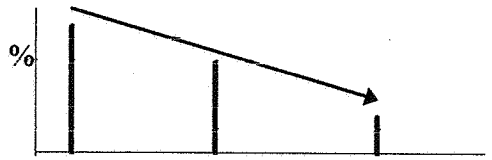
The relationships between calcium content of the soil and mineral content is discussed in detail in Section F: Bound Calcium:Mineral Ratios and Section F: Unbound Calcium:Mineral Ratios. Calcium can affect mineral content by (1) directly increasing content (2) antagonistically decreasing content or (3) with no obvious pattern if Ca is not a primary control factor.

F4-3. Mycorrhizal Abundance Parameters

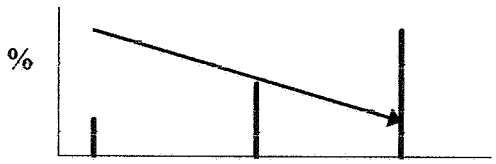
F4-3A. Mycorrhizal Abundance & Depth

The mycorrhizal complements in unlimed and limed soil were examined in detail in Section B: Ectomycorrhizal Abundance and Distribution. In summary, the mycorrhizal mass greatly declines with depth in both unlimed and limed soils. (1) If uptake of a mineral is directly dependent upon mycorrhizal abundance, then the relative content of the mineral should decline with depth, or (2) if a mineral is mostly barred from entry into the cytosol by the mycorrhizae then the reverse trend should be apparent with the mineral increasing in concentration with depth or (3) with no obvious pattern if mycorrhizal influences are not primary factors in nutrient uptake. (Diagram 3.3)

Diagram F4-3A: Potential mineral abundance (lines) at various soil depths relative to mycorrhizal abundance (arrows). If a mineral is accumulated in mycorrhizae, then its concentration should increase relative to an increase in mycorrhizal numbers (A). Conversely, if the mycorrhiza inhibits or blocks the mineral or if the mineral is primarily controlled by other environmental factors, then as the mycorrhizae disappear, the mineral concentration should rise (B).



(A) Mineral correlated to mycorrhizae



(B) Mineral inverse to mycorrhizae

F4-3B. Seasonal Mycorrhizal Abundance

The abundance of mycorrhizae is seasonally dependent with the relative abundance lowest in the very dry fall of 1999 and highest in fall 2000. (1) If mineral uptake is dependent upon mycorrhizal presence, then its concentration should be low in fall 1999 and high in fall 2000, but (2) if mycorrhizae inhibit uptake then the reverse trend should be apparent and (3) no obvious pattern will be evident if seasonal mycorrhizal abundance is irrelevant.

F4-3C Mycorrhizal and Moisture, pH and Liming Effects

In a natural ecosystem, all the above factors are dynamic and can interact synergistically or antagonistically altering physical and physiological nutrient uptake, translocation and sequestration by living mycorrhizal roots. The diversity, seasonal and successional complement of mycorrhizal species may be the single largest factor affecting net intake of minerals. The cumulative effect of mycorrhizae may in turn be dependent upon growth limiting parameters such as moisture and pH.

F5: BOUND ALUMINUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-1 to F3-3)

F5-1. Moisture Responses - Aluminum

F5-1A. In the unlimed forest seasonal oscillations were somewhat evident with the lower Al concentrations in the spring relative to the following fall at all depths except for 50-60 cm in 1999. In the limed plot there was no obvious seasonal oscillation pattern. Both forest zones had an annual increase in Al content at all depths. After a stress event (dry fall 1999), the limed mycorrhizal roots sequestered more Al than the unlimed. But it is important to note, as will be shown later, the limed roots had less free unbound Al present.

F5-1B. In unlimed soil at both 0-10 and 30-40 cm depths, the Al content was low in the wet spring 1999, equilibrating (but consistently a little higher) in the dry fall 1999 and following spring 2000. This dry period was followed by a jump in Al content in the succeeding moister fall 2000. At 50-60 cm depth, Al content dropped slightly in the dry fall but rose steadily thereafter and was also highest in fall 2000. In limed soil, the Al content was most similar in spring and fall 1999 but experienced an increment rise in spring 2000 which was maintained through fall 2000 at all depths. Liming clearly altered mycorrhizal seasonal and moisture responsiveness and was tied to some post-winter, early-spring triggering event which allowed Al uptake. In shallow soil, the fall 2000 increase in both the unlimed and limed zones may have been, in part, moisture related.

F5-1C. With respect to depth, in both forest zones, Al content dramatically rose in deeper soil. At 50-60 cm depth, there was no significant difference between the two forest areas in uptake concentrations or patterns. At 30-40 cm depth, in the sandwiched dry soil

horizon, there was more variability in the Al content and uptake patterns but the concentrations were still very high. At 0-10 cm depth, the lowest Al uptake occurred despite the fact that the water content was similar to the 50-60 cm zone. Al uptake was therefore more directly affected by factors other than actual soil water content.

F5-2. Acidification Responses - Aluminum

F5-2A. With temporal soil acidification, Al content in both unlimed and limed probes increased at all soil depths. Over time, as more Al was released, more was stored.

F5-2B. With acidification, more Al is released from the soil matrix. According to Ulrich (1989) the Al buffer range of pH 4.0-4.4 results in Al stress, and between pH 2.8 to 3.8 both Fe and Al stress occur. Despite the fact that the soil was more acidic (N: 3.79 to 3.53 and L: 4.47 to 4.24) at the 0-10 cm depth horizon than any of the lower horizons (N: 4.54-4.49 and L: 4.69-4.66 at 30-40 cm) (N: 4.9-4.6 and L: 4.78-4.48 at 50-60 cm), the Al content of the roots was lower than that of the lower more alkaline horizons. This implies a factor other than the actual pH was primarily controlling Al uptake in the roots. This factor was most likely the effective mycorrhizal exclusion of Al in shallow soils where a greater diversity and abundance of mycorrhizal tips were present. According to the fluorescence studies it is probable that in deeper soil where fewer mycorrhizal were present to act as barriers, Al is sequestered to a greater extent in the cortex and xylem.

F5-2C. At 0-10 cm depth, in spring 1999 there was no significant difference between the unlimed and limed probes, despite pH and Ca content differences. After the very dry fall 1999 and with increasing acidification followed by enhanced moisture, the Al accumulation in the limed probes was significantly higher (about 35 to 25% more) than in the unlimed probes. At the same time Ca content was declining but it was still (about 50 to 10 %) higher than in the unlimed samples. In the unlimed probes, the Ca content increased with time and so did the Al content. In the fluorescence study, more Al was evident in the majority of limed probes but it was not possible to determine how much. From the bound mineral analysis it was discovered that the average bound Al content at 0-10 cm depth was insignificantly higher in 1999 (L:4602 vs. N:4580 mg/kg in spring 1999)

(L:6284 vs. N:6235 mg/kg in fall 1999) but significantly higher in 2000 (L:9878 vs. N 6425 mg/kg in spring 2000) (L:12256 vs. N:9360 mg/kg in fall 2000). These results confirm the qualitative observations made with fluorescence microscopic analysis.

The control or modification of Al and Ca uptake and the ratio relationships was lost in deeper soil as mycorrhizal roots disappeared. At 30-40 cm depth, in the spring 1999, the limed probes had both a greater Al and Ca content than the unlimed. Up to this point there seemed to be a positive correlation between Ca and Al uptake. But in drier soils at 30-40 cm depth and in wet soils at 50-60 cm depths the relationship became inverse. Al content rose as Ca content declined over time in both forest zones. In deeper soils (except for spring 1999) there was no significant difference between the unlimed and limed forest. The concentration of Ca and Al sequestration was directly related at low concentrations (below 5000 PPM Ca) in shallow acidic soil in the presence of heavily mycorrhizal roots, but became inversely related as the mycorrhizal roots disappeared or as the Ca content rose or fell above the critical concentration point (near 6000 PPM Ca).

F5-3. Mycorrhizal Responses - Aluminum

F5-3A. Al content dramatically increased as mycorrhizal abundance declined with depth in both the unlimed and limed soils. Mycorrhizae in the shallow rhizosphere effectively excluded Al whereas the non-mycorrhizal roots could not eliminate or control Al entry.

F5-3B. Mineral sequestration was dependent upon changing mycorrhizal abundance. In both forest zones, at all depths, the Al content rose as mycorrhizal numbers increased from the dry 1999 to the wet fall 2000. However the primary function of the shallow soil mycorrhizae was Al exclusion. Despite greater mycorrhizal abundance than in deeper soils, considerably less Al was present. Liming seemed to improve sequestration but at 0-10 cm depth the Al was still preferentially excluded to being sequestered.

F5-3C. After a seasonal drought (fall 1999) a combination of increasing acidification (Al release), and moisture rehydration (allowing Al uptake) along with enhanced mycorrhizal presence resulted in greater Al content in both the unlimed and limed zones at all depths. At 0-10 cm depth however, the limed probes had greater sequestration of Aluminum.

F5-4. Summary - Aluminum

The relationship between mycorrhizal species and Al sequestration was dealt with in the Section C. From the fluorescence study it was shown that Aluminum can be sequestered in the fine mycorrhizal root tips and that the degree of sequestration, translocation or blockage is determined by the species of mycorrhizae. The initial qualitative results indicated that, in the same species, the samples from the limed forest zone often had brighter, faster fluorescence reactions implying higher bound Al content. The question that could not be answered by the fluorescence studies was how much Al was being held. Due to the microscopic nature of the mycorrhizae only 4 of the isolated species were directly quantified with respect to Al content (Section F18 & Graphs F3-43 to F3-53). From the general results just presented, the qualitative fluorescence results were confirmed. A brighter, faster reaction with Morin was associated directly with increased Al content in the limed roots.

In the above comparative analysis of bound Al, between unlimed and limed forest zones, fine unsorted whole mycorrhizal oak roots were used. The results are therefore general and not specific for any given species. In general conclusion, the primary control of Al exclusion was mycorrhizal presence. As the mycorrhizae disappeared with depth, the control was lost and more Al is accumulated in the bound form within the walls of the root cortex and xylem. Prior to the fall 1999 seasonal drought, the Al content of the unlimed and limed soils were not significantly different at 0-10 cm depth but were very different at 30-40 and 50-60 cm depths. After the fall 1999 dry spell, the Al content of the limed roots was higher than the unlimed at 0-10 cm but in deeper soils there was no significant difference between the zones. Drought definitely affected mycorrhizal metabolism with respect to Al and liming improved mycorrhizal sequestration of Al after the mini-drought.

F6: BOUND CALCIUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-4 to F3-6)

F6-1. Moisture Responses - Calcium

F6-1A. The limed probes exhibited seasonal oscillations with lower Ca content in the fall relative to the preceding spring (0-10 cm depth) implying a faster, but incomplete recovery after the fall 1999 dry spell. The unlimed probes (0-10 cm depth) did not have

the same trend but rather gradually increased in Ca content over time following the 1999 drought. Seasonal oscillations were not evident in deeper soils from either zones.

F6-1B. In comparing the dry and wet fall accumulations, the limed probes were not significantly different while the unlimed probes definitely increased their stored Ca at 0-10 cm depth. In deeper soils, increasing moisture content between fall 1999 and fall 2000 did not improve bound Ca content but rather in both cases there was a marked decline in the wet fall. Other than spring 1999, there was no significant difference in Calcium uptake between unlimed and limed soils at either 30-40 or 50-60 cm depths. Soil moisture was not a primary controlling factor in Ca uptake and binding. In fact with more moisture, less Ca seemed to be present in the bound form.

F6-1C. Despite the fact that the soil moisture content at 0-10 and 50-60 cm depth were nearly equivalent, the Ca content in the heavily mycorrhizal zone at 0-10 cm depth in the limed forest was consistently higher. Calcium content in the limed plot nicely declined with depth and lower mycorrhizal abundance but the unlimed probes were not as consistent. In the spring and fall 1999, the Calcium content was actually higher with depth and in the fall 1999 even exceeded that of the limed probes. But in the spring and fall 2000 during the recovery period, there was no significant difference between the unlimed and limed probes at 30-40 and 50-60 cm depth. Moisture content of the soil was therefore not a primary factor in Ca content of the unlimed or limed roots with respect to soil depth.

F6-2. Acidification Responses - Calcium

F6-2A. With temporal acidification at 0-10 cm depth, Ca content declined in the limed soils but rose in the unlimed soils and at 30-40 and 50-60 cm depth, Ca content declined in both forest zones over time.

F6-2B. In unlimed soils from pH 3.79 to 3.53 over time Ca content rose at 0-10 cm depth. While at pH 4.47 to 4.24 with acidification, the Ca content dropped in the limed soils at 0-10 cm depth. In deeper soils where the pH dropped (from 4.53 to 4.49 (N) and 4.69 to 4.66 (L) at 30-40 cm and from 4.9 to 4.6 (N) and 4.78 to 4.48 (L) at 50-60 cm), in all cases, the Ca content also dropped. Calcium seems to be lost with increasing

acidification, but at very low pH levels in the unlimed forest Ca rose, most likely due to the augmented presence of acid-adapted mycorrhizal species.

F6-2C. While liming improved the Ca content of roots at 0-10 cm depth, it altered the diversity and abundance of mycorrhizal species. Amelioration effects of liming were lost at 30-40 and 50-60 cm depths.

F6-3. Mycorrhizal Responses - Calcium

F6-3A. As depth increased and mycorrhizal abundance declined, Ca content of the roots declined especially in the limed forest zone. In the unlimed acidic soils the Ca content actually increased with depth in 1999 possibly due to the mycorrhizal selection present but after the dry period in the fall 1999 these species seem to have been lost and with them the Ca uptake in deeper soils with a shift in primary Ca uptake to the 0-10 horizon.

F6-3B. At 0-10 cm depth in both the unlimed and limed soils Ca uptake improved from the dry fall 1999 to the wet fall 2000, most likely due to enhanced mycorrhizal presence rather than moisture or pH changes. In deeper soil in both zones, with fewer species and lower mycorrhizal abundance, Ca declined from the dry fall 1999 to the wet fall 2000.

F6-3C. In the case of Ca content, mycorrhizal presence seemed to be the controlling factor and when mycorrhiza were lost, such as in deeper soils, Ca uptake declined. Temporal soil acidification tended to result in Ca loss but was probably modified by acid-tolerant mycorrhizal species. Moisture alone had a modest influence with more impact in the unlimed forest at 0-10 cm depth. Additional stress such as a short term drought however seemed to negatively impact the already stressed mycorrhizal complements resulting in major Ca losses in deeper soils where recovery can be very slow.

F6-4: Summary - Calcium

The primary controlling factor in Ca uptake was mycorrhizal abundance followed by the probable positive influences of acid - tolerant mycorrhizal species in very acidic soils. Continued acidification was detrimental to bound Ca presence. Short term dry periods

negatively impacted Ca content for several seasons after the drought at all soil depths. Liming improved Ca content in the 0-10 cm horizon but not necessarily in deeper soils.

F7: BOUND MAGNESIUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-7 to F3-9)

F7-1. Moisture Responses - Magnesium

F7-1A. No distinct seasonal oscillation patterns were evident in either unlimed or limed zones at 0-10 or 30-40 cm depth. A weak oscillation pattern was present only at 50-60 cm depth with slightly more Mg present in the fall than the previous spring for both forest zones. Mg content was consistently higher in limed probes.

F7-1B. In limed probes Mg content was very slightly higher in the dry fall 1999 and lower in the wet fall 2000 at all depths. In unlimed probes the Mg content was followed a similar pattern at 30-40 and 50-60 cm depths but had the opposite trend at 0-10 cm depth with Mg content low in the dry fall and higher in the wet fall. Mg was consistently more abundant in the limed samples but leached easily the soil water content rose. By contrast, Mg content was considerably less in the unlimed probes but tended to leach less easily in deeper soils and actually accumulated in the 0-10 horizon as water content rose.

F7-1C. The soil water content at 0-10 and 50-60 cm depths was similar but the total Mg content was not. In the limed probes, the Mg content declined with depth with slightly more loss during the wetter seasons (20-30%) than the dry fall 1999 (10-15%) at each horizon. In the unlimed probes Mg content increased (18-23%) with depth in the dry fall 1999 but otherwise declined with depth during the wetter seasons. The loss of mycorrhizae with depth appeared to have more impact than moisture. The unlimed probes were less capable of bounding Mg and more responsive to desiccation.

F7-2. Acidification Responses - Magnesium

F7-2A. With temporal acidification, Mg declined at all depths in limed soil. In unlimed soil, at 0-10 cm depth, Mg content increased with acidification but declined in the deeper horizons with acidification implying that other factors such as mycorrhizal abundance may mitigate Mg content.

F7-2B. In deeper unlimed soil when the pH was actually higher than in comparable samples from the limed forest, the Mg content occasionally rose in the unlimed roots but was still significantly lower in comparison. The pH alone does not seem to be responsible for the low Mg content of unlimed probes.

F7-2C. In shallow soil one might attribute the enhanced bound Mg presence to liming but in deeper soil horizons where it was discovered that the Calcium levels were occasionally higher in the unlimed soil, there is no comparable enhancement in the Mg content. Liming seems to be a key contributing factor to improved Mg binding it is more likely that the mycorrhizal species promoted by the liming treatment may play a more significant role. In the unlimed forest, the average Ca and Mg contents of the probes follow very similar patterns of storage and loss from season to season and with soil depth with an average Ca:Mg ratio of 3.8:1. In the limed forest, there was no similar pattern consistency and the average Ca:Mg ratio was lower (3.4:1) indicating that liming somewhat improved Mg content. But the lack of consistency in the accumulation patterns indicated other factors were operating.

F7-3. Mycorrhizal Responses - Magnesium

F7-3A. In limed soil, Mg content declined as mycorrhizal abundance declined with depth. In unlimed soil, the mycorrhizae present seemed to be less efficient at binding Mg at all depths. It was possible that minimal levels (633-1075 mg/kg) of Mg content were being maintained in the unlimed probes since the drops in content were not consistent with depth nor as dramatic as in the limed probes.

F7-3B. The Mg content in the limed samples was not as dramatically affected by the fall 1999 dry period as that of the unlimed samples at 0-10 cm depth. In the recovery period, while the limed mycorrhizae dropped in average content from 2170 to 1727 mg/kg (20%), the unlimed mycorrhizae doubled their Mg content (665 to 1244 mg/kg). In deeper soil, Mg content declined from the dry fall 1999 to the wet fall 2000 despite an increase in mycorrhizal abundance in both the unlimed and limed areas. At these depths, leaching may have played a more important role.

F7-3C. In the case of Mg content, the particular mix of mycorrhizal species may have been of more importance than the total general abundance of mycorrhizal roots. Despite the fact that in the fall 2000 both the unlimed and limed zones had the greatest abundance of mycorrhizal roots at 0-10 cm depth, the limed samples lost Mg and the unlimed samples gained Mg. In the former case, enhanced leaching could have been a contributing factor but since both zones had the same moisture levels this possibility needs to be discounted. Acidification changes may have affected mycorrhizal competence in the limed zone but this explanation still leads us to suspect a species specific Mg binding capacity.

F7-Summary - Magnesium

In most cases Mg content was enhanced with liming, but indirectly so. The most likely explanation for the trends observed in Mg content probably involve species-specific liming-induced variations in Mg-binding capabilities of the mycorrhizae. Mg uptake improved with better soil moisture in acidic soils most likely due to better functioning of the acid-tolerant mycorrhizal species. In deeper soil horizons, rising water content promoted leaching of Mg which probably overrode the mycorrhizal functions. Both Mg and Ca are known base exchangers but there was not an indication from the results that they were competitively exclusive or inversely related in any way. Both Mg and Ca declined with time at all depths and both were augmented by liming. In unlimed soil the Ca: Mg ratio averaged 3.9:1 and in limed soil 3.4:1 so Mg content was definitely improved with liming.

F8: BOUND POTASSIUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-10 to F3-12)

F8-1. Moisture Responses - Potassium

F8-1A. The limed soil samples had strong seasonal oscillations at 0-10 cm depth and weak ones at 30-40 cm. The unlimed soil samples lacked seasonal oscillation patterns.

F8-1B. In both unlimed and limed forest zones, and at all depths, K content was improved with enhanced soil moisture. At 0-10 cm depth the unlimed probes were the most responsive to desiccation during the 1999 fall drought with the lowest K content.

F8-1C. In the spring and fall 2000, the K content for limed probes at 0-10 and 50-60 cm depths were not significantly different from each other but were both higher than the 30-40 cm depth contents. The trends were less prominent in the unlimed probes but still present. Potassium content was directly and strongly influenced by soil moisture.

F8-2. Acidification Responses - Potassium

F8-2A. At 0-10 and 50-60 cm depths, temporal acidification resulted in increased K presence in both the unlimed and limed forest zones. There was no significant increase however at 30-40 cm depth. Acidification was to be secondary to soil moisture in controlling K content.

F8-2B. At 0-10 cm depth the unlimed, more acidic soil samples generally contained less K except for spring 1999 when the unlimed pH 3.79 soil probes had more K than the limed pH 4.47 probes. In deeper soil where the average pH exceeded 4.48 there was for the most part no significant difference between the unlimed and the limed zones in K content. In all cases, K concentration rose with temporal acidification with the highest contents occurring in limed soils at pH 4.24 at 0-10 cm depth and in unlimed soils at pH 4.6 at 50-60 cm depth. Clearly more factors were controlling K content than just the pH.

F8-2C. At 0-10 cm depth, the limed mycorrhizal probes had significantly more K content than the unlimed. In deeper soil, where the liming effects were lost, the unlimed and limed zones were not significantly different. However, Ca content alone did not explain the fluctuations in K content. The seasonal and soil depth patterns of Ca accumulation did not correspond directly to the K content, in fact, they seemed to be most frequently inversely related. Improved Ca content was more important than pH at 0-10 cm depth but its impact may have been more directly related to its effects upon mycorrhizal diversity.

F8-3. Mycorrhizal Responses - Potassium

F8-3A. In unlimed probes in 1999 there was either no or relatively little significant difference in K content regardless of soil depth. In 2000 the differences were somewhat greater with the most significant differences between depths occurring in spring 2000

between the 0-10 and 50-60 cm depths. But overall, the loss of mycorrhizae with depth did not dramatically affect K binding in unlimed probes.

The limed probes were most variable in the 0-10 horizon. In the dry fall 1999, K content was higher in the 0-10 than the 50-60 horizon but in spring and fall 2000 the trend reversed. Liming and moisture changes therefore affected K content. It is quite likely that the mixture of mycorrhizal species with their individual responses to K and microclimatic parameters in the 0-10 cm horizon may have contributed to the fluctuations in K content to at least in part account for the discrepancies between the unlimed and limed zones. In deeper soil where fewer species were present there were no real differences between the unlimed and limed samples. In the 50-60 cm depth horizon, both the forest zones had improved K retention when the mycorrhizal complements and moisture levels were the highest. But in the drier 30-40 cm zone, even with enhanced mycorrhizal presence, K content did not improve. So it would seem that moisture (or lack of it) played a key role.

F8-3B. The most fluctuation occurred in limed soil 0-10 cm depth. When the mycorrhizal abundance was good in spring 1999, K was lowest, but it rose in the fall 1999 dry spell when fungal abundance dropped to its lowest level. K dropped again when the mycorrhizal abundance was higher in spring 2000 but rose to its highest levels in fall 2000 when mycorrhizal abundance was the greatest. It would seem more likely that changes in the complement of mycorrhizal species present and their relative abilities to take up K had more to do with the fluctuations than the overall abundance of mycorrhizal tips. The actual soil moisture content at least at 0-10 cm depth may indirectly have affected K uptake by selectively altering mycorrhizal diversity. In deeper, more stabilized soil environments, at 30-40 cm, much less fluctuation was evident. At 50-60 cm depth, microclimatic changes and lower mycorrhizal abundance with fewer well adapted species increasing in number may have been responsible for the increase in K retention.

F8-3C. From the demographic study, it was shown that liming promoted a more unstable and dynamic mycorrhizal environment with numerous species but with low individual abundance. The unlimed forest zone with its acidified soil had fewer, more well adapted,

and abundant species in the upper soil horizon. In deeper horizons, the unlimed and limed forest zones were more similar with fewer, but differing, mycorrhizal species. It seems evident that each mycorrhizal species, in response to moisture and other survival parameters such as liming, may vary in storage potential for element K resulting in fluctuations in K content dependent upon the actual species present at any given moment in time. If this was really the case, then it would account for the unusual fluctuations seen in the limed probes at 0-10 cm depth. Where a few well adapted species were present, the primary control factor was soil moisture content which directly affected mycorrhizal abundance and K content. With improved surface area and subsequently the number of potential K bonding sites, more K uptake and sequestration would be possible.

F8-4: Summary - Potassium

The primary controlling factor in bound Potassium content seemed to be moisture. In unlimed soil where there were fewer mycorrhizae, improvement in moisture resulted in increased K content. In deep soil where few select mycorrhizal species existed, moisture which improved general abundance also improved K content. In limed soil numerous mycorrhizal species co-existed in close competition on the shallow roots and these were more directly affected by varying moisture levels. The resulting seasonal changes in the complement of mycorrhizal species with some disappearing and others reappearing may have been primarily responsible for the unusual fluctuations in K content seen at 0-10 cm depth. Temporal soil acidification, actual soil pH and actual Ca content were less important than mycorrhizal diversity and soil moisture.

F9: BOUND SODIUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-13 to F3-15)

F9-1. Moisture Responses - Sodium

F9-1A. Sodium content did not exhibit traditional seasonal oscillations in either of the forest zones at 0-10 cm but did have modest associations at 30-40 and 50-60 cm depth.

F9-1B. Na content improved with moisture accumulation at all depths for both regions.

F9-1C. In spring 1999 there was no significant difference between 0-10 and 50-60 cm depth in either moisture or Na content nor between the two forest zones. During the dry

fall 1999, the limed probes contained more Na at 0-10 cm depth and less in deeper soil. In contrast, the unlimed probes had much less Na at 0-10 cm depth and significantly more in the deeper soil. In spring and fall 2000 both zones had more Na in the upper horizon and less in the lower horizons with Na content improving as moisture levels rose.

F9-2. Acidification Responses - Sodium

F9-2A. Na content rose in conjunction with temporal acidification in both zones at all depths but due to other factors discussed below, it is more likely that moisture changes were the primary control factors.

F9-2B. Changes in pH relative to soil depth did not have an impact upon Na uptake. Despite the differences in pH between the limed and unlimed regions, there was often no significant difference in Na uptake. The temporal increase seen in Na content then is most likely not exclusively related to acidification.

F9-2C. Liming seemed to impact Na content only during the spring 1999 at 30-40 cm depth, in the fall 1999 dry spell and in the very wet fall 2000. Otherwise Na content was relatively low with an average variance of between 162 and 426 mg/kg and a Ca:Na ratio of 17:1 in unlimed soil and 27:1 in limed soil. Over all it can be stated that Ca presence affected Na content more so than actual pH.

F9-3. Mycorrhizal Responses - Sodium

F9-3A. The fact that Na content generally declined with depth implies that mycorrhizal action was involved but given the very low concentrations present it is likely that mycorrhizal exclusion may also have played a factor. In the very dry fall species loss and changes in the diversity may have contributed to the differences between the zones.

F9-3B. As seasonal mycorrhizal abundance increased, so did Na content at all depths.

F9-3C. Improved moisture and improved mycorrhizal abundance were directly correlated to Na content. Liming played an important role during drought stress most likely through

changes in mycorrhizal diversity rather than directly due to Ca content of the mycorrhizal roots. Of all the factors soil pH was probably the least significant.

F9-4: Summary- Sodium

The primary control over Na content was soil moisture with liming acting as a secondary factor that expressed itself in very dry and very wet periods through changes in mycorrhizal diversity. Na uptake was not as closely related to pH variations. Although Na and K are well known base exchangers, no inverse patterns were evident. Both were strongly responsive to moisture, but Na content (averaging 200 to 400 mg/kg) was very low in comparison to K content (averaging 1000-2500 mg/kg) with and average K:Na ratio of 5.5:1. Sodium probably played a lesser role in the mycorrhizal root metabolism and in view of the low predrought status, may even have been actively excluded.

F10: BOUND IRON (Appendices 10A, 10B, 10C, 10D; Graphs F3-16 to F3-18)

F10-1. Moisture Responses - Iron

F10-1A. Iron lacked distinct seasonal oscillation patterns at 0-10 cm depth but exhibited weak seasonal oscillations at 30-40 and 50-60 cm depth for both forest regions with slightly more Fe in the fall probes.

F10-1B. For both forest zones, at 0-10 cm depth iron increased content from the dry fall 1999 to the wet fall 2000, but in deeper soil there was no significant difference between the two seasons despite improved moisture content. The moisture content of the soil therefore was not a primary controlling factor.

F10-1C. For both forest zones, the mycorrhizal roots dramatically dropped in Fe content with depth despite the high moisture content of the soil at 50-60 cm depth. Iron concentrations in the roots is not primarily responsive to soil moisture.

F10-2. Acidification Responses - Iron

F10-2A. With temporal acidification at 0-10 cm depth, iron content increased but the increases at 30-40 and 50-60 cm depths were minimal and well within the range of demonstrated seasonal oscillation.

F10-2B. At pH 3.79 to 3.53, less Fe was found bound to the unlimed mycorrhizal roots than in the limed soil probes at pH 4.47 to 4.24 but in both cases more Fe became bound as the soil acidified. In deeper unlimed soils where the pH exceeded 4.48, the lowest Fe contents were recorded. According to Ulrich (1989) the Fe/Al buffer range is 2.8 to 3.8. At these pH levels, Fe becomes freed into solution to potentially enter the rhizosphere. In theory, more Fe was present and should have been bound by the unlimed roots. It is puzzling that this was not the case unless the presence of low pH compromises the cell wall structure to allow free passage to the xylem for translocation, or alternately the damaged root are somehow incorporating active exclusion (not likely). Jumping ahead a little to the unbound Fe content (Section G), it was discovered that in fact, in unlimed roots where less Fe is sequestered, more Fe was unbound or free in the cytosol while in the limed roots, at a higher pH, better sequestration occurred and less Fe was unbound in the cytosol. Hence acidification can increase internal, but distal, Fe root stress.

In the deeper soils, at even higher pH, the Fe may simply have not been easily solubilized or available for root uptake. These roots contained about 25% less Fe. Even so, very small drops in pH which occurred each fall were associated with very small rises in Fe content of the mycorrhizal roots. For example: At 50-60 cm depth the pH H_2O levels dropped from 4.9 (spring 1999) to 4.68 (fall 1999) and from 4.71 (spring 2000) to 4.6 (fall 2000) and these small seasonal drops were directly associated with small increment rises in Fe content in the limed probes (Graph 3-18).

F10-2C. In reviewing the Ca:Fe ratios, it was found that there was no overall significant difference between the unlimed and limed probes. The average ratio for unlimed probes was 1.5:1 and for limed probes 1.8:1. Reviewing the bound iron content however it was evident that there was a significant difference in many of the comparative probes with the limed samples consistently containing more Ca and Fe. Ca seemed to improve Fe binding.

F10-3. Mycorrhizal Responses - Iron

F10-3A. Iron content was high where mycorrhizal abundance was high at 0-10 cm depth, and low where mycorrhizal roots were rarer in deeper soils.

F10-3B. In the very dry fall 1999 when the mycorrhizal abundance was the lowest, Fe content declined only in the unlimed roots at 0-10 cm depth and not the limed, and in deeper soils the Fe content actually rose in both forest zones. The roots from the limed probes had the most significant increases in Fe content despite comparable Ca content and soil pH between the forest zones in deeper soil. This leaves the distinct possibility that specific fall species of mycorrhizae may have been responsible for selective Fe binding. Additional work would need to be done to determine which species are preferential sequesters of Fe of those species now known to be present at those soil depths (See: Section B). In the wet fall 2000, when the mycorrhizal abundance was at its highest, at 0-10 cm depth, Fe binding was also at its highest in both zones and as will be seen in the next section, free Fe was the lowest. But in deeper soils, the bound Fe concentrations were not significantly different from the previous, dry fall. Most likely, very little Fe was available to be bound at this depth due to the higher soil pH.

F10-3C. Limed mycorrhizal roots sequestered more Fe than unlimed at all depths and seasons but the actual Fe binding was directly and intimately associated the small increment changes in pH into the more acidic range.

F10-4: Summary - Iron

The primary control of Fe in the soil was pH mediated solubilization. Based upon the sensitivity of the deep root uptake to small increment changes in pH it can be said that the free Fe was most likely immediately sequestered by the mycorrhizal roots. With increasing acidification and greater mycorrhizal abundance, more Fe was bound. The limed mycorrhizal roots were more effective at sequestering the iron than unlimed roots in more acid soils. Soil moisture content was not relevant.

F11: BOUND MANGANESE (Appendices 10A, 10B, 10C, 10D; Graphs F3-19 to 21)

F11-1. Moisture Responses - Manganese

F11-1A. Manganese content was not obviously associated with seasonal oscillations.

F11-1B. In 0-10 cm depth soil, the limed probes had only a tiny increase in Mn over time. The unlimed probes had a definite drop in Mg content in the dry fall 1999 but a

rapid return to pre-drought levels which was maintained through the wet fall 2000 with no significant change. In deeper soil, Mn was lost as soil moisture improved over time with the wet fall 2000 having the lowest Mn content for both forest zones.

F11-1C. In the spring 1999 there was no significant difference in the Mn content of the limed roots regardless of the soil depth, pH or moisture content. In the dry fall 1999, the limed probes lost Mn as depth increased, but conversely, the unlimed probes increased in Mn content as depth increased. Liming clearly affected mycorrhizal functioning during the dry period. During the subsequent recovery, for the most part there was no significant difference between the unlimed and limed probes with respect to Mn content. In both zones, Mn content declined with depth regardless of moisture content. Other than the dry spell, changes in moisture content do not significantly affect Mn concentrations.

F11-2. Acidification Responses - Manganese

F11-2A. With increasing temporal acidification at 0-10 cm depth there was only a tiny increase in Mn in the limed probes and no effective change in the unlimed. In deeper soils, both regions lost Mn over time.

F11-2B. At first it appeared that there was a lower Mn bonding in the unlimed probes and a greater sequestration in the limed but statistic analysis showed that the only real differences occurred during the fall 1999 dry spell at 0-10 and 50-60 cm depths. Part of the explanation for the perceived differences may lie in the fact that we were dealing with averages of already very low concentrations with overlapping standard deviations.

F11-2C. Liming did not significantly affect Mn sequestration except during the drought period when it functioned to maintain Mn content at the levels consistent with pre and post drought concentrations. The unlimed probes however suffered a Mn loss during the dry spell especially in the critical growing zone at 0-10 cm depth.

