ABSTRACT

MOYER-HENRY, KARI ANNE. Plant Responses to Stress in Acid Environments: An Assessment of the Role of Mycorrhizal Fungi. (Under the direction of Thomas W. Rufty.)

The purpose of the research was to gain further understanding of the responses of plants to stress in acid environments and the role of mycorrhizal fungi in stress tolerance. Acidic soils of the southeastern coastal plain typically have low fertility, but weeds remain problematic even in soybean and peanut fields where no fertilizer nitrogen is applied. Field experiments using ^{15}N natural abundance examined whether nitrogen might be transferred between the N_2-fixing crop species and neighboring weeds. A five year field study demonstrated that substantial nitrogen transfer did occur. Because nitrogen transfer was largely dependent on the presence of arbuscular mycorrhizae, the results strongly suggested that N moved from plant to plant through mycorrhizal hyphae that connected plant root systems. Another characteristic of acid soils is the presence of high levels of aluminum. When soil pH is below 5.0, the presence of Al^{+3} in soil solution can cause aluminum toxicity to occur in many plants. Aluminum toxicity inhibits root growth and predisposes crop plants to drought and nutrient deficiencies. Loblolly pine was determined to be extremely tolerant to aluminum. In a series of experiments, we examined the ability of loblolly pine to exclude aluminum from root tip meristems which are known to be the main sites of aluminum toxicity. The primary and secondary roots of pine exhibited high degrees of Al tolerance. Tolerance was associated with Al exclusion from the root tips and, of the Al accumulating in the root, exclusion from the root meristem. Ectomycorrhizal colonization was found to contribute to aluminum tolerance, evidently by providing an extra barrier to Al entry into the root. Additional experiments

examined Al relations of arbuscular mycorrhizal fungi. The root systems of more than 80% of all plant species are colonized by arbuscular mycorrhizae. In the presence of aluminum, colonization by arbuscular mycorrhizal fungal species was inhibited. The results suggested alterations in root function caused lower colonization, as mycorrhizal infection potential appeared unaffected. Aluminum also inhibited fungal spore germination, but only at very high Al levels. Much of the function of the mycorrhizal fungi was unaffected even as aluminum accumulated in fungal structures.

PLANT RESPONSES TO STRESS IN ACID ENVIRONMENTS: AN ASSESSMENT OF THE ROLE OF MYCORRHIZAL FUNGI

by
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APPROVED BY:

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DEDICATION

To my parents William and Kathleen Moyer and my husband Matt

BIOGRAPHY

Kari Anne Moyer-Henry was born in Glen Falls, NY in 1976 to William and Kathleen Moyer of Granville, NY. She graduated from Granville High School in 1994. She earned her B.S. in Biology and minor in Chemistry from the University of North Carolina at Greensboro in May 1999. While at UNC-Greensboro she was named a Glaxo-Wellcome Women in Science Scholar and received an Undergraduate Research Assistantship which she completed in Dr. C. Neal Stewart's molecular biology laboratory. In July of 1999 she was awarded a Research Assistantship from Dr. Thomas Rufty to pursue a Ph. D. degree in Crop Science and Plant Physiology at North Carolina State University.

The author is married to Matthew Henry.

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Special thanks go out to Michael Jennette, Dr. Eva Johannes, Dolly Watson and Dr. Wayne Robarge for their collaborative efforts which made my research successful.

I am wholeheartedly grateful for my wonderful parents William and Kathleen Moyer who encouraged me to continue my education to the highest degree possible. Without their love and support I would not have been able to make it this far.

Finally I wish to thank my colleagues and friends, Shannon Sermons, Laura Vance and Judy Edwards, not only for assisting with my research whenever help was needed but also for their friendship.

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INTRODUCTION

Crop and native plant species are continually exposed to environmental stresses in the field. The most common above-ground stresses include heat and cold. Stresses also can be edaphically based, and factors prevalent in highly weathered soils such as those found in the southeastern U.S. and tropical America can be particularly problematic for plant growth and development.

Ultisols and oxisols are the dominant soil types found in the Southeast. They are characteristically acid with very low fertility. Nitrogen is present at very low levels, as organic matter typically is < 1% and extensive leaching of inorganic N occurs. The main sources of N for cultivated plants are inorganic N applied as fertilizer or, in the case of leguminous species, fixation of atmospheric N. With the crop species soybean (*Glycine max*) and peanut (*Arachis hypogaea* L.), N_2-fixation provides adequate N for production of near maximal yields.

One of the mysteries in weed ecology has been the prevalence of weed problems in soybean and peanut fields of the southeastern coastal plain. Most often, little or no inorganic N is supplied (Israel and Burton 1997) but weed problems are intense. The obvious question arises: Where do the weed species get sufficient N to grow and develop, and compete for soil resources?

In the first chapter of this thesis, and the initial experiments of this physiological ecology project, we explore the possibility that weeds acquire N from the N_2-fixing crop plants. There is some experimental evidence that N might be exchanged between neighboring plants (He et al. 2003, Simard 2002), although considerable uncertainty

exists about the amounts that could be transferred and whether transfer plays an important role in the field. The experiments build on observations from studies conducted in the plant-breeding program of J.W. Burton (Burton et al. 1983), who found that non-nodulating lines were larger and contained more N when grown with N_2-fixing lines. Our experimental approach used the ^{15}N natural abundance technique (Shearer and Kohl 1988) to estimate the amounts of N transferred and the possible dependence on arbuscular mycorrhizae.

The majority of terrestrial plant root systems are successfully colonized by one of two types of mycorrhizal fungi, either ectomycorrhizal fungi or arbuscular mycorrhizal fungi (Smith and Read 1997). The symbiotic relationship enhances root access to previously unavailable soil resources while the plant provides the fungus with carbon that it cannot acquire on its own. Fungal hyphae serve as extensions of the plant root system, increasing the absorptive surface area in the soil. Mycorrhizal colonization often leads to improved plant growth resulting from the ability of the fungi to reach nutrients that are otherwise inaccessible to plant root systems, such as insoluble forms of phosphorus (Smith and Read 1997, Harrison 1999).

Another characteristic of the highly weathered soils is the presence of high levels of Al in the acid soil environment. Acidic soils are a large-scale problem throughout much of the world, as they occupy approximately 40% of the world's arable land, and 67% of total acid soils contain problematic levels of Al (Fig.1). Agronomic studies have shown that the major problem on the acid-Al soils is Al toxicity. Thus, it can be estimated that productivity is negatively impacted by Al on as much as 26 % of arable land (Eswaran et al. 1997).

The normal management strategy to control Al toxicity is to add lime to soil. Aluminum toxicity is exerted by the Al^{3+} ion, which becomes the dominant Al molecular form as pH decreases below 5.0 (Kinraide 1991). Lime does not solve the Al problem, however, as it typically does not migrate downward below the plow layer. The unaffected subsoil Al limits deep root penetration, which limits access of roots to deeper soil resources and predisposes plants to drought and nutrient deficiencies.

In a series of research projects, our research group has been attempting to understand the mechanisms controlling tolerance to Al. An important goal has been to identify species with high levels of Al tolerance. It is always difficult to make comparisons among published results. No experimental systems are exactly the same; even small differences can alter results. Factors such as solution composition, pH or the precision of pH control, and nutrient supply can cause the amount of Al in the toxic Al^{+3} form and its presence at the root surface to be unpredictable. In the species screening experiments shown below (Fig.2), solutions contained the same nutrient concentrations and pH was precisely controlled at 4.3±0.1, resulting in similar experimental conditions

An important finding of screening experiments is that loblolly pine is extremely tolerant to Al. It is considerably more tolerant to Al than other crop plant and tree species. Inhibition of primary root extension of loblolly pine does not begin to occur until Al^{+3} concentrations are increased into the 450-600 μM range. Results from experiments a number of years ago had suggested that pine might have a high degree of Al tolerance (Raynal et al. 1990). As a plant that is intimately involved in old field succession, it should not be surprising that pine trees would be extremely Al tolerant.

The Al tolerance of pine is the main focus of Chapters 2 and 3 in this thesis. We examine the ability of loblolly pine to exclude Al from the meristematic zone at roots tips, which is the main site of Al toxicity. The research also considers a possible protective role for ectomycorrhizae, which almost always is associated with pine lateral roots in the field. Because ectomycorrhizae did seem to be part of the Al tolerance mechanisms, further research was conducted to examine a possible role for arbuscular mycorrhizae in Al tolerance of the crop species soybean.

An important part of this Al research effort was the development and use of the Al-lumogallion methodology (Fig. 3). Precise detection of Al in plant tissues has always been problematic, and it has held back the Al-physiology field. The high degree of specificity for Al coupled with detection of the lumogallion fluorescent signal by confocal laser microscopy has allowed precise spatial localization of Al inside root tissues that was not possible before. Up to this time, only two labs have used the lumogallion method. The other is Kataoka's group (1997) in Japan, who borrowed the concept from research in analytical chemistry and first developed the procedure for use with plants.

RESEARCH HYPOTHESES

Chapter II.

1. Nitrogen transfer does not occur between N_2-fixing soybean and non-nodulating soybean.

2. Nitrogen transfer does not depend on arbuscular mycorrhizal colonization.

Chapter III.

1. Loblolly pine is not tolerant to high aluminum levels.

2. The aluminum tolerance observed in loblolly pine is not dependent on the ectomycorrhizal fungus *Pisolithus tinctorius.*

Chapter IV.

1. The lateral roots of loblolly pine are not differentially tolerant to aluminum.

2. Organic acids do not play a role in the aluminum tolerance of loblolly pine.

Chapter V.