F11-3. Mycorrhizal Responses - Manganese

F11-3A. Manganese concentrations declined slightly with depth in the limed forest zone from a maximum of 660 to a minimum of 251 mg/kg. In unlimed forest zone there was

narrower range of variation with a maximum of 566 to minimum of 282 mg/kg. The drop in Mn content was loosely tied to a loss in mycorrhizal abundance with depth.

F11-3B. In 0-10 cm depth soil, the limed probes maintained Mn content with only a slight rise from 622 to 660 mg/kg from fall 1999 to fall 2000. The rise in Mn content in the unlimed probes was greater (300 to 480) during the same time period. However, despite an increase in mycorrhizal abundance at 30-40 and 50-60 cm depths, from fall 1999 to fall 2000, both the unlimed and limed probes lost Mn. Mycorrhizal abundance alone was not enough to maintain Mn content. It is likely that in the wet fall 2000, either the deeper horizons experienced a Mn leaching effect, or the deep soil species present in the fall had lower Mn retention ability.

F11-3C. Liming may have influenced mycorrhizal diversity and abundance but overall did not affect Mn retention except perhaps in the dry fall 1999 when the limed probes had more effective maintenance.

F11-4: Summary - Manganese

Manganese was definitely a micronutrient. At 0-10 cm depth the mycorrhizae seemed to be able to maintain minimum concentrations of Mn with liming providing a slight edge over the unlimed probes. As mycorrhizae were lost with depth, Mn was also lost. Temporal soil acidification and increasing moisture were detrimental in deeper soil but had no impact in the 0-10 horizon.

F12: BOUND PHOSPHATE (Appendices 10A, 10B, 10C, Graphs F3-22 to 24)

F12-1. Moisture Responses - Phosphate

F12-1A. Seasonal oscillation patterns were not evident.

F12-1B. At 0-10 and 50-60 cm depths, more P was present in the wet fall 2000 than the preceding dry fall 1999 for both zones. Phosphate binding was related to improved soil moisture content over time

F12-1C. Average P content was highest (647-975 mg/kg) in the 0-10 cm horizon; while the P levels were lower at 30-40 cm depth (541-785 mg/kg) and at 50-60 cm depth (436-693 mg/kg). The difference in P content between the 0-10 and 50-60 cm depths concentrations indicated that soil moisture level was of secondary importance to the drop in mycorrhizal abundance. During the dry spell in fall 1999, despite a large loss in mycorrhizal abundance the P concentrations dropped only modestly. Considering the importance of P to cell metabolism it is not unexpected that it would be protected from wild changes in moisture fluctuations but still somewhat subject to long term vacillation.

F12-2. Acidification Responses- Phosphate

F12-2A. Temporal acidification resulted in a small increase in P content in both unlimed and limed probes but only at 0-10 cm depth.

F12-2B. Unlimed acidified probes retained more P than the limed probes at most depths and seasons. The limed probes from 30-40 and 50-60 cm depths in the spring of 1999 were an exception. In addition, in the very moist fall 2000 there was no significant difference between the unlimed and limed probes at 0-10 cm depth. In more alkaline pH, generally less Phosphate was present in the mycorrhizal roots. For example: As the pH dropped from 3.79 to 3.53, P content rose from 800 to 1000 mg/kg in the unlimed probes. As the pH dropped from 4.47 to 4.24, P content rose from 650 to 950 mg/kg in the limed probes At pH 4.5 the P content averaged 620 mg/kg in unlimed probes at 30-40 cm depth. At pH 4.7 the P content averaged 613 mg/kg in limed probes at 30-40 cm depth. With increasing (more alkaline) pH, P content declined.

F12-2C. The unlimed probes generally contained more P than the limed.

F12-3. Mycorrhizal Responses- Phosphate

F12-3A. Phosphate declined with depth but could not be related to the mycorrhizal loss.

12-3B. At 0-10 cm depth, P content increased with mycorrhizal abundance but there was no similar trend at 30-40 or 50-60 cm depth. Phosphate content could not be exclusively related to mycorrhizal abundance.

F12-3C. Liming reduced P content at 0-10 cm, but in deeper soils where the Ca was higher in some instances than in the unlimed probes, the relationship did not persist.

F12-4: Summary- Phosphate

Phosphate content in the mycorrhizal roots was most directly related to small changes in the soil pH with less P being bound as the pH becomes more alkaline. Abundance of mycorrhizal species, Ca and soil moisture were less important than soil acidification. The Phosphate content of the roots seems to be tightly maintained even during drought and other seasonal and biotic fluctuations.

F13: BOUND SULFUR (Appendices 10A, 10B, 10C, 10D; Graphs F3-25-27)

F13-1. Moisture Responses - Sulfur

F13-1A. Distinct seasonal oscillations were not evident.

F13-1B. At 0-10 cm depth there was a significant 33% accumulation of S in the unlimed mycorrhizal roots from the dry fall 1999 to the wet fall 2000 but only a 9% change in the limed roots. At similarly wet 50-60 cm depth during the same time interval there was a 20 % increase in the unlimed and 27% in the limed roots and no significant difference between the two in total S content. In the dry soil at 30-40 cm depth the unlimed roots had a 16% S loss while the limed roots gained 15% and while there was no significant difference between the forest zones in the wet season, there was during the dry. Soil moisture increases from the dry to wet fall affected S content in both zones with the most distinct change occurring in the unlimed probes at 0-10 cm depth.

F13-1C. Compared to the deeper soils, the 0-10 cm depth the S content was persistently higher (above 1100 mg/kg) in both zones. Despite similar moisture content between the 0-10 and 50-60 cm depths, the Sulfur levels were significantly different. Lack of mycorrhizal roots in the deeper soil had a greater impact than soil moisture content.

F13-2. Acidification Responses- Sulfur

F13-2A. With temporal acidification, S content increased in both soil zones with the greatest increase occurring in the unlimed probes at 0-10 cm depth.

F13-2B. In more acidic pH (3.79 to 3.53) at 0-10 cm depth in unlimed roots, more S was present than in mycorrhizal roots from limed soils (pH 4.47 to 4.24). In deeper soils (above pH 4.48) however the patterns of S accumulations did not follow the empirical moment to moment changes in pH. Other than spring 1999, there was no significant difference between the unlimed and limed zones in deeper soils. The pH alone was not a contributing factor but slow long term acidification was.

F13-2C. Liming seemed to stabilize S content in the 0-10 cm depth horizon even in the dry spell in fall 1999. The effects of liming were lost in deeper horizons where the actual Ca content of the unlimed and limed mycorrhizal roots were very similar.

F13-3. Mycorrhizal Responses- Sulfur

F13-3A. Sulfur content decreased with depth and the concurrent loss of mycorrhizae.

F13-3B. When the mycorrhizal abundance increased, so did the S, but the dramatic loss of mycorrhizae in the dry fall 1999 only had a minor impact on the total S content.

F13-3C. The effects of liming upon the mycorrhizal sequestration of S are most evident in the 0-10 cm horizon. In the dry fall the limed mycorrhizal root S content was higher, but in more acidified and wetter soils it was lower than that of the unlimed mycorrhizal roots which were steadily climbing in S. The overall ratio of Ca:S in the unlimed zone was 2.8:1 and 5.6:1 in the limed. Liming overall reduced S content.

F13-4: Summary- Sulfur

At 0-10 cm depth, liming seemed to stabilize S content even in the dry fall 1999, but the S content of the unlimed roots rose steadily in stronger response to enhanced moisture, acidification and mycorrhizal abundance. Liming overall reduced S content. In deeper soils, except for the anomalies in spring 1999, there was no significant difference between the unlimed and limed soils in S content. In deeper soil the greatest control factor seems to have been mycorrhizal abundance followed by moisture and weak responses to pH.

F14: BOUND ZINC (Appendices 10A, 10B, 10C, 10D; Graphs F3-28 to F3-30)

F14-1. Moisture Responses - Zinc

F14-1A. Seasonal oscillations were seen at 0-10 cm and 50-60 cm depths, but only in the limed probes. In these cases Zn was more abundant in the spring than the fall varying between 100-400 mg/kg. Seasonal oscillation was not seen in the unlimed probes. At 0-10 cm depth the Zn content rose steadily form 75 to 148 mg/kg but in deeper soils the content steadily dropped from a maximum of 244 to 104 mg/kg.

F14-1B. At 0-10 cm depth, both zones had increased Zn content from the dry fall 1999 to the wet fall 2000 in conjunction with moister soil (N: 30%, L 17%). In deeper soils however, both lost Zn despite an improvement in soil moisture (N: 9%, L: 18% at 30-40 cm) and (N: 28%, L: 13% at 50-60 cm). Seasonal soil moisture alone could not account for the Zn content.

F14-1C. Although varying with season, the soil moisture at 0-10 and 50-60 cm depth was roughly equivalent with the middle zone consistently having lower moisture levels. The pattern of Zn accumulation could not be matched. Soil moisture content was not a primary controlling factor.

F14-2. Acidification Responses- Zinc

F14-2A. With temporal acidification, the unlimed probes at 0-10 cm depth in more acidic soil tended to accumulate Zn, but in deeper more alkaline soils, they tended to lose the Zn despite continued acidification. The limed probes followed roughly the same pattern in the fall but not the spring.

F14-2B. At 0-10 cm depth, differences in pH had an impact only in the spring with the unlimed acidic probes having significantly less Zn. In the fall there were no significant differences between the forest zones despite continued pH distinctions. This would imply that succession in seasonal mycorrhizal species was more important than either moisture or pH. (Some spring species may be better at Zn sequestration, or conversely some fall species may be more adept at exclusion, especially in the limed forest zone.) At 30-40 and 50-60 cm depths in more alkaline pH (>4.48), variations in Zn content could not be

related to moment to moment empirical pH changes. In unlimed forest probes, more Zn was associated with roots from more alkaline soils. In general, more Zn was present in more alkaline soil probes but the precise uptake was most likely mycorrhizal species mediated.

F14-2C. In unlimed soil probes, the overall Ca:Zn ratio was 36:1 and in limed probes it was 51:1. Zinc was clearly a minor component. Because of the intense spring sequestration, the limed probes contained more Zn than the unlimed. For the limed probes, at 0-10 and 30-40 cm depths, the Zn uptake pattern was very similar to that of Ca, and likewise for 50-60 cm depth with one exception (Spring 2000). For the unlimed probes the Zn uptake patterns for 30-40 and 50-60 cm depth were comparable to Ca but not at 0-10 cm depth. Calcium seemed to have a direct influence upon Zn sequestration in all but the most acidic soils.

F14-3. Mycorrhizal Responses- Zinc

F14-3A. Zn content sometimes rose (1999), sometimes fell (2000) with depth in both the unlimed and limed probes despite a consistent drop in mycorrhizal abundance with depth. In drier deeper soils, the mycorrhizae seem less able to exclude Zn.

F14-3B. Mycorrhizae tend to inhibit Zn uptake. This was especially evident in deeper soils. The trend was less obvious in shallow soil, but overall, despite the greater mycorrhizal abundance in 0-10 cm depth, the Zn content was relatively lower than in the deeper horizons. In the limed probes, in the spring, Zn content was higher than in the fall at 0-10 cm depth and the drier spring probes had a higher Zn content than the wetter spring probes. The mycorrhizal abilities therefore were linked to varying seasonal species diversity (succession). With improved soil moisture content they were more able to exclude Zn..

F14-3C. Calcium content seemed to have direct influence upon Zn sequestration in all but the most acidic soils. Despite the greater abundance of mycorrhizae at 0-10 cm depth, the Zn uptake was limited and in most cases relatively low. In limed probes, the spring mycorrhizal species had the least capacity to eliminate Zn.

F14-4: Summary- Zinc

The primary control of Zn limitation was mycorrhizal activity. This activity was impaired by liming, enhanced Ca content, higher pH and drier soils. In limed probes, the spring species were less effective in inhibiting Zn binding than the fall species. There was no similar distinction in the less diverse unlimed samples.

F15: BOUND CADMIUM (Appendices 10A, 10B, 10C, 10D; Graphs F3-31 to F3-33)

F15-1. Moisture Responses - Cadmium

F15-1A. Seasonal oscillations were most evident in the unlimed probes but were not consistent. In the 0-10 cm depth Cd was most evident in the spring while in deeper probes Cd was more abundant in the fall. The limed probes lacked obvious seasonal oscillation.

F15-1B. In comparing the dry to the wet fall, in the limed probes at every depth more Cd was present in the wet fall. In the unlimed probes, in the very acidic soils at 0-10 cm depth, more Cd was present in the wet fall but in deeper, less acidic soils, more Cd was present in the dry fall.

F15-1C. With respect to soil depth, despite the fact that the 0-10 and 50-60 cm zones had similar water content, the total Cd was considerably less in the 0-10 cm depth implying better mycorrhizal exclusion.

F15-2. Acidification Responses- Cadmium

F15-2A. With temporal acidification, Cd increased at all depths in the limed soils. In the unlimed soils, Cd content increased only at 0-10 cm depth. In reviewing the following data, this increase may have been more likely related to improved moisture and mycorrhizal abundance than acidification effects.

F15-2B. At all depths, the Cd content of the unlimed probes was consistently less than that of the limed probes except for the dry fall 1999 at 30-40 and 50-60 cm depths. Since the pH of the unlimed probes in deeper soil was often higher than that of the limed probes, pH alone cannot be a primary control factor. Empirical pH readings could not be tied

directly to Cd concentrations. But very generally, in deeper soils with higher pH levels (>4.48), up to three times more Cd was stored than in the more acidic upper soil horizons.

F15-2C. The Calcium:Cadmium ratios were very large. For the unlimed probes Ca:Cd was 6343:1 with an average concentration of 0.63 mg/kg at 0-10 cm depth, and for the limed probes the ratio was 8983:1 with an average concentration of 0.93 mg/kg at 0-10 cm depth. Ca improved Cd binding. In deeper soil where the liming effects were lost, there was frequently no significant difference in the average Cd concentrations between the forest zones. However, except for the dry fall 1999 in deeper soil, the limed probes generally accumulated more Cd than the unlimed. Overall liming improved Cd retention.

F15-3. Mycorrhizal Responses- Cadmium

F15-3A. As mycorrhizal abundance declined with depth, Cd content rose for both forest zones. The unlimed probes seemed better at excluding Cd and the limed seemed better at binding it. Since this element was found in such low concentrations and as such an unlikely candidate for toxicity, it was not selected for comparative study with respect to the unbound component. As a result it is not possible to confirm whether the ion was being excluded or bound. But regardless of the mode of control, at 0-10 cm depth the mycorrhizal species present were successfully able to limit Cd presence in the roots. In deeper soil, where fewer mycorrhizal tips were present, more Cd was in evidence.

F15-3B. With increases in the mycorrhizal abundance from fall 1999 to fall 2000 at 0-10 cm depth, Cd content likewise increased. In deeper soils the Cd content increased in the limed probes but decreased in the unlimed probes. This dichotomy may be directly related to differential species diversity induced by liming since all other factors were consistent.

F15-3C. The primary role of mycorrhizae seemed to be to exclude Cd. The unlimed mycorrhizal roots seem to be better at this while the limed roots seem to have better binding powers. In both cases the mycorrhizal actions were improved by moisture and conversely damaged by drought. Where fewer mycorrhizae were present, pH played a larger role with more Cd present when the soil pH is greater than 4.48.

F15-4: Summary- Cadmium

Cadmium was present only in extremely low concentrations. In the heavily mycorrhizal forest soils it is primarily excluded from the roots but significantly increases in concentration in deeper more alkaline soils where fewer mycorrhizae are present. More Cd was present in the limed probes and with improved soil moisture. Variations in Cd uptake were related to mycorrhizal species diversity induced by liming.

F16: BOUND LEAD (Appendices 10A, 10B, 10C, 10D; Graphs F3-34 to F3-36)

F16-1. Moisture Responses - Lead

F16-1A. Seasonal oscillation was seen at 30-40 cm depth in the unlimed probes and at 50-60 cm depth in the limed probes. But in both cases, the Pb content was very low.

F16-1B. In unlimed probes, Pb content increased from the dry fall 1999 to the wet fall 2000 greatly at 0-10 cm depth and only slightly in deep probes. In the limed probes, Pb content increased only at 0-10 depth during this same time interval. At least at 0-10 cm depth, Pb content seems to be related to moisture changes.

F16-1C. At 0-10 cm depth, the Pb concentrations were the highest (70-120 mg/kg) and despite similar moisture content, at 50-60 cm depth, the Pb concentrations were extremely low (10-22 mg/kg). Pb uptake was not directly related to soil water content.

F16-2. Acidification Responses - Lead

F16-2A. At 0-10 cm depth, with temporal acidification, Pb content rose in both zones.

F16-2B. With respect to empirical pH, Pb content was highest in the most acidic soils with the greatest presence in the unlimed probes. Where pH exceeded 4.48 the content dropped dramatically. Even so, the Pb pattern could not be matched with moment to moment changes in soil pH at 0-10 cm depth. At 30-40 and 50-60 cm depths in unlimed soils the pH and Pb patterns were fairly close but the narrow range in Pb readings (10-22 PPM) and standard deviation overlaps made the comparison untenable.

F16-2C. In unlimed probes, the Ca:Pb ratio was 48:1 and in the limed probes 86:1, and overall there was 8.2 % less total Pb present in the limed samples at 0-10 cm depth. At 30-40 cm depth the differences were mostly not significant and at 50-60 cm depth there was 3% more Pb in the limed samples. Overall liming did not have a very dramatic effect on Pb uptake.

F16-3. Mycorrhizal Responses - Lead

F16-3A. Lead uptake dramatically declined with depth and lower mycorrhizal abundance in both forest zones.

F16-3B. At 0-10 cm depth, the Pb content increased with mycorrhizal abundance. There was however no significant difference in deeper soils.

F16-3C. At 0-10 cm depth, the limed mycorrhizal roots had somewhat reduced Pb content. In deeper soils, the differences were mostly not significant.

F16-4: Summary - Lead

In 0-10 cm depth soils, mycorrhizal abundance was the primary control factor with respect to Pb presence. Improved moisture and acidification improved Pb content. Liming resulted in better exclusion of Pb. In deeper soils where fewer mycorrhizal species were present, the primary control factor seemed to be pH. The more alkaline the pH, the less Pb present.

F17: BOUND MINERALS GENERAL CONCLUSIONS

The relative abundance of all the minerals in unlimed and limed soils at various depths can be found in Graphs F3-37 to F3-42. In nearly every instance, Aluminum was the most abundant mineral. In 0-10 cm soil depth, Calcium and Iron competed for second place while the other minerals were considerably lower in abundance [Graphs F3-37 (unlimed) & F3-40 (limed)]. At 0-10 cm depth, the Al content in the limed probes (5000 to 13000 ppm) was usually higher than that of the unlimed probes (4500 to 8500 ppm). At 30-40 and 50-60 cm depths, the unlimed and limed roots were more similar than different with Al still number one in abundance, and more prevalent than in Horizon A. In unlimed

roots Al varied from 10000 to 19000 ppm at 30-40 cm depth while limed roots vacillated between 12000 and 19000 ppm. At 50-60 cm depth the unlimed Al varied from 12000 to 19000 ppm and limed Al varied from 12000 to 17500 ppm. The Ca (and Fe) level were low(< 2000 ppm), approaching the concentrations of the other minerals in the deep soils.

According to the numerous observations cited above, Aluminum exclusion or control was primarily monitored by mycorrhizal presence. Where mycorrhizal roots were abundant, less potentially toxic Al was present and where the mycorrhizae were less abundant, more Al was present. Drought played havoc with Al control initially maintaining strongly bound Al at pre-drought levels with no significant difference between the limed and unlimed mycorrhizae. Following the dry spell however Al accumulated as it was freed into solution from the acidic soils. Liming improved binding of this freed acidic Al in Calcium rich mycorrhizal roots and so as a result, the limed mycorrhizal roots at 0-10 cm depth accumulated more Al than the unlimed despite the lower pH levels in the unlimed forest zone. In deeper more alkaline soils, where fewer mycorrhizae were present, the unlimed roots were richer in Calcium approaching or exceeding the probes from the limed forest region. After a drought, as more solubilized acidic Al was released it accumulated in both the unlimed and limed forest regions in the fine roots with no significant difference between the regions. Roughly equivalent Calcium content seemed to be the controlling factor. However with advancing acidification, Aluminum content continued to increase while Ca content steadily dropped in both zones. With time and prolonged acidification a displacement effect seemed to occur where Al replaced Ca.

Calcium uptake was primarily controlled by liming and mycorrhizal abundance with most of the essential Calcium being bound effectively in the 0-10 cm depth and very little filtering down to deeper horizons. As a result, in deeper soil the Calcium content in the unlimed and limed forests was not significantly different. But as soil became increasingly acidified, Ca was lost from the deeper roots. In shallow limed soil the same trend in Ca loss over time was seen but conversely in the unlimed forest at 0-10 cm depth Ca content was improved. The most probable explanation was that the acid-tolerant species present in the unlimed forest zone had better compensatory mechanisms.

Magnesium content improved with liming. Both Ca and Mg had very similar patterns of accumulations implying some similar operating mechanisms but with more gradual changes in Mg content in response to drought, acidification and rehydration. Once bound, Mg seemed to stabilize in mycorrhizal roots but it slowly leached away with prolonged acidification. Mg was consistently higher in the probes from the limed forest soils and less affected by drought. Mg content was lower in the unlimed probes and more affected by drought. The fact that Mg content was lower in the unlimed probes at all depths despite similarities in Ca and pH in deeper soils implies that long term species selection processes may be involved.

Potassium content was primarily controlled by improved moisture levels. In deeper soils where few mycorrhizal species were present, the K content was fairly stable, not declining in drought and with low mycorrhizal numbers, but rising with improved moisture and mycorrhizal abundance. In 0-10 cm depth there was more variation between the limed and unlimed forest zones. The K levels in the limed zone were generally higher but much more flexible most likely due to greater diversity in mycorrhizal species co-existing in close competition on the shallow roots. In unlimed probes at 0-10 cm depth, K content did decline with drought but steadily improved there after. Despite the differences in mycorrhizal abundance between the 0-10 and 50-60 cm horizons, the K content was similar. So although mycorrhizal abundance changes were associated with K changes, soil moisture was the primary controlling factor.

Sodium content was likewise primarily controlled by soil moisture with liming acting as a secondary factor that expressed itself in very dry periods and very wet periods through changes in mycorrhizal diversity that improved Na binding. Na uptake was not closely related to pH variations. Na content was low overall and so probably plays a lesser role in mycorrhizal root metabolism and in view of the low pre-drought status, may even be preferentially excluded in unstressed roots.

Iron content was directly related to pH mediated solubilization. Small increment changes in pH acidification were directly correlated to Fe sequestration by mycorrhizal roots.

With increasing acidification and greater mycorrhizal abundance, more Fe was bound especially at the 0-10 horizon. The Fe concentrations in shallow acid soil may be problematic but in deeper more alkaline horizons very little Fe was incorporated. The limed mycorrhizal roots were more effective at sequestering the iron than the unlimed roots in more acidic soils. Soil moisture content was not relevant.

Manganese is definitely a micronutrient. At the 0-10 cm depth the mycorrhizae seem to be able to maintain minimum concentrations of Mn with liming providing a slight edge over the unlimed probes. In shallow soil, temporal acidification and increasing moisture have no impact upon Mn content. As mycorrhizae are lost with depth, Mn is also lost. In deeper soil, temporal acidification and increasing moisture are detrimental to Mn content.

Phosphate content was directly related to small changes in soil pH with less P being bound as the pH becomes more alkaline. The Phosphate content was very stable with only small changes occurring even during drought or with varying moisture or seasonal biotic fluctuations. Less P was present in the limed mycorrhizal roots than the unlimed and less P was present with increasing depth.

Sulfur content was stable at 0-10 cm depth in the limed probes even in the dry fall 1999, while in the unlimed roots S concentration rose steadily stronger in response to enhanced moisture, acidification and mycorrhizal abundance. Liming overall reduced and stabilized S content in shallow soil. In deeper soils, except for the anomalies in spring 1999, there was no significant difference between the unlimed and limed soils in S content. In deeper soil the greatest control factor seems to have been Calcium content, then mycorrhizal abundance followed by moisture changes and weak responses to pH.

Zinc limitation was primarily controlled by mycorrhizal activity. This activity was impaired by liming, greater Ca content, higher pH and drier soils. In limed probes, the spring species were less effective in inhibiting Zn binding than the fall species. There was no similar seasonal species distinction in the less diverse unlimed samples. Zinc contents were lowest in the unlimed probes.

Cadmium was present only in extremely low concentrations. In the heavily mycorrhizal forest soils it is primarily excluded from the roots but significantly increases in concentration in deeper more alkaline soils where fewer mycorrhizae are present. More Cd was present in the limed probes and with improved soil moisture. Variations in Cd uptake may be related to mycorrhizal species diversity induced by liming.

Lead was primarily present in 0-10 cm depth soils, where mycorrhizal abundance was highest in association with improved moisture and acidification levels. Enhanced Ca content in shallow soils resulted in better exclusion of Pb. In deeper soils where fewer mycorrhizal species are present, the primary control factor seems to be pH. The more alkaline the pH, the less Pb present.

Chart F17-1: Ranking of the factors that had the greatest influence upon bound mineral content followed by the relative overall comparative content of the mineral in unlimed and limed soil at 0-10 cm depth.

	Factor Ranking				Unlimed	Limed
	1	2	3	4	Content	Content
Al	Mycorrhizae	Liming	pH	Moisture	Low	High
Ca	Liming	Mycorrhizae	pH	Moisture	Low	High
Mg	Liming	Mycorrhizae	pH	Moisture	Low	High
K	Moisture	Mycorrhizae	Liming	pH	Low	High
Na	Moisture	Liming	Mycorrhizae	pH	Equal	Equal
Mn	Mycorrhizae	Liming	pH	Moisture	Low	High
P	pH	Liming	Mycorrhizae	Moisture	High	Low
S	Liming	Mycorrhizae	Moisture	pH	High	Low
Zn	Mycorrhizae	Liming	pH	Moisture	Low	High
Fe	pH	Mycorrhizae	Liming	Moisture	Low	High
Cd	Mycorrhizae	pH	Liming	Moisture	Low	High
Pb	Mycorrhizae	pH	Liming	Moisture	High	Low

F18: Bound Mineral Content of Individual Mycorrhizal species.

(Appendix 10E, Graphs F3-43 to F3-53)

The four most abundant ectomycorrhizal species in spring and fall 2000 (*Cenococcum geophilum, Lactarius subdulcis, Piceirhiza chordata* and *Quercirhiza fibulocystidiata*), isolated from 0-10 cm depth from both the unlimed and limed plots, were examined for Ca and Al content (Graphs F3-43 to F3-50). *Piceirhiza chordata* (limed plot) and *Cenococcum geophilum* (limed & unlimed plots) were abundant enough at 30-40 cm depth to be tested for Ca and Al content in spring and fall 2000 (Graphs F3-51 to F3-53). At 50-60 cm depths however even fewer samples were available. *Cenococcum geophilum* (unlimed soil only, spring and fall 2000) and *Piceirhiza chordata* (limed soil only, fall 2000) could be tested for Ca and Al content (Graphs F3-51 & F3-53). Of all the mycorrhizae, only *Cenococcum geophilum* was abundant enough to be examined for additional minerals (Fe, K), at nearly all depths, in the wet spring and wet fall 2000 (Graphs F3-51 & F3-52). There was insufficient *Cenococcum geophilum* at 50-60 cm depth in the *limed* soils to run comparative analysis. This dramatically underscores the previously mentioned shift of root biomass and the majority of mycorrhizal species to the upper soil horizons in *limed* soils (Section B3). In this discussion, the mineral (Al, Ca, Fe, K) content of mycorrhizal tips will be compared to that of the whole fine roots followed by a discussion of intraspecies variation for each of the four species examined.

F18-1A: Aluminum.

Four individual mycorhizal species (*Cenococcum geophilum, Lactarius subdulcis, Piceirhiza chordata* and *Quercirhiza fibulocystidiata*), isolated from 0-10 cm depth, from unlimed and limed plots in spring and fall 2000, were examined for Al content. When compared to the whole unlimed (Graph F3-37) and limed (Graph F3-40) fine roots from the same seasons, it was determined that these mycorrhizal tips (Graphs F3-47 to F3-50) contained the majority of the Aluminum (Figure 18-1). It was further observed, that with the exception of *Piceirhiza chordata,* in fall 2000 (Graph F3-49), less Al was held in the mycorrhizal tips from the *unlimed* probes than in similar seasonal samples from the *limed* probes (Graphs F3-47 to F3-50).

In the **limed** probes, at 0-10 cm depth from spring 2000 to fall 2000, the Al content in the *long* fine roots (< 1 mm diameter, tips removed) ranged from 6180 to 16600 ppm and averaged 11072 ppm (Appendix 10A-2) while the mycorrhizal tips (from all 4 species) ranged from 3740 to 16360 ppm, averaging 9950 ppm (Graphs F3-47- F3-50). If we estimate that the total average root aluminum content (long roots + tips) was approximately 21000 ppm, then approximately 48 % of the Al was in the mycorrhizal tips (<<10mm) while 52 % was found in more distal (<10 to 100 mm) fine root regions. If Al were evenly distributed, one would expect the Al content of the tips to be only 1-10 % of that of the total fine root, based upon the length ratios. Considering that the whole fine roots (< 1 mm diameter) averaged ≤ 100 mm in length and the mycorrhizal tips averaged ≤ 1 mm in length (i.e. *Cenococcum geophilum, Quercirhiza fibulocystidiata*) to ≤ 10 mm (i.e. *Lactarius subdulcis, Piceirhzia chordata*), one can see that the majority of the Aluminum is present in the very tips of the mycorrhizal roots.

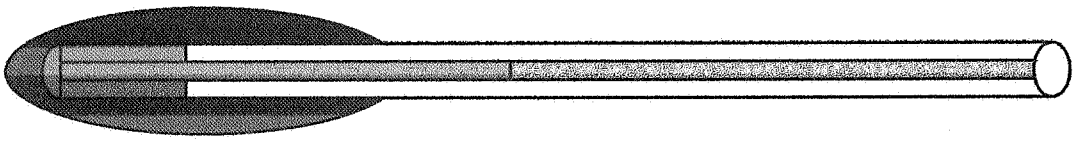
In the **unlimed** probes, at 0-10 cm depth from spring 2000 to fall 2000, the Al content in the long fine roots ranged from 2350 to 15300 ppm and averaged 7893 ppm (Appendix 10A-1) while the mycorrhizal tips (from the 4 species), Al ranged from 1830 to 17770 ppm and averaged 8464 ppm (Graphs F3-48 to F3-50). If we estimate that the total root Al content (long roots + tips) averaged approximately 16400 ppm, then approximately 52% of the Al was in the mycorrhizal tips while 48% was found in the more distal fine root regions. The distribution pattern was the same in both limed and unlimed roots with the majority of the bound Al found in the mycorrhizal tips of the long fine roots.

In **comparing limed to unlimed** probes at 0-10 cm depth from spring 2000 to fall 2000, the ratio of *total* bound Al in the limed roots (L) to total bound Al in the unlimed roots (N) averaged 21000 ppm (L):16400 ppm (N) or **1.3:1**. The ratio of bound Al in the long roots (minus tips) averaged 11000 ppm (L): 7900 ppm (N) or **1.2:1**. The ratio of bound Al in the mycorrhizal tips averaged 10000 ppm (L): 8500 ppm (N) or **1.4:1**. In all three cases, although the distribution patterns were similar, there was consistently and significantly more bound Aluminum found in the limed roots than the unlimed.

From the fluorescence work (Section E), we know that most of the Al in the distal (non-mycorrhizal portion) fine roots was located in the xylem walls. At the mycorrhizal tips, the majority of Al was located either in the fungal Hartig net *or* in the root cortex but the location was dependent primarily upon the ectomycorrhizal species (Section E). For example, acccording to the fluorescence study, the mantle and Hartig net of *Cenococcum geophilum* retained negligible amounts of Al, but the adjacent root cortex fluoresced brightly with Morin implying the presence of large amounts of bound Al, especially in the limed probes (Appendix 12-5) demonstrating lack of fungal Al-sequestration (Figure F18-1B). In contrast, *Lactarius subdulcis* had fainter Morin reactions than *Cenococcum,* but contained some Al in the mantle and Hartig net with less Al generally present in the adjacent root cortex in both the unlimed and limed samples (Appendix 12-25) demonstrating fungal Al-sequestration (Figure F18-1A). In both fungal species, according to the fluorescence studies, declining amounts of Al were found bound (primarily in the xylem) in the distal fine roots >1 to 5 mm from the tip. These qualitative results correspond directly to the quantitative results of the bound mineral analysis (Graphs F3-47 & F3-48) and will be discussed in more detail (Sections F18-2 to F18-5).

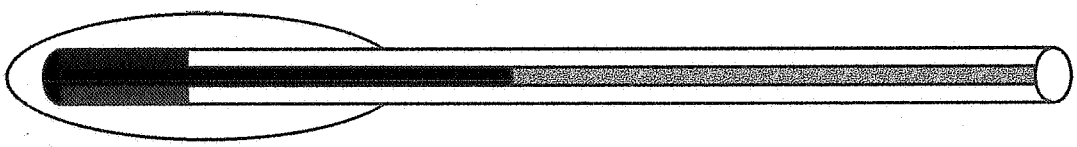
Figure F18-1: Al sequestration in mycorrhizal and non-mycorhizal fine root area

A. *Lactarius subdulcis* : Al sequestration (dark gray) in mantle with lower Al accumulation in cortex (lighter gray) and lowest content (lightest gray) in the xylem.



← Mycorrhiza & Root tip →← Non-mycorrhizal Fine Root (≤ 1 mm diameter) →

B. *Cenococcum geophilum*: Lack of Al sequestration in mantle, but increased Al accumulation in the adjacent root cortex (dark gray) and xylem (black to light gray).



← Mycorrhiza & Root tip →← Non-mycorrhizal Fine Root (≤ 1 mm diameter) →

F18-1B: Calcium

Over 2 years, the seasonal Ca content of the whole unlimed roots fluctuated between 2500 and 4500 ppm, averaging 3700 ppm (Graph F3-37), while in limed roots it generally declined from 10000 to 5000 ppm, averaging 6500 ppm (Graph F3-40). While the Al content increased 200% (unlimed) to 300% (limed) over 2 years, the Ca content declined by 37% (unlimed) to 50% (limed) following the short 1999 drought, followed by a gradual recovery only in shallow unlimed soils but no recovery in deeper soils or in the limed plot. Despite the lack of recovery to pre-drought Ca levels in the limed probes, the declining Ca concentrations remained higher than in the unlimed probes. Before the drought the Ca:Al ratios were < 1 in only the unlimed soils, after the drought they were < 1 in both zones.

More specifically, in the unlimed mycorrhizal species isolated, the Ca content ranged from 2120 to 7250 ppm with an average of 2659 ppm, and the limed mycorrhizal apices ranged from 3330 to 9560 ppm with an average of 6694 ppm (Graphs F3-43 to F3-46). An estimated 2.5 times more Calcium was present in the limed mycorrhizal apices. In the whole roots from the same depth (0-10 cm) and time (spring & fall 2000) the range of Ca content in unlimed roots was 1890 to 5620 (normal high) to 13420 (an aberrant high) ppm with an average of 3798 ppm, excluding the aberrant reading (Appendix 10A-1). Limed roots ranged from 3040 to 9550 with an average of 6070 ppm (Appendix 10A-2). Limed roots contained 1.6 times more Ca than the unlimed. Interestingly, while the whole limed roots lost Ca over time, the limed mycorrhizal tips actually increased bound content. As the Ca resource became limited (declined over time), the mycorrhizae increasingly stored and limited release to the roots. Clearly, Ca was sequestered in the mycorrhizal tips.

While *Cenococcum geophilum* (Graph F3-43) was not significantly different in its ability to bind Ca in the unlimed or limed soils in spring or fall, *Piceirhiza chordata* was dramatically affected (Graph F3-45). In unlimed soils *Piceirhiza* contained 75% less Ca. *Lactarius subdulcis* varied seasonally with 50% less Ca in unlimed tips in spring to no significant differences between the plots in the fall (Graph F3-44). No definitive trend could be established for *Quercirhiza fibulocystidata* (Graph F3-46). Clearly, as with Al, each mycorrhizal species is independent in its physiological acquisition of Calcium.

F18-2: *Cenococcum geophilum*

Cenococcum geophilum is a Class 1 mycorrhiza (Section E) that does not bind Al in the mantle but can sequester negligible amounts in its mature Hartig net region. It allows Al to easily move into the cortex where the Al was found to strongly bond to the cortical walls (Section E2, Appendices 7-5 and 12-5). *From the intensity of the fluorescence color reactions it was established that more Al entered the limed Cenococcum tips than the unlimed at 0-10 cm depth (Appendices 7-5 & 12-5).* In deeper soil probes it was determined fluorescently that Al more strongly accumulated in the limed tips (especially at 50-60 cm depth), but frequently individual unlimed tips were free of *bound* Aluminum. Seasonal accumulation of Al occurred in most cases with augmentation in the fall, especially the dry fall. The *bound aluminum* tended to accumulate in the limed roots where the lime was most abundant and the soil was less acidic (at 0-10 and 30-40 cm) and in the unlimed roots where the soil was less acidic in the deeper horizons (Section C).

Being the single most abundant (Appendix 8A- Graph B3-5) and adaptable species (Section B), *Cenococcum geophilum* was isolated in large enough quantities to run multiple mineral analyses (Graphs F3-51 & F3-52). Comparatively, *Cenococcum geophilum* was most abundant in the *unlimed* probes (Appendix 8A- Graph B3-5). In limed probes the relative abundance increased during the wet spring and fall of 2000 but despite this there was still not enough material to run a comparative analysis for 50-60 cm depths. Analyses were run on all possible samples collected in 2000.

In all the probes, bound Al was single most abundant mineral, followed by either Fe or Ca. Potassium was consistently the lowest in concentration. *While the bound Al content was lowest at 0-10 cm depth in unlimed probes, it was higher in concentration in the deeper soil samples, relative to the limed probes.* Conversely, in all the probes, the secondary minerals Fe, Ca and K were more abundant at 0-10 cm depth, but less abundant in deeper soils. With respect to temporal trends from spring to fall 2000, in the unlimed soil, the bound Al remained consistently low at 0-10 cm depth (it dropped slightly in limed probes

but was still abundant), but in deeper soil the Al content rose in the fall at 30-40 cm depth for both. In the unlimed probes, the Al content rose at 50-60 cm from spring to fall.

The quantitative results confirm the general fluorescent observations. Since there was not enough raw material to run complete samples for all seasons and depths, the dry / wet seasonal fluctuations could not be confirmed, but the trend to accumulation in the fall and in deeper soils was confirmed. The fluorescence observations were much more precise with respect to the exact location of Al.

If we accept the fluorescence results at face value, the lower numbers of *Cenococcum geophilum* in the limed probes, especially at 50-60 cm depths may be directly related to toxic accumulations of Al. Looking closely at the actual concentrations of Aluminum in all the *Cenococcum geophilum* probes examined, the minimum reading was 7660 mg/Kg (unlimed probe 0-10 cm depth) and the maximum reading was 29290 mg/Kg (unlimed probe 50-60 cm depth) (Appendix 10E). In the limed probes the concentrations were intermediate with a minimum of 13270 (0-10 cm depth) and maximum of 19190 (30-40 cm depth) mg/Kg . As the bound Al concentrations increased, the abundance of *Cenococcum* decreased, especially in limed soils.

F18-3*: Piceirhiza chordata*

Piceirhiza chordata was one of the most common ectomycorrhizal species after *Cenococcum geophilum* (Table B3-1) often competing with *Quercirhiza fibulocystidiata* and *Lactarius subdulcis* for second place. It was present in every season and soil depth with the lowest numbers in the dry fall 1999 and at 50-60 cm depth and the highest numbers in the wet fall 2000 (Appendix 8A - Graph 3-36). *Comparatively, it was most common in limed probes, especially at 30-40 cm depth, and in unlimed probes it was most prevalent at 0-10 cm but otherwise low in numbers* (Appendix 8A - Graph 3-36). Typically this mycorrhiza naturally had an evenly brown "old looking" outer mantle so it was difficult to macroscopically determine damage but under the microscope disturbances in the outer mantle of older areas (further from the apex) were more readily visible (Appendix 12- Figures 12-29A to 12-29H).

Piceirhiza chordata was a Class 2/3 ectomycorrhiza which sequestered some Al within the mantle walls and the Hartig net (Section E2- Figure E2-3F-6A) but the degree of bound Al content was dependent upon liming, season and soil depth (Appendices 7-29 and 12-29). Where the mantle sequestration was strongest, the content within the cortex of young roots was strongly reduced. Conversely, if the fungal cells contained little Al, in young or shallow roots, the cortex contained more Al. (Figures 12-29A-H). Upon aging or with depth, *Piceirhiza* developed an orange- or green- fluorescent pigmentation which reduced Al sequestration (Figure 12-29E & 12-29F). Liming affected this ectomycorrhizal resulting in greater general sequestration of Al which fluctuated with season, soil depth and individual root tips. Many tips in both the limed and unlimed samples were Al-free or contained only spotty regions of accumulation in the cortex. These isolated regional accumulations within the root were found mostly in younger growing tips, near the apex, in shallow soils, in the spring and especially the fall of the moist year 2000. In older roots the regional Al accumulations were associated with lateral root emergence points or areas of mantle damage.

At 0-10 cm depth, in *Piceirhiza chordata,* the unlimed tips contained very little Calcium (< 2320 ppm) relative to the limed (> 7920 ppm) tips in spring and fall 2000 (Graphs F3-43 & 45). In the *limed* tips, the Al content (> 7500 ppm) remained constant (Ca:Al = 1:1) (Graphs F3-49 and F3-53), but in the unlimed tips, the Al content varied from extremely low (< 2000 ppm, Ca:Al = 1.16:1) in the spring to extremely high (>17770 ppm, Ca:Al = 0.13:1) in the fall 2000 (Graph F3-49). These seasonal variations in Al correspond to the fluorescence observations (Appendices 7-29 & 12-29) and possible Al-induced damages.

Unfortunately only one *unlimed* probe (0-10 cm depth, spring 2000) had enough material for mineral analysis (Appendix 10E). The remaining observations apply only to probes from the limed soils. Liming improved the abundance of this species. The low numbers of *Piceirhiza* in the unlimed soils may be related to the dramatic fluctuations and sudden aluminum accumulations mentioned in the previous paragraph. Further study may be needed to confirm this but it is logical to assume that any major shock to a living system can contribute to its demise.

In limed probes (Graph F3-53), Ca may have acted to stabilize the physiological functions of this species. In shallow soil with abundant Ca, the Ca:Al ratio was ≥1, which is a healthy indicator, but in deeper soils where the Ca levels were very low, the Ca in the *Piceirhiza* tips was also very low and the Al concentrations became outrageously high, causing the "healthy" Ca:Al ratio of 1:1 to drop below 0.13:1. In the fluorescence study, those ectomycorrhizal tips that developed a green or orange autofluorescent pigmentation in the outer mantles contained less Al. In deeper soils, even with this chemical alteration, the *Piceirhiza* tips were in trouble. The fluorescence work supports the observation that more Al accumulated in roots from deeper soils. Without the lime, or in the increasingly acidic deeper soils, control of Al was lost and blockage was incomplete.

F18: 4: *Lactarius subdulcis*

In comparing the quantitative mineral content (Graph F3-44) of *Lactarius subdulcis* to the qualitative fluorescence (Appendices 7-25 & 12-25) results, it can be stated that they support each other. In both the mineral and fluorescence analysis, the strongest Al accumulation was in the fall 2000. In many cases the Al content of the limed roots was less than that of the unlimed at 0-10 cm depth in the spring, but the total Al abundance was higher in the limed roots at 30-40 cm depth and about equivalent at 50-60 cm depth. The content of Al was highly variable in both the mantle and the root cells in unlimed and limed soils. Like *Piceirhiza chordata, Lactarius subdulcis* deposited orange and green-autofluorescent pigments in the mantle walls with age and with desiccation, but unlike *Piceirhiza*, the chemical modifications could not be related to Al control or blockage. The green (aging/desiccation) chemical modification did however interfere with interpretation of the Morin reactions. Based upon the fluorescent results, the green autofluorescent regions often fluoresced brighter green or yellow-green in the presence of Morin, which was assumed to be due to moderate Al sequestration. (Bright yellow being strong accumulation). But according to the mineral analysis, these areas actually contained strong Al content. Liming probably then affected the chemical constitution of the mycorrhizal roots, altering (increasing) its ability to bind Al.

F18-5: *Quercirhiza fibulocystidiata*

The mineral content of *Quercirhiza fibulocystidiata* in the fall 2000 (Graph F3-46) was comparable to the estimated abundance determined by fluorescent analysis (Appendices 7-35 & 12-35). The unlimed probes tended to develop a green autofluorescence which was at first thought to be associated with declining Al content since, in the presence of Morin, the reactions were greener or yellow-green instead of bright yellow. But, according to the mineral results we must question this assumption. The green autofluorescence that developed may mask the intense Al accumulation that actually occurred in the unlimed roots. Further research is needed to determine the chemical make up of this mysterious green component. Best guess: suberin.

F18-6: Summary - Bound Minerals in Individual Mycorrhizal Species

The primary function of the bound mineral analysis was to confirm the fluorescence observations. The quantitative findings were directly correlated to the Al fluorescence observations (Section E). From the combination of these findings, it can be definitively stated that mycorrhizae do indeed affect mineral uptake. For example, *Cenococcum geophilum* (Class 1) were the most effective when they were the healthiest in general appearance, size and color, in the A horizon. Control of Al translocation was lost in the deeper horizons where the mycorrhizae were less frequent, isolated instead of in large groupings, less healthy in appearance with respect to color (brown spots) and smaller in size (Section B). *Piceirhiza chordata* (Class 2/3) were much more effective in their abilities to sequester or block Al than *Cenococcum* in the A horizon. Effectiveness was lost however with increasing depth, acidification or loss of lime, despite Al-blocking chemical modifications which were common in this species upon maturation. The effects of lime were somewhat beneficial for *Piceirhiza* resulting in a stabilization of mineral content and exclusion abilities, but for *Cenococcum* the liming was more detrimental resulting in a serious decline in species abundance. In general liming increased **BOUND** aluminum content of all the OAK-mycorrhizal species used in the mineral analysis. It can be further stated that acidification and liming both alter the physiological action of the mycorrhizal roots.

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SECTION G: ENIGMA OF UNBOUND MINERALS

INDEX

SECTION G: ENIGMA OF UNBOUND MINERALS

G1: INTRODUCTION

There are significant differences in the uptake of various minerals into the cytosol of fine roots depending upon their forest location (unlimed or limed soil), soil depth, moisture and pH (Section E). The minerals Al, Ca, Mg, K, Na, Mn, P, S, Zn and Fe were extracted from very fine mycorrhizal oak roots isolated from unlimed and limed forest soils from 0-10 cm, 30-40 cm and 50-60 cm depths. The ultimate purpose was to compare the ratios of bound (Section F) to unbound (Section G) minerals to determine if roots from unlimed and limed soils differed in their uptake and sequestration of translocatable minerals (Section H). This section deals solely with the nature of the unbound minerals. Of the greatest interest in this section is the relationship between Aluminum and Calcium as they appear unbound in the root cytosol.

G1-1: Original Hypothesis

If mycorrhizae are the primary site of nutrient uptake, then the concentration of ions should generally decline as the % of mycorrhizal roots declines at increasing depth. It was expected that the concentration of unbound Al and other ions would be higher in the limed soil due to the enhanced uptake of ions in the presence of additional Calcium.

G1-2: Modified Hypothesis

Where the mycorrhizae control nutrient uptake, several modes of action are possible. In shallow soil where the mycorrhizae are abundant, blockage or sequestration of Al ions may reduce the unbound Al content in the cytosol. Opposing effects may be seen in deeper soil regions where the mycorrhizae are less common. Variations in ion presence may occur depending upon complex interactions of soil moisture, pH, depth, seasonal climatic variations, and liming and their effects upon species-specific mycorrhizal physiology.

G2: MATERIALS AND METHODS

G2-1: Root Isolation

After sample collection (Section B), the oak roots were extracted from the soil by sieving, followed by rinsing in cold distilled water to remove the adhering soil particles. The fine root tips (<1 mm diameter) and their associated mycorrhizae were separated from the remaining roots and placed in a 60°C drying chamber for 12 hours (overnight) (Section F). The separated, dry fine mycorrhizal roots were weighed and stored in plastic ziploc bags at room temperature until they were ground up to a fine powder using a porcelain mortar and pestle. A small quantity of liquid nitrogen was added to aid the grinding process. The entire root powder sample for each probe was placed into 2 ml plastic safe-lock Ependorf caplets and stored at room temperature until extraction.

G2-3: Extraction Medium

The extraction medium was made using HPLC water (ROTI sol v HPLC, Roth Inc., Karlsruhe) to which two standard reagents were added. Exactly 400 mg of Digitoxose and 100 mg of Propionic acid were placed into a volumetric flask which was then filled with HPLC water to the one liter mark. The extraction medium can be refrigerated, but it was either freshly made or used within 2 days.

G2-2: Homogenization

The procedures for homogenization and extraction of fine roots were adapted from Kleinschmidt (1993) and Rommel (1998). The dried fine root powders (≤ 100 mg) were transferred to new 2 ml Safe-Seal Micro-centrifuge tubes (BioScience Inc.). The actual amount of dry material was measured very precisely using a Sartorius weighing scale to obtain exactly 100 mg where possible. According to Kleinschmitt (1993), a dry mass between 75 and 100 mg should be used with 1.3 ml of extraction medium and masses under 75 mg need only 0.65 ml of medium while masses under 20 mg in weight were considered insufficient for this type of analysis. As suggested, probes less than 20 mg dry material were not used. Using 1.3 ml per 100 mg of sample provided sufficient extracted fluid for mineral analysis. However, it was found that by using only 0.65 ml of extraction medium for probes ≤ 75 mg, too much liquid medium was lost due to absorption by the

dry root material. For the sake of uniformity in the *Extraction Fluid : Fine root powder* (1.3ml:100mg = 0.013:1) ratios, and to obtain adequate liquid sample, the amount of extraction fluid to be used was calculated by multipying 0.013 ml x the number of mg of root powder. So, for example while 1.3 ml would still be added to a 100 mg sample, 0.975 ml (instead of 0.65ml) of extraction fluid would be added to a 75 mg sample.

G2-4: Extraction Procedures

A small (8 x 3 mm) plastic coated magnetic rod was placed into each micro-centrifuge reaction caplet containing the root powder and extraction medium. The safe-seal cap was securely closed and the reaction tube was not reopened until after the centrifugation process was completed. The labeled caplet was placed on a magnetic stirrer for about 30 seconds to thoroughly mix the contents. Immediately following this the caplet was placed into a plastic holder and set into a water bath resting upright with warm water extending half way up the outside of the tube. The extract was processed for 30 minutes at 80° C with a vibrating frequency of 250 cycles per minute in the vibrating water bath (GFL 1086 manufactured by Labortechnik mbH & Co, Hannover and Burgwedel). Following the warming bath, the reaction tube was placed into a centrifuge (Minifuge GL, Heraeus-Christ, Heraeus Instruments GmbH, Rhein-Main and Osterode) and processed for 30 minutes at 20° C with a rotation speed of 4000 cycles per minute. According to Rommel (1998) at temperatures less than 20° C, the extract becomes viscous and so hinders the next pipetting step in the extraction process.

G2-5: Deproteinization & Ultrafiltration

In order to deproteinize the sample, the supernatant of the centrifuged sample was ultrafiltrated using a Centrisart 1 System (Sartorius AG, Göttingen) with a millipore barrier of 20 kD (kilodalton) (Figure G2-5). The Centrisart system cap was removed and the inner tube (floater) was removed by sliding it out and then standing it on a clean surface with the open end down to avoid contact with the delicate millipore membrane. The empty Centrisart centrifuge tube (outer tube) was weighed and then the supernatant from the caplet was pipetted into the clean Centrisart centrifuge tube. The outer tube with supernatant was re-weighed to obtain the weight of the supernatant. The floater was then

reinserted into the Centrisart outer tube with the membrane side down by allowing it to slide down onto the surface of the liquid sample. The system was allowed to rest standing for 5 minutes to ensure complete wetting of the membrane. The closing cap was NOT re-inserted for the centrifugation process. The prepared system was then centrifuged in the Minifuge GL for 45 minutes at 20° C and 5000 revolutions per minute. Following centrifugation, the filtrate was decanted from the floater chamber into new 2 ml Ependorf tubes and capped. Since the filtrate will slowly diffuse back into the concentrate in the outer chamber, this step was done as soon as possible after centrifugation. The extract was frozen in liquid nitrogen and stored frozen at –35° C in an AEG Öko-Arctic Super freezer until ICP analysis.

G2-6: Procedure Notes:

1. The Ependorf reaction caplets were not reused but the magnetic rods removed from the caplets were carefully washed and rinsed with distilled water 2x and dried before reuse.

2. Before the first time use of the floater, the Centrisart centrifuge chamber should be filled with 2.5 ml of double distilled water, the floater set inside and the system centrifuged for 15 minutes at 4° C and 4000 cycles per minute. This procedure should be repeated 3 times. The washing serves to remove glycerin that the membrane was embedded with before use and in between uses. (J. Schwanz, personal advice, In: Rommel, 1998). Replace the cap to avoid desiccation of the membrane prior to use.

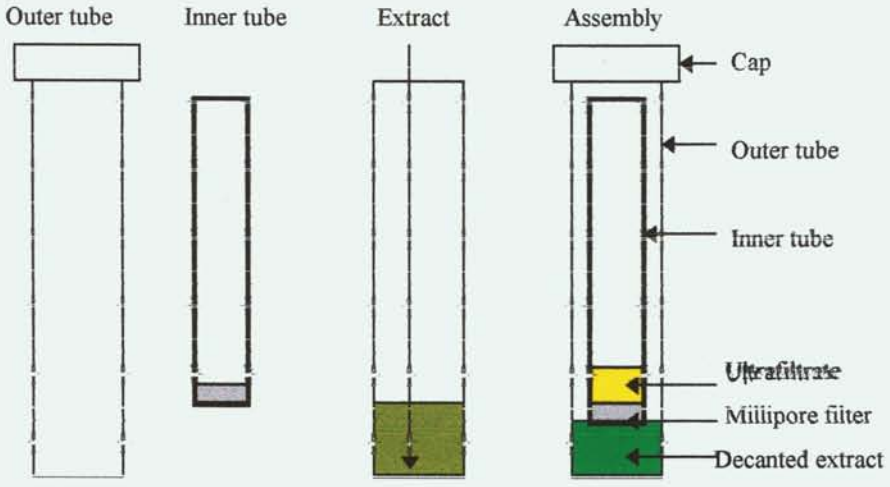
3. In between uses, the Centrisart floater can be cleaned overnight by soaking in a 0.1 M Triton X-100 (t-Octylphenoxypolyethooxyethanol) solution. Care should be taken to protect the hands and eyes from this chemical and it should be disposed of in a special waste receptacle. The floater should then be thoroughly washed with double distilled water and stored in double distilled water until the next day's use. The membrane must remain moist to retain its filtering function. For longer times between use, the floater can be left in a glycerin solution and then cleaned as if for first time use.

4. The Centrisart outer chamber can be cleaned between uses with a small brush and then rinsed with double distilled water, drained and oven dried at 60° C for 30 minutes, and stored dry at room temperature.

G2-7: Ultrafiltrate Mineral Analysis

Frozen samples were delivered to the Forstwirschaftliche Versuchsanstalt Rheinland-Pfalz, Trippstadt and through them the extracts were analyzed by Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA), Speyer (Obere Langgasse 40, 67346 Speyer) using Inductively Coupled Plasma (ICP) procedures. The mg/l concentrations of Ca, Al, Mg, K, Na, Mn, P, S, Zn and Fe were determined for each probe.

Figure G2-5: Centrisart 1 – Ultrafiltration System (Millipore filter barrier 20 KD)



G3: RESULTS

The mg/l concentrations of Ca, Al, Mg, K, Na, Mn, P, S, Zn and Fe were determined for each probe by LUFA, Speyer (Appendix 11A). From this data, each mineral was graphed to show its average abundance in unlimed (N) and limed (L) soils at various depths and seasons (Graphs G3: 1 -30; **Al**: G3:1-3, **Ca**: G3:4-6, **Mg:** G3:7-9, **K:** G3:10-12, **Na:** G3:13-15, **Mn:** G316-18, **P:** G3:19-21, **S:** G3:22-24, **Zn:** G3:25-27, **Fe**: G3:28-30). In addition, the seasonal significant differences between the unlimed and limed samples were determined and recorded below each graph as percentage confidence units. The significant differences for the mineral concentrations from various depths and other

statistical analyses were calculated to assist in interpretation of the effects (Appendices 11B & 11C). In addition, the Calcium/mineral ratios were compared (Appendix 11D) and summarized (Table: G3-2). Various parameters including: moisture, acidification, and ectomycorrhizae will be discussed first in general and then in context with each mineral and summarized in Figure 15-1.

G4: INTERPRETATION OF RESULTS

The results of the mineral analysis were determined using the same parameters for moisture, acidification and mycorrhizal abundance as described in Sections F4-1, F4-2 and F4-3. Each unbound mineral element will be discussed individually with respect to all three of these parameters with a summary to follow.

G5: UNBOUND ALUMINUM (Appendices 11A-D, and Graphs F3-1 to F3-3)

G5-1. Moisture Responses - Aluminum

G5-1A. For all depths, a seasonal oscillation pattern emerged for both the unlimed and limed plots which, despite being slightly out of synchronization, tended to rise in the fall and decline in the spring at all depths. If mineral accumulation was solely a function of mechanical hydration we would expect lower concentrations in the drier fall soil than in the wetter spring soil. The fact that unbound content increased in the wet fall may have more to do with physiological changes in xylem flow, than hydration levels of the soil. As explained previously, as the minerals dehydrate they tend to stop translocating and previously mobilized minerals may then begin to accumulate in the cytosol eventually becoming bound to cytoskeletal structures as dehydration progresses. Al accumulation was related to moisture, but was moderated by root physiology.

B5-1B. In the very wet fall 2000, the unbound Al content was not significantly different in the two forest plots. During this time period, the moisture content in both plots were the most similar, averaging 26.5% at 0-10 cm depth, 15% at 30-40 cm depth and 22.5% at 50-60 cm depth. From this it might be assumed that there is an upper moisture limit for each soil horizon beyond which point the physiological changes induced by liming are discounted. In the very dry fall 1999 more Al accumulated in both forest zones but with

greater differences so conversely, it might be stated that soil desiccation exasperates the physiological differences in Al uptake and translocation induced by liming treatments.

G5-1C. Interestingly, for each season, the relative Al uptake at 0-10 cm was not significantly different from that at 50-60 cm depth in the separate forest zones. However at the drier 30-40 cm depth, enhanced accumulations usually differed considerably from the wetter horizons immediately above and below. The combination of these factors implies a strong moisture requirement for solubilization and mobilization which is supported by the literature (Haug, 1984; See Section A3-8).

G5-2. Acidification Responses - Aluminum

G5-2A. The limed samples from 0-10 cm depth showed a general increase in unbound aluminum content (3.7 to 6.8 mg/l) corresponding to long-term acidification (from spring 1999 to fall 2000) but the unlimed samples which were higher and more consistent in content and tended to drop slightly in Al concentration over time (7.9 to 6.8 mg/l) despite a lower relative pH. At pH levels below 5, as was the case for both forest zones, relative moisture content of the soil may play a larger role in differential Al accumulation than pH.

G5-2B. The Al buffer stress range is from pH 4.0 to 4.4 (Ulrich, 1989, p.21). At 0-10 cm depth, roots from the more acidic (pH 3.53 to pH 3.79), unlimed soil contained more unbound Al than those from the limed soil (pH 4.24 to 4.47). This was the expected response. More Al was mobilized as it was acidified.

At 30-40 cm depth however, more unbound Al was present in the limed probes despite the fact they were more alkaline (pH 4.66 to 4.75) than the unlimed probes (pH 4.46 to 4.56) ! This implies, that liming, and more importantly, the soil moisture content, which is much lower here, has a more definite impact upon Al presence than does acidification. The greatest concentration at this depth occurred with the lowest soil moisture.

At 50-60 cm depth, once again, the unlimed probes had a greater unbound Al uptake however it is extremely important to note that at this depth, the pH of the unlimed soil was actually higher (pH 4.6 to 4.9) than that of the limed soil (pH 4.48 to 4.78). What this

means is that the physiology of Al uptake is much more complex than first thought and also that it is more strongly related to the calcium levels than to the actual pH of the soil.

G5-2C. It was determined from the Ca / Al ratios (Appendix 11D) that the unbound Al content was inversely related to the Ca concentrations. Despite similar moisture trends, at 0-10 cm and 50-60 cm depths there were significant differences in the uptake of Al between unlimed and limed samples, with more Al present in the unlimed root cytosol at every season, except for the very wet Fall 2000 soil when there was no significant difference between the forest zones. The only time a lowered presence of Al could be directly correlated to lower moisture content in limed soil relative to the unlimed soil, was in the very dry Fall 1999, when the limed soil averaged 3-4% less moisture. At other times, the limed probes had less unbound Al present despite frequently higher moisture levels in the soil.

At 30-40 cm depth, the soil moisture ranged between 11 and 17 % with the greatest Al accumulations in roots from both forest zones at the lowest moisture points (11%). The differences in Al content between the plots mostly disappeared, at this intermediate (drier) depth. The unlimed probes often had slightly more unbound Ca present in the overlying horizon. In support of the inverse relationship between Ca and Al, the limed probes had their greatest accumulations of Al here, modestly exceeding that of the unlimed probes.

G5-3. Mycorrhizal Impact - Aluminum

G5-3A. If unbound Al uptake was exclusively promoted by mycorrhizae we would expect much higher concentrations at 0-10 cm depth with lower concentrations in deeper soil. Conversely if mycorrhizae were important in exclusion of Al, then as they disappear the unbound Al content should rise. Neither trend was found. In the 30-40 cm horizon more Al was found than either above or below. Despite the strength of the abiotic relationships to Al presence, the impact of the mycorrhizal community cannot be eliminated.

G5-3B. In the unlimed soil in the dry fall, more Al was present than in the wet fall, this implies that with sufficient moisture, the mycorrhizal roots were able to exclude some Al from the cytosol but the total accumulation was still higher than in the limed probes. In

the limed soil there was no significant difference between dry and wet fall at 0-10 cm depth but some exclusion at 30-40 cm depth and enhanced uptake at 50-60 cm depth. Despite the variable responses there was less overall accumulation than in the unlimed probes. Seasonal fluctuations were evident but the limed roots differed in response.

G5-3C. The limed roots accumulated the least unbound Al at 0-10 cm and 50-60 cm depth and the most at 30-40 cm depth while the unlimed probes had more unbound Al at 0-10 cm and 50-60 cm depths and approximately the same as the limed roots at 30-40 cm depth. Despite similarities in the moisture content of the soil, liming definitely affected Al uptake. Generally, the unlimed roots tended to have very little bound Al, but abundant unbound Al, and conversely, the limed roots tended to have abundant bound Al but relatively less in the unbound form. This indicates that the mycorrhizal roots are functioning differently in the two forest zones.

Mineral responses that cannot be consistently attributed to specific abiotic factors indicate that biotic interactions are occurring. These interactions may be dependent not just on the presence of mycorrhizae but also upon the species distribution within the rhizosphere. This would especially be the case in otherwise inexplicable differences between the unlimed and limed forest regions, such as the lower than expected accumulation of Al in the 0-10 cm depth probes, especially in the limed probes. Individual mycorrhizal responses, specifically the ability to exclude Al, may be dependent upon complex interactions with abiotic factors such as Ca content, moisture, leaching, pH, and soil depth all playing roles. Forest liming has affected individual mycorrhizal responses and the overall chemical interactions in the rhizosphere.

G5-4: Summary - Aluminum

The primary factors enhancing the presence of Al were soil moisture followed by the acidification. (All probes were less than pH 5). Very generally, the presence of unbound Al in oak roots was lower if soil moisture levels dropped slightly but as they approached a critical moisture point (possibly as xylem translocation rates declined) the unbound Al content stabilized and proceeded to increase in the cytosol as desiccation progressed.

Mycorrhizae seemed to play a secondary role in unbound Al accumulation, but also acted to counteract the passive ion flow. Exclusion of Al occurred most effectively in moist limed soil, regardless of the pH. This exclusion ability tended to be lost where mycorrhizal numbers were lowered, and in very dry soil, especially at 30-40 cm depth. Limed probes exhibited the greatest increases in unbound Al content as desiccation progressed, but in moister soils, the unlimed probes had significantly higher Al. In acidified unlimed soil at 0-10 cm depth, and in less acidic soil at 50-60 cm depth, the mycorrhizae were less capable of excluding Al than their counterparts in the limed forest.

G5-5: Discussion - Aluminum

In the unlimed forest, certain species of mycorrhizae were less capable of excluding Al and some even promoted symplastic or apoplastic movement of the ion rather than sequestration. According to the fluorescence and bound mineral results, Al was not as effectively bound to cell walls in unlimed roots. According to the unbound mineral results Al was generally higher in concentration in the cytosol in unlimed roots. Unbound Al mobilized by the correct moisture and pH complements, was free to move in the cytosol and to potentially be translocated via the symplast to the xylem and up to sensitive photosynthetic areas where it may promote leaf dieback and tree mortality. According to the Tripptstadt Forestry Department surveys (2003) crown damage was more extensive and mature tree mortality rate was higher in the unlimed zone (Appendix 3A & 3B) (Block 1999, Schöck 2003).

In the limed probes, considerable fluorescence evidence indicated that mycorrhizae, depending upon the species, sequestered more Al in their walls than the same species from the unlimed forest, but conversely, they contained less Al in the unbound form in the cytosol. (If Al is tied up in the cytoskeleton, it cannot be free in the cytosol to translocate to other regions of the plant via the xylem). So despite similar moisture levels, the limed mycorrhizal roots had less Al available for translocation to sensitive above ground growing tips which would correspond to healthier looking crowns.

What will the future hold however for the limed trees? If the roots are sequestering potentially toxic Al and so protecting the trees from the poisonous effects of translocated ions, then as the soil continues to acidify, at some point the accumulated and stored Al ions may be freed enmass resulting in sudden death of a seemingly healthy tree. Liming is apparently delaying tree mortality but may also be setting the stage for mass dieback. Unless the temporal acidification can be stopped or the soil can be modified with acid-neutralizing humus, the oaks will continue to grow until threshold is reached (80% of maximum Al toxin accumulation ?), and then may begin the sudden downwards spiral to death with or without the advent of additional stressors.

One way of determining the potential for this sudden death would be to determine a critical accumulation point for the mycorrhizal roots. Because of the complex nature of the interactions, the additional factors beyond soil acidification need to be considered. For example, if a drought period is followed by moderate to heavy precipitation, accumulated Al may suddenly be released from the soil, and from the root cytoskeleton storage areas, during the wet period. A massive inoculation of Al into the symplast may translocate to the leaves and initiate the downwards spiral prior to sudden oak death. Another problem, associated with liming, has to do with the shifting of root biomass to the upper soil horizons. This shift promotes increased frost and drought sensitivity. Prolonged drought or severe frosts which often precede a dieback may be a function of both biomass shift and Al accumulation in the roots cell walls. Added Ca may slow down the mortality rate by promoting Al sequestration, but sudden Al release after a major stress could in effect initiate sudden mortality.

Liming alone is not the answer. Returning humic material, in the form of mulched, composted and well rotted logging debris, to the forest would improve the soil. Logically, additional humus would help to reduce the shock of liming and fertilizer treatments especially if the additives were incorporated into the compost prior to spreading. This would provide the needed micronutrients and ultimately improve the bacterial, arthropod and macroinvertebrate webs and slow down acidification damage.

G6: UNBOUND CALCIUM (Appendices 11A-D; Graphs 4.4-4.6)

G6-1: Moisture Responses - Calcium

G6-1A. At 0-10 cm depth, the abundance of Ca closely followed seasonal moisture variations for both unlimed and limed probes. However, at 30-40 and 50-60 cm depths a seasonal oscillation pattern was less distinct. Soil moisture content was a secondary factor with respect to Ca uptake by the roots in deeper soil, but had an impact in shallow soil, especially in the limed forest.

G6-1B. With regard to seasonal accumulation, the unbound Ca accumulation in the dry fall was lower than that observed in the wet fall for both zones at 0-10 and 30-40. But at 50-60 cm depth there was no significant difference between the total Ca content of the unlimed and limed probes, but interestingly in apposition to the accumulations in shallow soil, in the dry fall more Ca was present than in the wet fall for both. The presence of Ca in the roots was not dependent upon seasonal moisture levels especially in deeper soil

G6-1C. At 0-10 cm depth, the unbound Ca concentration was significantly lower in the unlimed (10 to 25 mg/l) relative to the limed (20 to 40 mg/l) probes, as expected. However, in lower horizons, Ca was sometimes more abundant in the unlimed than the limed! This was unexpected. At 30-40 cm depth, the Ca content in the unlimed exceeded that of the limed roots each fall. At 50-60 cm depth, Ca contents were very low and interestingly, there was no significant content difference between the plots. Abundant moisture in deeper soil had no obvious impact on Ca uptake but surface moisture may have affected displacement to the lower horizons, especially in the unlimed forest.