1. Aluminum does not accumulate in the structures of arbuscular mycorrhizal fungi.

2. Aluminum does not interfere with the function of arbuscular mycorrhizal colonization.

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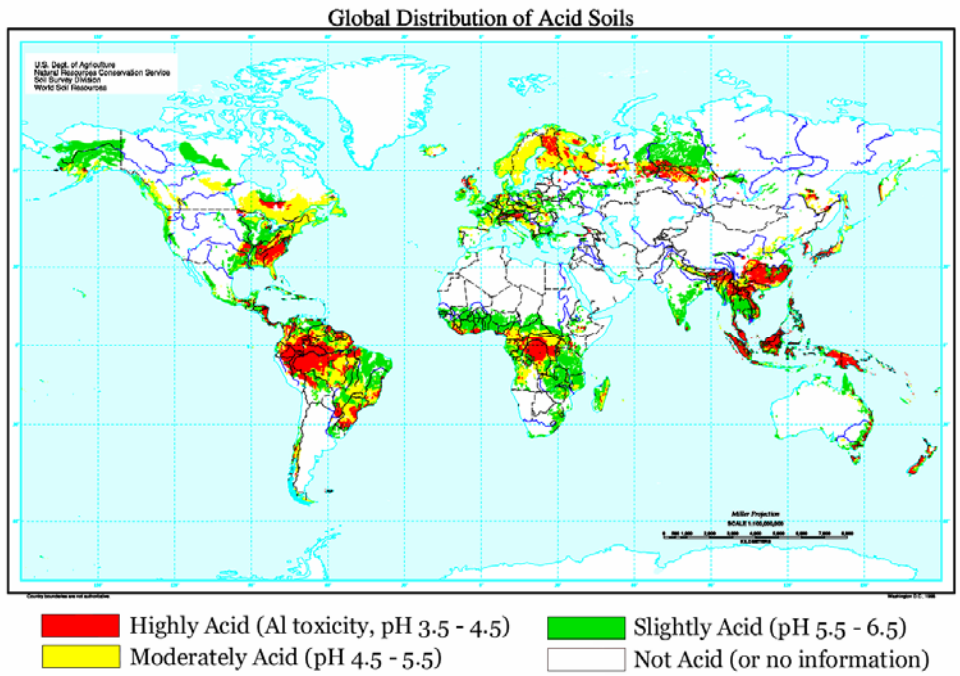

Figure 1. Map depicting the extensive nature of acid soil throughout the world, as compiled by the U.S. Soil Conservation Service.

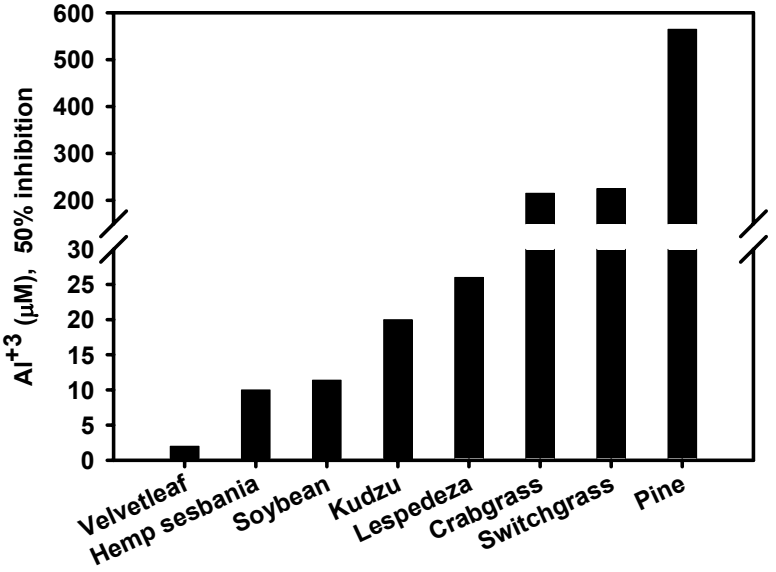

Figure 2. Species comparison of Al tolerance.

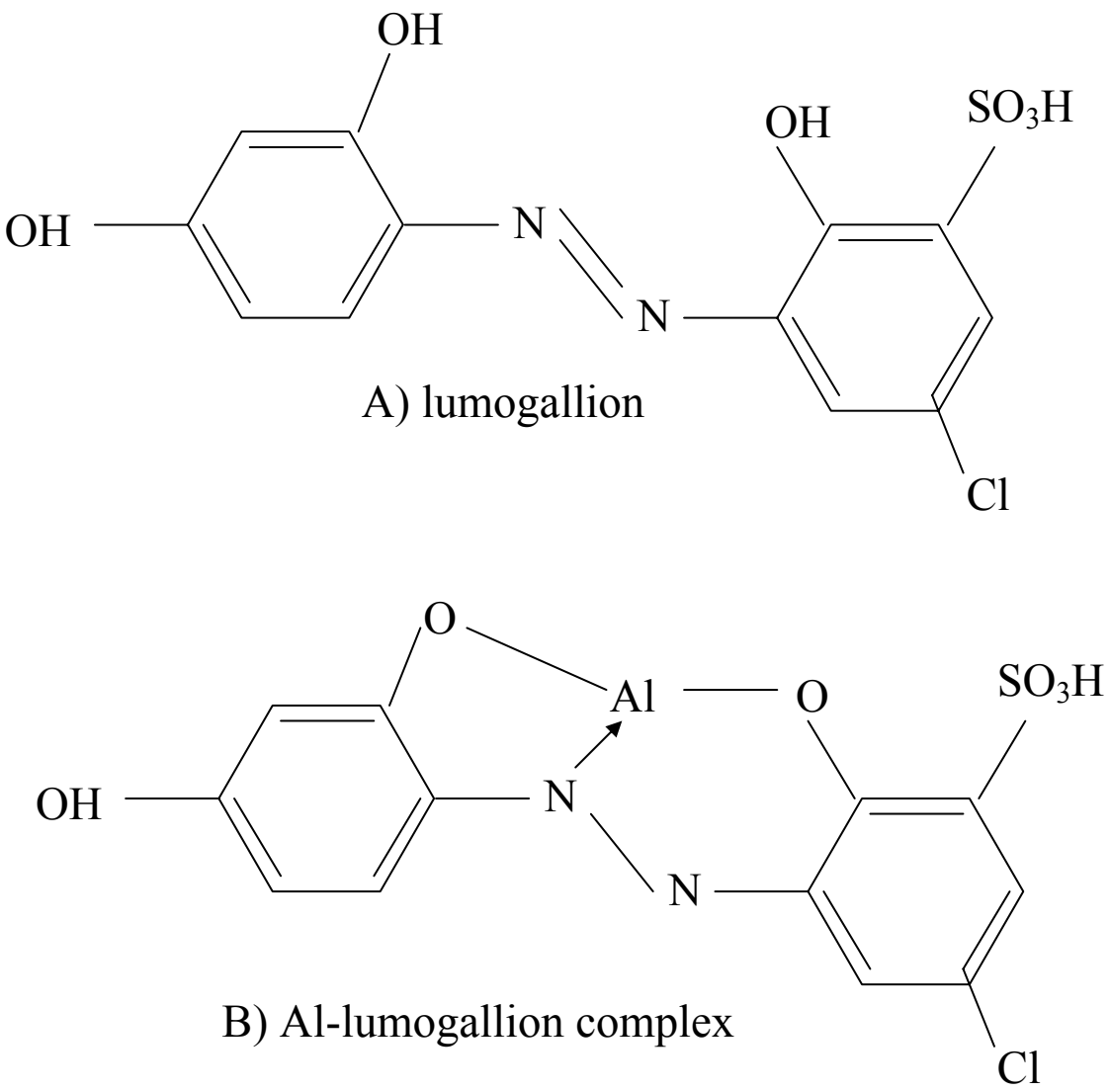

Figure 3. Molecular structure of lumogallion molecule. A) lumogallion molecule; B) Al-lumogallion complex.

CHAPTER II

NITROGEN TRANSFER BETWEEN PLANTS: A ^{15}N NATURAL ABUNDANCE STUDY WITH CROP AND WEED SPECIES

ABSTRACT

An increasing amount of evidence indicates that N can be transferred between plants. Nonetheless, a number of fundamental questions remain. A series of experiments was initiated in the field to examine N transfer between N_2-fixing soybean (*Glycine max* [L.] Merr.) varieties and a non-nodulating soybean, and between N_2-fixing peanut (*Arachis hypogaea* L.) or soybean and neighboring weed species. The experiments were conducted in soils with low N fertilities and used differences in N accumulation and/or ^{15}N natural abundance to estimate N transfer. Mixtures of N_2-fixing and non-nod soybean indicated that substantial inter-plant N transfer occurred. Amounts were variable, ranging from negligible levels to 48% of the N found in the non-nod at maturity. Transfer did not appear to strongly penalize the N_2-fixing donor plants. But, in cases where high amounts of N were transferred, N content of donors was noticeably lowered. Differences were evident in the amount of N transferred from different N_2-fixing donor genotypes. Results of experiments with N_2-fixing crops and the weed species prickly sida (*Sida spinosa* L.) and sicklepod (*Senna obtusifolia* [L.] Irwin & Barneby) also indicated substantial N transfer occurred over a 60-day period, with amounts accounting for 30 to 80% of the N present in the weeds. Transfer of N, however, was generally very low in weed species that are known to be non-hosts for arbuscular mycorrhizae (yellow nutsedge, *Cyperus esculentus* L. and Palmer amaranth, *Amaranthus palmeri* [S.] Watson). The results are consistent with the view that N transfer occurs primarily through mycorrhizal hyphal

networks, and they reveal that N transfer may be a contributing factor to weed problems in N_2-fixing crops in low N fertility conditions.

INTRODUCTION

Acquisition of nitrogen (N) is crucial to plant growth and development. Plants can obtain N in several ways, the most obvious being uptake of inorganic N from the surrounding soil and N_2 fixation by leguminous plants (Haynes and Goh, 1978). There is evidence to suggest that plants also might be able to acquire N transferred from neighboring plants (He et al., 2003; Simard et al., 2002).