G6-2. Acidification Responses.

G6-2A. With temporal acidification, Ca content tended to increase in both unlimed and limed soil at 0-10 cm depth but not in lower horizons. It is likely that this increase was more closely associated with increased mycorrhizal abundance than pH or moisture.

G6-2B. Probes from heavily acidified unlimed soil at 0-10 cm depth, had lower unbound Ca content than corresponding probes from limed soil. This implies an antagonistic

relationship between pH and Ca content. At 30-40 cm horizons however, despite a rise in the pH, the Ca levels were inconsistent, rising in the fall and dropping in the spring, regardless of moisture content. The rise in pH with depth in unlimed soil and the occasionally higher probe content implies a displacement of Ca to the lower horizons which altered the pH but did not consistently improve Ca presence in the fine roots.

G6-2C. In limed soil, the 0-10 cm and 30-40 cm horizons were more alkaline than in the unlimed forest plots but at 50-60 cm depth, the soil was more acidic and the probes had the lowest Ca content. A drop in pH then seems to be associated with lower fine root Ca content. Liming improved Ca content significantly, but only in the 0-10 cm horizon.

G6-3. Mycorrhizal Responses - Calcium

G6-3A. The general decline in Ca with depth positively corresponded to lower mycorrhizal abundance in limed forest plots. The trend was not as evident in the unlimed soil where the Ca levels at 30-40 cm depth often exceeded that of the limed plots. But this trend may be correlated to greater relative mycorrhizal abundance in the unlimed soil at this depth. At 50-60 cm depth, where very few mycorrhizae were present in either forest zone, there was no significant difference in the typically low Ca contents of the roots.

G6-3B. Ca uptake was found to seasonally improve from fall 1999 to fall 2000 which was directly correlated to the increase in mycorrhizae at 0-10 and 30-40 cm depths.

G6-3C. The unbound Ca content of roots in the upper soil horizons was primarily correlated to mycorrhizal abundance and secondarily related to moisture and pH as they affected myccorhizal abundance, distribution and physiology.

G6-4: Summary - Calcium

In limed forests, while the upper horizon was Ca-rich, the differences between unlimed and limed forest disappeared quickly in the lower horizons and were completely lost at 50-60 cm depth. The presence of Ca in fine roots was primarily dependent upon the mycorrhizal presence and secondarily upon the effects of moisture and pH upon the individual mycorrhizal species.

G7: UNBOUND MAGNESIUM (Appendix 11A-D; Graphs G3-7 to G3-9)

G7-1. Moisture Responses - Magnesium

G7-1A. At 0-10 cm depth, Mg tended to increase over time with acidification and increasing moisture in unlimed roots but declined in limed roots. At 30-40 cm depth, the same general trend was present except that there was more seasonal oscillation. In the unlimed roots, at 50-60 cm depth, there was no significant difference between unlimed and limed roots except fall 2000 when the limed samples had more Mg. There was a weak relationship between Mg and seasonal oscillations only for the unlimed probes at 0-10 and 30-40 cm depths.

G7-1B. In unlimed probes, at 0-10 and 30-40 cm depth, more Mg was present in the wet fall than the dry fall, but in limed probes the opposite occurred and Mg was lost. Moisture responses were altered by liming.

G7-1C. In unlimed soil in the spring there was no significant difference between the 0-10 and 50-60 cm depth horizons with respect to Mg content implying that at least in the spring, Mg uptake was related to soil moisture levels at these depths. The relationship did not hold for the fall. Different trends were seen in limed soil where there was consistently less Mg present as depth increased. Clearly soil moisture content with respect to soil depth was not a primary factor governing Mg presence in the root cytosol in limed roots. Relative moisture was more important in the unlimed probes but not consistent enough to be a primary governing factor.

G7-2. Acidification Responses - Magnesium

G7-2A. As the upper horizon became more acidic with time, Mg content of the unlimed roots (pH 3.79 to 3.53) rose but Mg content in the limed roots (pH 4.47 to 4.24) dropped. Temporal acidification was not a primary controlling factor, but definitely affected the mycorrhizae in limed soil more than in the unlimed.

G7-2B. As the pH dropped with depth in the *limed soil*, the Mg content generally declined. As pH rose and fell with depth in *unlimed soil*, the Mg variably rose and fell. Stronger Mg content was associated with higher pH and Ca content, at least at 0-10 cm

depth, but not consistently in deeper soils. At 50-60 cm depth, where the pH was most alkaline, the least Mg was found. If pH is a factor in Mg content, then it is mitigated by other stronger influences at this depth.

G7-2C. In reviewing the Ca:Mg ratios it was determined that Ca augmented Mg uptake. Less Mg was present in mycorrhizal roots from acidic unlimed soils. Mg presence was affected by mycorrhizae, acidification, soil depth and in a very limited sense, to moisture.

G7-3. Mycorrhizal Responses - Magnesium

G7-3A. There was a strong positive correlation between mycorrhizal abundance at 0-10 cm depth and Mg presence in both unlimed and limed soil. At 50-60 cm depth, all samples had low Mg. Overall there was more Mg present in the cytosol of limed roots.

G7-3B. In unlimed soil, the Mg uptake improved as the mycorrhizal abundance improved with time at 0-10 and 30-40 cm depths. In limed soil, Mg declined with time at 0-10 cm and 30-40 cm depths but in most cases was still relatively more abundant than in similar probes from the unlimed forest.

G7-3C. The causes for the Magnesium decline in the limed forest were generally related to increased soil acidification. Soil moisture content was not a seriously contributing factor to changes in mycorrhizal Mg content.

G7-4: Summary

Overall, liming improved unbound Mg presence. The concentration of Mg declined with depth in limed soil directly correlating to the mycorrhizal decline. Drop in Mg concentration was associated with mycorrhizal responses to increasing soil acidification but not specifically to moisture variations. Generally the unlimed probes had less Mg present and seemed less dependent upon mycorrhizal complements and more responsive to abiotic factors.

G8: UNBOUND POTASSIUM (Appendices 11A-D; Graphs G3-10 to G3-12)

G8-1. Moisture Responses - Potassium

G8-1A. At 0-10 cm depth there was a strong correlation between seasonal oscillations in moisture and K content for both forest zones. Both had less K present in the spring than the fall. At 30-40 and 50-60 cm depths, the relationship was not as obvious in limed soil but was strong in the unlimed zone.

G8-1B. In unlimed soil, at each depth, the unbound K content was greater in wet fall than in the dry fall. In limed soil at 0-10 and 30-40 cm depths there was no significant difference in K content between dry and wet fall weather but at 50-60 cm depth K concentration was significantly higher in the wet fall.

G8-1C. The presence of K was strongly related to soil moisture content at 0-10 and 50-60 cm depth, but since the dry soil at 30-40 cm also had high K content, moisture cannot be the only factor controlling uptake. In spring 2000 (limed) or the fall 2000(unlimed) there was no significant difference in K content in the relative samples regardless of soil depth, pH or moisture content. Between the spring and fall there was an increase in mycorrhizal abundance and root biomass in both forest regions and a corresponding increase in K.

G8-2. Acidification Responses - Potassium

G8-2A. In the unlimed roots temporal acidification resulted in increased K content at all depths. The trends for the limed roots were less consistent: With temporal acidification, the limed roots had higher K each fall. At 30-40 cm, K content declined over time and at 50-60 cm it increased over time. In limed soil, a separate factor, beyond pH, controlled K.

G8-2B. At 0-10 cm depth in the spring and fall 2000 there was no significant difference between the unlimed and limed samples with respect to K content despite a distinct pH segregation. In deeper soil, the K levels in the unlimed probes increased in correlation to more alkaline pH levels. Inconsistencies in the relationship between K concentration and pH levels indicates that pH is not a primary limiting factor.

G8-2C. Liming tended to reduce unbound K levels in the cytosol but at 0-10 cm depth the differences were only weakly significant. At 30-40 cm depth, the strongest differences occurred in the spring 1999 and fall 2000 but these differences were unrelated to pH.

G8-3. Mycorrhizal Responses - Potassium

G8-3A. K content either remained constant or increased with depth in the unlimed probes, so either the mycorrhizae do not directly affect K uptake or have some capacity to block uptake. In the limed probes, K content either remained constant or else increased (spring 1999, 30-40 cm depth) or decreased (fall 1999, 50-60 cm depth). The actual uptake may be depend upon the complement of species present at any particular moment in time.

G8-3B. In the moist fall 2000, when the most mycorrhizae were present, both unlimed and limed roots had highest K content. At 0-10 cm depth, in moist soil there was less difference between the probes than there was in drier soil. At 30-40 cm depth there was no significant difference between the zones in the very driest period but greater significant difference in the moist seasons. At 50-60 cm depth, more K was present in the unlimed probes, but both probes had a similar seasonal pattern and low mycorrhizal abundance. It is likely that mycorrhizal influences affect K presence only in the upper horizons.

G8-3C. The limed mycorrhizae seem to be better at excluding K at all depths but their ability was not directly correlated to moisture or pH. It is possible then that the cumulative ability to exclude K may be due to liming and more specifically due to the diversity of mycorrhizal species present and their unique contributions to control of unbound K movement. The unlimed mycorrhizae had lower K presence in 0-10 cm depth soil and higher content at 50-60 cm depth. Since K was enhanced in deeper unlimed soil, the loss of mycorrhizae at this depth may be a significant factor.

G8-4: Summary - Potassium

In limed soil, K uptake was variably influenced by mycorrhizal complements present at each soil depth with moisture and pH acting as distant secondary factors. In unlimed soil, temporal acidification and moisture were stronger factors, both generally resulting in increased K content. Maximal K presence may stimulate extrusion activities.

G9: UNBOUND SODIUM (Appendices 11A-D, Graphs G3-13 to G3-15)

G9-1. Moisture Responses - Sodium

G9-1A. Seasonal variations were strongly evident at 0-10 cm depth for both forest zones and at 30-40 cm especially in the limed soil. At 50-60 cm depth seasonal oscillations were weak but present for both zones. Sodium content was related to seasonal moisture levels.

G9-1B. Very generally, Na content was positively related to improved soil water content when comparing the dry fall 1999 to the wet fall 2000 at every soil depth in both zones.

G9-1C. The sodium content declined with depth in both forest zones. The lowest Na content was present in the 50-60 cm depth probes in limed soil despite a moisture content equivalent to the 0-10 cm depth. This may irregularity may be due to lower mycorrhizal numbers. Sodium easily dissociates in water, even so, soil moisture was not the only factor associated with Na presence in the root cytosol.

G9-2. Acidification Responses - Sodium

G9-2A. With long term acidification, both unlimed and limed probes increased in Na content at all depths. This trend was more precisely related to the improvement in soil moisture content and mycorrhizal numbers than to the actual pH.

G9-2B. At 0-10 cm depth, the Na content was higher in limed probes than in the acidic unlimed probes. In unlimed soil, Na content exceeded that of the limed probes only in the 50-60 cm depths where the pH levels were actually relatively more alkaline. Enhanced sodium in the cytosol is associated with rising pH.

G9-2C. In unlimed soil, the Na content remained relatively constant despite changes in Ca content. In limed soil, total Ca had the greater impact upon Na uptake at 0-10 cm depth but a reduced impact in lower soil horizons.

G9-3. Mycorrhizal Responses - Sodium

G9-3A. There was a strong correlation between soil depth, mycorrhizal abundance, and Na content for the limed soil region. As depth increased, and mycorrhizal numbers

decreased, Na content dropped. In unlimed soil, there was a weaker but still obvious relationship between mycorrhizal numbers and Na content.

G9-3B. As the numbers of mycorrhizae increased from the very dry fall 1999 to the wet fall 2000, Na presence also increased in limed probes at all depths. The same general trend existed in the unlimed probes but with more variation in the deeper soil horizons.

G9-3C. In shallow soil, the application of lime improved Na presence but the ameliorating effects were lost in the lower horizons.

G9-4: Summary - Sodium

Sodium content was tied primarily and directly to mycorrhizal numbers in limed soil with a much weaker relationship evident in the unlimed soil. Moisture content played a major role in Na uptake while actual pH was of less strategic importance. The moister and more alkaline the soil, the more Na tended to be present in the unbound form in the cytosol. Liming tentatively improved Na presence but only at 0-10 cm depth.

G10: UNBOUND MANGANESE (Appendices 11A-D, Graphs G3-16 to G3-17)

G10-1. Moisture Responses - Manganese

G10-1A. In both the unlimed and limed forests, direct seasonal oscillations in Mn presence were strongly evident at 0-10 and 50-60 cm depth, but inconsistent at 30-40 cm. At 30-40 cm depth there was no significant difference in Mn presence between the unlimed and limed plots but Mn content was influenced by seasonal moisture.

G10-1B. In both unlimed and limed probes, Mn was less prevalent in the dry fall and more prevalent in the wet fall with the greatest uptake in the limed soil probes at 0-10 cm depth. In deeper soil, the seasonal trends are more complex. At 30-40 cm depth, Mn declines from dry to wet fall in limed probes, and increases in unlimed probes. At 50-60 cm depth, Mn declines in the unlimed probes and increases in the limed. Clearly, more than just moisture factors are affecting Mn presence.

G10-1C. In both unlimed limed probes there was a distinct drop in Mn content with depth. Therefore, moisture content is important but not a primary factor in Mn presence.

G10-2. Acidification Responses - Manganese

G10-2A. At 0-10 cm and 50-60 cm depths the limed roots had more Mn with increasing acidification, but the temporal trend was not present at 30-40 cm depth. In unlimed probes, there was no trend at 0-10 cm depth, a positive correlation at 30-40 cm and a negative correlation at 50-60 cm depths. It was evident that pH alone was not enough.

G10-2B. At 0-10 cm depth, unbound Mn was usually lower in unlimed more acidic probes but in spring 2000 it was not significantly different. Despite the low concentrations at 50-60 cm depth, Mn was more prevalent in the more alkaline unlimed probes than the limed. Mn was more common in more alkaline soil probes.

G10-2C. Liming enhanced Mn presence at least at 0-10 cm depth. The effects of liming were lost in deeper horizons.

G10-3. Mycorrhizal Responses - Manganese

G10-3A. Unbound Mn declined with depth in direct correlation to mycorrhizal loss in both forest zones. At 30-40 cm depth there were irregularities in Mn presence implying potentially impaired mycorrhizal functioning or altered diversity effects. In the dry fall more Mn was retained in both forest zones at 30-40 cm depth than at 0-10 cm. In the wet fall at 30-40 cm depth, the unlimed probes retained the most Mn but the limed probes had a major Mn loss in comparison to the abundance in probes from the overlying horizon. The lowest Mn levels were recorded at 50-60 cm depth in correlation to the lowest mycorrhizal numbers.

G10-3B. At 0-10 cm depth, Mn fluctuated in spring and fall directly with the mycorrhizal abundance or loss while soil moisture and pH acted as secondary factors. In deeper soil, while moisture and pH still behaved as secondary factors, there were unexplained fluctuations in the Mn contents which may be partially due to the cumulative effects of various species of mycorrhizae at those depths and seasons.

G10-3C. Liming increased Mn levels only in the fall in the limed probes at 0-10 cm depth. Otherwise, Calcium content could only be loosely tied to Mn content.

G10-4L Summary - Manganese

Unbound Mn fluctuations were most directly correlated to mycorrhizal abundance with seasonal soil moisture and pH levels acting as secondary factors. Mn presence seemed to be modestly enhanced by Calcium presence and slightly more alkaline soils.

G11-UNBOUND PHOSPHATE (Appendices 11A-D; Graphs G3-19 to G3-21)

G11-1. Moisture Responses - Phosphate

G11-1A. Seasonal oscillations in P content were present at all depths but the unlimed and limed probes were slightly out of synchronization especially in the spring and fall of 2000. At 0-10 and 30-40 cm depths, despite the major difference in content, the unbound P levels were tied to seasonal moisture fluctuations.

G11-1B. At 0-10 cm depth more P was present in the wet fall than the dry fall for both unlimed and limed probes. In deeper soil, the unlimed probes had a similar relationship but not the limed. Phosphate levels were only moderately related to soil water content.

G11-1C. P levels were not directly related to soil moisture content relative to depth. Average P content was highest at 0-10 cm depth, with the unlimed probes (N = 6 mg / L) exceeding the limed probes (L = 4.8 mg / L) and dramatically lower at 30-40 (N = 1.4 mg / L and L = 0.9 mg / L) and 50-60 cm depths (N = 0.9 mg /L and L = 0.8 mg / L).. The relationship to moisture content disappeared at 50-60 cm depth.

G11-2. Acidification Responses - Phosphate

G11-2A. More unbound P was present in probes from moist more acidic soil than from drier more alkaline soil at 0-10 cm depth. In addition, both unlimed and limed probes increased in P content with temporal acidification. The same trend was seen in unlimed soil at deeper depths but not in the limed probes.

G11-2B. In shallow unlimed soil (pH 3.53 to 3.79) and limed soil (pH 4.24 to 4.47) very large amounts of P were present in the mycorrhizal cytosol. In deeper soil, P levels were extremely low despite moderation of the pH in the unlimed probes(above pH 4.49 to 4.53) and limed probes (pH 4,66 to 4,75) at 30-40 cm depth. In deeper soil, P content more closely corresponded to moisture and Ca fluctuations than pH changes.

G11-2C. Enhanced Calcium presence reduced P levels in the cytosol at 0-10 cm depth and somewhat at 30-40 cm depth but not at 50-60 cm depth where the Ca levels in the unlimed soil often exceeded that of the limed. Liming was associated with reduced P levels but as an insignificant secondary factor.

G11-3. Mycorrhizal Responses - Phosphate

G11-3A. Unbound P presence was strongly tied to the abundance of mycorrhizal species in the A horizon with a slight advantage going to those in more acidic soil. All advantages were lost in the deeper soils where the P and mycorrhizal abundance were both minimal.

G11-3B. The P content declined when the mycorrhizal abundance declined in the very dry fall but did not bounce back as quickly in the limed soil when the moisture levels and mycorrhizal numbers increased the following wet spring at 0-10 cm depth. By the wet fall there was no significant difference between the unlimed and limed zones in the A horizon.

G11-3C. Soil depth made the largest impact upon mycorrhizal number and P content with pH and moisture acting as secondary factors. Liming reduced P concentration at 0-10 cm and in wet soil at 30-40 cm depth but had a negligible impact at 50-60 cm depth.

G11-4: Summary - Phosphate

The greatest cytosolic P presence occurred at the 0-10 cm horizon, especially in acidic soil. Less P was present in the limed probes directly related to the higher pH levels but probably also related to the specific mycorrhizal species present and their moisture requirements. Liming elevated pH and reduced P content in the cytosol especially at 30-40 and 50-60 cm depths. The lack of P in deeper soils (possilby due to higher uptake at 0-10 cm) is a serious limiting factor affecting energy production and mycorrhizal survival.

G12: UNBOUND SULFUR (Appendices 11A-D; Figures G3-22 to G3-24).

G12-1. Moisture Responses - Sulfur

G12-1A. In both the unlimed and limed forest zones the S content was directly correlated to seasonal moisture fluctuations at every depth. Although the seasonal trends were out of synchronization at 30-40 cm depth they were nearly identical in the B and C horizons.

G12-1B. More S was present in the wet seasons than the dry in both forest zones at nearly every depth. At 0-10 cm and 50-60 cm the increases in content were modest, predictable and often not-significantly different in the two forest areas, but at 30-40 cm depth there was more unexplainable variation and greater significant difference.

G12-1C. Sulfur content was slightly higher at 0-10 cm depth than at 50-60 cm depth for both forest regions. In the shallow horizon there was generally no significant difference in S content between the zones but at 50-60 cm depth, in drier seasons, a little less unbound S was present in the limed probes. With respect to soil depth, S content was often higher at drier 30-40 cm depth than in the corresponding wetter horizons either above or below. This might be correlated to a lower cytosol mobility (translocation) in the roots from the drier soil in the dry fall 1999. However, this logic cannot explain the extremely high concentrations in limed soil in the spring 1999 relative to the extremely low levels in the unlimed probes in the same season. At 30-40 cm depth the unlimed and limed probes were the least similar in S content. Moisture then plays a role in homeostatic balance and loss of fluid plays havoc with the root system S content.

G12-2. Acidification Responses - Sulfur

G12-2A. There was a timid trend of increased S content with time in both the unlimed and limed forest zones. However, the lower pH of the unlimed soil with respect to the limed soil did not have an impact. In fact, at 0-10 cm depth, there was no significant difference in S presence between the unlimed and limed probes. The acidification trend was secondary to moisture and mycorrhizal interactions. Since both forest zones were already below pH 5 due to acid rain input they could well be near their maximum S content for living mycorrhizal roots.

G12-2B. At 0-10 cm depth, where the most significant pH difference occurred, there was no significant difference in the presence of S between the unlimed and limed probes from fall 1999 to fall 2000. The overall effect of lowered pH is negligible. At 30-40 cm depth wild variations in the S content and distinct differences in unlimed and limed soil content occurred At this depth, the pH of the soil was also the most variable, but mycorrhizal abundance and distribution were also highly flexible. At 50-60 cm depth the unlimed soil probes (with a higher pH) had more S than the limed. With the loss of modifying mycorrhizal action, pH here may play a role.

G12-2C. At 0-10 cm depth, in unlimed soil, low S content was fairly closely tied to the low Ca and low pH levels while in limed, more alkaline soil, the Ca content had both a positive and a negative influence on the total S present. At 30-40 cm depth, frequently a drop in Ca was associated with a rise in S. At 50-60 cm depth with the lowest Ca, the lowest S levels were recorded.

G12-3. Mycorrhizal Responses - Sulfur

G12-3A. The S levels did not obviously correspond to the abundance of mycorrhizae in either the unlimed or limed soil as depth changed. At 50-60 cm depth there was a little less unbound S present than at 0-10 cm depth which loosely correlates to lower mycorrhizal numbers. However, the higher S content at 30-40 cm depth negates the relationship. The other possibility is that in the 0-10 cm horizon, the mycorrhizae were more capable of excluding S from the symplast than similar mycorrhizae in the mid range or deeper horizons.

G12-3B. At all depths there was slightly higher S content in the wet fall relative to the dry fall which corresponds to enhanced myccorhizal numbers. Sulfur content was tied to seasonal increases in mycorrhizal abundance.

G12-3C. At 0-10 cm depth, there was no significant difference in S content in unlimed and limed mycorhizal roots despite the variance in soil pH and Ca content. Moisture did play a role at this depth. With adequate moisture, the mycorrhizal species may be better able to exclude S. At 30-40 cm depth physiological changes in the mycorrhizae in

response to liming, acidification and low moisture may account for the unusual and significant differences in S presence. The 30-40 cm depth mycorrhizal probes were characterized by a loss of control. Balance was returned at 50-60 cm depth despite lower mycorrhizal numbers. At this depth more water was present.

G12-Summary - Sulfur

Soil moisture and mycorrhizal abundance had a definite but moderate impact upon the relative amount of S present in the roots in the unbound form. In shallow soil, mycorrhizae were able to exclude S, regardless of the soil pH or Calcium content. In the 30-40 cm horizon, lowered abundance and diversity of mycorrhizae and a loss of moisture played havoc with the root sulfur content. With the loss of modifying mycorrhizae in deeper soil, pH plays a larger role. Lowered pH was associated with lowered S content.

G13: UNBOUND ZINC (Appendices 11A-D; Graphs G3-25 to G3-27)

G13-1. Moisture - Zinc

G13-1A. Unbound Zinc content did not follow typical seasonally oscillating patterns. The unlimed and limed probes were distinctly different at 0-10 and 30-40 cm depth but shared a similar seasonal trend at 50-60 cm. At 50-60 cm depth, the highest Zn concentrations occurred in the very dry fall 1999 and were lowest in the wet fall. At 0-10 and 30-40 cm depth, the trend in unlimed soil was the same, but in limed soil, the highest Zn levels occurred in the spring 1999. In regards to seasonal changes, Zn content was inversely related to moisture levels: As soil moisture increased, Zn was excluded.

G13- 1B. In unlimed soil, at each depth, the Zn content was consistent in all the samples in the spring but less consistent in the fall. In the dry fall, the highest Zn levels were recorded at every depth. At 50-60 cm depth, the limed soil probes had a similar pattern but with significantly less Zn present. At 0-10 and 30-40 cm depth the limed probes varied most obviously in their Zn content being highest in the spring 1999 and generally declining each season there after. While the unlimed samples closely followed seasonal variations, at least in shallow soil, the limed probes did not.

G13-1C. In unlimed probes, as seasonal moisture levels rose, more Zn was excluded. However, with respect to depth, more Zn was present in the wet 0-10 and 50-60 cm horizons and less in the drier 30-40 cm horizon. In limed probes, as seasonal moisture levels rose, more Zn was excluded except for Spring 1999 which had unusually high Zn levels. With respect to depth the Zn concentrations were highest at 0-10 cm depth. At 30-40 cm depth, except for spring 1999, the Zn concentrations were at their lowest points. In general, as soil moisture rose, Zn levels dropped but at 30-40 cm depth factors other than moisture content must be operating.

G13-2. Acidification Responses - Zinc

G13-2A. With temporal acidification there was a decline in Zn levels in all samples but in reviewing the other data the decline in Zn may be more closely associated with increased soil moisture content and enhanced ability of mycorrhizae to exclude Zn ions.

G13-2B. At all depths the unlimed soil probes had somewhat higher Zn concentrations except for spring 1999. At 0-10 cm depth, the unlimed soil was definitely more acidic than the limed in every case, despite this in the wet spring and fall of 2000 there was no significant difference in Zn content. At 50-60 cm depth, the unlimed probes, which were more alkaline than the limed probes, had more Zn. So the relationship to pH was not clean. If pH played a role, it was only in the deeper horizons where fewer mycorrhizal roots were present to control Zn exclusion.

G13-2C. At 0-10 cm depth, despite the significant difference in Ca:Zn ratios there was no significant difference in average Zn content (1.5 mg / L). At 30-40 cm depth, the Zn content definitely decreased in direct relationship to a decrease in Ca. At 50-60 cm depth, an inverse relationship existed where Zn content increased as Ca content dropped in the unlimed probes and decreased as Ca content increased in the limed probes.

G13-3. Mycorrhizal Responses - Zinc

G13-3A. In unlimed soil, there was a general increase in Zn content with depth. It was probable that with the loss of mycorrhizae, exclusion ability was lost also. Unbound Zn

was frequently higher in the unlimed samples implying impaired ability to exclude this ion. The limed mycorrhizae seem more successful in excluding Zn at each depth. At 30-40 cm depth, odd responses to variations in pH, and moisture may be a function of individual mycorrhizal physiology in response to the combination of environmental factors and the lower Ca levels. At 50-60 cm depth, where the fewest mycorrhizae were present, the unlimed probes had the least control of Zn exclusion possibly due to physiological changes associated with the increase in pH and drop in Ca. At this depth the limed species were better at excluding Zn.

G13-3B. Mycorrhizae actively excluded Zn. From the dry fall 1999 to the wet fall 2000, mycorrhizal abundance increased dramatically. Along with the increase there was a drop in Zn content at all depths. The fact that there was no significant difference between the readings at the highest concentration levels in the dry fall 1999, regardless of depth or pH, may indicate that the unlimed roots were near their maximum Zn levels when the mycorrhizae were at their lowest functional abilities.

G13-3C. With respect to Zn exclusion abilities, the mycorrhizae operated best in moister soils. At 0-10 cm depth, pH and Calcium content were of secondary importance. At 30-40 cm depth the lowest Zn uptakes were recorded. In this drier soil, the mycorrhizal species present seem more responsive to the lower Calcium content and higher pH. At any rate, the physiological reaction of mycorrhizal species in both the unlimed and limed zones were definitely altered and Zn exclusion definitely improved. At 50-60 cm depth, despite the high moisture content at this depth, the few mycorrhizal species left in the unlimed soil had less control over Zn uptake than those in the limed soil which had a slightly higher Ca content. Liming seemed to improve Zn exclusion ability at every depth.

G13-4: Summary - Zinc

Zinc was mobilized in water but mycorrhizae had some ability to exclude Zn given ideal, moisture, calcium and pH levels, in that order. Liming improved Zn exclusion best at 50-60 cm depth.

G14: UNBOUND IRON (Appendices 11A-D; Graph G3-28 to G3-30)

G14-1. Moisture Responses - Iron

G14-1A.. No obvious seasonal variations were evident at 0-10 cm depth and in both the unlimed and limed zones. Fe content declined with time irrespective of seasonal moisture variations but in indirect correlation to overall improved moisture levels over time. In deeper soil, seasonal oscillations were present but in opposing patterns. Both probes had low spring and higher fall Fe content but the limed probes declined in Fe content over time irrespective of seasonal moisture variations. Unlimed probes were more responsive to moisture variations.

G14-1B. The limed probes had more Fe in the dry fall than the wet fall at every depth. The unlimed probes had a similar trend at 0-10 and 50-60 cm but not at 30-40 cm. The fall mycorrhizae in limed soil generally had a better ability to exclude Fe.

G14-1C. The unbound Fe levels were highest in the 0-10 cm depth while the deeper horizons has extremely small quantities in the cytosol. Because of the large difference in the Fe content, a different scale was used on Graph 4.28 relative to Graphs 4.29 and 4.30. Since the Fe content at 0-10 and 50-60 cm depths were not similar, no relationship between moisture and soil depth and unbound iron content could be established.

G14-2. Acidification Responses - Iron

G14-2A. With temporal acidification, Fe content generally declined. At 0-10 cm depth, this decline was also correlated to increased moisture and mycorrhizal abundance in both the unlimed and limed zones (better exclusion). In deeper soil the same trend existed for the limed probes, but despite increasing acidification, the unlimed mycorrhizal roots were more variable in their Fe exclusion. For the unlimed mycorhizal roots this may be an indication of lower adaptability in deeper soil.

G14-2B. With respect to empirical pH changes, the acidic unlimed probes had more Fe than the limed at 0-10 cm depth but as the pH changed with depth, the unlimed probes still had more Fe. Despite a much higher pH at 50-60 cm depth than their counterparts in

the limed soil the Fe levels were still higher. If pH was a factor, then between pH 4.46 and 4.9, it was secondary to Ca content and mycorrhizal exclusion abilities in moist soil.

With respect to the dramatic difference in Fe content in the 0-10 horizon relative to the lower reaches, the solubility point for iron, or the iron buffer range may be of greatest significance. In the acidic unlimed soil, the pH H_20 range varied from 3.53 to 3.79, while the limed soil range was 4.24 to 4.47 with some probe samples dipping into the Fe / Al buffer range. Iron is freed into solution only as the pH drops into the Fe / Al buffer range of pH 2.8 to 3.8 (Ulrich, 1989, p.21). (The greatest single increase in Fe cytosol content occurred at 0-10 cm depth in unlimed soil at pH H_2O 3.79 in spring 1999). The lower relative content of Fe in limed probes then may be more of a function of ambient soil pH than any ability on the part of the mycorrhizae to exclude the ion. In deeper soil, as the pH rises above the Fe / Al buffer range in both the unlimed and limed forests, insignificant amounts of Fe are found in the cytosol. At these very low iron levels, root Calcium content had an impact.

G14-2C. At 0-10 cm depth, more Fe was present in the unlimed soil than in the limed. Except for Spring 1999, the same trend was seen for deeper soil probes. Limed mycorrhizal roots seemed more capable at excluding Fe than unlimed. Calcium content was a primary control factor especially in more alkaline soil.

G14-3. Mycorrhizal Responses - Iron

G14-3A. Very generally, the greatest Fe uptake occurred in the 0-10 cm deep horizon where the most mycorrhizae were found and where the soil was the most acidic. The limed samples had better exclusion abilities probably associated with their Ca metabolism but also associated with lower iron ion release from the soil due to a slightly higher pH. In deeper soil, the low Fe content probably had more to do with reduced release of iron ions from the soil than mycorrhizal action. In deeper soil however, improved Ca content improved the mycorrhizal control of Fe content.

G14-3B. Over time as the soil acidified, less Fe was present in the cytosol so it would seem that in the presence of adequate moisture the mycorrhizae have better Fe exclusion capacities. Adjustments and adaptation likely occurred over the 2 year time span with survival of the fittest mycorrhizal species.

G14-3C. Mycorrhiza growing in limed soil were better able to exclude Fe at all depths except for in Spring 1999 in the deeper soil horizons.

G14-4: Summary - Iron

Iron is known to be highly mobilized between pH 2.8 and 3.8. The assault on the roots was severe in the unlimed soil (pH 3.53 to 3.79) and a little less severe in the limed soil (pH 4.24 - 4.47) but still strong. In the presence of adequate moisture mycorrhizae were more capable of excluding Fe in both cases as time progressed. In deeper or more alkaline soil, above pH 4.49, the Fe assault was minimal. At these low levels, the limed mycorrhizal roots had more of an advantage.

G15: UNBOUND MINERALS - GENERAL CONCLUSIONS (Figure 15-1)

Aluminum was released into the rhizosphere when the pH of the soil becomes acidic and when the moisture levels were high enough to allow solubilization. In very moist conditions, the mycorrhizal roots from the unlimed soil had a reduced ability to exclude unbound Al. The limed mycorrhizae were more effective at excluding aluminum when the soil was moist but lost it in the drier 30-40 cm horizon.

Calcium presence was primarily dependent upon mycorrhizal abundance and secondarily upon the effects of moisture and pH. The greatest uptake occurred in the 0-10 cm horizon in the limed soil. Below that level, differences between the unlimed and limed zones disappeared.

Magnesium content improved with liming. In limed soil mycorrhizal abundance was the most important factor followed weakly by changes in pH but not moisture. In unlimed soil the Mg presence was also related to mycorrhizal abundance but with stronger responses to pH and moisture variations. Mg content generally dropped with acidification.

Potassium was variably influenced in limed soil by mycorrhizal complements present at each soil depth, with moisture and pH acting as distant secondary factors. In unlimed soil, temporal acidification and moisture were stronger factors, both resulting in increased K content.

Sodium presence was tied primarily and directly to mycorrhizal numbers, to a greater extent in limed areas and a lesser extent in the unlimed. Liming improved Na content at 0-10 cm depth. The moister and more alkaline the soil, the more Na tended to be present in the cytosol. The pH was of lesser strategic importance than soil moisture.

Manganese presence was directly correlated mycorrhizal abundance with seasonal moisture and pH acting as secondary factors. Mn presence was modestly enhanced by Ca and a more alkaline pH.

Phosphate content was most strongly related to the soil pH. It was heavily released in the more acidic 0-10 cm deep horizon and minimally released in the deeper more alkaline reaches. Liming further reduced P content in mycorrhizal roots. Soil moisture variations were not very relevant

Sulfur content was moderately enhanced by improved soil moisture and mycorrhizal abundance. Low Sulfur content was associated with lower pH values. There was little difference between the unlimed and limed plots.

Zinc was mobilized in water but mycorrhizae had some ability to exclude Zn given ideal moisture, calcium and pH levels, in that order. Liming improved Zn exclusion best at 50-60 cm depth.

Iron was heavily released in acidic soil at 0-10 cm depth but not in more alkaline, deeper horizons. In the presence of adequate moisture mycorrhizae were more capable of excluding Fe as time progressed. In deeper soil, with low Fe levels, limed roots had an advantage.

Figure 15-1: Ranking of the factors that had the greatest influence upon unbound mineral content is followed by the relative overall content of the mineral in unlimed and limed soil at 0-10 cm depth.

	Factor Ranking				Unlimed	Limed
	1	2	3	4	Content	Content
Al	pH	Moisture	Mycorrhizae	Liming	high	low
Ca	Liming	Mycorrhizae	Moisture	pH	low	high
Mg	Liming	Mycorrhizae	pH	Moisture	low	high
K	Mycorrhizae	Moisture	pH	Liming	equal	equal
Na	Mycorrhizae	Moisture	Liming	pH	low	high
Mn	Mycorrhizae	Moisture	Liming	pH	low	high
P	pH	Mycorrhizae	Liming	Moisture	high	low
S	Moisture	Mycorrhizae	pH	Liming	low	high
Zn	Moisture	Mycorrhizae	Liming	pH	high	low
Fe	pH	Moisture	Mycorrhizae	Liming	high	low

In unlimed roots the mycorrhizal tips tended to sequester less aluminum than the limed tips (Section F). However, the unlimed roots contained significantly more unbound aluminum than the limed (Section G). This unbound component is of the greatest importance because it is in this form that the Aluminum can be translocated to the xylem and subsequently via mass flow to the leaves where symptoms of aluminum toxicity become evident. The trees from the unlimed forest has an average of 25% less crown coverage and a higher mortality rate than trees from the adjacent limed plot. Liming then improves binding of potentially toxic aluminum in the roots, preventing its translocation and lowering its toxicity in the distal leaves.

The effectiveness of aluminum sequestration is dependent upon the diversity and abundance (Section B) of the mycorrhizal complement on the roots with each individual species contributing to aluminum control in its own unique way (Section E).

G: References

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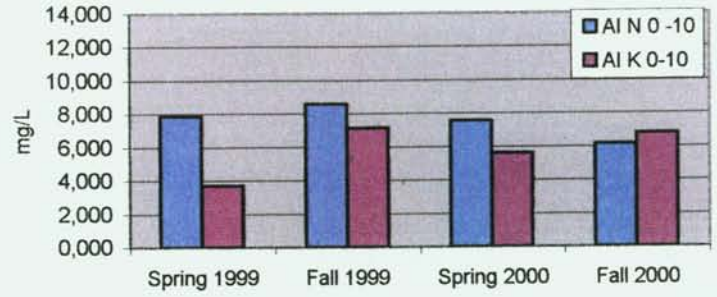
G3

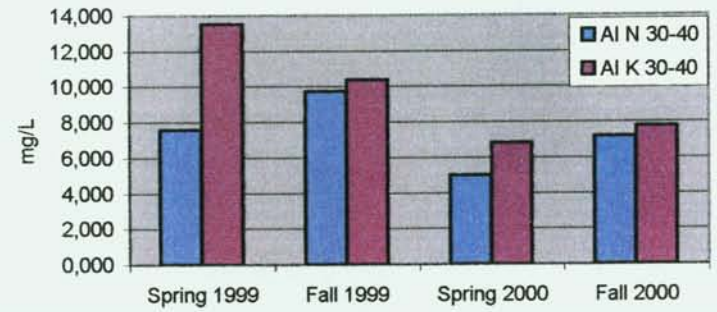
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph . . 1: Average unbound **Aluminum** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth.



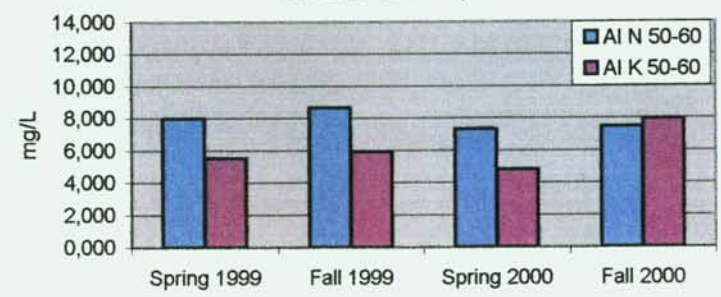
90% 50% 80% NS

Graph . 2: Average unbound **Aluminum** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth.



NS NS 70% NS

Graph . 3: Average unbound **Aluminum** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

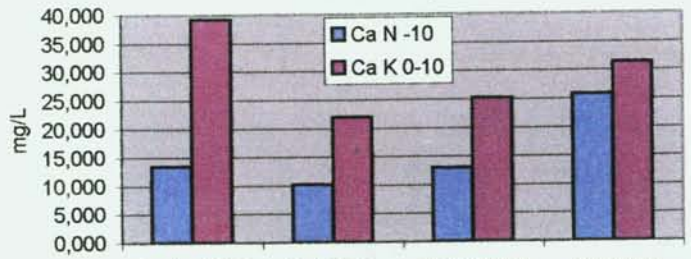


70% 50% 80% NS

G3

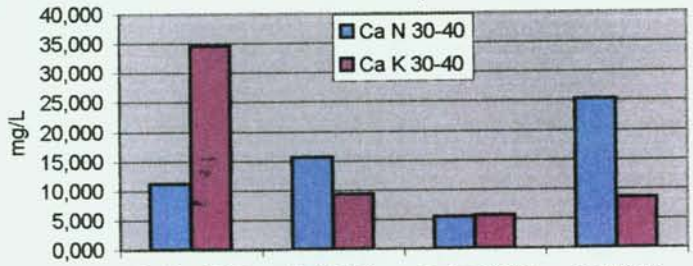
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph 4: Average unbound **Calcium** content of the cytosol of fine mycorrhial oak roots from unlimed (N) and limed (K) forest soil from 0-10 cm depth



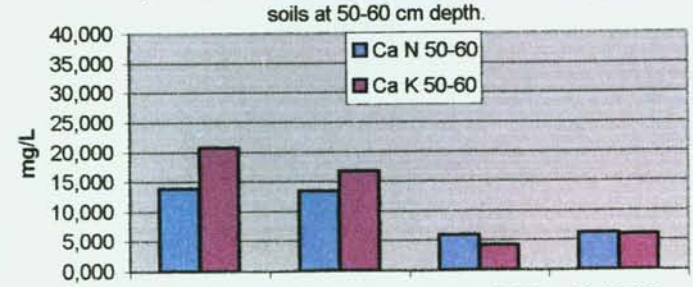
95% 99% 80% 50%

Graph 5: Average unbound **Calcium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth



80% 60% NS 70%

Graph 6: Average unbound **Calcium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

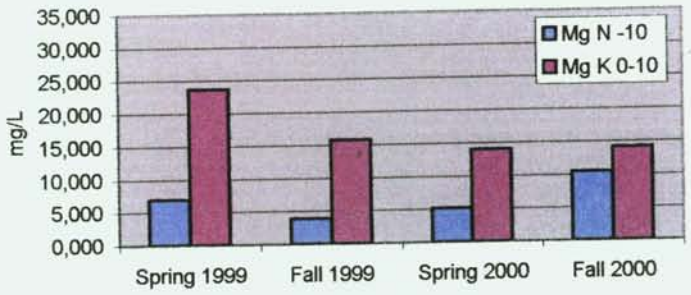


NS NS NS NS

G3

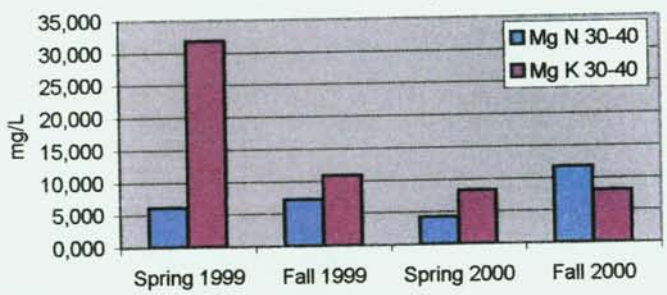
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph 7: Average unbound **Magnesium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth.



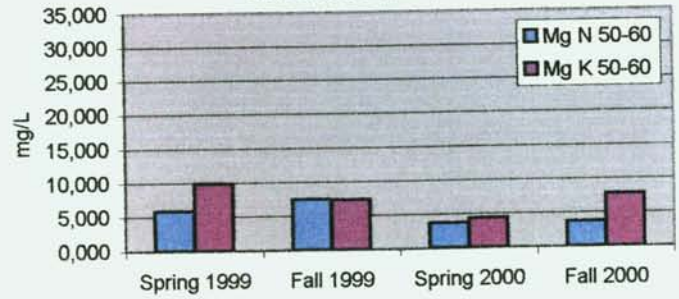
99% 99% 99% 70%

Graph 8: Average unbound **Magnesium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth.



80% 80% 99% NS

Graph 9: Average unbound **Magnesium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

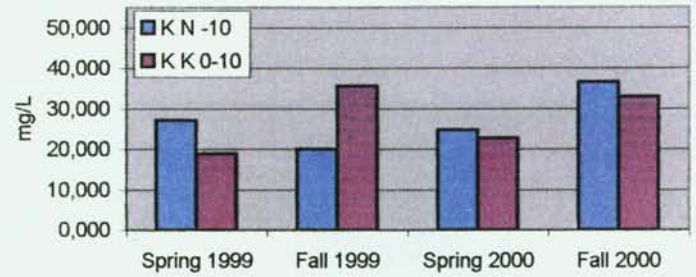


50% NS NS 95%

G3

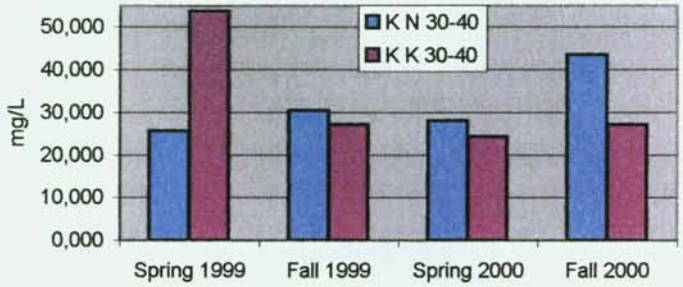
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph 10: Average unbound **Potassium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils from 0-10 cm depth.



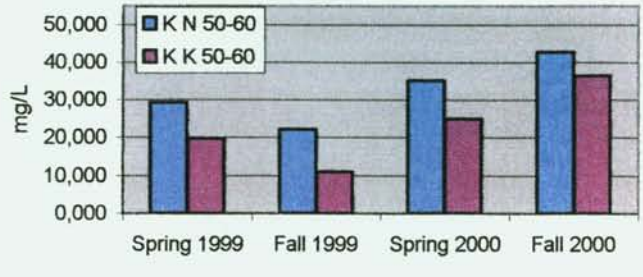
60% 60% NS NS

Graph 11: Average unbound **Potassium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils from 30-40 cm depth



70% NS 60% 90%

Graph 12: Average unbound **Potassium** content of the cytosol of fine oak mycorrhizal roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth

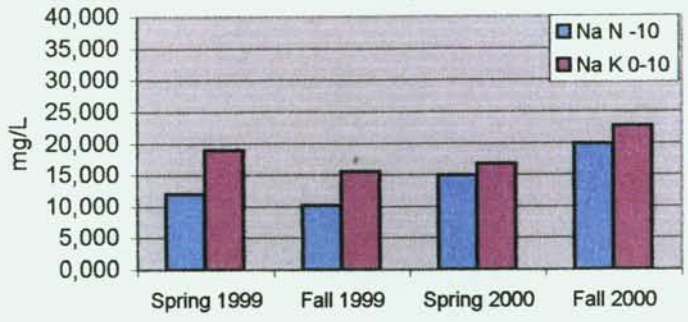


60% 80% 80% 60%

G3

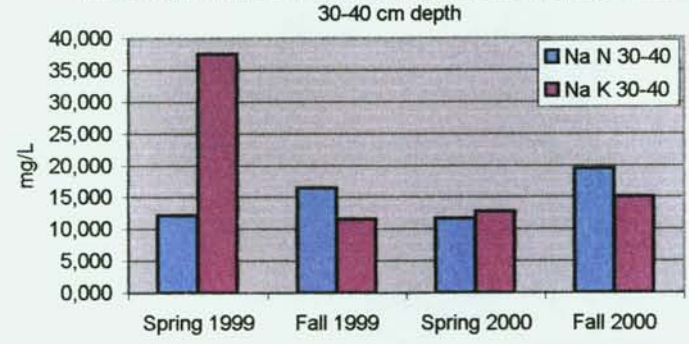
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph .13: Average unbound **Sodium** content of the cytosol of fine mycorhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth



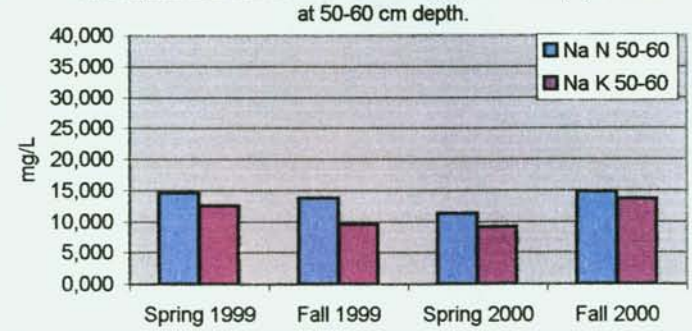
90% 70% NS 60%

Graph .14:Average unbound **Sodium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth



90% NS 70% 80%

Graph .15: Average unbound **Sodium** content of the cytosol of fine mycorrhizal oak root from unlimed (N) and limed (K) forest soil at 50-60 cm depth.

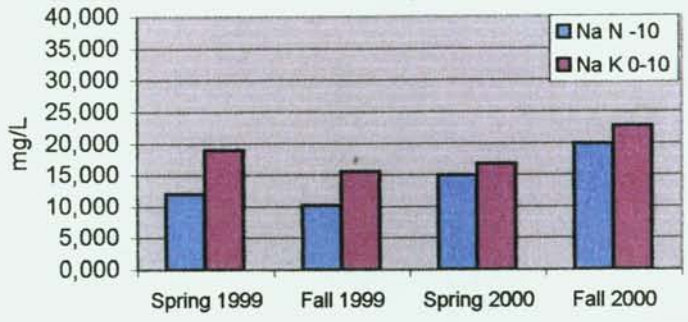


NS 80% 60% NS

G3

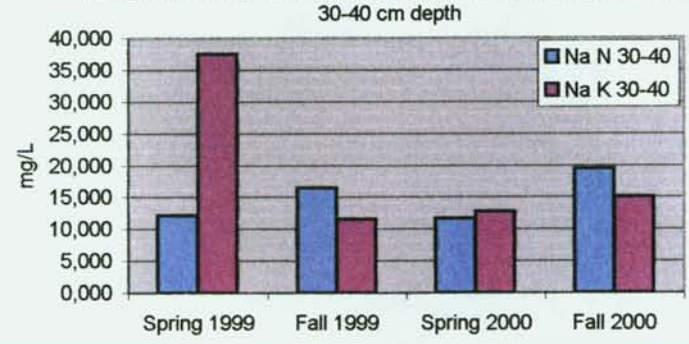
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph .13: Average unbound **Sodium** content of the cytosol of fine mycorhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth



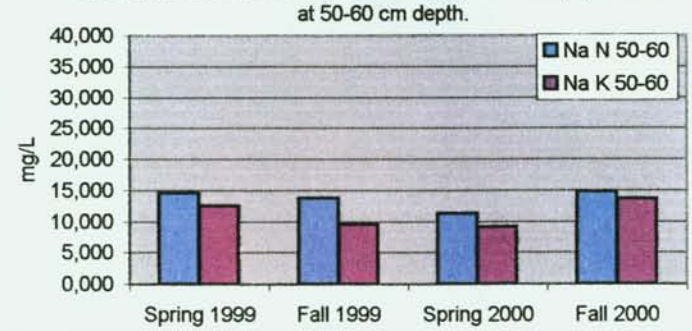
90% 70% NS 60%

Graph .14:Average unbound **Sodium** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth



90% NS 70% 80%

Graph .15: Average unbound **Sodium** content of the cytosol of fine mycorrhizal oak root from unlimed (N) and limed (K) forest soil at 50-60 cm depth.

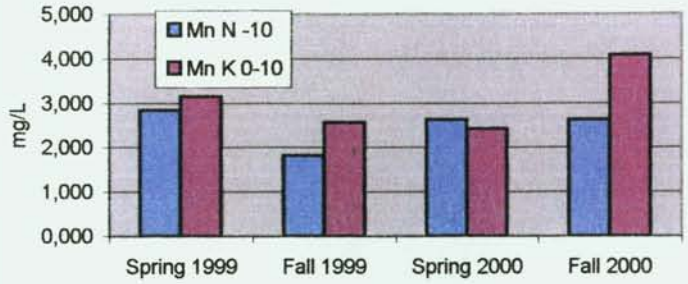


NS 80% 60% NS

G3

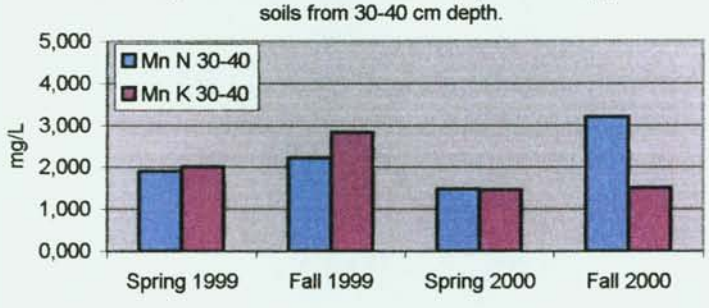
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph . 16: Average unbound **Manganese** content of the cytosol of fine mycorrhizal oak roots of unlimed (N) and limed (K) forest soils from 0-10 cm depth



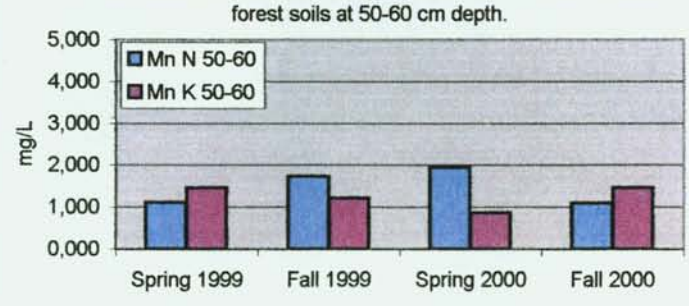
NS 60% NS 60%

Graph . 17: Average unbound **Manganese** content of the cytosol of fine mycorrhizal oak roots of unlimed (N) and limed (K) forest soils from 30-40 cm depth.



NS NS NS 50%

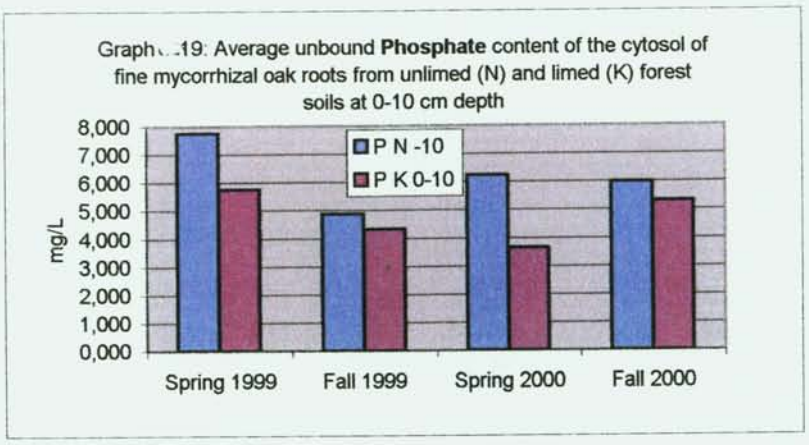
Graph . 18: Average unbound **Manganese** content of the cytosol of fine mycorrhizal oak roots of unlimed (N) and limed (K) forest soils at 50-60 cm depth.



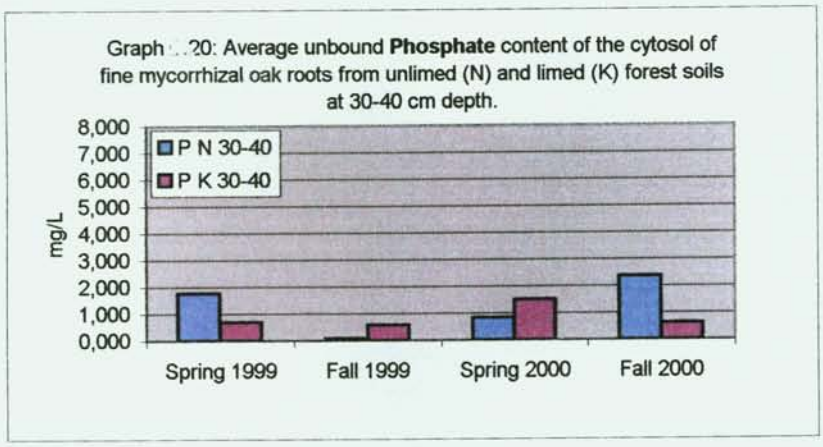
80% 60% 90% 50%

G3

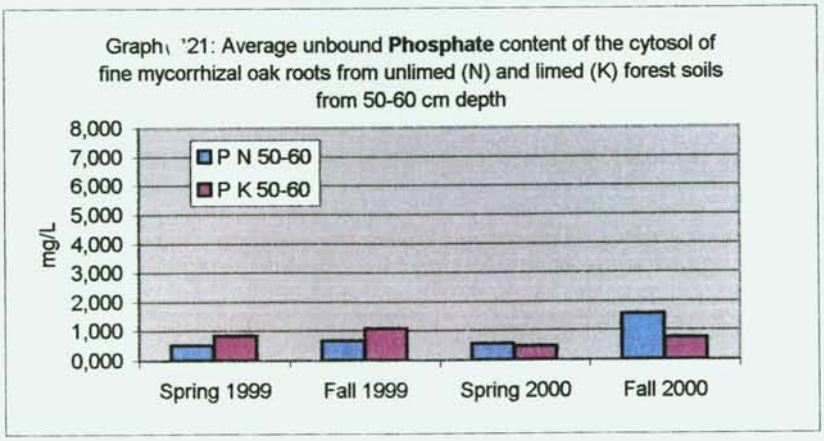
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.



NS 60% NS 60%



NS NS NS 50%

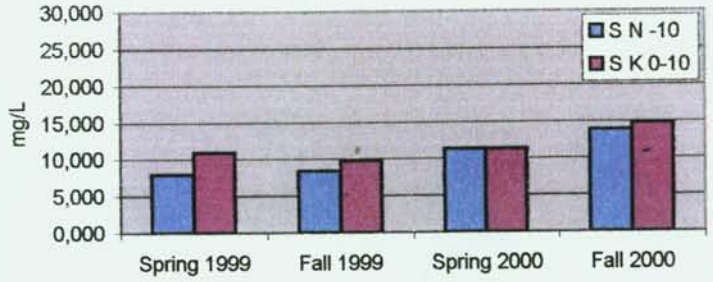


80% 60% 90% 50%

G3

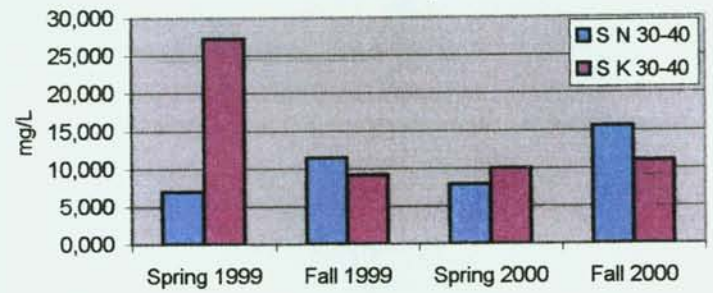
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph 22: Average unbound **Sulfur** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth.



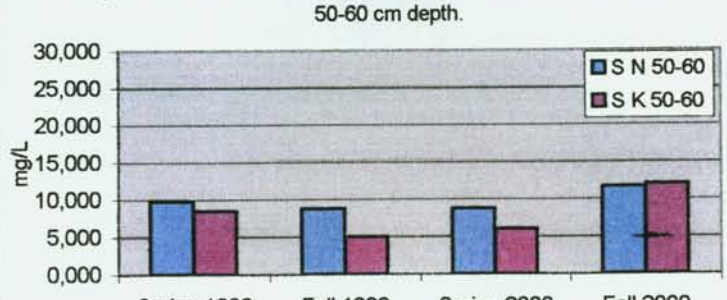
80% NS NS NS

Graph 23: Average unbound **Sulfur** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth



99% 90% 80% 90%

Graph 24: Average unbound **Sulfur** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

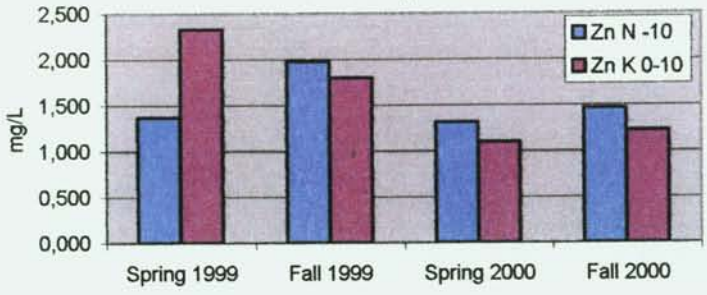


NS 80% 90% NS

G3

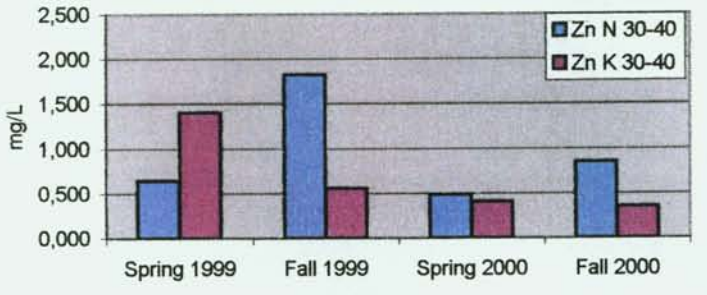
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph . 25: Average unbound **Zinc** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth.



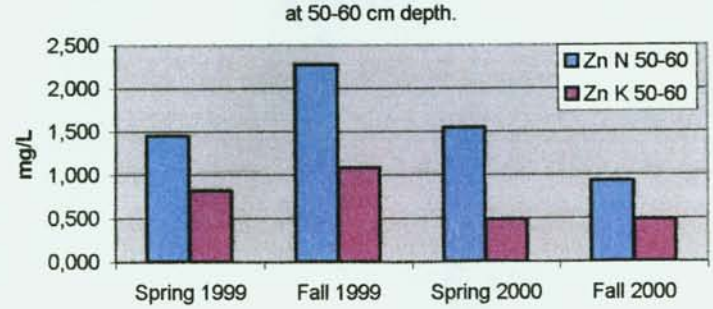
90% 95% NS NS

Graph . 26: Average unbound **Zinc** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth.



50% 90% 70% 99%

Graph . 27: Average unbound **Zinc** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

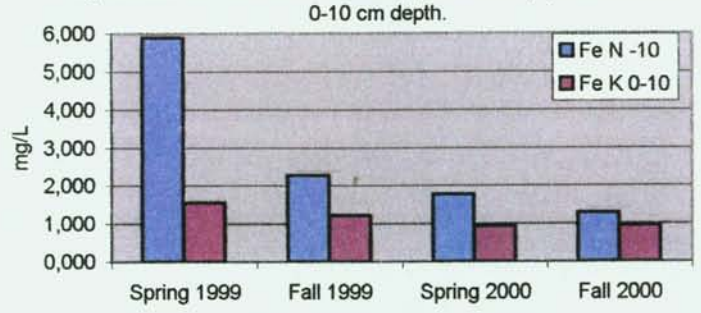


80% 70% 80% 99%

G3

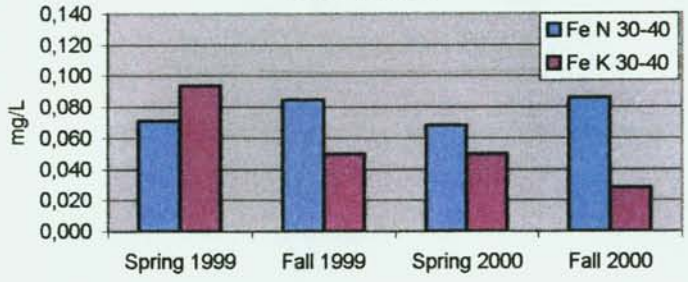
Analysis of unbound root cytosol minerals with % Significant Difference between unlimed and limed samples.
NS = No significant difference.

Graph - 28: Average unbound **Iron** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 0-10 cm depth.



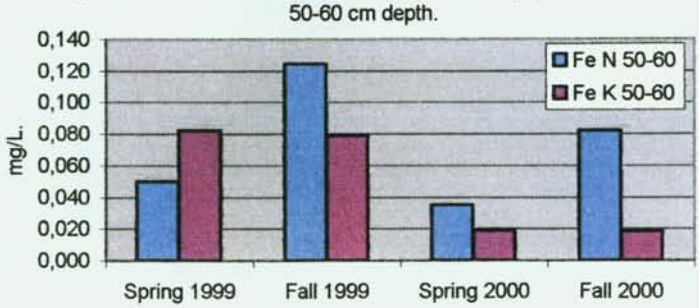
80% 80% 80% NS

Graph - 29: Average unbound **Iron** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 30-40 cm depth.

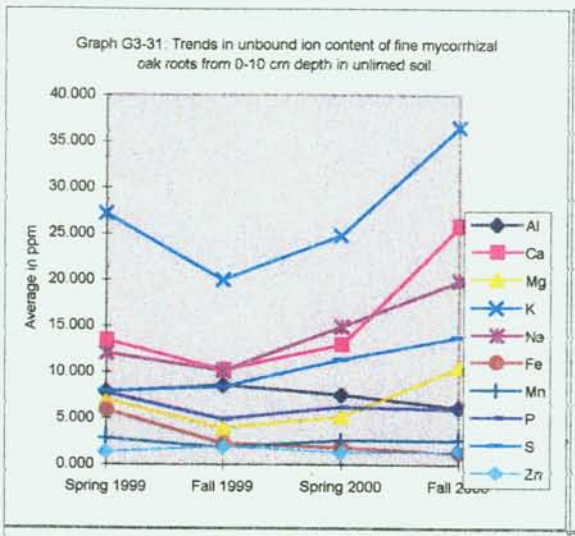


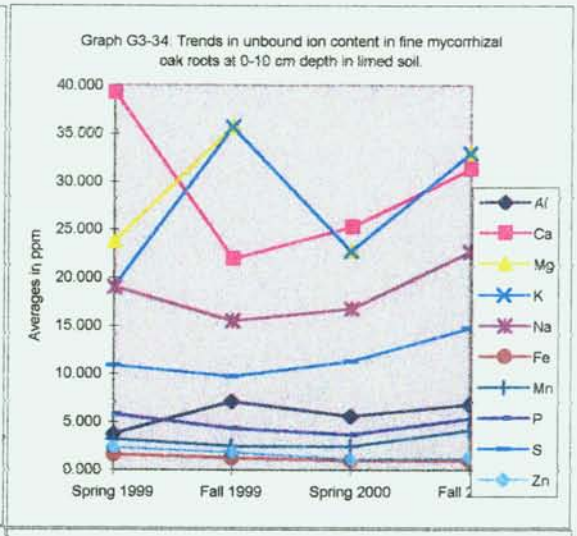
NS 50% NS 60%

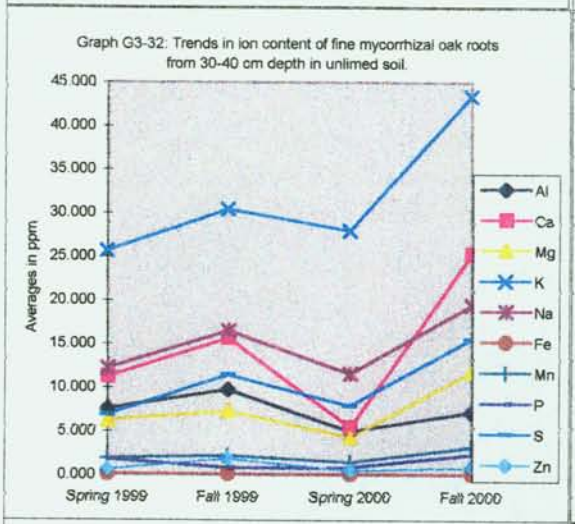
Graph - 30: Average unbound **Iron** content of the cytosol of fine mycorrhizal oak roots from unlimed (N) and limed (K) forest soils at 50-60 cm depth.