The evidence for inter-plant N transfer comes from studies in pot culture and in the field. Pot culture experiments typically have used labeled ^{15}N and split-root systems with 'donor' and 'receiver' plants. Results show that N transfer occurs, but there has been no agreement on the amount. In some cases N transfer was small (≤ 5%) (Frey and Schuepp, 1992; 1993; Johansen and Jensen, 1996; Martin et al., 1991b), but in others N transfer accounted for as much as 10 to 20% of the N in the receiver plant (Bethlenfalvay et al., 1991; Haystead et al., 1988; Martins and Cruz, 1998; Van Kessel et al., 1985).

The split-root experiments also have been used to examine the possible involvement of mycorrhizae and their interconnecting hyphal network (Newman, 1988; Smith and Read, 1997) in the transfer process. The inclusion of fine mesh (25 – 60 μm) screens in the pot containing donor and receiver roots separated roots from one another but allowed arbuscular mycorrhizal hyphae to form linkages between the root systems. Results generally indicate that mycorrhizal linkages enhance and may be required for N transfer (Bethlenfalvay et al., 1991; Frey and Schuepp, 1992; 1993; Haystead et al., 1988; Martins and Cruz, 1998).

The bulk of the evidence for N transfer between plants in the field comes from experiments showing that growth and N yield of non-N_2-fixing plants can be increased

when grown in mixed culture with N_2-fixing plants (Brophy et al., 1987; Elmore and Jackobs, 1986; Heichel and Henjum, 1991; Hogh-Jensen and Schjoerring, 2000; Martin et al., 1991a; 1991b). The mechanism of N exchange, however, has been somewhat ambiguous. As an example, an experiment with four types of forage legumes grown in mixed culture with a grass species used the ^{15}N isotope dilution method to examine N movement from legumes to the grass (Heichel and Henjum, 1991). Since transfer was greatest in year 2, it was concluded that release of N from breakdown of belowground residues of the legume coupled with grass uptake of mineralized N may have been a dominant factor in the N exchange, and not necessarily direct transfer between plants. Transfer decreased in years 3 and 4, which was thought to reflect progressive loss of the legume in the sward and an associated decline in residues.

A few field experiments have used ^{15}N applications to examine N transfer within a growing season, where involvement of decaying residues would be expected to be less. Nitrogen-fixing donor plants were labeled with ^{15}N by solution application to leaves (Hogh-Jensen and Schjoerring, 2000), cut petioles (Martin et al., 1991b), or adventitious roots on stems (Hamel et al., 1991; Hamel and Smith, 1992) and grown with non-nodulating receiver plants. Generally, results have shown that ^{15}N transfer can occur, although amounts have been relatively low. In addition, field experiments using soil fumigation and additions of mycorrhizal inoculum have indicated that ^{15}N transfer was enhanced by mycorrhizal presence and positively correlated with mycorrhizal hyphal density in the soil (Hamel et al., 1991).

Even though evidence of N transfer between plants is accumulating, a number of fundamental questions remain (He et al., 2003). We attempted to gain insights into some

of those through a series of field experiments using the ^{15}N natural abundance technique (Shearer and Kohl 1988). An initial set of experiments involved growing N_2-fixing soybean genotypes and a non-nodulating soybean line in mixed culture. The experiments addressed the following questions: 1) does inter-plant transfer occur within a single season; and if so, 2) does N transfer penalize the reproductive performance of donor plants, and 3) do individual varieties, acting as N donors, transfer differing amounts of N? A second set of field experiments examined N transfer in mixed culture experiments with N_2-fixing peanut or soybean and different weed species. The questions were: 4) what is the extent of N transfer between crop and weed species, and 5) is transfer to weeds dependent on the weed-receiver plants being mycorrhizal host species?

MATERIALS AND METHODS

Nodulating and non-nodulating soybean

Seeds of four nodulating, N_2-fixing (nod) soybean (*Glycine max* [L]. Merr.) genotypes (cultivars Hood, Pickett 71, and Young, and the breeding line N93-1264) and a single non-nodulating (non-nod) line (D68-0099) were mechanically planted at 24 seeds/m in early June of 1997 and 1998 at the Central Crops Research Station, Clayton, N.C. The soil type was a Dothan Loamy Sand in 1997 and Norfolk/Marlboro series Loamy Sand in 1998. The soils typically have low levels of inorganic N and organic matter levels less than 0.5 %. Phosphorus fertilizer additions keep P indices close to 100 (> 240 kg ha^{-1}), and regular lime additions maintain pH between 5.5 and 6.2.

Treatments consisted of individual lines grown alone and of each nodulating, N_2-fixing line grown in mixed culture with the non-nod line, arranged in a complete block

design with 3 replications in 1997 and 4 replications in 1998. Each replication consisted of 9 plots, each consisting of 3 rows 5.8 m long and 3 m wide. For the mixtures, seeds were randomly planted in approximate 50:50 mixtures in the rows. After germination, plants in the rows were about 3.8 cm apart. At maturity, plants in the middle 4.8 m of the middle row of each plot were harvested for yield and ^{15}N abundance measurements. The non-nod soybean line had a tawny pubescence that was clearly distinguishable from the gray pubescence of the N_2-fixing cultivars (Burton et al., 1983). In mixed plots, the number of plants of each genotype within the row was counted, and the shoots of non-nods were harvested by hand and seeds mechanically removed using a stationary thresher. The remaining nodulated plants were then mechanically harvested and threshed separately. Individual component yields were adjusted (whole plot component yield = yield within mixture / plant frequency within mixture) and expressed on a per hectare basis to permit comparison of yields and N accumulation with those when the soybean lines were grown alone.

Weeds and nodulating peanut and soybean

Weed seeds were germinated in a greenhouse in 20 cm 'cone-tainers' (Stuewe and Sons, Inc., Corvallis, Or.) containing field soil several days prior to crop planting in the field. The soil had been collected from the top 15 cm of soil profile in the fields where weeds subsequently were transplanted. During and after germination 'cone-tainers' received water daily. After approximately 3 weeks, weed seedlings were transplanted into field plots containing peanut *(Arachis hypogaea* L.*)* at the Peanut Belt Research Station, Lewiston, N.C., or soybean at the Central Crops Research Station, Clayton, N.C. In all cases, soils had low native N fertilities.

In all experiments, transplanted weeds were watered by hand initially and the entire plot was irrigated periodically. Unwanted weeds were controlled by tillage early in the experiments and removed manually as the experiments progressed. After 60 days of growth, whole leaf canopies of weed and crop species were harvested separately, dried, and ground for later analyses.

Experiments with peanuts were conducted in 1998, 1999 and 2000 in a Goldsboro Sandy Loam. Five treatments were established: prickly sida (*Sida spinosa* L.) ± peanut, yellow nutsedge (*Cyperus esculentus* L.) ± peanut, and peanut alone. Prickly sida is known to be a host and yellow nutsedge a non-host for arbuscular mycorrhizae (Muthukumar et al., 2004). Peanut seeds, cultivar NC-7 (Mozingo et al., 1987), were planted at a rate of 16 seeds/m in early May in plots consisting of 4 rows 3 m long and 91 cm apart. For mixed plots, about 2 weeks after planting and 1 week after germination of the peanut, 3 weed seedlings were transplanted into each of the 2 center rows of the plot several feet apart, establishing 6 weed seedlings per plot. Weeds were approximately 3 cm from adjacent peanut plants. In separate plots, where weeds were grown alone, all peanut seedlings were removed from the four plot rows just after emergence, prior to transplanting the weeds. Treatment plots were established in a randomized complete block design with 10 replications for a total of 50 plots.

Experiments with soybean were conducted in 1998, 2000 and 2001 on a Dothan Loamy Sand. The treatments included N_2-fixing soybean lines Pickett 71, Young, or N93-1264, prickly sida or sicklepod (*Cassia obtusifolia* [L.] Irwin & Barneby) as mycorrhizal host weed species, and in 2001 also included Palmer amaranth (*Amaranthus palmeri* [S.] Watson) (Gerdemann, 1968). Again, the plant types were grown alone and

in mixed culture. Soybean seeds for each experiment were planted in early June. Experimental plots consisted of 3 rows 3 m long and 91 cm apart. Treatments were assigned to individual plots following a randomized complete block design, with four replications in 1999 and 10 replications in 2000 and 2001. Six weed seedlings were transplanted into the center row of each plot. Treatment plots with weed species growing alone were cleared of all soybean plants, even in the border rows, ensuring that the weeds were separated from soybean plants by at least 2 m of fallow ground.

Tissue protein and ^{15}N analyses

After harvest and drying of plant tissues, a bulk sample was ground and mixed. With N_2-fixing and non-nodulating soybean experiments, a portion of each ground bulk sample was used for protein analysis using a Leco automated N analyzer (St. Joseph, MI). Other subsamples were removed from the bulk sample, and ground a second time to a fine powder using a Crescent "Wig-L-Bug" grinder. Multiple samples of 2 to 3 mg were then weighed into tin capsules using a Mettler AT20 micro-balance (Toledo, OH), and analyzed using ratio mass spectrometry (Isotope Services, Inc., Los Alamos, NM). A similar procedure was followed with tissues harvested in the weed experiments.

The amount (%) of fixed N in plants was calculated (refer to Shearer and Kohl, 1986) using the formula:

$$\text{Fixed N \% of total} = \frac{\delta^{15}N_o - \delta^{15}N_t}{\delta^{15}N_o - \delta^{15}N_a} \times 100,$$

where $\delta^{15}N_o$ is the $\delta^{15}N$ value from non-nod soybean or non-mycorrhizal weeds grown alone and dependent on soil N; $\delta^{15}N_t$ is the $\delta^{15}N$ value from plants grown in mixed culture with N_2-fixing plants where fixed N and soil N are available as an N source; and

$\delta^{15}N_a$ is the $\delta^{15}N$ value for N_2-fixing plants when they are totally dependent on fixed N as an N source.