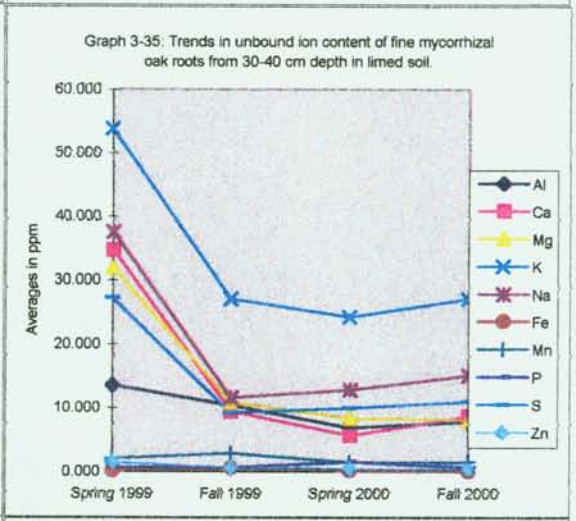


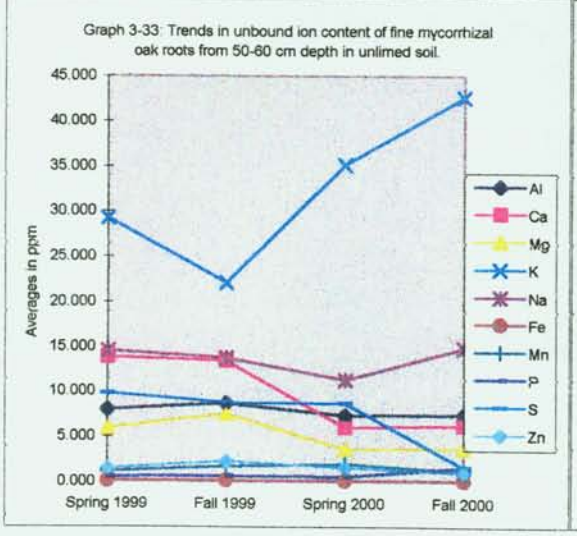
50% NS 70% 70%

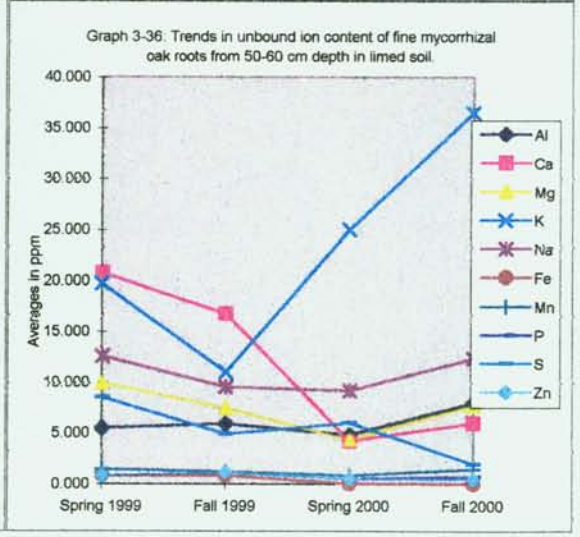












Analysis provided by UKS Merzalben: LUFA Speyer

Section H: Bound and Unbound Mineral Ratios

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Section H: Bound and Unbound Mineral Ratios

H 1: Introduction

In December, 1988, 3 tons per hectar of Dolomite lime was applied to the experimental plot examined in this study (Block, 1999, p.c.). Dolomite lime ($CaMg(CO_3)_2$) consists of equal parts $CaCO_3$ and Mg CO_3 or (approximately 33% Ca, 33% Mg). From past trials (Section H2-1) it is known that the objective of liming is to be high enough to stimulate nutrient uptake (Sections H2-2, H2-3), but low enough in concentration to avoid calcium toxicity (Section H2-4) and reduce Al stress (Section H2-10). From the literature it is known that Ca is involved in many similar physiological processes in plant and fungal cells (Sections H2-5 to H2-9). Unfortunately, knowledge of the actual functioning of the Ca system in specific oak ectomycorrhizae is limited. The precise locations of mineral ions other than Al (Section E) were not determined but potential localizations of Ca and the minerals it affects, based upon information from recent publications, will be reviewed and compared. This particular section deals primarily with bound and unbound Ca ratios (Section H4-1), Ca:Al ratios (Sections H2-9, H4-2), and how Ca affected the binding and uptake of selected nutrients and non-essential minerals. The bound and unbound ratios of the minerals Mg, K, Na, Fe, Mn, P, S, and Zn will be generally discussed.

H 2-1: Past Liming Trials

Acid rain and enhanced atmospheric N deposition are both considered crucial factors in accelerating soil acidification and nutritional disturbances in forest trees (Ulrich et al, 1979, Zoettl & Huettl, 1986). Liming was seen as a useful mitigation tool to stop or reverse anthropogenic influences to alleviate acidification, Al toxicity and Mg deficiencies (Ulrich 1986, Huettl, 1989). Between 1953 and 1965, more than 100,000 ha of southwestern Germany's Black Forest were limed (Aldinger, 1987). The primary goal was to improve the humus form of topsoil layers which had been disturbed by logging and other human activities (Hausser 1958, Schairer 1958, Mitscherlich and Wittich, 1958). Some P and Mg fertilization accompanied the liming, not just with the hope of improving nutrient content, but rather with the idea that Ca, P and Mg would improve cycling of

existing nutrients. In the 1970's most trials were discontinued because the forests not only did not show signs of improved growth but also showed a "new type of forest damage" and "critical questions arose related to the ecological side-effects of liming" (Ulrich, 1972, Evers, 1976, Hildebrand & Schack-Kirchner, 1990, Marschner & Wilcyznski, 1991, Huettl and Zoettl, 1993).

Huettl and Zoettl (1993) determined that analysis of older liming trials indicated that liming generally lead to a long-term decrease in soil acidity, improvement of cation exchange capacity, base saturation, increased content of exchangeable Ca (when dolomite is used, also of Mg) and better Ca/Al (Mg/Al) ratios. However an increase in forest productivity due to a faster turnover of organic matter was not achieved. Furthermore, they determined that liming caused acidification of the subsoil and displacement of heavy metal ions. Liming also appeared to stimulate fine root development in the uppermost soil layers, possibly increasing the danger of frost and drought damage (Huettl and Zoettl, 1993). In trials performed in October 1995, in same Merzalben plot used in this study, liming did stimulate fine root development in the uppermost soil layers (Rommel, 1998). As expected, these fine roots had more Ca and Mg after liming than the unlimed roots, and both ions decreased in concentration with depth. But, possibly because of the small sample size (30 probes), time of year (fall), soil moisture (unknown) or higher pH values (Unlimed pH 4.36 and limed pH 4.62 at 0-10 cm depth) there was no significant difference in bound Al concentration between the unlimed and limed plots, nor were there any clear trends in concentration increases with depth as were observed in this study.

In an extensive series of literature reviews done by Singh (1999, 2000a,b,c) the following observations were made in studies on tree seedlings: (1) decreasing pH over the short term enhanced mycorrhizal formation while alkalization frequently did not. (2) at pH 4 mycorrhizae were very efficient at Ca uptake but as the pH rose the uptake of labeled ions was reduced (3) heavy metals released in acidified soils frequently damaged roots (4) in drought mycorrhizal complements declined (5) in low fertilization mycorrhizal inoculations produced faster colonization and (6) mycorrhizae were very variable in their responses to applied treatments. In addition to these general observations, seasonal cycles

have been observed for mycorrhizae of Norway spruce (Rothe & Vogelei, 1991). As much as is relevant, seasonal variations in mineral content have been noted in this study.

One of the primary observations made in this study was that liming significantly altered the diversity, abundance and distribution of Oak ectomycorrhiae. A shift in ectomycorrhizal community structure as the result of liming was observed in boreal Norway spruce forest (Jonsson et al, 1999). An increase in the mass of mycorrhizal roots in humus, and a general shift to upper soil horizons after liming of Norway spruce, were both attributed to an increase in pH (fungal preference for higher pH), rather than an alleviation of Al toxicity (Notwotny et al, 1998a). However, under conditions of low Ca/Al or (Ca + Mg + K)/Al ratios both non-mycorrhizal and mycorrhizal roots of Norway spruce seedlings are adversely affected and can strongly bind Al, especially within the cortical cell walls (Vogelei & Rothe, 1993). Rost-Siebert (1983) felt that besides lower Ca/Al ratio and low pH (< 4.0), the chemical form of Al (Al^{3+}) also played a part in determining the degree of root toxicity. Eeckhaoudt et al (1992) noted the unquantified presence of Al in the mantle, Hartig net and hyphae of unidentified ectomycorrhizae using Laser microprobe mass analysis. Although Al can enter the root cells (Vogelei & Rothe, 1993), the "free" Al may be quickly neutralized (stored), which may be why visibly undamaged mycorrhizal roots of Norway spruce may contain more Al than visibly damaged trees (Notwotny et al, 1998b). In the mycorrhizal roots of healthy trees, uptake of "free" Al ions may also be compensated for by higher proton excretion rates (Rothe et al, 1987). It has been suggested that Al is not toxic because it is selectively immobilized by some ectomycorrhizal fungi (Martin et al, 1994). This thesis has supported this last contention. This last section will concentrate on the effects of liming on the relationships between the bound and unbound "free" mineral content of mycorrhizal roots.

H2-2: Calcium & Aluminum General Storage Locations

In plants, Calcium is poorly stored in lignified (xylem) and suberized tissues (cork) (Orgeas & Bonin, 1996), but it is stored in large quantities at leaf bases (Martin-Prevel, 1978) and in pectin rich zones within cell walls and in the middle lamella from which it can be moved short distances via the cytosol (Ridolfi et al , 1996). Internal fungal cell

binding sites and vaculolar storage areas have been investigated (Gadd, 1993). Although low in affinity, Ca^{2+} has been located in fungal mitochondria (Carafoli et al, 1970; Gadd, 1993), and the endoplasmic reticulum (Morales and Ruiz-Herrara, 1989, Gadd, 1993; Belde et al, 1993). Calcium may also be stored extracellularily within a polyanionic matrix (root secretion) or as insoluble calcium oxalate crystals (Jellison et al, 1997). The insoluble calcium oxalate crystals associated with brown rot fungi may be waste products precipitated outside the hyphae, tying up the calcium and preventing it from interfering with the normally acidic fungal metabolic functions (Jellison et al , 1997). However, calcium oxalate crystal surfaces, which can bind iron, can also be sites for damaging radical generation (Ghio et al ,1992) so this is not an ideal solution to an excessive calcium concentration. In the root preparation processes, the Calcium oxalate crystals which are adhesive and poorly soluble were most likely retained and if present then will contribute to the bound ion concentrations.

Relative estimates of bound Ca and Al within fungal walls using the microclimatic parameters set up in this study, were not found in the literature. However, in general Al shares many of the same general storage locations often displacing Ca (Section A3-8; Godbold et al, 1988, Stienen and Bauch, 1988, Hüttermann, 1983, Tischner et al, 1983). Middle lamella pectin-rich layers contain anionic carboxylate (COO-) groups that can attract positively charged ions such as Ca^{+2}, Mg^{+2}, H^{+}, and Al^{+3} (Mengel, 1984, Rothe 1993; Nowotny et al, 1998). The Al^{3+} forms are 1000 times better bound to pectin than Ca^{2+} or Mg^{2+} (Rothe, 2004, pc). Mucilage which contains oxalic acid or other cation-chelating secretions can also bind Al (Marschner et al, 1986). Limed roots in this study often contained more Al than unlimed despite the high Ca content. This would indicate that not all the sites are shared and that improved Ca may alter cellular metabolism increasing the number of sites for Al acquisition, rather than just being displaced.

H2-3: Vacuoles and Ion Storage

Fungal vacuoles appear to be a major storage site for extra ions with most of the vacuolar calcium is stored as calcium phosphate (Ohsumi et al 1988, Gadd, 1993). The major storage site for aluminum ions is also phosphate based with the aluminum precipitating

the phosphate out of solution to form granules (Haug, 1984, Manning & Goldberg, 1996, Section A3-9). Energy-dependent transport of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) and elemental P, across the tonoplast channels into vacuoles plays an important role in regulating the cytoplasmic concentration, sequestering potentially toxic ions, and thus allowing normal, controlled, activation of essential metabolic processes (Gadd, 1993).

H2-4: Cytosolic Concentrations

Consistent with eukaryotic cells, divalent calcium is tightly controlled and maintained at a concentration of about 0.1 μM (10^{-7}M) in the fungal cytosol and sequestered in various membrane bound compartments at higher concentrations (Belde et al, 1993, Bush 1993). The Ca^{2+} concentration can fluctuate, (i.e. From 10^{-7} to 10^{-5} M), without significantly affecting cellular ionic balance and at the same time still selectively binding to appropriate substrates (Carafoli, 1987; Rasmussen and Rasmussen, 1990). In general, within fungal vacuoles and the endoplasmic reticulum, calcium can be sequestered at magnitudes three to four times higher than the cytosolic concentrations (Jellison et al, 1997). Vacuolar concentration of calcium in *Saccharomyces cerevisiae* for example can be up to 1.3 mM ($1.3x10^{-3}$M) (Belde et al, 1993). Compartmentalizing the calcium allows growth in abnormally high Ca^{2+} concentrations (i.e. >100 mM $CaCl_2$) (Ohya et al, 1986).

One Mole of Calcium contains 40.08 g/L, so based upon the assumption that mycorrhizae in general have a similar Ca balancing mechanism to those of fungal cells previously studied, then one could estimate the "balanced Ca" cytosol component to contain a minimum of 10^{-7} M = (40.08 $x10^{-7}$grams) = 0.04 mg/L of Ca and a maximum of 1.3 mM = (1.3 x 10^{-3}M) = (40.08 x 1.3 x 10^{-3}g) = 53.04 mg/L. Defects in the vacuolar storage mechanism can lead to serious metabolic lesions (Ohya et al, 1991). As long as the mg concentrations of cytosolic (unbound) Ca remain below 53 mg/L, the problem of Ca induced lesions and leakage damage would be unlikely. In the roots used in this study the unbound Ca component was below 40.000 mg/kg = 40 mg/L (Graphs G3-4 to G3-6) and the bound Ca was below 10.000 mg/kg = 10 mg/L (Graphs F3-4 to F3-6), so no Ca

damage should have been evident. Any damage visible then would have been due to Al which was the most prevalent ion present.

H2-5. Calcium Diffusion

By sensing of the environment, receptors that control membrane channel proteins can allow Ca^{2+} to enter (Harold, 1991, c.f. Chapter 13). Passive influx of Ca can occur through ligand or voltage-gated channels, allowing the ion to enter along its electrochemical gradient (Pietrobon et al, 1990). This influx can be stimulated by intracellular acidification associated with glucose uptake (Eilam et al, 1990) or stretch-activated. Stretch-activated Ca^{2+}, K^+, and Mg^{2+} channels have been described in *Saprolegnia ferax* (oomycete) hyphal tips (Garrill et al, 1992,1993). Calcium can also enter the fungal cell via a general active cation carrier transport system with electroneutrality maintained by efflux of H^+ ions, which may result in opening of the passive Ca^{2+}- activated K^+ (voltage gated) channels for the influx of additional cations (Fuhrmann and Rothstein, 1968), or in other words, indirectly drive the passive uptake system for Al entry into the cytosol.

H2-6: Calcium Transport

Although Ca is generally considered to be not readily soluble in water it can be moved slowly within the hydrological system and once within the vicinity of a fungal hypha it can be transported. Connolly and Jellison (1995) found that *Resinicium bicolor* (brown rot fungus) translocated Ca several centimeters away from red spruce wood blocks along mycelial cords climbing up the sides of a glass jar implying internal transportation. No studies have apparently been done to actually demonstrate labeled ion movement. Most, have depended upon transport implied by relative changes in ion concentration. Like Al, translocation from the roots to the leaves has been implied but not demonstrated.

H2-7: Calcium Export

Because cytosolic free Ca^{2+} is potentially toxic (largely because of its ability to bind phosphate groups, particularly those of ATP, ADP and AMP) fungal cells possess several

mechanisms for maintenance of low Ca concentrations (Gadd, 1995, p184). Besides vacuolar sequestration, internal Ca $^{2+}$ levels are kept low by continuous Ca $^{2+}$ expulsion via Ca $^{2+}$/ H^+ antiports (Belde et al, 1993). The antiports are coupled to the transmembrane pH gradient by plasma membrane ATPase (Strooband & Scarbourgh, 1979). Both Ca dependent ATPases and Na/Ca exchangers are found on the membranes of animal and plant cells (Belde et al, 1993) however, the P-type Ca^{2+}ATPases are thought to be most important in maintaining the low cytosol Ca^{2+} concentrations (Bush, 1993). Oxalic acid produced and secreted by fungi can readily precipitate Ca ions externally forming insoluble Ca-oxalic acid crystals depending upon the thermodynamics of the microenvironment (Connolly & Jellsion, 1995). Since Al and Ca share similar binding sites, it is possible they have some of the same exclusion processes. Extrusion of anions can reduce cation uptake (Godbold et al, 1988), and thus toxicity symptoms.

H2-8: Calcium Physiology - A Secondary Growth Signaler

Calcium from stores can be mobilized by signaling systems. A signal transduction cascade can begin with a cell receptor binding to a ligand at the plasma membrane, which in turn activates G-proteins and releases phosphoinositides which release Ca from intracellular stores (Jellison et al, 1997). In this system, Inositol 1,4,5-trisphosphate, produced by the catabolism of inositol lipids, can act as an agonist to release Ca from intracellular storage, and may even promote influx from the external bathing solution (Berridge 1993), afterwhich the inflowing Ca ions bind to calmodulin. (CaM) (called such because it binds and modulates calcium). In combination with another hydrolysis product, diacylglycerol, a sequence of events can be synergistically triggered to promote growth and differentiation (Berridge, 1993). In brown rot fungi, secretion of cellulolytic (wood digesting) enzymes requires calmodulin activation (Highley, 1989).

Once the Calmodulin is activated, it can react with protein kinases, phophatases, adenylate cyclases, Ca^{+2}-ATPases and phophodieseterases (Pietrobon et al , 1990, gg188) and in doing so, regulate cell proliferation, cell cycle and nuclear division (Anraku et al, 1991). Calcineurin is a phosphatase that is regulated by Ca^{+2}-CaM, with several of its substrates

regulating other phosphatases and kinases *in vitro*, the implication is thus that Ca plays a role in the mediation of protein phosphorylation in response to Ca^{+2} fluctuations (Cyert and Thorner, 1988, Hiraga et al, 1993). Al is known to interfere with the Ca-calmodulin complexing system (Haug 1984) but the precise mechanism for this interference is unknown. Despite this, Al toxicity syndrome appears to be directly related to the Ca-Al interactions that disturb Ca homeostasis (Rengel, 1992, Delhaize & Ryan, 1995). High Al concentrations are known to reduce Ca root content in *Fagus sylvatica* (Bengtsson, 1991).

In mutant strains of *Saccharomyces cerevisiae* which had an impaired Ca^{+2} - CaM binding, the cells were still capable of good growth (Geiser et al, 1991), whereas disruption of the calmodulin gene resulted in the loss of cell proliferation ability (Davis et al, 1986, Ohya and Anraku, 1988). Which means that the calmodulin is first in importance with Ca acting only as a replaceable secondary regulator or co-factor. But even so, a tiny Ca influx can act as a powerful secondary signaler. It is quiet possible that where Al is a normal nutrient at low concentrations, it may replace Calcium as a co-factor and in higher concentrations it may inhibit the systems by interfering with the gene action, but further research would be needed to confirm this.

There is also evidence that cytosol-free Ca^{2+} operates as a messenger in light-mediated regulatory processes such as stomatal closure and photosynthesis (Ridolfi et al , 1996). In healthy oak leaves the % concentrations of N, K, Ca and Mg were higher than in unhealthy leaves (Simon and Wild, 1996). In healthy oak leaves, Al concentrations were lower than in unhealthy leaves (Section A3-8-3).

H2-9: Calcium Physiology - Cytoskeleton Regulation

Internally, cytosol-free Ca^{2+} can be bound by the protein Calmodulin, which can then regulate actin functions in cytoskeleton formation (Brockerhoff & Davis, 1992). There is also evidence of regulation of fungal actin-myosin interactions, which affects cytoskeletal flexibility, through the binding of Ca to tropomyosin proteins (Liu and Brescher, 1989b, 1992). With increased cytoskeleton flexibility, modifications of the membrane surface

can occur. In the VAM fungi *Zoophthora radicans*, symbiotic appressoria membrane formation occurs especially at presumptive sites where the Ca^{+2}-CaM binding concentrations are highest on the adjacent membranes (Magalhaes et al, 1991). High concentrations of free cytoplasmic Ca have also been seen in some fungal tips and so because of this circumstantial evidence and the known calmodulin and tropomyosin binding sites, it is assumed that the Ca^{2+} influences on hyphal growth are therefore likely to be cytoskeleton mediated, affecting both extension (Jackson & Heath, 1993b) and branching (Robson et al, 1991a,b). Calcium is generally considered to be a membrane stabilizer/regulator but its role in hyphal tip growth of basidiomycete fungi has yet to be confirmed (Jellison et al, 1997).

In summary, homeostatic control is essential since Ca has a wide range of secondary triggering activities, all based upon its ability to bind with phosphate groups and proteins at very low concentrations. At excessively low concentrations, Ca deficiencies (or displacements by Al ions) can lead to some serious metabolic problems and poor growth. At very high concentrations it can bind P groups, effectively inhibiting metabolic interactions and energy accessibility.

H2-10: Ca:Al & BC:Al Ratios & Stress Risks

In soil, Ca/Al quotients of 1 or less indicates a degraded soil. Depleted Ca levels and elevated Al availability set the stage for inherent Al-toxicity (Abrahamsen, 1983). In comparing soil leaching values, a Ca/Al quotient of less than 1 implies loss of soluabilized, potentially toxic Al, to the dissipating soil water (Moreno et al, 1996) which can negatively affect deeper roots. This soluabilized Al, now in the free ion form can potentially bind with cell wall components or enter the cell cytosol and interfere with normal metabolic activities. According to Cronan & Grigal (1995) Al stress risks increase as the Ca/Al (Ca^{+2}/Al^{3+}inorganic [mol $\cdot mole^{-1}$]) ratios drop in the soil *and within the fine roots* (Table H2-9). Toxicity symptoms can occur when ratios of Ca/H $\leq$ 0.1 (Boudot et al, 1994) or when Ca/Al $\leq$ 0.1 (Cronan & Grigal, 1995) or when (K + Mg + Ca): Al $\leq$ 1 (Swerdrup & Warfvinge, 1993).

The BC/Al ratio [Base Cations (Ca + Mg + K):Al] can also be used as an indicator of potential risk. In controlled lab experiments and from a literature search, Svedrup and Warfvinge (1993) determined the critical BC:Al ratios for various tree and plant species basing their critical risk ratio upon a >80% reduction in root growth. *Quercus petraea* was not listed, but for *Quercus robur* and *Fagus sylvatica*, tree species that share very similar habitats, the high risk ratio for Al stress was 0.6 and for *Fagus sylvatica*, the slight risk ratio was 1.5, and middle risk ratio was 0.8. The BC/Al ratio of 0.6:1 usually refers to soil solutions (BELF, 2000). In this case we are examining fine mycorrhizal roots rather than soil but since no data is available concerning the roots themselves, the assumption will be made that the BC/Al ratios must be related. For the intent of this report, BC/Al root ratios below 0.6 will be considered high risk ratios for Al stress. In reality, due to compensation, the true risk ratio is possibly << 0.6.

Table H 2-10: General Aluminum stress risk assessment based upon the Ca:Al ratio in the forest ecosystem *soil solution*, and in *fine roots* as derived from Cronan and Grigal (1995)

Forest Soil Solution Ca/Al	Risk	*Fine roots* Ca/Al	Risk
<0.2 ± 0.1	100%	0.1	80%
0.5 ± 0.25	75%	0.2	50 %
1.0 ± 0.5	50%		

According to Schröck (2003, pc) of the Tripptstadt Forestry Department, in the unlimed forest zone, since 1998, 15 trees have died, while in the limed zone only 3 have been lost. In a survey of the crown structures the limed trees have experiences an average thinning of 22% while the unlimed oaks have lost 35% of their leaves over the same time period. The question that arises then is how are the bound and unbound Al and other mineral contents within the roots related to these losses. ***For the purposes of this report, a risk ratios of BC:Al ≤ 0.6 and Ca:Al ≤ 0.1 will be used as critical points for the fine roots.***

H 3: MATERIALS & METHODS

Refer to the Sections B, E, F & G for details concerning mycorrhizal root extraction, preparation and mineral analysis. Using statistical analysis (Appendix 9), the mineral readings for each individual probe were compared and the individual bound (Appendices 10b, 10c & 10d) and unbound (Appendices 11c, 11c & 11d) mineral ratios (Appendices 11e & 11f) were calculated. The bound and unbound Ca:Mineral ratios (Table H4-1B) and Base Cation: Al ratios (Table H4-2D) of fine mycorrhizal root tips at each soil horizon for the time period from spring 1999 to fall 2000 were averaged and statistically analyzed to produce the % significant differences between the unlimed and limed probes.

H4: RESULTS

The basic data sets for bound (Appendix 10) and unbound (Appendix 11) minerals were combined to produce Bound Calcium:Mineral ratios (Appendix 10D) and Bound:Unbound mineral ratios (Appendix 11F). Unlimed and limed probes were compared. The relationship between the averaged bound and unbound Calcium content is provided in Table H4-1A with a summary of the bound and unbound Ca:Mineral ratios provided in Table H4-1B. A summary of the BC:Al ratios is presented in Table H4-2D. A graphic summary of the bound (Graphs F3-37 to F3-42) and unbound (Graphs G3-43 to G3-36) minerals was previously presented to demonstrate relative abundance. Bound and unbound Ca:Mineral and Mineral:Mineral ratios will be individually discussed.

H4-1: CALCIUM

H4-1A: Bound and Unbound Calcium Ratios

In both the unlimed and limed plots, the bound Ca content declined with depth. In the unlimed forest the loss was relatively minor (6.5-17 %) with the Ca B:U (bound:unbound) ratios remaining fairly stable (232-240):1. In the limed forest however, the loss with depth relative to the A Horizon was much larger (37-48 %) with destabilized bound : unbound ratios (233-403):1. Despite the fact that the Ca content in the limed probes was nearly double that of the unlimed, at 0-10 cm depth where the greatest concentration of mycorrhizal roots were found, the B:U calcium ratio was nearly identical. This comparison did not hold for the deeper soil probes. At 30-40 cm depth,

the unbound Ca levels in the limed probes dramatically fell, often to levels below those of the untreated forest probes. Looking at the total unbound Ca in the limed probes, the average (translocatable) was about 20% greater than in the unlimed probes (Table H4-1A). Despite the huge difference in Ca content of the limed soil, most of the excess was bound in the mycorrhizal roots and only a very limited amount was actually freed in unbound form to potentially interact and affect the physiology of the roots or potentially be translocated to other growing regions. As will be seen shortly, the additional Ca did affect the concentrations of other minerals but rarely (2 readings) exceeded the expected maximum (53 mg/L) discussed in the introduction to this section.

Table H4-1A: Average bound and unbound Calcium. Content of fine mycorrhizal roots from unlimed and limed forest soil at various depth from spring 1999 to fall 2000.

Depth	Unlimed Forest			Limed Forest		
	Bound	Unbound	Ratio	Bound	Unbound	Ratio
0-10 cm	3857 mg/kg	16.6 mg / L	232:1	6810 mg/kg	29.2 mg / L	233:1
30-40 cm	3607 mg/kg	15.0 mg / L	240:1	4276 mg/kg	10.6 mg / L	403:1
50-60 cm	3212 mg/kg	9.5 mg / L	238:1	3554 mg/kg	11.6 mg / L	306:1
Total Ave.	3558 mg/kg	13.7 mg / L	236:1	4880 mg/kg	17.1 mg / L	314:1

H4-1B: Calcium : Mineral Ratios

From the data analysis it was determined that the higher the Ca:Mineral ratio was, the more Ca was present relative to the mineral. If the ratio fell below 1:1 then the mineral was more prevalent than the Ca. Once the Ca concentration reached threshold, further Ca uptake did not necessarily occur and so the ratios varied then as the comparative mineral content changed. In addition, if a mineral was directly affected by Ca concentration, then the Ca:Mineral ratio stabilized but did not necessarily reflect actual changes in concentration which equally rose and fell with no ratio change. In addition, a high Ca: Mineral ratio did not mean that the average total content of the mineral was lower in one region than in another. For these reasons comparisons between the mineral ratios and average total mineral content will be discussed. Table H4-1B is a summary of the

averaged data for the two year period studied. Each mineral will be discussed individually with heavy emphasis on the Ca:Al ratios.

Table H4-1B : Comparison of two-year averaged bound and unbound Ca : Mineral ratios. SD = % Significant Difference between the unlimed and limed Ca:Mineral ratios. All the values are presented but only values ≥ 95% are considered highly significant. NS = No Significant Difference (< 50%). C = Concentration comparison (2 year average) where the dark tone square represents the sample with the higher overall mineral concentration.

		Bound Mineral Ratios						Unbound Mineral Ratios				
	Depth	Unlimed	C	Limed	C	SD		Unlimed	C	Limed	C	SD
Ca/Al	0-10 cm	0.8:1		1.2:1	■	80%		4.3:1	■	7.8:1		70%
	30-40 cm	0.3:1		0.4:1	■	NS		16.7:1		1.4:1	■	60%
	50-60 cm	0.2:1	■	0.3:1		60%		1.5:1	■	3.6:1		70%
Ca/Mg	0-10 cm	3.9:1		3.4:1	■	70%		2.3:1		1.8:1	■	99%
	30-40 cm	4.2:1		2.7:1	■	99%		2.0:1		0.9:1	■	99%
	50-60 cm	3.7:1		2.6:1	■	90%		1.9:1		1.4:1	■	95%
Ca/K	0-10 cm	3.0:1		5.0:1	■	80%		0.8:1		1.3:1	■	99%
	30-40 cm	2.5:1	■	3.0:1		50%		0.5:1	■	0.4:1		60%
	50-60 cm	2.4:1		2.1:1	■	60%		0.4:1	■	0.9:1		80%
Ca/Na	0-10 cm	17.2:1		27.2:1	■	95%		1.1:1		1.6:1	■	99%
	30-40 cm	16.5:1		17.1:1	■	NS		0.8:1		0.6:1	■	70%
	50-60 cm	13.5:1	■	16.7:1		80%		0.7:1	■	1.0:1		80%
Ca/Fe	0-10 cm	1.5:1		1.8:1	■	NS		34.1:1	■	54.6:1		80%
	30-40 cm	2.8:1		2.8:1	■	NS		434.4:1	■	280.5:1		70%
	50-60 cm	2.7:1		1.8:1	■	80%		1639.2:1	■	394.3:1		60%
Ca/Mn	0-10 cm	10.5:1		15.2:1	■	90%		8.7:1		14.2:1	■	90%
	30-40 cm	11.3:1	■	11.8:1		NS		9.2:1	■	7.0:1		NS
	50-60 cm	9.7:1	■	15.0:1		90%		7.6:1	■	9.8:1		NS
Ca/P	0-10 cm	4.5:1	■	9.3:1		99%		4.1:1	■	7.3:1		99%
	30-40 cm	6.0:1	■	7.0:1		50%		19.8:1	■	16.9:1		NS
	50-60 cm	5.9:1	■	6.4:1		60%		17.3:1	■	15.5:1		50%
Ca/S	0-10 cm	2.8:1	■	5.6:1		99%		1.6:1		2.7:1	■	99%
	30-40 cm	4.3:1		4.8:1	■	60%		1.4:1		0.8:1	■	80%
	50-60 cm	3.8:1	■	4.7:1		90%		1.1:1	■	1.8:1		60%
Ca/Zn	0-10 cm	38.0:1		51.0:1	■	90%		14.1:1		22.1:1	■	98%
	30-40 cm	23.0:1		26.0:1	■	60%		16.2:1	■	20.5:1		NS
	50-60 cm	24.0:1		26.0:1	■	NS		7.4:1	■	19.9:1		80%
Ca/Cd	0-10 cm	6343.0:1		8983.0:1	■	95%						
	30-40 cm	2367.0:1	NS	3612.0:1	NS	NS						
	50-60 cm	2823.0:1		3470.0:1	■	80%						
Ca/Pb	0-10 cm	48.0:1	■	86.0:1		99%						
	30-40 cm	322.0:1	■	311.0:1		NS						
	50-60 cm	451.0:1		362.0:1	■	70%						

H4-2: ALUMINUM

H4-2A: Bound Calcium : Aluminum Ratios

Aluminum was the most common bound mineral, usually followed by Ca (Graphs F3-37 to F3-42). According to the statistical analysis, the Al content of the unlimed and limed roots, at 0-10 cm depth, was not significantly different in spring 1999, fall 1999 and fall 2000, even though it was visibly more abundant in the fluorescence observations (Section E) and frequently higher in the mineral analysis (Section F) in the limed roots. The Al content was however highly significantly (> 95%) different in spring 2000 (Appendix 10B-2). Cumulatively , at 0-10 cm depth (Appendix 10D-1), when the bound Al content for *all seasons* and *all probes* was calculated and analyzed, there was found to be a highly significant difference (>96%) between the limed probes (Averaged total = 8504 mg/kg Al) and the unlimed probes (Averaged total = 6758 mg/kg Al).

At 0-10 cm depth, in the limed samples, bound Ca:Al ratios ranged from 0.25:1 to 6.1:1 with an average ratio of 1.2:1 with 52 % of the samples dropping below 1:1 with 38% falling below 0.6 into the high risk category. In the unlimed probes, the ratios ranged from 0.16:1 to 3:1 with an average ratio of 0.8:1 with 77% of the samples dropping below 1:1 and with 68% falling below 0.6 into the high risk category for Al stress. None of the probes tested went below the critical Ca:Al value of 0.1 (Appendix 10D-1). Despite the dramatic tendencies, there was enough variation to render the differences less than 80% significant.

At 30-40 cm depth (Appendix 10D-5), in the limed samples, bound Ca:Al ratios ranged from 0.09:1 to 4.4:1 with an average ratio of 0.4:1 with 96 % of the samples dropping below 1:1, and with 88% below 0.6 in the high stress risk category. In the unlimed probes, the ratios ranged from 0.04 to 1.3:1 with an average ratio of 0.3:1 with 92 % of the samples dropping below 1:1 and with 88% in the high Al-stress category. In the unlimed probes, 5 samples, and in the limed probes, 2 samples, went below the critical Ca:Al value of 0.1 (Appendix 10D-5). Even though the average bound Al content was not significantly different in the limed probes (16289 mg/kg) relative to the unlimed probes (15420 mg/kg) at 30-40 cm depth, it was about double that of the probes at 0-10 cm depth.

At 50-60 cm depth (Appendix 10D-9), in the limed samples, bound Ca:Al ratios ranged from 0.09:1 to 1.1:1 with an average ratio of 0.08:1 with 96 % of the samples dropping below 1:1, and with 92% in the high stress risk category. In the unlimed probes, the ratios ranged from 0.06:1 to 0.6:1 with an average ratio of 0.2:1 with 100 % of the samples dropping below 1:1, and with 96% below 0.6 in the high risk category. In the limed probes, 5 samples and in the unlimed probes, 8 samples dropped below the critical Ca:Al ratio of 0.1 (Appendix 10D-9). The average bound Al content was lower in the limed probes (15220 mg/kg) relative to the unlimed probes (15573 mg/kg), but the values were not significantly (<80%) different. The concentrations at this depth were about double that of the 0-10 cm depth probes but approximated that of the 30-40 cm depth samples. Despite the similarity to the 30-40 cm depth samples, fewer mycorrhizae were present and more fine root samples dropped below the critical Ca:Al ratio at 50-60 cm depth. Relative to the deeper probes, the mycorrhizal presence at 0-10 cm depth was associated with lower Al sequestration, despite higher soil acidity implying either bioactive Al blockage or greater translocation or both.