The most straightforward way to understand calculation of the amount of fixed N in donor or receiver plants is by visualizing ^{15}N abundance on a linear scale with a pole on each end (Shearer and Kohl, 1986). As an example, for nod/non-nod soybean experiments, the left hand pole is the $\delta^{15}N_o$ for soil N, assigned the value 4.2 (± 0.3) as measured by the average $\delta^{15}N$ value from leaf tissues of non-nod soybean growing alone. The right hand pole is biologically fixed N, assigned a value of -1.1 (± 0.1) as determined by the average $\delta^{15}N_a$ value of leaf tissues from nodulated, N_2-fixing soybean grown in perlite and entirely dependent on N_2 fixation. Therefore, the range of ^{15}N abundance equals 5.3, and that becomes the denominator in the above formula. If non-nod soybean has a $\delta^{15}N_t$ value of 2.1 when grown in mixed culture with N_2-fixing soybean, it can be calculated that 40% of the N originated from N_2 fixation and 60% originated from the soil.

In weed experiments, $\delta^{15}N$ of the mycorrhizal non-host weed species growing alone was used as the indicator of soil N ($\delta^{15}N_o$) when included in the experiment. This allowed estimation of soil $\delta^{15}N$ separate from possible fractionation effects associated with mycorrhizae itself (see arguments in Discussion below). When mycorrhizal non-host weed species were not included, the $\delta^{15}N$ of mycorrhizal host weeds growing alone was used.

The field sites used for the experiments were selected for soil uniformity and crop history, with no N_2-fixing crops having been grown for at least 2 seasons. Preliminary examination of tissues from non-nodulating plants grown in soil from the sites revealed

that soil $\delta^{15}N$ values in some cases were as low as 3.5. This is somewhat lower than would be preferred, because it limits the range between soil and fixed-N $\delta^{15}N$ values used to estimate the amount of N coming from either source in harvested plant tissue. In studies in the Midwestern U.S. and in Australia, for example, soil $\delta^{15}N$ values were only as low as 6 to 7, allowing a scale of 7 to 8 $\delta^{15}N$ units (Herridge et al., 1990; Kohl et al., 1980). The limited range of differences in our experiments dictated the use of relatively large plots whenever possible to limit field micro-site variability, and in experiments with weeds, as many as 10 replicated plots were used. Also, large amounts of plant tissue were harvested, and tissues were double-ground and thoroughly mixed before the mass spectrometry analyses. The analytical error of the mass spectrometry itself in the natural abundance δ range is about 0.1 to 0.3 units per mil.

The total amount of seed protein in soybean was the product of seed yield and percent protein. Total N in seeds was calculated from total seed protein by dividing by the conversion factor 6.2 (Garrett, 1974). Israel and Burton (1997) reported that ~ 30% of whole crop N remained in stover at harvest in experiments involving different soybean varieties. Therefore, total plant N was calculated as 1.3x total seed N. In all experiments, data were adjusted to account for the total N present in the N_2-fixing plant (fixed N + soil N; Tables 3 and 4). This assumes that N taken up from the soil was as readily transferred to the weeds as N acquired from N_2-fixation.

Mycorrhizal colonization

In 2000, seeds of soybean cv. Young and prickly sida were planted adjacent to existing soybean plants in the border rows of field plots at the Central Crops Research Station, Clayton, N.C. to determine the initial rate of arbuscular mycorrhizal colonization of seedlings growing in the field. Ten seedlings of soybean and prickly sida were

harvested at 3-day intervals for 24 days after emergence. To assess mycorrhizal colonization, standard methods of root staining were used (Phillips and Hayman, 1970). Colonized root length was determined using the grid-line intersect method (Giovannetti and Mosse, 1980). The same methods were used to determine the extent of colonization in the mixed plot experiments at 45 to 60 days after planting. When 2 genotypes or species were growing together, it was not possible to separate the degree of mycorrhizal colonization in the different root systems.

Statistical analyses

The covariances between components of the mixed plots for the various traits measured were determined to be non-significant. Thus, each component of the mixed plots (adjusted as previously noted) was treated as an independent plot in the analysis of variance. Analysis of variance was performed on individual plot data from the N_2-fixing and non-nod soybean in mixed culture experiments using the GLM procedure in SAS software (SAS Institute, Cary, NC 2000). Least significant differences with 5% significance levels were calculated using the error variance from the analysis. Student's t-test was performed on pairs of means to determine if growth parameters of mixture components were significantly different from the growth of the same component grown alone. Curves in figures were determined using the regression curve-fitting function of Sigmaplot (SPSS Inc., Chicago, IL 60611). From an appropriate category (e.g. polynomial or power), the equation with the highest r^2 was used.

Experimental results were evaluated based on biological significance as opposed to relying solely on the arbitrary 5% level to determine their validity, as has been argued by others (refer to Bethlenfalvay et al., 1991). This approach allows for more direct comparison of data since it reduces the likelihood of incorrectly disregarding important

treatment effects because of slightly lower statistical probability. In all cases, relevant probabilities are presented.

RESULTS

Transfer to non-nodulated soybean from N_2-fixing soybean

In combined analyses of the field studies with N_2-fixing and non-nod soybean lines, there were significant treatment by year interactions. Therefore, yield characteristics are presented separately (Tables 1 and 2). Scaling data up to a hectare basis, N_2-fixing lines grown alone produced yields of 2800 to 3200 kg/ha in both years, and total seed protein ranged from about 1000 to 1300 kg/ha, typical ranges for soybean grown in Southeastern conditions.

When N_2-fixing lines were grown in mixed culture with the non-nod soybean line, changes in yields and total N of the N_2-fixing plants were variable. For the most part, they were similar to or higher than those when the lines were growing alone. Increases in yields would reflect lower inter-plant competition from the non-nods than would be experienced in the control plots. This was most evident in 1998 (Table 2). In 1997, even though competitive pressures may have been lower, decreases occurred in yields and total N of Pickett 71 and N93-1264 in the mixed cultures. Yields were 88 and 83% of their controls in pure cultures, respectively, and total N was decreased 39 and 38 kg/ha (Table 1).

Yields and total N of the non-nod line were increased in mixed culture with the N_2-fixing lines, and differences were evident among different 'donor' soybean lines (Tables 3 and 4). Little additional N was obtained by the non-nod when growing with Young, for example, as non-nod N increased by only 13% in 1997 and then decreased in

1998. In contrast, the non-nod had 20 to 41% more N when grown in mixed culture with Pickett 71 and N93-1264 during the two seasons. In absolute terms, increases in the non-nod line in 1997 resembled decreases in N_2-fixing 'donor' plants in the mixed cultures, with a gain of 32 to 41 kg/ha, compared to the donor line losses of 38 to 39 kg/ha (cf. Table 1). The increase in the non-nod in 1998 was lower, 14 to 24 kg/ha, when N did not decrease in the N_2-fixing donors.

The $\delta^{15}N$ values of non-nods in mixed cultures were noticeably lower than the soil $\delta^{15}N$ value of 4.2 (same value in both years), which is consistent with transfer of N from the N_2-fixing lines (Table 5). The $\delta^{15}N$ of the non-nods was not different among the different donor lines, but once yield was considered, differences in the amount of N transferred from donor genotypes became evident. Transfer of N from N_2 fixation was as high as 41.2 kg/ha in 1997, accounting for 41 to 48% of the total N in non-nod plants. The $\delta^{15}N$ indicated that although the N_2-fixing donor lines obtained a similar proportion of their N from N_2 fixation in both years, the total N transferred to the non-nod receivers in 1998 was less than half that transferred in 1997.

The estimates of N transfer using $\delta^{15}N$ (Table 5) and yield N measurements (Tables 3 and 4) are summarized in Figure 1. The $\delta^{15}N$ estimates generally were similar to or somewhat higher than estimates based on N accumulation. From both estimates, however, there were separations in the amounts of N transferred from the different soybean donor lines. Cultivars Young and Hood lost little or no N to the accompanying non-nod line, while cv. Pickett 71 and the breeding line N93-1264 transferred larger amounts.

Transfer to mycorrhizal and non-mycorrhizal weeds from N_2-fixing peanut or soybean

To determine whether significant amounts of N could be transferred from N_2-fixing donor plants to weeds, weed seedlings were transplanted into recently planted fields of peanut or soybean and harvested after 60 days. Analysis of the leaf tissues of mycorrhizal host weeds revealed that $\delta^{15}N$ was noticeably lower than the background soil $\delta^{15}N$, suggesting substantial N transfer (Table 6). For prickly sida grown in mixtures with N_2-fixing peanut, 31% of its N was estimated to originate from the N_2 fixation process in 1998, 83% in 1999, and 74% in 2000. For prickly sida or sicklepod in soybean mixtures, estimates of the proportion of N in the weed tissues originating from N_2 fixation ranged from 38 to 60% over three growing seasons. No differences in $\delta^{15}N$ of receiver plants could be distinguished between soybean 'donor' cultivars.

The tissue $\delta^{15}N$ profiles of the non-hosts for mycorrhizae, yellow nutsedge and Palmer amaranth, differed from those of the mycorrhizal host species. The $\delta^{15}N$ values generally were similar when the weeds were grown in mixtures and when they were grown alone (Table 6). There were two exceptions, the most notable being yellow nutsedge growing in peanut in 2000, when about 31% of the N in the nutsedge originated from N_2 fixation. And in 2001, about 9% of the leaf N in Palmer amaranth originated from N_2 fixation when it was growing with one of the N_2-fixing soybean cultivars.

The experiments did not include evaluation of weed growth. Field experiments to examine inter-species competition are inherently difficult (Cousens, 1996; Radosevich, 1987) and beyond the scope of the present study. A few observations were notable. At the 60-day harvest, weeds in the mixed plots typically were at or just below canopy height of the crop. Periodic sampling indicated that weeds growing in the mixed plots generally

were only about 10 to 20 % of the mass found in the plots where weeds were growing alone. This was true for both mycorrhizal and non-mycorrhizal species, and presumably reflected the competitive pressures exerted by the crop plants.

In all the field experiments, with non-nod soybean and with weeds, soil cores were taken from the plots at 45 and 60 days to examine the extent of mycorrhizal colonization. Of the roots recovered, colonization always was about 40 to 60% of root length when mycorrhizal host species were present. On the other hand, little colonization and no arbuscules were ever found in roots of yellow nutsedge and no colonization was found in the Palmer amaranth in the plots when they were growing alone.

Separate plots were planted with seed of soybean and prickly sida at the Clayton location in 2000 to determine how quickly colonization was established. Colonization could be detected soon after weed species emerged, reaching 12 to 15% of total root length within 21 days (data not shown). Presumably, colonization steadily increased to the 40 to 60% level observed in the longer running experiments.

DISCUSSION

The results of these experiments provide evidence of substantial N transfer from N_2-fixing plants to non-N_2-fixing plants, and large-scale transfer, for the most part, was confined to mycorrhizal host species. Perhaps the best examples of the magnitude of transfer come from the experiments with non-nodulated soybean growing in mixed culture with N_2-fixing genotypes, which used large plots and extended to plant maturity. The increases in seed N contents of the non-nod plants in the 1997 experiment and the $\delta^{15}N$ abundance measurements showed that up to 41 kg/ha of N could be transferred, which accounted for as much as 48% of the N in the receiver plants. With weed species

that were mycorrhizal hosts, transferred N accounted for 30 to 83% of the N present over the 60-day growth periods. Furthermore, the positive correlation between N transfer and yields of the non-nod soybean (Figure 1) would suggest that extra increments of N obtained by the transfer process were quantitatively important for growth and development of the 'receiver' plants.

Enhancement of non-nod soybean growth and N content in mixed culture with N_2-fixing soybean lines can be seen in results of earlier studies (Singh et al., 1974; Vest, 1971), even though those experiments were designed for somewhat different reasons. Our field design, purposely, was similar to that of the previous study by Burton et al. (1983), which also found that yields of non-nod soybean lines were increased when growing in mixed plots with nodulating lines, implying N transfer.

The contention that N transfer is largely dependent on mycorrhizae rests upon observations with $\delta^{15}N$ abundance that the fixed-N_2 'fingerprint' was always present in tissues of mycorrhizal host species, but rarely present in non-host weed species. The most notable exception with the non-host species was found in the third year of the experiments with peanut, where calculations indicated that ~ 30% of the N present in yellow nutsedge was associated with the N_2 fixation process. In this instance, it is certainly possible that N movement involved N leakage or tissue breakdown and subsequent uptake of inorganic N by the weed plants.

It is rather surprising, in our view, that yellow nutsedge and Palmer amaranth did not accumulate more N from the N_2-fixing crop species if much leakage of N or tissue breakdown were occurring. Weeds were transplanted into the peanut and soybean rows, within inches of the potential N donor plants, so roots would have become intensely

interwoven over a 60-day period. Yellow nutsedge and Palmer amaranth both have morphologies dominated by fine roots. Studies using digital imaging have shown, for example, that more than 60% of Palmer amaranth's roots have diameters less then 100 μm (Wright et al., 1999a). One would expect that fine roots would closely approach neighboring root and nodule surfaces. Nonetheless, the weed $\delta^{15}N$ data, as a whole, indicate little N was acquired from the N_2-fixing plants. Evidently, the ability of mycorrhizal hyphae to penetrate the root cortex (Smith and Read, 1997) and nodule cell walls (Scheublin et al., 2004) and closely approach cell membrane surfaces is crucial for N transfer to occur.

In experiments like these, one must be aware of the quantitative limitations in using the natural abundance method. From the first evaluations of the method in agronomic systems, it was determined to generate 'semi-quantitative' but reasonably accurate estimates of N_2 fixation, assuming certain precautions were taken during experimental design and sampling (Herridge et al., 1990; Kohl et al., 1980; Shearer and Kohl, 1986; and see Mariotti et al. 1981 for an early application of the method). An additional difficulty was encountered in our experiments on highly weathered soils, where soil ^{15}N abundance levels were sometimes relatively low, limiting the scale between soil and N_2 fixation derived N. A number of steps were taken to minimize the impact of the low soil $\delta^{15}N$. Fields for the experiments were chosen for uniformity and extra replication was included in the weed experiments where variability tends to be greater (treatments included up to 10 replications). The extent of variability in the amounts of N transferred from nodulated to non-nodulated soybean (Figure 1) is reminiscent of that seen in some of the original $\delta^{15}N$ evaluation experiments (Herridge et

al., 1990; Kohl et al., 1980). In those studies, $\delta^{15}N$ estimates of N_2 fixation also tended to be somewhat higher than comparative estimates from the ureide/xylem sap method or N accumulation measurements.

While the consistency of the results indicates that substantial N transfer occurred in our experiments, the 'semi-quantitative' nature of the natural abundance technique and the reliance on several assumptions dictate that absolute values be viewed with some caution. The conversion of measured seed N to whole plant N (Israel and Burton 1987), for example, may have varied somewhat among the soybean genotypes and with environmental interactions each season. Also, estimates of the soil background (N_o) $\delta^{15}N$ are somewhat problematic and variation would influence all of the transfer calculations using $\delta^{15}N$. The variation could result from isotopic variation within a field or the soil profile, and it is conceivable that mycorrhizae themselves may have physiological processes that discriminate between the ^{14}N and ^{15}N isotopes. In experiments with ectomycorrhizae, evidence suggested that $\delta^{15}N$ values of the fungal N pool available for transport into plants might be lowered (Emmerton et al., 2001; Evans, 2001; Hogberg et al., 1999). An indication of mycorrhizal bias in our experiments would be lower estimates of soil $\delta^{15}N$ in mycorrhizal host weeds compared to non-host weeds when they were growing alone. The data, however, were inconclusive on this point, as soil $\delta^{15}N$ was not always higher in the non-mycorrhizal weeds (data not shown). It should be recognized, nonetheless, that the use of non-mycorrhizal weed tissues to estimate soil $\delta^{15}N$ (N_o) would lead to over-estimates of N transfer if mycorrhizal discrimination were to occur.

Donor cost and genotypic variability

The data do not provide a definitive answer to the question of whether transfer of N penalizes a donor plant. Results from the nod/non-nod soybean experiments in 1997 (Table 1) indicated that amounts of N transferred from some of the soybean lines to the 'receiver' non-nod plants were similar to the amounts of N lost from the donor plants (~ 38-39 kg/ha), which would be consistent with a penalty. The relationship was not present in 1998, however, when lower amounts of N were transferred and the N content of the donor plants did not decline.

It is probably overly optimistic to think that a quantitative relationship would consistently occur in the amounts of N lost by donors and those gained by receivers. The N_2 fixation system itself is elastic and can respond to changes in demand for N by adjustments in the feedback system regulating N_2 fixation (King and Purcell, 2005; Oti-Boateng and Silsbury, 1993; Parsons et al., 1993; Silsbury et al., 1986). Conceptually, if a receiver plant began drawing off N from the donor plants through cell leakage and then transport through the mycelial network, and no additional stresses were present, donor plants would up-regulate N_2 fixation by increasing nitrogenase activity and increasing nodule number. However, a 40 kg/ha loss from N_2-fixing soybean, like that occurring in 1997, would account for as much as a 20% of total N_2 fixation (calculated from Tables 1-5). Evidently, elasticity in the N_2 fixation process could not compensate to that extent.

The results of the soybean non-nod experiments appeared to indicate that differences existed in the ability of donor plants to transfer N. Based on seed N yields and $\delta^{15}N$ data, Young transferred lower amounts of N than did Pickett 71 and the breeding line N93-1264 (Figure 1). Pickett appeared to transfer larger amounts of N to non-nods in the earlier experiments of Burton et al. (1983). The absence of genotypic donor effects in

the soybean to weed transfer experiments should not necessarily be taken as contrary evidence (Table 6). The length of the experiments was limited to 60 days. The N_2 fixation system and its activity are not well developed in soybean until 3 to 4 weeks after germination (Sa and Israel, 1998), so there may not have been sufficient time for the genotypic differences to emerge.

Logically, one might expect that donor differences could be related with at least 3 factors. One would be elasticity of the feedback system, as mentioned above. The feedback process is linked with stress responses (King and Purcell, 2005), thus a donor genotype able to physiologically minimize stresses presumably would be better able to adjust feedback controls and sustain N transfer over a broader range of field conditions. In the case of a water limitation, for example, a key might be depth of rooting or a finer root system that acquires water more efficiently. In our experiments, rates of N_2 fixation alone evidently were not the determinant of N transfer, because donor differences occurred even though soybean genotypes fixed similar amounts of N. A second factor could be the extent of mycorrhizal colonization and the number of inter-specific hyphal connections. In a previous study with soybean and maize, hyphal density was positively correlated with transfer (Hamel et al. 1991). Our examination of mycorrhizal colonization was limited to sampling late in the experiments, to check that significant colonization was present, so it was not intensive enough to evaluate a role for hyphal density. The key issue, however, is not simply hyphal density, but rather *inter-specific* hyphal density, which would be extremely difficult to determine experimentally in the field.

A third factor that could be involved in N transfer differences among donors is the relative competitiveness of donor and receiver plants. Current thinking is that transfer through the mycorrhizal network is driven by source-sink effects (He et al., 2003; Simard et al., 2002; Simard and Durall, 2004; Smith and Read, 1997). One could envision that donor plants that become established and grow more quickly might become dominant spatially and in acquisition of resources from the soil, decreasing the growth potential and sink strength of competing receiver plants, which would limit N transfer. Nonetheless, the competitive relationship between donor and receiver plants would be complex, strongly influenced by environmental interactions, difficult to resolve experimentally (Cousens 1996; Radosevich 1987), and one can devise possible scenarios where they might be positive or negative. Unfortunately, none of our data, or any in the literature, allow evaluation of the impact of competitive effects on N transfer.

N transfer to weeds: agronomic considerations

Soybean and peanut are two of the most important crops in the southeastern U.S. and much of the acreage is on highly weathered, sandy soils with low fertility, such as those used in our experiments. Most often, fields are fertilized with all major nutrients except N, and N_2 fixation provides sufficient N to maximize yields (Israel and Burton, 1997). As with other crops in the Southeast, both soybean and peanut have substantial weed problems (Dowler, 1995). It has been unknown how weeds obtain enough N to survive and compete for other resources. The $\delta^{15}N$ abundance data show that weeds can acquire substantial amounts of N through transfer from the N_2-fixing crop plants.