H4-2B: Unbound Ca:Al Ratios

Despite being the most common mineral in the bound forms, Al in the unbound form ranked 6th (L) or 7th (N) in abundance in the cytosol at 0-10 cm depth and 5th (L&N) in deeper soils, relative to the other minerals (Graphs G3-31 to G3-36). At 0-10 cm depth (Appendix 11C-1), in the limed samples, unbound Ca:Al ratios ranged from 1.5:1 to 73:1 with an average ratio of 8:1 with no samples falling below 1:1. In the unlimed probes, the ratios ranged from 0.4:1 to 32:1 with an significantly different average ratio of 4:1, with about 29% of the samples falling below 1:1 and with only 4% below 0.6 into the high risk category. In the limed probes, the average free Al content was 0.087% relative to the total amount of bound Al, and despite the differences in Ca content, the free Al in the unlimed probes averaged 0.088%. This 0.001 % difference in volume translates into a significantly higher average total soluble Al content in the unlimed probes (7.4 mg/L) than in the limed (6 mg/L) probes. *The enhanced presence of Calcium improved Al sequestration (binding) within the mycorrhizal and root walls, but effectively lowered the total Al cytosol content*

at 0-10 cm depth. At this depth, where the majority of roots were found, this reduction in total aluminum in the cytosol was a significant event.

At 30-40 cm depth (Appendix 11C-4), in unlimed soil, the unbound Ca:Al ratio ranged from 0.4:1 to 6:1 with one aberrant reading of 374:1 resulting in an average ratio of 17:1 with a average mean of 1.7:1 which is closer to reality. Nearly 36 % of the probes fell below 1:1, while 24 % fell below 0.6 into the high stress range. In limed soil, the ratio ranged from 0.4:1 to 5:1 with an average of 1.4:1, with 44 % less than 1:1 and with 22 % falling below the 0.6 range. The average Ca:Al ratio was therefore lower in the unlimed than in the limed probes. Despite this, the average total unbound Al content was also lower in the unlimed probes (7.4 mg/L) compared to the limed (8.5 mg/L) probes. In the unlimed probes, the average free Al content was 0.048 % and in the limed probes it was 0.052 % relative to the total bound Al. On one hand, the lower unbound Ca:Al ratio implies greater Al-stress but on the other hand the total unbound Al content was also lower implying reduced stress (due to translocation?) in the unlimed relative to the limed probes. Considering the dramatically reduced mass of mycorrhizal roots at this depth relative to 0-10 cm depth, the overall Al uptake (translocation) was probably relatively small and the overall differences between the limed and unlimed probes were negligible.

At 50-60 cm depth (Appendix 11C-7), in unlimed soil the Ca:Al ratio ranged from 0.4:1 to 4:1 with an average of 1.5:1, with 36 % below 1:1 and with 18% below 0.6 in the high risk category. In limed soil, the range was 0.15: 1 to 40:1 with an average of 3.5:1, with 42 % below 1:1 and with 13 % below 0.6 in the high Al-stress category. The total unbound Al content was highest in the unlimed probes (7.8 mg/L) relative to the limed probes (6 mg/L). In the unlimed probes, the free Al content was 0.050 %, while that of the limed probes was 0.039 % relative to the total bound Al. The differences between the unlimed and limed probes were found not to be significant. Compared to 30-40 and 0-10 cm depths, the root biomass at 50-60 depth was extremely small, and so while the free cytosolic Al per Liter of fluid is higher, the total fluid content of the roots at this depth is minimal and so not of critical importance. It is interesting to note that, in the absence of

extensive mycorrhizal coverage, the roots bind Al very effectively reducing total free cytosolic content. The cortex and the xylem formed the strongest binding areas.

None of the probes from any soil depth or soil source fell below the critical Ca:Al value of 0.1 (Appendix 11C).

H4-2C: Bound Al : Unbound Al Ratios

The averaged ratios of bound to unbound aluminum (Appendix 11F-1) varied from about 582:1 to 3309:1, (averaging 1707:1) in the unlimed probes and ranged from 882:1 to 3623:1, (averaging 1941:1) in the limed probes. The differences between the limed and unlimed samples at 0-10 cm depth were 90% significant, at 30-4 cm there was a 95% significant difference, but at 50-60 cm depth there was no significant difference in the bound Al: unbound Al ratios. Overall, more Al was bound in the limed probes and more Al was free in the unbound probes in the unlimed probes at 0-10 and 50-60 cm depths, but the reverse was true at 30-40 cm depth. It is important to note that at 30-40 cm depth the biomass in the limed soil plot was severely reduced, with a shift to the upper soil horizon.

H4-2D: Base Cation : Aluminum Ratios

The *unbound* base cation: aluminum ratio (BC:Al) (Table H4-2D), within the cytosol was well above estimated critical level 0.6 (Section H2-10), enough for maintenance of existing internal cell integrity at all depths in both forest zones. The ratio of base cations to Al in the *bound* form was less certain with the limed probes fairing significantly better than the unlimed at 0-10 cm depth, but both were still above the estimated value critical (0.6) for growth. In general observation and actual mycorrhizal quantification (Section B) the root biomass at 0-10 cm depth was the most abundant. In deeper soils (30-40, 50-60 cm) the roots from both plots were similarly lacking in sufficient base ions, with ratios well below 0.6, to maintain good root growth. This data supported the general observation of greatly reduced root biomass and reduced mycorrhizal presence at these depths (Section B).

Table H4-2D: The bound and unbound base cation ratios : (Ca^{2+}+ Mg^{2+} + K^{+}) : Al^{3+} [mol ·mol^{-1}] for unlimed and limed fine mycorrhizal root probes from various soil depths as averaged over a 2 year period from 1999 to 2000.

	Unlimed Roots	Limed Roots
Depth	Bound BC:Al	Bound BC:Al
0-10 cm	0.947	1.273
30-40 cm	0.400	0.416
50-60 cm	0.382	0.458
	Unbound BC:Al	Unbound BC:Al
0-10 cm	7.013	12.394
30-40 cm	7.402	5.980
50-60 cm	6.058	7.144

H4-2E: Summary of Al Ratios

A homeostatic balance was effectively maintained by the roots with the mycorrhizae acting as barriers to Al, lowering total access to binding points (but binding up to an average of 8500 mg/kg of Al) while also controlling systemic uptake. While the mycorrhizal-free roots lacked the ability to block the Al from entering the root walls, once it was there, the root cells very effectively bound it (up to about an average of 16500 mg/kg) within the cellulosic matrix of the cortex and the lignified xylem walls. In both cases, with or without mycorrhizae, the end result was an extremely low content of free Al within the cytosol. Different mechanisms, similar end results, with the end - cytosolic Al content varying between 6 to 8 mg/L.

The heavy accumulation of Al within the walls especially in deeper soils was associated with poor root growth and lower mycorrhizal abundance and diversity. So while the transportable, cytosolic Al was tightly controlled, the roots, especially the mycorrhizal roots performed defensive duties with the cortical tissues and the xylem acting as backup systems for the sequestration of Al which breached the mycorrhizal barriers. Liming affected Al sequestration and translocation primarily in the 0-10 cm depth horizon where it enhanced binding within the cellulosic matrix and thus reduced total Al within the

cytosol by approximately 1 mg/L. Understanding that the greatest total biomass of roots occurs at this depth, this is a significant reduction in potentially *translocatable* Aluminum. In deeper soils, the amelioration effects of Calcium were lost.

H4-2F: Bound vs. Unbound Aluminum - Discussion

The current theory holds that as long as the Ca:Al ratio is greater than 1 ± 0.5, there should be no signs of Al toxicity. Although the focus of this study was not on the indicators of Al toxicity but rather on the presence and location of the Al ions, it needs to be noted that, in both forest zones, some signs of root damage (stunted tips, necrosis) were seen but not quantified. Instead, the diversity and abundance of the mycorrhizal species was recorded as indicators of rhizosphere health (Section B). The results of this study show that in both the unlimed and limed soil, the roots were stressed.

Upon initial inspection, the bound Al content of the mycorrhizal roots was extremely high, especially in the Calcium-rich probes. Both R and K selection occurred in response to environmental stress. The stress of short term, sudden liming may in part account for the R selection observed in the limed rhizosphere where the mycorrhizal diversity was large but the relative abundance (gram weight) of each individual species was low (Section B). Augmented accumulation of Al in the limed roots (Sections E & F) may have been a contributing factor to lower relative abundance while the "good" Ca:Al ratios were favorable to increased diversity. In the unlimed soils, long term acid stress, combined with lower bound Calcium, seems to have produced K selection pressure resulting in low diversity but high relative abundance of individual species. Relatively lower bound Al accumulation in the unlimed mycorrhizal roots may have contributed to strong individual species survival with unfavorable Ca:Al ratios contributing to diversity loss.

It would be logical to assume that a lower bound Al content in the roots should be associated with better tree rhizosphere and crown health, and lower mortality but this was not entirely the case. While the mycorrhizae in the unlimed probes were numerous and the few individual species were very abundant, the oak crowns were thinning more rapidly and the whole tree mortality rate was higher than in the limed zone despite the lower

bound Al content. The limed mycorrhizal roots, with higher bound Al content, were associated with better tree survival. This may be due to the differential selection of mycorrhizal species present on the limed plot which may have higher tolerance levels or better sequestration abilities. Despite this, the general assumption was that if more Al was present in the limed roots, the oak trees in the limed plot should have shown poorer crown growth and more indications of Al toxicity. The lack of symptoms presented a dilemma, prompting further study.

In stark contrast to the enormous amount of bound Al, the free cytosolic component was extremely small (Section G, Graphs G3-1 to G3-3). An effective combination of Al blocking and sequestration by mycorrhizal roots kept the concentrations of free unbound Al extremely low even in highly metabolic zones (0-10 cm depth) with abundant Ca present. In the unlimed forest zone, however, the Al was not as efficiently blocked or sequestered by the mycorrhizal roots and as a result more was present in the cytosol in the free, potentially toxic, form. As a result of this discovery, the lower health status of the unlimed trees (Hans Werner Schöck, 2003, pc) could be indirectly related to the inability of the unlimed mycorrhizal roots to adequately block and sequester Al and directly related to an increased cytosolic free Al. In addition, the bound and unbound Ca:Al ratios more frequently fell below 0.6, into the high Al-stress risk, and below 0.1 into the critical risk categories in the unlimed probes In contrast, even though the bound (stored) Al component continued to rise, even in relatively healthy trees in the limed forest, the unbound portion remained extremely low and fairly stable. As a result, the relative ratio of bound to unbound could be used as a general indicator of increasing Al sequestration but not as a direct health indicator. Small changes in unbound Al content were of greater importance. Besides lower Ca:Al ratios, higher unbound Al content > 6 mg/L could be arbitrarily used as an indicator of poor health.

According to the prevalent theory, as the Ca/Al and BC/Al ratios decline in the soil solution and as shown here, also in the roots, we should see more root damage or loss. This seems to be the case since, at both 30-40 and 50-60-cm depths, the mycorrhizal abundance and root biomass dropped, along with the ratios. The bound Al was highest at

these depths probably directly affecting the health of the mycorrhizae and contributing to their low numbers. While the unbound content was extremely low in all cases, unbound Al increased very slightly with depth, while the unbound Ca content declined very slightly resulting in declining Ca:Al and BC:Al ratios. Unbound Al was also higher in unlimed probes than limed probes in shallow soil. *The very small increases in unbound Al within the cytosol may be more devastating to daily root health and long term tree survival than the huge amount of bound aluminum sequestered in the walls.*

The cytosolic content of Al was frequently inversely related to the Ca content. A declining Ca:Al ratio (through either Ca loss, Al accumulation, or both) contributed to the chronic mycorrhizal mortality and diversity loss seen in the unlimed forest. In limed roots, as the Ca:Al ratio continues to decline with prolonged soil acidification, roots with high residual bound Al content may be at great risk. Augmenting natural Ca seemed to moderate the toxic effects of Al by reducing the symplastic concentrations, but it also enhanced bound Al sequestration.

According to Huettle & Zoettle (1993) and Block (1999 p.c.), with $CaCO_3$ and $MgCO_3$ liming, soil Al content was known to decline and was assumed to have leached into the ground water and surface water. So originally, it was assumed that with liming, the bound and unbound Al concentrations within the limed roots should decline, while the content of the roots in the unlimed soil should increase. But, it is clearly shown here that, although freed Al ions can leach, they can also move into the mycorrhizal roots, with liming actually improving the uptake and sequestration of potentially toxic Al, but more interestingly, in the unlimed probes, the expected heavier accumulations of bound Al ions did not materialize, but rather the acidic soils promoted increased concentrations of free (unbound) cytosolic Al. This cytosolic Al may possibly be a critical factor contributing the "new type of forest damage" mentioned by Huettle and Zoettle (1993).

With aging, many years after liming, roots with high residual Al content may reach a critical point when a number of interacting factors may initiate Al release directly from the mycorrhiza to the root translocation system, to sensitive growing areas, contributing to

sudden oak death. *The factors that may lead to the "new type of forest damage" may include: BC:Al ratio < 0.6 (in fine roots or soil solution), Ca:Al ratio < 1 (in soil solution), Ca:Al ratio < 0.1 (in fine roots), Calcium cytosol content < 10 mg / L, Al cytosol content > 6 mg/L, bound Al content > 20,000 mg/kg, temporal acidification, soil pH <4, prolonged reduced soil moisture content < 20%. The most likely triggering event to sudden oak death in an Al-stressed forest would be prolonged drought followed by sudden, short-term post-drought hydration release of stored Al to the symplast.*

H4-3: MAGNESIUM

H4-3A: Bound Calcium: Magnesium Ratios

Overall, bound Mg in the limed (L) probes was nearly double that of the unlimed (N) at all depths (Appendix 10D-1,5,9), a fact that was somewhat reflected by the Ca:Mg ratios which ranged below 3.4:1 in the limed probes and above that in the unlimed (Table H4-1B). The amount of Mg which dropped slightly with depth (L:N=2.1:1 at 0-10 cm; L:N=1.8:1 at 30-40 cm; L:N=1.6:1 at 50-60 cm), was not exclusively related to the Ca content of the roots (Appendix 10D-1,5,9).

H4-3B: Unbound Calcium : Magnesium Ratios

Very generally, in unlimed soil, the unbound Ca was roughly twice that of Mg at each depth with the Ca:Mg ratios generally > 1.9:1 while in limed soil there was more variability in the Ca:Mg ratios which were mostly < 1.8:1, but with greater total Mg uptake. (Table H4-1B; Appendix 11C-1,4,7). More specifically, at 0-10 cm depth, over 2 years, in unlimed soil the Ca:Mg ratio ranged from 1.1:1 to 3.5:1 with an average of 2.3:1 while in limed soil the ratios ranged from 1.1: 1 to 3:1 with an average of 1.8:1 and, while the 2 year average total Mg levels were 7 mg /L in unlimed soil, they were over twice that (16.4 mg/L) in limed soil. Clearly, liming augmented Mg presence in the cytosol. The Ca advantage sometimes dissipated when the Ca cytosolic content exceeded 35 mg /L .

Ca:Mg ratio generally dropped with depth. At 30-40 cm depth , in unlimed soil the Ca:Mg ratio ranged from 0.5:1 to 4:1 with an average of 2:1, while in limed soil the ratios ranged from 0.4:1 to 1.4:1 with an average of 0.8:1. The average total Mg in the cytosol of

unlimed roots at this depth was 7.6 mg/L and for limed roots 11.2 mg/L. The greatest Mg presence occurred at this depth in both forest zones.

At 50-60 cm depth, in unlimed soil, the Ca:Mg ratio ranged from 1:1 to 2.6:1 with an average of 1.9:1 while in limed soil the ratios ranged from 0.4:1 to 4.7:1 with an average of 1.4:1. The average total Mg in the cytosol of unlimed roots at this depth was 5 mg / L and for limed roots 7.4 mg / L. The lowest Mg presence occurred at this depth probably associated with reduced mycorrhizal presence or perhaps enhanced leaching.

H4-3C: Bound Mg :Unbound Mg

The averaged ratio of bound to unbound Mg (Appendix 11F-1) was significantly higher in the unlimed probes (150:1) than the limed probes (125:1) at 0-10 cm depth. Since Mg is an essential plant nutrient, and relatively deficient in most forest soils (Huettle & Zoettle, 1993), it would make sense that the unlimed roots would have stronger ratios of bound to unbound Mg, relative to the Mg-augmented, limed soil probes. In deeper soils the ratio differences were not significantly different, possibly due to similarities in soil leaching effects. In the A horizon, liming significantly affected Mg primarily by storing approximately 60% more bound Mg (by weight) in the cell walls but also releasing 60% more Mg to the cytosol relative to the unlimed probes from the same horizon. As expected, probably as the result of dolomite liming, the Mg content of the limed plot was significantly higher than that of the unlimed.

H4-4: POTASSIUM

H4-4A: Bound Calcium:Potassium Ratios

The Ca:K ratios bore no relationship to total bound K content (Table H4-1B), K varied independently of Ca. Despite this, more K was present in the bound form in the limed probes at 0-10 and 50-60 cm depth.

H4-4B: Unbound Calcium : Potassium Ratios

At 0-10 cm depth, in unlimed soil, the unbound Ca:K ratios ranged from 0.1:1 to 3.8:1 with an average of 0.8:1 while in limed soil the ratios ranged from 0.3:1 to 3.8:1 with an average of 1.3:1. The potassium content exceeded that of calcium 81% of the time in the

unlimed samples and 44 % of the time in the limed probes. The K content averaged 28 mg/L in both the unlimed and limed probes. K presence was independent of Calcium.

At 30-40 cm depth, in unlimed soil, the Ca:K ratios ranged from 0.02:1 to 1.6:1 with an average of 0.5:1 while in limed soil the ratios ranged from 0.06:1 to 1:1 with an average of 0.4:1. The potassium content exceeded that of calcium 84% of the time in the unlimed samples and 95 % of the time in the limed probes. The average K content was 33 mg/L in unlimed probes and 29 mg / L in limed probes.

At 50-60 cm depth, in unlimed soil, the Ca:K ratios ranged from 0.1: 1 to 1.4:1 with an average of 0.4:1 while in limed soil the ratios ranged from 0.02:1 to 3:1 with an average of 0.9:1. The potassium content exceeded that of calcium 91% of the time in the unlimed samples and 67 % of the time in the limed probes. The average K content was 33 mg/L in unlimed probes and 25 mg/L in limed probes.

H4-4C: Bound K : Unbound K

Potassium presence was independent of the Ca content. The K uptake was not significantly affected by soil depth nor mycorrhizal abundance. The ratio of bound to unbound K remained fairly constant in the unlimed probes, ranging from 44 to 74:1 and averaging 58:1 but was volatile in the limed probes raging from 28 to 147:1 and averaging 75:1 (Appendix 5-2). *If anything, liming may have destabilized K metabolism resulting in a cytosolic loss in deeper soil probes.* It was interesting to note that while Ca was the more prominent bound form (Graphs F3-37 to F3-42), unbound K replaced Ca as a primary mineral within the cytosol (Graphs G3.31-G3-36).

H4-5: SODIUM

H4-5A: Bound Calcium: Sodium Ratios

More bound Na was present in limed probes at 0-10 (15%) and 30-40 (10%) cm depths which roughly corresponds to the higher Ca content but the Ca:Na ratios were also high (L ≥17:1≥ N) relative to the unlimed probes (Table H4-1B). Enhanced Ca was related to enhanced Na presence but the correlation was not direct. At 50-60 cm depth, the bound

Na content was 6% higher in the unlimed probes, but so was the Ca content ratio (N=14:1; L=17:1). With respect to the other minerals examined, bound Na was minimally present (Graphs F3-37 to F3-42) averaging 255 mg/kg (N) and 276 mg/kg (L) over a 2 year period (Appendix 10D-2,6,10).

H4-5B: Unbound Calcium : Sodium Ratios

In unbound cytosolic form, Na content was more significant, sometimes being the 2nd or 3rd most common mineral after potassium and sometimes calcium (Graphs G3-31 to G3-36, Appendix 11C). At 0-10 cm depth in unlimed soil, the Ca: Na ratios ranged from 0.2:1 to 3:1 with an average of 1.1:1 while in limed soil, the ratios ranged from 0.9:1 to 2.4:1 with an average of 1.6:1. The Na content exceeded that of Ca, 52 % of the time in unlimed roots, and 4 % in limed roots. The average Na content was 14.9 mg /L in unlimed roots and 18.8 mg/l in limed roots. Calcium content was associated with a minimally improved Na presence.

At 30-40 cm depth in unlimed soil, the Ca:Na ratios ranged from 01.:1 to 3.6:1 with an average of 0.8:1 while in limed soil, the ratios ranged from 0.15:1 to 1.3:1 with an average of 0.6:1. The sodium content exceeded that of calcium 60% of the time and 95 % in limed roots. The average Na content was 15 mg/L in unlimed roots and 11 mg/L in limed roots. Loss of Calcium was associated with declining Na content.

At 50-60 cm depth in unlimed soil, the Ca:Na ratios ranged from 0.2:1 to 1.4:1 with an average of 0.7:1 while in limed soil, the ratios ranged from 0.1:1 to 3.9:1 with an average of 0.97:1. The Na content exceeded that of calcium 82% of the time in unlimed roots and 67% in limed roots. The average Na content was 14 mg/L in unlimed roots and 11.5 mg/L in limed roots. Small increases or decreases in Calcium content were not effectively related to Na content changes at this depth.

In unlimed soil, the total average content of unbound Na remained fairly consistent regardless calcium content which averaged between 9.5 and 16.6 mg /L. In limed soil at 0-10 cm depth (29.2 mg / L Ca), the Na was augmented but it dropped in concentration in deeper soils as the Ca levels dropped (10 mg / L Ca). Sodium content may be loosely

tied to anthropogenically limed soils but enhanced bound Na in the upper horizon roots may be directly tied to lime-altered mycorrhizal influences.

H4-5C: Bound Na : Unbound Na

In unlimed soil, the ratio of bound to unbound sodium was fairly consistent, averaging 18:1 regardless of season, soil depth or calcium levels. In limed soil, the B:U ratio was more variable, generally increasing with time but over the 2 year period, also averaged to be 18:1. While the bound sodium content was minimal, in the cytosol, Na was often the second most abundant mineral. *In general, liming did not alter the sodium content.*

H4-6: IRON

H4-6A: Bound Calcium: Iron Ratios

Bound iron was high in content at 0-10 cm depth with a relatively low presence in roots from deeper soils, and was more generally abundant in the limed probes over the 2 year period (Table H4-1B, Appendix 10D-2,6,10, Graphs F3-36 to F3-42). At 0-10 cm depth there was no significant difference between the Ca:Fe ratios (N=1.5:1, L=1.8:1), over 2 years however, in the limed zones, because (43%) more bound Ca was present, (26%) more bound Fe was present. In the A horizon, bound Fe was the 2nd (N ≅ 4000 mg/kg when Ca:Fe ≅ 0.6:1) or 3rd (L ≅ 5500 mg/kg when Ca:Fe ≅ 1:1) most common mineral after Al and Ca. *Very generally, a Ca:Fe ratio of 1.8:1 or less was associated with very high bound iron levels. Improved Calcium definitely improved Fe sequestration.*

In deeper horizons, over the 2 year time span, Fe was much less evident while Al and Ca were still abundant. At 30-40 cm depth the averaged Ca:Fe ratios were identical (2.8:1 for both zones) but the limed probes had 20% more Fe which roughly corresponded to a 16% higher calcium content than the unlimed probes (Table H4-1B, Appendix 10D-6).

At 50-60 cm depth there were greater ratio differences between the two zones since more calcium was often present in the unlimed probes relative to higher soil areas, but in the 2-year averaging, the limed probes still had 10% more Ca and 30% more Fe (Table H4-1B,

Appendix 10D-10). Calcium was a contributing factor, with an averaged ratio of 1.82:1 in the limed zone relative to the unlimed (2.7:1).

H4-6B: Unbound Calcium : Iron Ratios

Iron, although extremely low in unbound content, was more evident in the unlimed cytosol than the limed over the 2 year period. (Table H4-1B, Graphs G3-31 to G3-42, Appendix 11C-2,5,8). At 0-10 cm depth, in unlimed soil, the Ca:Fe ratios ranged from 0.1:1 to 222:1 with an average of 34:1, while in limed soil the ratio ranged from 6:1 to 219:1 with an average of 55:1. The average unbound Fe content in roots from unlimed soil was 2.7 mg/L and in limed soil 1.1 mg/L. The Fe content in acid unlimed forest soil was often over twice that of the limed forest. In unlimed soil, Fe exceeded the Ca content in one probe in spring 1999. High Ca content moderated Fe presence, but did not control it.

At 30-40 cm depth, in unlimed soil, the Ca / Fe ratio ranged from 2:1 to 3082:1 with an average of 434:1 while in limed soil, the ratio ranged from 77:1 to 984:1 with an average of 280:1. In every case, Ca exceeded Fe in total concentration. In unlimed soil the average unbound Fe concentration was 0.08 mg/L and in limed soil it was 0.05 mg / L. At this depth, the Calcium and iron content were both higher in the unlimed soil probes and so the perceived moderating effect of Calcium seen at 0-10 cm no longer existed.

At 50-60 cm depth in unlimed soil, the Ca / Fe ratio ranged from 0:1 to 31018:1 with an average of 1639:1 while in limed soil, the Ca / Fe ratio ranged from 24:1 to 1053:1 with an average of 394:1. The average Fe present in unbound form in the root cytosol was 0.08 mg /L in unlimed roots and 0.05 mg/L in limed roots. Despite variations in Ca content, the Fe content remained constant at 30-40 cm and 50-60 cm. Fe content was more related to pH levels than Ca concentrations.

Clearly, Fe was not easily taken in to the root cytosol but where it was, it was more evident in the very acidic unlimed probes than the limed probes. Liming may have moderated Fe presence in shallow soil (0-10 cm depth) by altering soil pH rather than directly interacting with Fe. Acidified soil and associated mycorrhizae was correlated to enhanced Fe presence.

H4-6C: Bound Fe : Unbound Fe

Very large amounts of iron (i.e.-4000 mg/kg) were bound in the mycorrhizal roots but only extremely small quantities (i.e.- 1.5 mg/L) were available in the free form in the cytoplasm (Appendix 11F). In the acidic A horizon, up to a maximum of 6 (N) to 10 (L) time more Fe was bound in the mycorrhizal roots than in the more basic lower soil reaches (Appendix 10D-2,6,10). In a similar fashion, considerably more (36x (N) to 40x (L)) unbound Fe was found in the cytosol of roots from the A horizon than in the lower reaches but the total unbound Fe content remained extremely low (Appendix 11C-2,5,8). While the limed mycorrhizal roots bound more Fe overall, they had lower cytosolic Fe content than similar samples from the unlimed forest (Table 5.3). The B:U Fe ratios varied from a low of 710:1 (N) to a high of 7665:1 (L) in the A horizon but in the lower horizons the ratios were much larger (i.e.- 22734:1 (N) and 165401:1 (L)) reflecting the tiny amount of Fe found in the cytoplasm (Appendix H4-1B). *Generally, B:U Fe ratios below 8000:1 were associated with unbound Fe contents > 0.5 mg/L. In acid stressed unlimed soils, an unbound Fe content > 1.2 mg/L (B:U Fe 4000:1 or less) was common.*

H4-7: MANGANESE

H4-7A: Bound Calcium:Manganese Ratios

In limed soil with a high Ca:Mn (15:1) ratio at 0-10 cm depth, the greatest bound Mn content was found with a 2 year average of 615 mg/kg relative to the unlimed soil (Ca:Mn = 10:1; 447 mg/kg) (Table H4-1B, Appendix 10D-2). The total bound Mn fell only slightly with depth but was slightly more abundant in the unlimed probes, with no significant difference at 30-40 cm depth (418 (L) to 470 (N) mg/kg), and a 90% SD at 50-60 cm depth (368 (N) to 350 (L) mg/kg) despite the higher Ca content in the limed probes (Appendix 10D-6,10). Calcium seemed to be mildly antagonistic to Mn sequestration. Relative to the other minerals, Mn was consistently, but minimally present (Graphs G3-37 to G3-42).

H4-7B: Unbound Calcium : Manganese Ratios

At 0-10 cm depth in unlimed soil, the Ca:Mn ratio ranged from 2.1:1 to 28.5:1 with an average of 8.7:1 while in limed soil, the range varied from 2.9:1 to 65:1 with an average of

14:1. The average unbound Mn in unlimed root cytosol was 2.5 mg/L and in limed was 3.1 mg/L. Calcium content weakly increased Mn.

At 30-40 cm depth in unlimed soil, the Ca:Mn ratio ranged from 1.2:1 to 51:1 with an average of 9:1 while in limed soil the range varied from 2:1 to 22:1 with an average of 7:1. The average unbound Mn in unlimed root cytosol was 2.3 mg/L and in limed was 1.8 mg/L. Calcium loss was weakly associated with Mn loss.

At 50-60 cm depth in unlimed soil, the Ca:Mn ratio ranged from 1.3:1 to 16:1 (7.5:1 ave.) while in limed soil the range varied from 2:1 to 34:1 (9.8:1 ave.). The average unbound Mn in unlimed root cytosol was 1.5 mg/L and in limed probes was 1.3 mg/L. There was no significant difference between the samples.

Unbound Mn content was weakly proportional to Ca content except at 50-60 cm depth. The drop in Manganese content with soil depth could be loosely correlated to mycorrhizal decline. Relative to the other unbound minerals, Mn was consistently but minimally present (Graphs G3-31 to G3-36).

H4-7C: Bound Mn :Unbound Mn

The 2 year average, bound Mn content varied from 300-566 mg/kg in the unlimed probes and from 251-660 mg/kg in the limed probes (Appendix 11F). The unbound Mn content varied from 1.1-3.1 mg/L in the unlimed and 0.8 to 3.2 mg/L in the limed probes. Overall, the limed probes had a slightly wider range of Mn content which was also reflected in the B:U Mn ratios (N= 126:1 to 354:1; L = 161:1 to 429:1). The effects of liming upon Mn content were minimal to negligible with no significant difference between the averaged B:U Mn ratios (228:1 (N); 243:1 (L)). Ca improved Mn minimally in shallow acidic soils but seemed mildly antagonistic to both bound and unbound Mn in deeper basic soils.

H4-8: PHOSPHATE

H4-8A: Bound Calcium: Phosphate Ratios

Phosphate was low in concentration relative to the Al, Fe and the macronutrients Ca, K, Mg but more abundant than the micronutrients Mn and Zn (Graphs F3-37 to F3-42). In

unlimed probes, the bound P content was higher at all depths, corresponding to Ca:P ratios below 6:1 while in limed probes the bound P content was lower corresponding to Ca:P ratios exceeding 6:1 (Table H4-1B). (In an experiment by Bengtsson (1994), the presence of 0.1 mM Al in nutrient medium facilitated uptake and transport of Phosphorous, but reduced Calcium in *Fagus sylvatica* seedlings.) At 0-10 cm depth, the 2 year average for P from unlimed soil was 888 mg/kg relative to 784 mg/kg from limed soil, with a 23% loss at 30-40 cm depth and an additional 9% loss at 50-60 cm depth in limed soils, while the losses in the unlimed probes were 30% and 7% respectively (Appendix 10D-3,7,11). Calcium had a negative impact upon P accumulation.

H4-8B: Unbound Calcium : Phosphate Ratios

Unbound P content in the cytosol was consistently greater in the unlimed probes (Table H4-1B). At 0-10 cm depth in unlimed soil, the Ca:P ratios ranged from 0.3:1 to 15.6:1 with an average of 4:1 while in the limed soil the ratios ranged from 2.8:1 to 15:1 with an average of 7:1 (Appendix 11C-3). Unbound P content exceeded Ca content in 20% of the unlimed roots (6.3 mg/L ave.) and 0% of the limed (4.7 mg/L ave.). The differences were significant. Calcium was antagonistic to P presence. Acidic unlimed forest soil probes, lowest in Ca, had the greatest augmentation in P content in the A horizon.

In deeper soil probes, despite a lower Ca:P ratio in the limed forest zones, the unlimed roots continued to have more P content (Table H4-1B; Appendix 11C-6). At 30-40 cm depth, in unlimed soil, the Ca:P ratios ranged from 0.2:1 to 74:1 with an average of 19.8:1 while in limed soil the range varied from 0.6:1 to 55:1 with an average of 16.9:1. Unbound P content exceeded Ca content in 8% (1.4 mg/L ave.) of the roots from unlimed soil and 6 % (0.9 mg/L ave.) from limed soil.

At 50-60 cm depth, in unlimed soil, the Ca:P ratios ranged from 1:1 to 56:1 with an average of 17:1 while in limed soil the range varied from 1.5:1 to 57:1 with an average of 16:1 (Table H4-1B; Appendix 11C-9). Unbound P content never exceeded Ca. The average P content in unlimed root cytosol was 0.9 mg/L and in limed probes, 0.8 mg/L

and although slight, the differences were significant. There was an inverse relationship between Ca and P.

Overall, liming tended to reduce the phosphate content of the cytosol especially at 0-10 cm depth although the presence there was still stronger here than in deeper soil implying a strong mycorrhizal role. At 30-40 cm depth there was no significant difference between the samples. As the effects of liming disappeared with depth, the similarities between the forest zones increased but the inverse relationship between Ca and P held.

H4-8C Bound P : Unbound P

In shallow soil, B:U P ratios (Table H4-1B, Appendix 11C-3) indicated that significant amounts of P were bound and stored and entered the cytosol, but from the previous information, the uptake was hindered by Ca but promoted by mycorrhizal activity. In deeper soils, where mycorrhizal abundance declined, P uptake dropped to very low levels and again was somewhat hampered by Ca. In the deepest soils there was no significant difference which correlated to similarities in Ca content and low mycorrhizal abundance.

Table H4-8C: Bound and Unbound P. Mycorrhizal roots from various soil depths in unlimed and limed forest zones. (Appendix 11C-3)

Depth	**Unlimed Forest**			**Limed Forest**		
	Bound P	Unbound P	Ratio	Bound P	Unbound P	Ratio
0-10 cm	885 mg/kg	6.2 mg/L	147:1	773 mg/kg	4.8 mg/L	168:1
30-40 cm	620 mg/kg	1.4 mg/L	526:1	613 mg/kg	0.8 mg/L	840:1
50-60 cm	574 mg/kg	0.8 mg/L	844:1	551 mg/kg	0.8 mg/L	780:1

H4-8D: Phosphate Summary

Phosphate is an essential macronutrient required for ATP production and is involved in Al sequestration via cytoplasmic Phosphate-rich granular inclusions. The surprisingly low P concentrations, especially in the limed roots, may have a long-term negative impact upon stress response mechanisms and survival.