The possibility that N_2-fixing soybean genotypes have different potentials to transfer N to receiver plants raises the possibility that a degree of weed control could be

achieved through plant breeding. An examination of troublesome weed species in peanut and soybean in the southeastern U.S. reveals that about two-thirds are mycorrhizal hosts (Dowler, 1995; Webster, 2001) and could be influenced by lowered N transfer. From previous reasoning, overall competitiveness of a soybean or peanut donor plant would be an important trait, along with any traits associated with direct suppression of the transfer process.

A major frustration in these experiments has been the inability to conduct valid experiments that evaluate the extent that transferred N increases weed competitiveness. Growth of mycorrhizal weeds in plots alone cannot be compared to mixed plots with the N_2-fixing crop plants. Crops like soybean have a much larger seed and much higher seed nutrient content than the weed species (Wright et al., 1999b). Thus, early competition may reduce weed growth, even though the weeds may be receiving transferred N. A useful experiment to evaluate competitiveness would have the same weed species plus and minus mycorrhizae, a treatment used previously by others that involves soil fumigation (Hamel et al., 1991; Hamel and Smith, 1992). We have resisted this approach because of the potential confounding effects on the soil microbial population as a whole and particularly on the nodulation process. Another consideration with the weed experiments is that the growth period was limited to 60 days because plants had to be removed prior to seed maturation. We had the opportunity to measure $\delta^{15}N$ abundance in seed tissues of mature volunteer sicklepod plants in soybean fields near the end of the growing season, and the results indicated that transfer accounted for as much as 80% of the N present (data not shown). Thus, the $\delta^{15}N$ values seen here over shorter time periods are likely to be minimal estimates of N transfer in weed species approaching maturity.

One can reasonably assume that breeding advances that suppress N transfer could markedly lower weed competitiveness and reproduction, and thus lower weed pressures over time.

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Table 1. 1997 yield, seed protein, total seed protein (yield x seed protein), and total plant N for nod soybean lines grown alone and with non-nod (Mix) line

	Yield	Seed Protein	Total Seed Protein	Total N
	kg/ha	% protein	kg/ha	kg/ha
Young	3092	39.6	1224	256.6
Young (Mix)	3455	39.5	1365	286.2
Hood	2977	37.6	1119	234.6
Hood (Mix)	3443	37.7	1299	272.4
Pickett	2870	37.0	1062	222.7
Pickett (Mix)	2528	28.7*	878	184.1
N93-1264	2965	40.2	1191	249.7
N93-1264 (Mix)	2465	40.9	1009	211.6
$lsd_{.05}$	576	1.08	241	

Total N in plants is (total seed protein/6.2) x 1.3 (see text)
* indicates significant difference (P≤0.05 using the GLM procedure for LSD) between soybean line grown with the Nn and grown alone

Type III mean squares and significance of the F test statistics for each performance parameter

	df	Yield	Seed Protein	Total Seed protein
Rep	2	22353	0.0048	5532
Treatment	8	350548*	12.2528**	67057*

* P≤0.05 ** P≤0.01

Table 2. 1998 yield, seed protein, total seed protein (yield x seed protein), and total plant N for nod soybean line grown alone and with non-nod (Mix) line

	Yield	Seed Protein	Total Seed Protein	Total N
	kg/ha	% protein	kg/ha	kg/ha
Young	3136	42.1	1321	276.9
Young (Mix)	4255*	41.8	1779*	373.0
Pickett	3186	39.7	1265	265.2
Pickett (Mix)	3557	38.3*	1368	286.8
N93-1264	2881	45.1	1299	272.4
N93-1264 (Mix)	3577*	44.9	1604*	336.3
$lsd_{.05}$	462	1.16	208	

Total N is (total seed protein/6.2) x 1.3 (see text)
* indicates significant difference (P≤0.05 using the GLM procedure for LSD) between soybean line grown with the Nn and grown alone

Type III mean squares and significance of the F test statistics for each performance parameter

	df	Yield	Seed Protein	Total Seed protein
Rep	3	164071	3.5737	44476
Treatment	6	782985**	24.3340**	142612**

* P≤0.05 ** P≤0.01

Table 3. 1997 yield, seed protein, total seed protein (yield x seed protein), and total plant N for non-nod (Nn) soybean line grown alone and with nod lines.

	Yield	Seed Protein	Total Seed Protein per plant	Total N in Plants	Total N transferred
	kg/ha	% protein	kg/ha	kg/ha	kg/ha
Pure Nn	1152	25.0	287	60.2	0.0
Nn (Young)	1253	26.0	327	68.5	8.3
Nn (Hood)	1239	27.2*	338	70.9	10.7
Nn (Pickett)	1699*	28.7**	483†	101.3	41.5
Nn (N93-1264)	1612†	27.1*	437*	91.6	31.4
$lsd_{.05}$	478	2.15	130		

Total N is (total seed protein/6.2) x 1.3 (see text). Total N transferred is the difference between total N in the Nn grown with a nod variety and total N in Nn plants grown alone
* indicates significant difference (P≤0.05 using the GLM procedure for LSD) between Nn grown with soybean line and Nn grown alone
** P≤0.01 † P≤0.1

Type III mean squares and significance of the F test statistics for each performance parameter

	df	Yield	Seed Protein	Total Seed protein
Rep	2	32419	1.9326	1697
Treatment	4	181898†	5.6523*	20099*

* P≤0.05 ** P≤0.01 †P≤0.1

Table 4. 1998 yield, seed protein, total seed protein (yield x seed protein), and total plant N for non-nod (Nn) soybean line grown alone and with nod lines.

	Yield	Seed Protein	Total Seed Protein per plant	Total N in Plants	Total N transferred
	kg/ha	% protein	kg/ha	kg/ha	kg/ha
Pure Nn	1021	25.8	265	55.6	0.0
Nn (Young)	847	30.2*	258	54.1	0.0
Nn (Pickett)	1395	27.2	380	79.7	24.1
Nn (N93-1264)	1169	28.2	332	69.6	14.0
$lsd_{.05}$	469	3.15	158		

Total N is (total seed protein/6.2) x 1.3 (see text). Total N transferred is the difference between total N in the Nn grown with a nod variety and total N in Nn plants grown alone
* indicates significant difference ($P \leq 0.05$ using the GLM procedure for LSD) between Nn grown with soybean line and Nn grown alone

Type III mean squares and significance of the F test statistics for each performance parameter

	df	Yield	Seed Protein	Total Seed protein
Rep	3	172342	4.4783	13291
Treatment	3	215636‡	13.47*	13423$^{\Sigma}$

* $P \leq 0.05$ ** $P \leq 0.01$ $^{\ddagger}P \leq 0.12$ $^{\Sigma}P \leq 0.31$

Table 5. Estimates of total N transferred to the non-nod soybean line grown in mixed culture with nod N_2-fixing soybean lines using the ^{15}N abundance method. Data are from soybean seed harvested at maturity. Individual values are means of 3 (1997) or 4 reps (1998). Soil $\delta^{15}N_o$ values were 4.2 (+/- 0.3) both years. The $\delta^{15}N_a$ value from N_2 fixation only equals -1.1.

A. 1997

	Nod in Pure Culture		Non-nod in Mixed Culture		Non-nod N obtained from the nodulated varieties	
	$\delta^{15}N_t$ values	% of N from fixation[1]	$\delta^{15}N_t$ values	% of N from fixation[1]	% N acquired by transfer[2]	Total N transferred (kg/ha)[3]
Young	-0.6	93.0	2.1	42.0	45.0	30.8
Hood	0.0	80.0	2.3	38.0	48.0	34.0
Pickett	-0.6	93.0	2.3	38.0	41.0	37.9
N93-1264	-0.5	91.0	2.1	41.0	45.0	41.2
$lsd_{.05}$	n.s.	11	n.s.	n.s.		

B. 1998

	Nod in Pure Culture		Non-nod in Mixed Culture		Non-nod N obtained from the nodulated varieties	
	$\delta^{15}N_t$ values	% of N from fixation[1]	$\delta^{15}N_t$ values	% of N from fixation[1]	% N acquired by transfer[2]	Total N transferred (kg/ha)[3]
Young	-0.4	90.0	3.5	16.0	17.0	9.2
Pickett	-0.4	89.0	3.3	18.0	20.0	15.9
N93-1264	-0.8	95.0	3.4	17.0	18.0	12.5
$lsd_{.05}$	n.s.	n.s.	n.s.	n.s.		

[1] % N from fixation is [(4.2 - $\delta^{15}N_t$ value) / ($\delta^{15}N_o$ value + 1.1)] x100

[2] % N acquired from transfer corrects for N in nod lines originating from soil.
It is calculated from [%N from fixation (non-nod mixed)] / [% N from fixation (nod pure)]

[3] Non-nod total N transferred is total plant N (non-nod mixed, Table 2) x % N acquired by transfer.

Table 6. Estimates of N_2 fixation and N transfer between crop and weed species grown together in mixed culture as determined by the ^{15}N natural abundance method.

Location	Year	Nod Host	Weed	$\delta^{15}N$ ± s.e.m.	fixed N (%)[1]	N acquired by transfer (%)[2]
Lewiston	1998	Peanut		1.3 ± 0.4	58	
			prickly sida	3.6 ± 0.4	18	31
			yellow nutsedge	4.6 ± 0.2	0	0
	1999	Peanut		0.8 ± 0.4	59	
			prickly sida	1.3 ± 0.2	49	83
			yellow nutsedge	3.5 ± 0.2	0	0
	2000	Peanut		1.1 ± 0.4	65	
			prickly sida	2.1 ± 0.2	48	74
			yellow nutsedge	3.8 ± 0.5	20	31
Clayton	1998	Pickett 71		(-0.6) ± 1.5	95	
			sicklepod	5.1 ± 0.6	39	41
	2000	N93-1264		(-0.8) ± 0.2	95	
			prickly sida	2.7 ± 0.2	38	40
		Young		(-0.5) ± 0.1	90	
			prickly sida	2.9 ± 0.2	34	38
	2001	Pickett 71		(-1.1) ± 0.2	100	
			prickly sida	0.8 ± 0.2	60	60
			Palmer amaranth	3.6 ± 0.2	0	0
		Young		(-1.1) ± 0.2	100	
			prickly sida	0.9 ± 0.2	57	57
			Palmer amaranth	3.2 ± 0.2	9	9

Soil $\delta^{15}N$ values (± s.e.) as determined from ^{15}N measurements of weeds grown in pure sand: Lewiston 1998 = 4.6 (0.2), 1999 = 3.0 (0.4) and 2000 = 5.1 (0.4);
Clayton 1998 = 9.0 (1.5), 2000 = 5.0 (0.5) and 2001 = 3.6 (0.2)

[1] Fixed N is (soil $\delta^{15}N$ - plant $\delta^{15}N$) / (soil $\delta^{15}N$ + 1.1)

[2] N transferred is fixed N (weed) / fixed N (crop)

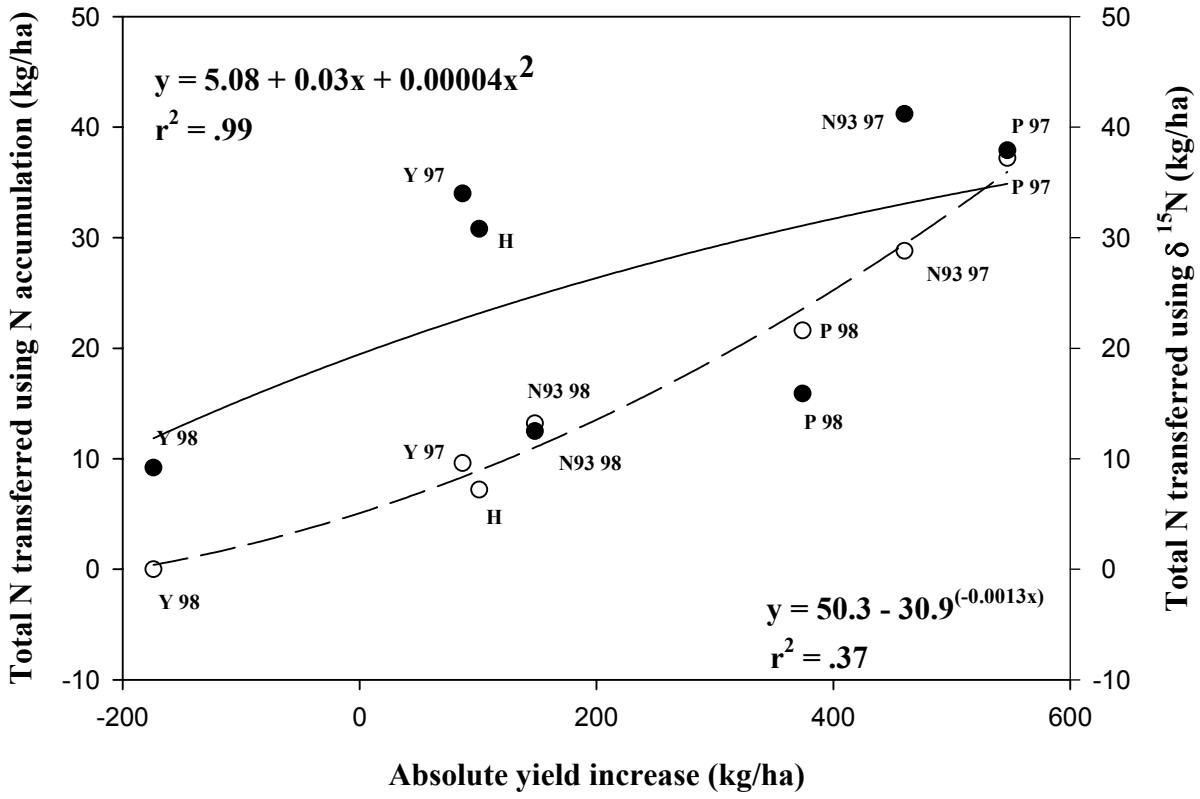

Figure 1. Relationship between absolute yield increase of non-nod line and total N transferred to non-nod determined by the N accumulation method (○, broken line) or by the $\delta^{15}N$ abundance method (●, solid line). Insets indicate soybean donor lines and experiment year.

CHAPTER III

ACCUMULATION AND LOCALIZATION OF ALUMINIUM IN ROOT TIPS OF LOBLOLLY PINE SEEDLINGS AND THE ASSOCIATED ECTOMYCORRHIZA *PISOLITHUS TINCTORIUS*

ABSTRACT

Evidence from past studies suggests that loblolly pine may be tolerant of Al. The experiments described in this manuscript were initiated to examine Al tolerance and Al accumulation in the pine root and the degree of Al accumulation in fungal hyphae when pine roots were colonized with the ectomycorrhiza *Pisolithus tinctorius*. The experiments used lumogallion staining and confocal microscopy to localize Al in root and fungal structures. The results clearly showed that loblolly pine seedlings were highly resistant to Al. A decrease in primary root extension could not be detected until Al^{+3} activities approached 40 μmol L^{-1}, and extension was suppressed only 30% at an Al^{+3} activity of 580 μmol L^{-1}. This contrasted with the response of the Al sensitive 'check' species soybean, where primary root extension was severely restricted at Al^{+3} activities lower than 5 μmol L^{-1}. Tissue Al measurements and lumogallion fluorescence of longitudinal sections of the pine root tip indicated that tolerance was associated with both Al exclusion from the tip region and compartmentalization of absorbed Al in peripheral cell areas outside of the meristem. In lateral roots colonized with ectomycorrhizae, lumogallion fluorescence showed that large amounts of Al accumulated at the fungal mantle and in areas with the Hartig net. At higher magnification, lumogallion indicated substantial Al accumulation inside hyphae. Little Al could be detected in lateral root cells. The results show that pine possesses multiple mechanisms that can contribute to Al tolerance in acid field soils.

INTRODUCTION

Aluminum toxicity is a constraint to plant growth in highly weathered, acid soils in many parts of the world (von Uexkull & Mutert 1995; Eswaran, Reich & Beinroth 1997). Problems have been aggravated in developed countries by acid precipitation, which can accelerate the breakdown of Al-containing minerals and leaching of basic cations (McBride 1994). As a result, plant roots face an environment that is poor in essential nutrients and has an increased presence of toxic Al^{+3} and H^{+}. Such alterations in soil chemical properties have been implicated in reduced productivity and decline of temperate forests (Godbold, Fritz & Hutterman 1988; Cronan 1994).

Even though forest productivity can be adversely impacted by acidity and Al toxicity, there is some evidence to suggest that loblolly pine (*Pinus taeda* L.) might be relatively tolerant to Al. It is problematic to compare results of Al studies from the literature, because experimental differences strongly influence apparent sensitivities. That caution notwithstanding, experiments using solution culture and crop plants such as wheat (Tice, Parker & DeMason 1992; Samuels, Kucukakyuz & Rincon-Zachary 1997), soybean (Yang et al. 2000; Silva et al. 2001a), and corn (Pellet, Grunes & Kochian 1995) have indicated strong inhibition of root growth at Al^{+3} activities between 5 and 15 μmol L^{-1}. Some solution culture experiments with loblolly pine, in contrast, indicated root growth restrictions did not become evident until Al^{+3} activities approached 150 μmol L^{-1} (Tepper, Yang & Schaedle 1989; Raynal et al. 1990), and pine appeared to be relatively tolerant to Al compared to other tree species (Nowak & Friend 1995).

An evaluation of Al toxicity in pine is not complete without consideration of the possible involvement of ectomycorrhizae. In most field conditions, ectomycorrhizal fungi form a symbiotic relationship with pine roots; the roots provide carbon for the fungus and

the fungus aids the plant in nutrient acquisition (Smith & Read 1997). Experiments have shown that ectomycorrhizal colonization can ameliorate Al toxicity effects (Hentschel et al. 1993; Godbold 1994; Schier & McQuattie 1995, 1996; Godbold et al. 1998). It has been suggested that the positive effects of ectomycorrhizae might result from blocking Al entry into root cells (Jentschke & Godbold 2000). Even when ectomycorrhizae are present, however, Al sensitivity of the pine root itself remains an issue, because ectomycorrhizal colonization typically does not occur on the entire root system (Rosado, Kropp & Piche 1994; Schier & McQuattie 1995, 1996; Visser 1995), and Al can come into direct contact with non-colonized root tissues.

In this study, we examine several aspects of Al toxicity in loblolly pine. One is the degree of Al tolerance present in the primary root and its relationship with accumulation of Al in cells of the root tip. Studies with crop species (Ryan, Ditomaso & Kochian 1993; Sivaguru & Horst 1998) indicate that cell areas in close proximity to the root tip are the main site of Al toxicity, and one can assume that is true also with tree species. If Al penetrates into the meristem and adjacent areas in the root's interior, cell division and expansion can be disrupted. A high degree of Al tolerance in pine may imply that Al is efficiently excluded from the root tip and/or accumulated in cells separate from the growth center. In other experiments using pine colonized with ectomycorrhizae, Al accumulation in roots cells and ectomycorrhizal structures is examined. If ectomycorrhizae were to block Al entry into root cells, one might presume that large amounts of Al would be accumulated by ectomycorrhizal structures. Indeed, experiments using X-ray microanalysis have indicated that Al accumulates in ectomycorrhizal cell walls (Brunner & Frey 2000). The extent of Al accumulation within ectomycorrhizal cells is unknown.