H4-9: SULFUR

H4-9A: Bound Calcium : Sulfur Ratios

Sulfur content was fairly stable over the two year test period but some trends were evident. The average bound S content was highest in the A horizon (N = 1325 mg/kg; L = 1239 mg/kg). S varied directly with Ca (3867 mg/kg ave.) in the unlimed probes. When the Ca concentrations were approximately doubled in the limed probes, S varied inversely (Graphs F3-36 to F3-42). High Ca concentrations were associated with lowered S levels, but at high concentrations, any drop in Ca resulted in a rise in S. At very low Ca concentrations in the unlimed probes, on average, Sulfur dropped when Ca dropped and vica versa. But even at very low concentrations, despite the prevalent general long-term trend, minute changes in Ca content were often associated with similarly minute inverse S reactions. It was evident from this that two different physiological systems were operating, one at high Ca concentrations and the other at low levels.

At 0-10 cm depth the Ca:S ratios ranged from 0.8 to 8.5 (2.8 ave.) in unlimed probes and from 2.1 to 12.1 (5.6 ave.) in limed probes (Table H4-1B). The S content dropped significantly at 30-40 cm (N = 850; L=879) and 50-60 cm (N = 843, L = 791) depths (Appendix 10D-3,7,11). At 30-40 cm depth, the ratios ranged from 0.9 to 12.2 (4.3 ave.) in the unlimed and from 1.6 to 15 (4.8 ave.) in the limed. At mid-depth, S varied directly with Ca in the unlimed probes and indirectly in the limed probes. At 50-60 cm depth, the ratios ranged from 0.8 to 9.5 (3.8 ave.) in the unlimed and from 1.2 to 10 (4.7 ave.) limed. At this depth S varied more directly with Ca in both forest zones. Very generally, when the Ca:S ratios were less than 4.8, bound S varied directly with Ca in general with "fine tuning inverses" and when the ratios exceeded 4.8 the relationship became more definitely inverse as the ratio rose.

H4-9B: Unbound Calcium : Sulfur Ratios

At 0-10 cm depth in unlimed soil the Ca:S ratio ranged from 0.46:1 to 4.9:1 with an average of 1.6:1 while in limed soil, the range varied from 0.97:1 to 5.5:1 with an average of 2.7:1 (Table H4-1B, Appendix 11C-3). Unbound S exceeded Ca levels in 28% of the unlimed probes and 4 % of the limed probes, but despite this, the average unbound S

content of unlimed roots was 10.7 mg / L and 11.9 mg / L in limed roots. In limed probes, the S content was both positively and negatively associated with Ca presence (inverse relationship) with Ca having only moderate influence (Graphs G3-31 to G3-36). In unlimed, acidic soil, the S content was more closely related to the low Ca concentrations.

At 30-40 cm depth, the unlimed soil Ca/S ratio ranged from 0.15:1 to 3.9:1 with an average of 1.4:1 while in limed soil the range varied from 0.2:1 to 1.5:1 with an average of 0.8:1 (Appendix 4D-6). Unbound S content exceeded that of Ca in 36% of the unlimed probes and 60% of the limed probes. The average unbound S of unlimed roots was 10.8 mg / L and 12 mg/L in limed probes. With a drop in Ca content, S content rose in both cases. Soil pH had a complicating impact (Section C). The highest unbound S contents were recorded at this depth.

At 50-60 cm depth in unlimed soil the Ca / S ratio ranged from 0.3:1 to 2.5:1 with an average of 1.1:1 while in limed soil the ratio varied from 0.1:1 to 13:1 with an average of 1.7:1 (Appendix 11C-9). The unbound sulfur content of the root cytosol exceeds that of calcium in 60 % of the unlimed probes and 54 % of the limed probes. The average S content of the cytosol was 9.8 mg / L in unlimed probes and 8.3 mg /L in limed probes. The soil at this depth was the more alkaline than immediately above in the 30-40 cm horizon. With the lowest Ca levels, the lowest S levels were recorded at this depth.

The decrease in S content with depth was loosely associated with the decline in mycorrhizae. Overall, the sulfur content was not totally dependent upon either Ca nor pH, but not totally independent either. In unlimed acidic soil, the S content was fairly closely tied to Ca levels but in limed, more alkaline soil, the Ca content had less influence or an inverse effect on the total S presence. Fine physiological changes induced by liming or acidification in the mycorrhizae may account for the differences in S presence. For all intents and purposes, it will be assumed that S content was mostly independent of Ca content since the variations that existed were not large, but S was definitely affected by Ca on a microchemical scale.

H4-9C: Bound S : Unbound S

Very generally, the limed probes contained a less (-4%) bound S and a more (+10.5%) unbound S over the 2 year period with the greatest significant differences occurring at 0-10 cm depth. In the A horizon, in unlimed soil the average B:U S ratio was 128:1 and in limed soil it was 108:1. Liming altered S uptake.

In unlimed soil the B:U S ratios varied from 54:1 to 558:1, and in limed soils from 43:1 to 444:1 with the lowest ratios occurring at 30-40 and the highest at 50-60 cm depths (Appendix 10D-3). Although there was no significant difference between the forest zones, there definitely were differences in S metabolism with depth with the greatest overall accumulation of unbound S occurring at 30-40 and the least at 50-60 cm depths.

H4-10: ZINC

H4-10A: Bound Calcium : Zinc Ratios

Zinc concentrations did not exceed 404 mg/kg in the unlimed probes or 638 in the limed probes (Appendix 10D-3,7,11) and overall Zn was one of the least abundant minerals, but more common than Pb or Cd (Graphs F3-37 to F3-42). Despite the higher Ca:Zn ratios (51:1 vs 38:1) (Table H4-1B), at 0-10 cm depth Zn was 39% higher, 9% higher at 30-40 cm, and 21% higher at 50-60 cm in the limed probes over the two year study period (Appendix 10D-3,7,11). Zn frequently fell and rose directly with the Calcium concentration changes except for (1) the very dry fall 1999 in both forest zones and (2) at 50-60 cm depth in spring and fall 2000 in the limed forest only (Appendix 10D-3, 7,11). However, none of the changes were very great. The greatest variations in Zn content occurred in deeper soils resulting in large standard deviations and low significant differences between the unlimed and limed zones and very similar Ca:Zn ratios (23 to 26:1). Overall the changes in Zn concentrations were microchemical in nature.

H4-10B: Unbound Calcium : Zinc Ratios

At 0-10 cm depth in unlimed soil, the Ca: Zn ratio ranged from 3.3:1 to 74:1 with an average of 14:1 while in limed soil the range varied from 7:1 to 48:1 with and average of 22:1. Despite the disparity in ratios, the unbound Zn content was very similar in both

forest zones, averaging a very low 1.5 mg/L. Calcium content did not greatly influence unbound Zn presence but when it did, the relationships were mostly direct.

At 30-40 cm depth in unlimed soil, the Ca:Zn ratio ranged from 1.3:1 to 86:1 with an average of 16:1 while in limed soil the range varied from 6.8:1 to 44.5:1 with an average of 21:1. The unbound zinc averaged 0.99 mg /L in unlimed probes and 0.5 mg / L in limed probes. Overall, Zn varied directly with Ca.

At 50-60 cm depth in unlimed soil, the Ca:Zn ratio ranged from 1.8:1 to 21:1 with an average of 7.4:1 while in limed soil the range varied from 2.7:1 to 104:1 with an average of 19.9:1. The unbound zinc averaged 1.5 mg/L in unlimed soil and 0.7 mg / L in limed soil. Despite a drop in Ca content, Zn increased in the unlimed probes and with an increase in Ca content in the limed probes, the Zn content dropped. Zn tended to drop when the Ca:Zn ratio rose above 24:1 in the limed probes and above 10:1 in the unlimed, and Zn tended to rise when the Ca:Zn ratio fell below 7:1 in the limed probes and below 5:1 in the unlimed. These rough estimates apply only to 50-60 cm depth probes.

H4-10C: Bound Zn : Unbound Zn

The averaged B:U Zn ratios ranged from 53 to 381:1 in unlimed probes and from 67 to 424:1 in the limed probes (Appendix 11F-4). Since the bound concentrations were generally very low, the unbound Zn contents were also extremely low with the highest unbound Zn occurring in the A horizon in the limed probes. Except for 50-60 cm depth, Zn varied fractional, but directly with Ca concentrations.

H4-11: BOUND CADMIUM & LEAD

Because the elements Cd and Pb were found in such tiny quantities in the bound form, it was assumed that they would not be significant within the cytosol, and would not contribute to oak decline, so further study was discontinued. It was of interest to note however that Cd was more abundant in the limed probes at 0-10 cm depth, while Pb was more common in the unlimed.

H5A: SUMMARY OF BOUND CALCIUM : MINERAL RATIOS

Calcium was associated with strong binding of Fe, Mg, and of Al especially in deeper (slightly less acidic than normal) soils; was weakly associated with K, Na, Zn, and Cd; was mildly antagonistic to Mn, P, and Pb; and directly improved S when low in concentration but had an inverse effect when high (Table H4-1B).

Unbound cytosol minerals such as Mn and Mg were found to be strongly associated with the Ca implying an interactive relationship, while Na was weakly related to Ca (Table 5.6). Unbound Al had a strongly inverse relationship to cytosolic Ca concentrations, while P was weakly inversely associated. Sulfur had a complicated relationship, being directly associated to low Ca levels in unlimed probes but inversely or weakly associated to Ca levels in limed probes. In shallow limed soil, Fe was inversely related to Ca most likely due to pH effects while in unlimed acidic shallow soil, Fe was unaffected by Ca but strongly affected by the acid levels. The unbound cytosol contents of K, Fe and Zn were usually independent of the Ca levels. Mycorrhizal abundance or decline directly affected the symplastic content of Al, Mg, Mn, Na, Fe, P, S, Zn and Fe, but not K which remained fairly constant at every depth.

H5B: BOUND VS UNBOUND MINERAL SUMMARY

The Bound Ca: Mineral (cytoskeleton) content of mycorrhizal tips was considerably higher than that of the unbound (cytosolic) forms. Often, where an ion (Al, Fe) was heavily bound in the root cells, it was more weakly present in the cytosol and conversely when an ion was weakly bound it was often slightly more evident in the cytosol (Tables H4-1B, H4-2D, H4-8C). This was considered to be an inverse relationship. In some cases the ion concentrations (Mg, P, Na, Mn) were more consistent. When they were strong in the bound form they were also strongly present in the unbound form. The relationships are summarized in Table H5.

Table H5: Relationships between bound and unbound Calcium and minerals. Some minerals were weakly related to Calcium fluctuations while others were strongly related and still others were totally independent in their fluctuations. Sometimes the relationships were direct, where both rose or fell in time or inverse where one rose the other fell in concentration and vica versa. An antagonistic relationship was one where an ion was present in unlimed but very low in concentration in the limed soils.

	Bound Forms	Unbound Forms
Al	Strong, direct, alkaline soils	Strong, inverse
Mg	Strong, direct	Strong, direct
K	Weak, direct to independent	Independent
Na	Weak, direct	Weak, direct
Fe	Strong, direct	Inverse (high pH)
Fe		independent (low pH)
Mn	Medium, antagonistic	Strong, direct
P	Medium, antagonistic	Weak, inverse
S	Direct (low Ca)	Direct (low Ca)
S	antagonistic (high Ca)	Inverse (high Ca)
Zn	Weak, direct	Direct to independent
Cd	Weak, direct	-
Pb	Weak, antagonistic	-

H6: END DISCUSSION

In December 1988, a 60 square meter parcel of land (adjacent to a long term forest research station) consisting of mixed *Fagus sylvaticus* (Beech) / *Quercus petraea* (oak) forest, in the high elevation (523-591 meters above seal level) Merzalben Forest District 04/0705, Rheinland-Pfalz, South West Germany, was experimentally limed with 3 tons of powdered dolomite ($CaMg(CO_3)_2$) limestone per hectar to counter act the damaging effects of acid rain and sudden oak death (Block (Forstliche Versuchsanstalt, Rheinland-Pfalz), 1999 p.c.; Section A1; Appendix 5). In the decade that followed, the study region was free from logging activities, except for sanitizing (removal) of dead trees. While most oaks live 200-300 years, Sessile oaks (*Quercus petraea*) have an average life expectancy of 435 years (Section A1-1). Sudden death of 170 to 197 year old Sessile oaks (cf Block, 1993; Section 1; Appendix 4), was suspected to be associated with annual increment increases in soil acidification (Section A1-2), due to acid precipitation from anthropogenic sources and acidic condensation from low lying clouds (Section A2). A drop in soil pH (below pH 5) can lead to weathering and solubilization of potentially toxic Al^{3+} (BELF, 2000; Section A3, Appendices 5 & 6). Once released, the hydrated Al ions can be translocated along diffusion gradients in the soil to the rhizosphere into the highly absorptive region of the mycorrhizal roots (Section A2, Appendix 6). From earlier studies (Rost-Siebert, 1985; Vogelei & Rothe, 1993; Nowotny et al, 1998), it was known that Al could accumulate in non-mycorrhizal and mycorrhizal roots of Norway spruce (*Picea abies*), but little information was available concerning the mycorrhizal roots of oaks (Appendix 1). It was suspected that since the trees in the limed forest zone exhibited better crown development and leaf retention (Appendix 3), that liming may have beneficially influenced the activity of the mycorrhizal roots. Before investigating this hypothesis however, the diversity, abundance and distribution of mycorrhizal species in unlimed and limed the Sessile oak forest plots needed to be determined. Each of the 67 species found was examined for Al content using qualitative fluorescence analysis. Following this, quantitative measurements of the bound and unbound mineral content of the mycorrhizal tips, associated long fine roots, and 4 of the most abundant mycorrhizal species were made.

H6-1: LIMING EFFECTS ON ECTOMYCORRHIZAE

From this empirical study (1999-2000), it was determined that dolomite liming, significantly altered the diversity, abundance and distribution of the ectomycorrhizal species of *Quercus petraea,* even over 10 years after the one-time liming application (Section B). In the limed plot there was higher diversity but lower individual species abundance relative to the unlimed plot which had lower diversity but greater individual species abundance. As well as species-specific distribution changes *within* each soil horizon (0-10 cm, 30-40 cm and 50-60 cm depths), there was a marked shift in mycorrhizal biomass to the upper horizons in the limed plot.

In addition to the long-term effects upon mycorrhizal demographics, it was determined by fluorescence (Section E), bound (Section F) and unbound (Section G) mineral analysis that liming altered the chemistry of many of the individual mycorrhizal species by affecting, their abilities to exclude, sequester (bind and hold) or translocate Al and other minerals. The precise location (or absence) of Al within the mycorrhizal mantle layers, Hartig net and/or the associated root tip was species-specific and fairly consistent within a given soil sample (Section E), but the strength of Al deposition was more dynamic (Sections E & G). In most mycorrhizal species, the intensity of Al sequestration was augmented by liming, but the degree of augmentation was variably affected by extrinsic microclimatic and geophysical factors (pH, moisture & depth) (Section F; Chart F17-1).

Using Morin dye and fluorescence analysis, it was determined that each mycorrhizal species could be tentatively classified (Section E2-3F-6) based upon the precise location and relative colormetric intensity of Al fluorescence (Section E2-3F; Appendices 7 & 12). In 28 of the 67 species examined, the *morphological location* of the Al deposition remained relatively consistent regardless of the sample source (unlimed or limed soil) (Fig. E2-3F-5A&B, highlighted species). In the other 39 species, the location of Al depended upon whether the species was found in unlimed or limed soil. The intensity of the Al fluorescence, which was assumed to reflect the approximate abundance of the bound mineral within the fungal or root cell walls, was found additionally to vary with pH,

moisture, and depth, but in all cases, these variations were very species specific. Detailed micrographs of the 67 mycorrhizal species are provided in Appendix 12.

In order to confirm the qualitative observations, quantitative mineral analyses were done on cumulative samples of mycorrhizae and fine roots and on independent samples of four of the most abundant mycorrhizal species. The quantitative mineral results (Section F3 & F18), in all cases directly confirmed the qualitative fluorescence observations (Section E, Appendices 7 & 12). It was concluded that the intensity of fluorescence was indeed directly related to the relative abundance of Al.

For example, while the mantle of *Cenococcum geophilum* (and 23 other species) was nearly always Al-free, the cortical cell walls in the adjacent root tip usually contained variable (unlimed probes) to abundant (limed probes) Al, with the cortical Al fluorescence usually increasing in samples from limed soils (Figure E2-3F-5A; Appendices 7-5, 12-5). Overall INCREASES (and small variations) in BOUND Al content of *Cenococcum geophilum* mycorrhizal tips, was confirmed by quantitative mineral analysis (Graphs F3-47, 51,52; & F18-2). While lack of sequestration of Al within the mantle of *Cenococcum* probably contributed to its survival and relative abundance in both limed and unlimed soils (Section B), the marked accumulation of Al within the cortical cells and xylem of the root (Section E) may not be beneficial to the tree.

In *Lactarius acris* and 16 other species, the reverse trend was generally true with the mantle usually containing more Al than the cortex (which was sometimes totally Al-free) at 0-10 cm depth, but still Al fluorescence, tended to increase temporally, especially in limed soils (Figure E2-3F-5B; Appendices 7-35 & 12-35). There was insufficient material *Lactarius acris* (and the other 15 species) to confirm the classic fluorescent observations via quantitative mineral analysis, but *Piceirhiza chordata* (much less consistent, but more abundant) was examined.

In *Piceirhiza chordata*, from *limed* soil from Horizon A (0-10 cm depth), according to the fluorescent observations, the mantle cells all contained abundant Al, but the cortical cells (which can strongly bind Al) were Al-deficient, implying a strong blockage of Al transit to

the cortex. Quantitative analysis showed a temporal DECREASE in bound Al content at 0-10 cm depth, which supports the Al-blockage concept (Graph F3-49). In deeper limed soils, where the mantle cells had lower obvious Al fluorescence and stronger Al content in the cortex, there was a marked INCREASE in quantitative Al concentration, implying a loss of control of Al transit to the cortex and a stronger cortical accumulation (Graph F3-53). Accumulation of Al within the fungal mantle may be more detrimental to survival of some mycorrhizae (i.e. *Lactarius acris*) than others (*Piceirhiza chordata*), but somewhat protective of the roots in limed soils. Augmented Al accumulation in the deeper roots, may have contributed their death and to the shifting of biomass to the upper horizons.

Piceirhiza chordata mycorrhizal tips were quantitatively more abundant in *unlimed* soils, than limed, but only at 0-10 cm depth (Appendix B3-36, Appendix 12-36). In addition, the unlimed tips were more highly variable in Al localization and concentration (Section E; Graph F3-49). According to the fluorescence work, in the *unlimed* tips, the mantle cells were lower in Al than the cortex, in the spring, but higher in Al content than the cortex in the fall. In the *limed* probes the Al content of the mantle cells was consistently higher than the cortex, regardless of the season. *Piceirhiza chordata* have a complex dynamics, that cannot be explained by just liming. It would seem then that in unlimed soils, as a species, *Piceirhiza chordata* is more subject to the variegations in soil moisture and season, and pH, than those members of the same species found in limed soils. Liming seemed to have had a stabilizing effect in this species of mycorrhizae.

In *Lactarius subdulcis* and *Quercirhiza fibulocystidiata* (and the remaining 24 species), the Al presence within the mycorrhizal mantle, Hartig net and root cortex was the most highly variable and dependent, in part, but not exclusively, upon whether they were found in limed or unlimed soil (Figures F2-3F-5A & 5B). In *Lactarius subdulcis*, Al was usually present in the mantle and absent in the cortex, but in *Quercirhiza fibulocystidiata*, it was usually absent from the mantle but strongly present in the cortex. The very modest increases in Al content of the limed probes, observed in the fluorescence study of the *Lactarius subdulcis,* were confirmed by quantitative mineral analysis (Graph F3-48; Section F18-4; Appendix 12-25). Of the four species studied, *Lactarius* had the lowest

qualitative and quantitative Al content. In comparison, quantitative mineral analysis showed six times higher concentrations of Al in both unlimed and limed tips of *Quercirhiza fibulocystidiata.* Fluorescent fluctuations were much more difficult to discern in this later species, especially in the unlimed probes, because of the masking influence of a natural green fluorescence pigment (Graph F3-50, Section F 18-5; Appendix 12-35).

In general, some mycorrhizal species can effectively bind Al in their fungal cell walls (preventing or reducing its passage into the root cortex), others cannot bind Al (augmenting or at least allowing passage into the root cortex), and still others have changing physiologies that allow binding at some times but not others. The physiological functioning of the individual mycorrhizal species was a complex process that not only varied with liming and soil depth, and was also dependent upon external climatic factors and weathering phenomena such as: prolonged acid rain fall, variable seasonal soil moisture levels, desiccation events and intrinsic pH of the rhizosphere (Sections C, G, H). Ideally each species should be examined independently for their unique anatomical and physiological responses to soil acidification and liming (Appendices 7 & 12). But in reality, the cumulative effect of Al control was most likely dependent upon the complement of mycorrhizal species present on the host roots. Ten years after liming, this complement was significantly altered.

The unlimed, acidic soils had reduced ectomycorrhizal diversity but greater individual species abundance. According to the mineral analysis results (Sections F & G), the surviving selected species in the unlimed soils, cumulatively, had lower Al sequestration (bind & hold) potential but higher translocation ability (higher unbound Al). The reduced potential for storage of toxic Al may have contributed to the preferential survival of certain ectomycorhizal species, but at the expense of host roots and eventually the canopy.

The limed soils had greater diversity but lower individual species abundance. According to the mineral analysis results (Sections F & G), the surviving selected species in the limed soils, cumulatively, had higher Al sequestration potential, but lower translocation

ability (lower unbound cytosolic Al). The increased potential for mantle storage of Al may have contributed to the lower individual species abundance, allowing for increased competition. At the same time, lower translocation to the roots, xylem and eventually the leaves, may account for the better canopy cover in the limed forest plot. In increasingly acidified soils, liming of mycorrhizal roots may have delayed crown damage.

Why delayed? Delayed because the ectomycorrhizal roots, in storing high concentrations of potentially toxic Al within the wall structures, are at risk. When these roots were exposed to desiccation stress (dry fall 1999), the bound Al content increased and the mycorrhizal numbers and biomass declined dramatically. The following spring 2000, with the return of moisture, there was a drop in bound Al and an increase in unbound Al. The ectomycorrhiza recovered and multiplied. The stored Al was most likely released and with mass flow, the only direction it could reasonably go was up. Up to the canopy. It is beyond the scope of this paper to determine how much Al was translocated up, but with further study it would be reasonable to assume that it did indeed. With temporal acidification and with repeated mini or more serious droughts followed by periods of recovery, it is only a matter of time before the Al content in the canopy reaches critical mass and either directly or indirectly (by displacing Mg) induces sudden leaf loss, and sudden oak death.

H6-2: Soil Mineral Release to the Rhizosphere

Minerals may be freed from the soil matrix through seasonal abiotic processes including hydration, pH alteration and chemical interactions. Biotic influences can also effect mineral release including, but not limited to, mycorrhizal root proton, enzyme and gaseous secretions. Once free in the soil solution, minerals can move via hydraulic flow, and within the water in response to gravitational and biogeochemical gradients.

H6-3: External Ion Binding

Phosphate rich gels, globs and watery secretions from fungal mycelial masses may attract and hold the minerals within the hydrorhizosphere. As a mineral more closely approaches the mycorrhizal sheath it may be passively and selectively bound to adhering external

complexes such as gel (globular or watery) secretions produced by certain mycorrhizal species. These external storage sites may have several functions. They may act to temporarily and loosely hold essential minerals in close proximity to the roots to be released upon demand through Proton or Ca-mediated enzyme action. They may provide stronger preferential binding for micronutrients that are difficult to accumulate. They may act as semi-permanent storage for potentially toxic ions effectively preventing them from entering the apoplast when the concentrations are low. In addition, they may be protective in nature, providing a toxic barrier to macroinvertebrate phagocytosis or microbiological bacterial invasion. In the cleaning processes performed in preparation of the roots for mineral analysis, some of these components were most likely washed away. The presence of proximal and adhering external binding sites in the form of globules and thin biofilms however was confirmed for several species using fluorescence microsopy. In *Cenococcum geophilum* this was often the only location where Al was evident.

H6-4: Apoplastic Ion Movement

If a mineral reaches the mycorrhizal wall it may be blocked because of a lack of mineral-specific sites or by other minerals usurping the bonding points. When sites are open, the mineral may bind externally or within the wall matrix and will accumulate until the sites are filled or to the point of saturation if the mineral can displace less attractive elements. Where the strands are not tightly adhered, ions can penetrate along the diffusion gradient and travel apoplastically until a binding site irresistibly attracts and hold them or until a barrier is reached. At the barrier, ion accumulation tends to occur. These ions are not necessarily immediately bound but with sufficient accumulation may eventually displace other ions altering the ion balance.

In this study, fluorescence evidence shows that Aluminum can penetrate along the apoplastic path way and can bind in the fungal cell walls and in the pectin rich intercellular zones in some mycorrhizal species but not others. In addition, in some mycorrhizal species there were functional differences in adhesion between the outer and inner mantle sheath strands as well as between the root epidermal and hypodermal cells and the Hartig net hyphae which limits diffusion and binding of Al along the apoplastic

pathway. The addition of (dolomite) lime distinctly alters the accumulation patterns of Al in some mycorrhizal species, frequently enhancing Al concentrations in the fungal walls. In other mycorrhizal species, Al is not bound within the fungal walls unless the strands have been physically damaged. But often the mycorrhizal sheath which does not bind Al also does not prevent it from penetrating directly to the root cortical cells where it can accumulate. Aluminum was able to penetrate mycorrhizal barriers to enter the root areas especially in root meristematic areas and at wound sites or in other words in areas where the rapidly extending tips may not have full complete mycorrhizal coverage. Mycorrhizae sheaths were definitely acting as control entry points.

Certain mycorrhizal species which bind Al may therefore act as filters or control points, preventing the movement of potentially toxic ions to the root cells. In this way the dramatically increased surface area provided by the mycelial sheath over the roots can both improve mineral storage and filter undesirable ions. If the binding is strong, potentially toxic minerals can be held outside the fungal cell and outside the root, lowering the effective translocation to sensitive metabolic areas.

Controlled pH and fungal enzyme action may cause release of selected ions from the storage sites making them available for passage through the Calcium-mediated pores allowing active uptake upon demand. When the cell wall structure, intercellular connections or membranes are damaged by drought or seasonal dehydration, the control of mineral entry may be temporarily lost resulting in massive ion movement upon initial rehydration of the mycorrhizal structures. As a result, mycorrhizal species that store Aluminum externally, and are sensitive to Al when it enters the cytosol, will be the first to suffer and die in stressed microenvironments. Mycorrhizal species that may or may not store Al outside the cell but can also sequester it inside the cell within vacuoles may have a better chance of survival. Those species that do not store toxic ions in the external walls, but either completely block them or have rapid extrusion capabilities will not be as negatively affected during post-drought rehydration. Survival and demographic distribution of mycorrhizal species may be related not just to drought sensitivity but also to the soil pH and their relative abilities to store solubilized Aluminum.

Once the ions pass the mycorrhizal barrier and enter the cortical root zone they appear to move easily and quickly along the diffusion gradient right up to the endodermal barrier where they tend to accumulate and become concentrated in the cortical walls adjacent to the endodermis, but not within the suberized endodermal or pericycle walls. These outer steele cell layers are nearly always free of Al. Where some Al seems to be present, it is in the form of tiny specks and then only in areas where the pericycle or endodermal cells are immature, very close to the very tip of the root in the zone of elongation.

Within the steele, the phloem is nearly always free of Al. In rare cases where there was a positive reaction to the Morin complex, it nearly always was not in the phloem wall but rather in the cytoplasmic regions as a general hazy complex. In contrast, the xylem was extremely strongly reactive to the Morin forming a very bright yellow indicating strong Al complexing in the lignified walls. In rare cases where the xylem did not form strong yellow fluorescence, the roots were resectioned, restained with fresh Morin solution and reexamined to confirm the lack of aluminum. The xylem is probably the last real barrier to Al translocation from the roots to the leaves. Aluminum however has been localized in the heart wood of oak, assumably associated with old xylem elements. All of this indicates that oak trees and their associated mycorrhizae have extremely strong and effective filter / sequestration systems that prevent or at least control Al translocation.

H6-5: Symplastic Ion Movement

Under hydrostatic pressure, if of the correct size, shape and charge, minerals may pass through the selective fungal pores into the cytosol and then may translocate into other fungal sheath cells via symplastic pathways. Once inside the fungal or root cell, bonding may occur with free floating cytosol components, interior wall membranes or organelle membranes. Minerals may be actively sequestered within vesicles to control concentrations or actively extruded from the cell. Free floating unbound cytosolic minerals may be translocated via the symplast from their fungal entry points to root cells and in this way breach the endodermal filter system to reach the xylem. Once in the xylem, strong Al bonding tendencies were evident in oak roots. With abundant mineral

binding in the mycorrhizal and fine root structures, relatively little unbound mineral may be actually translocated to the sensitive aerial portions of the tree. If the mineral happens to be a potentially toxic element this preferential protective bonding in the roots should benefit the tree in general but be somewhat detrimental to the roots. If a mineral is essential for physiological functioning then selective bonding in the mycorrhizal roots may be important for controlled storage and release.

H6-6: Prognosis

During a prolonged drought, minerals become concentrated not just in the cell walls but also within cytoplasmic storage vesicles. Upon rehydration, drought damaged vesicles may rupture, leak or otherwise release their toxic contents. A mass ion release moving along a rapid rehydration front may not be able to bind effectively to normal sequestration points until sufficient rehydration occurs. When insufficient mineral binding occurs in the roots, such as during periods of high hydraulic pressure after a drought, an excessive amount of mineral may be translocated via the xylem to aerial tree zones potentially affecting their metabolism. Small amounts of toxic minerals may be stored in leaf zones, especially in the petioles, but from the root fluorescent studies, very little is translocated back to the roots via the phloem. Temporal accumulation of Al in the leaves combined with sudden mass flow of toxic ions may directly or indirectly precipitate a sudden die-back event in the crown of an otherwise healthy looking tree. The massive sequestration ability of the mycorrhizal roots and the minute amount of unbound Al available for symplastic transport suggests that concentration control is essential. Nature is very conservative and most organisms do not expend energy unnecessarily. Survival of an organism is dependent upon judicious energy allocations. Continuous acidification of forest soils resulting in solubilization of potentially toxic ions may tax the natural active mycorrhizal barrrier controls even in limed soils. Combine this with repeated or long term droughts and the trees may be at intensified risk of sudden mortality.

CONCLUSIONS

According to the original hypothesis: "The sessile oaks in a limed forest will exhibit greater seasonal diversity and abundance of their symbiotic mycorrhizal species in rootlets extracted from three soil horizons (A, B, and C); mycorrhizal root tips will have less evidence of incorporated aluminum; and whole roots will have quantitatively less aluminum present than in similar unlimed regions of oak forest". Other than the greater seasonal diversity observed in limed mycorrhizal roots, the original hypothesis must be rejected.

Mycorrhizal Diversity & Abundance

- Liming had long-term effects upon the ectomycorrhizae of *Quercus petraea*.
- Liming increased mycorrhizal diversity, but reduced individual species abundance.
- Unlimed mycorrhizae were lower in diversity, but surviving species were very abundant.
- Liming altered mycorrhizal dominance hierarchy and distribution patterns with respect to both soil depth and season.
- Mycorrhizal diversity and abundance in both plots declined with depth.
- Liming promoted a larger overall shift in mycorrhizal root biomass to upper soil horizons.
- Mycorrhizal diversity and abundance in both plots declined during the short term drought.
- Unlimed mycorrhizae had a stronger shift of biomass to the lower (moister) horizons during drought, and a faster recovery after drought.
- Limed mycorrhizae had less of a shift of biomass to the lower horizons and a delayed, but stronger recovery after drought.

Soil Acidity

- Both the unlimed and limed Merzalben forest plots have become more acidic since 1988.
- The pH $CaCl_2$ values (Limed forest - Horizon A) dropped to a low of 3.78 in fall 2000.
- The pH $CaCl_2$ values (Unlimed forest - Horizon A) dropped to a low of 2.95 in fall 2000.
- In both plots, lower horizons were less acidic, but all values fell below pH 5.0.
- The limed plot had a stronger relative acidification front in deeper soil.
- All the soils samples were within the solubilization range for aluminum.

Aluminum Localization

- Of the 67 mycorrhizal species isolated, each was unique in its Al binding capacity.
- 17 mycorrhizal species strongly bound Al, but the location was species specific.

- 24 mycorrhizal species never or rarely bound Al.
- 26 mycorrhizal species had variable Al binding abilities.
- Depending on the species, Al could be selectively bound (or not bound) in the cystidia, emanating hyphae, outer, middle or inner mantle, outer or inner Hartig net.
- Varying ability to bind Al was often related to liming, pH, moisture and soil depth.
- If Al was strongly bound in the mycorrhiza, it was less evident in the root cortex.
- If Al was absent or weakly bound in the mycorrhiza, it was more evident in the cortex.
- Once past mycorrhizal barrier, Al was very strongly bound in the cortex.
- A diffusion gradient from the outer to the inner cortex was frequently visible.
- Al tended to accumulate on the cortical side of the endodermis.
- Endodermal and pericycle cells nearly never bound Al.
- Al can apoplastically bypass the endodermis and pericycle in the meristem zone.
- Once in the steele, Al binds strongly within the xylem walls, but not the phloem.

Bound Aluminum Concentration

- Aluminum was confirmed to be present in *Cenococcum geophilum* using ICP analysis.
- Quantitative mineral analysis confirmed the observations made using fluorescence analysis.
- Bound Al ranged from 5000 to 2000 ppm in mycorrhizal roots.
- Al content of mycorrhizal roots in both forest plots increased over time, and with depth.
- Combining all horizons, seasons and depths, the limed probes had significantly more Al.
- The highest significant (>95%) difference between the unlimed and limed plots occurred in Horizon A, spring 2000, after the short term fall 1999 drought.
- Despite qualitative observations showing stronger fluorescence in most limed probes, many samples were different, but not HIGHLY (>95%) significantly different.
- Al content in Horizon B differed significantly (>90%) only in spring 1999.
- Al content in Horizon C was not significantly different.
- According to the mineral analysis, Calcium and Aluminum were antagonistic to each other.
- Liming (ameliorated pH) enhanced binding of Al within compliant fungal and root cell walls.

Unbound "Free" Cytosolic Aluminum Concentration

- In Horizon A, Al content was 84% different, averaging 7.5 (unlimed) and 5.8 (limed) ppm.
- Combining all horizons, seasons and depth, there was no significant difference between plots.

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