A key feature of these experiments with pine is the use of the fluorescent probe lumogallion to localize Al (Silva et al. 2000; Silva et al. 2001a; Kataoka et al. 1997; Kataoka & Nakanishi 2001). Lumogallion binding to Al is highly specific (Shuman 1992; Sutheimer & Cabaniss 1995; Gabriels, Van Keirsbulch & Engels 1981) and has a high degree of spatial resolution. This allowed visualization of Al accumulation in cells of the primary root tip and in fungal structures in colonized roots.

MATERIAL AND METHODS

Growth in Solution Culture

Experiments in solution culture examined Al tolerance of the primary root. Seeds from one open-pollinated family of loblolly pine (*Pinus taeda* L.) were stratified (moistened and refrigerated for 30 days at 4^{o}C) and germinated in paper for seven days at 30°C. Seedlings were placed into 12 L hydroponics units (custom built by our research group) with a rapid flow rate, ~ 4 L per minute, and with temperature and pH control. Temperature was maintained at 25°C and pH was strictly controlled at 4.3 ± 0.1.

The experiments included companion treatments with soybean, which served as an Al-sensitive 'check' species. Seeds of soybean (*Glycine max* L. Merr.) cv. 'Young' were germinated in paper rolls placed in a dark germination chamber at 25^{o}C and 98% relative humidity and kept moist by capillary action with 0.1 mM $CaSO_4$ solution. After 72 hours, seedlings were selected for uniformity and transferred into the hydroponics units.

The hydroponics units were located in a growth room programmed for an 8/16h light/dark period and an aerial temperature of 32/24^{o}C. A radiance level of 1200 μmol m^{-2} sec^{-1} was provided by a combination of metal halide (Philips, Maddox Supply, Durham, NC) and incandescent lamps (Philips Halogena, Maddox Supply). The complete nutrient

solutions contained (in μmol L^{-1}): 800 $CaSO_4$, 300 $MgSO_4$, 600 KNO_3, 75 KH_2PO_4, 0.055 $ZnSO_4$, 0.06 $MnCl_2$, 0.3 $B(OH)_3$, 0.065 $CuSO_4$, and 25 $FeSO_4$. Aluminum was added from an acidified 1M $AlCl_3$ (A573, Fisher Scientific, Suwannee, GA) stock solution to establish activities specified in the Results section. The Al^{+3} activities were calculated using Geochem-PC (Parker, Norwell & Chaney 1995). For pine, there was a 28-day adjustment period in solution culture before Al was added. Length of the primary root (8-12 cm) was measured at the time of Al addition and 14 days later. For soybean, using protocols established previously (Silva et al. 2000; Silva et al. 2001a, b), a 24 hr adjustment period was followed by Al exposure for 3 days. As with pine, roots were 8 to 12 cm in length, and individual root lengths were measured at the beginning and end of the Al exposure. Different adjustment periods were used for pine and soybean (28 day vs 24 hrs) to allow Al sensitivity comparisons at a similar stage of morphological development. For both pine and soybean experiments, each treatment consisted of 21 seedlings, and experiments were replicated over time.

Growth in Sand

Pine seedlings were grown in aseptic conditions in an environmental chamber at the Duke University Phytotron. Seeds were germinated and seedlings initially grown in a sterile agar medium for 2 weeks. Then, the seedlings were transferred to acid-washed sand bags (0.3 dm^{-3}) for an eight-week pretreatment period. Four weeks into the pretreatment, one-half of the seedlings were inoculated with a liquid culture containing mycelia of the ectomycorrhizae *Pisolithus tinctorius* and colonization was allowed to develop. Radiance was provided at 350 μmol m^{-2} s^{-1} during the 16 h photoperiod by a combination of fluorescent and incandescent lamps (Philips, Maddox Supply). All seedlings were

automatically irrigated daily with a solution containing (mmol L^{-1}) 7.0 NH_4NO_3, 0.1 KH_2PO_4, 2.0 K_2SO_4, 1.5 $MgSO_4$, 3.0 $CaCl_2$, and (μmol L^{-1}) 140 Fe-EDTA, 50 B, 10 Mn, 1.0 Zn, 1.0 Cu, 5.0 Mo, and 0.16 Co.

Aluminum treatments began at the end of the eight-week pretreatment period. Solutions (pH 4.0) containing Al activities of 0, 43, 94 and 1,180 μmol L^{-1} Al^{+3} activities, respectively, were applied to sets of plants daily. Control plants not exposed to Al were irrigated with only de-ionized water (pH 4.0). Nutrient solution similar to that in the pre-treatment phase (except without $CaCl_2$ and Fe EDTA) was applied weekly to all plants. The Al treatment period lasted for eight weeks. At the time of harvest, some seedlings were used for Al localization (see below). For the remaining seedlings, plant height and stem diameter were measured, roots were washed free of sand with de-ionized water, and shoot and root material were separated and dried in a forced-draft oven. Experimental units consisted of 20 seedlings per treatment.

Aluminum in Root Tips

Tips of primary roots (~1 cm) were excised, weighed, placed in net well trays, and rinsed in ice-cold 10 mM citric acid (BP399, Fisher Scientific) for 30 min. The citrate wash has become a standard procedure in Al experiments to remove loosely bound apoplastic Al and minimize metal redistribution (Zhang & Taylor 1990; Samuels et al. 1997; Silva et al. 2000; Watanabe, Osaki & Tadano 2001). After the citrate wash, tips were rinsed in de-ionized water, placed in a 60°C drying oven for 72 hr, transferred to Teflon bottles, digested overnight in 1 ml Optima grade HNO_3 (A467, Fisher Scientific), and then microwave-digested for 30 minutes under a steady stream of compressed N_2 gas. The root material and HNO_3 then were transferred into 15 ml tubes, taken to 5 ml with de-ionized

H_2O, and total Al was determined by inductively coupled plasma (ICP) atomic emission spectrometry (Model 2000DV, Perkin-Elmer, Norwalk, CT).

Microscopy

The procedure for visualizing Al distribution among cells at the root tip using lumogallion staining and confocal microscopy has been detailed elsewhere (Silva et al. 2000). Briefly, plants were carefully removed from the hydroponics system or the sand bags, and tips of primary and lateral roots (~1 cm) were excised and rinsed in ice-cold citric acid for 30 min. The tissue was then embedded in agarose and sliced in 100 μm-thick longitudinal sections using a vibratome (1000 Plus, The Vibratome Company, St. Louis, MO). The root tip sections were stained with the fluorescent Al-indicator lumogallion (10 mmol L^{-1}; TCI America, Inc., Portland, OR) and mounted on glass slides with a glycerol medium containing the nuclear stain DAPI (4',6-diamidino-2-phenylindole 2HCl; 1 μg mL^{-1}; Sigma, St. Louis, MO). The slides were examined using a Leica TCS-SP confocal system with an inverted microscope (model DMIRBE) and either a dry Plan Apochromat 20 x/0.60 numerical aperture (N.A.), a 40 x/1.25 N.A. oil immersion, or a 63 x/ 1.2 N.A. water immersion objective (Leica, Wetzlar, Germany). The only exceptions are figure 4 and figure 6, which were acquired on a Zeiss inverted microscope (Zeiss Axiovert TV100, Zeiss, Thornwood, NY), fitted with FITC and UV illumination. The Hartig net was visualized using dark field microscopy after staining root tip longitudinal sections with 4% (v/v) Chlorazol Black E (Sigma, St. Louis, MO) overnight. Images of intact mycorrhizal root tips were obtained under a dissecting microscope by dark field microscopy and simultaneous UV illumination (Leica MZ FLIII fluorescence stereomicroscope; Leica,

Wetzlar, Germany) with a Hamamatsu color chilled 3CCD camera (Model C5810, Hamamatsu, Bridgewater, NJ).

RESULTS

Al effects on primary root extension in solution culture

The initial experiments examined accumulation of Al in the root tip and the restriction of primary root extension when pine seedlings were exposed to a range of Al^{+3} activities. Aluminum accumulation in the root tip was negligible until the solution Al^{+3} activity approached 40 μmol L^{-1} (Fig 1A), and then it increased only to about 15 to 25 μmol g^{-1} DW even as the solution Al activity increased to 580 μmol L^{-1} (note log scale). Root extension was noticeably inhibited at about 40 μmol L^{-1}, but effects were gradual. Extension was inhibited only 30% at the 580 μmol L^{-1} Al^{+3} activity level.

Soybean was included in the experiments to allow comparison with an Al sensitive species. At Al^{+3} activities ranging from 1 to 50 μmol L^{-1}, soybean accumulated Al in a linear fashion (Fig 1B). Primary root extension was strongly inhibited below 5 μmol L^{-1} and little extension occurred above 10 μmol L^{-1}. Extension was inhibited 40 to 50% when the Al concentration in the root tip tissue was lower than 5 μmol g^{-1} DW and severely inhibited when tissue Al was 15 to 20 μmol g^{-1}.

Longitudinal sections of root tips from pine seedlings exposed to 40 μmol L^{-1} Al^{+3} activity were examined using the Al-lumogallion staining. Strong fluorescence occurred in particular cell areas in the root tip (Fig 2A). The highest Al levels were present in root cap and parenchyma cells, with fluorescence located primarily at the plasma membrane/cell wall membrane interface. Little Al penetrated into the meristematic region. An image of the

root section stained with DAPI shows the location of stained nuclei in the densely packed meristem (Fig 2B). An image of meristematic cells with increased magnification and laser strength to enhance visualization of the lumogallion/Al complex can be seen in Fig 2C. The lumogallion image again indicates the presence of Al at the plasma membrane/cell wall interface but little Al inside cells. A DAPI overlay (Fig 2D) confirms that the prominent nuclei within the meristematic cells were separate from the Al at the cell periphery. The detection of Al by lumogallion extends into the nM range (Katyal & Prakash 1977; Shuman 1992).

Al effects in sand culture with ectomycorrhizal seedlings

A series of experiments was conducted with pine seedlings growing in sand and colonized with the ectomycorrhizal fungus *P. tinctorius*. Only the highest Al treatment (10 mmol L^{-1} $AlCl_3$ = 1.18 mmol L^{-1} Al^{+3} activity) had a negative impact on growth, with root dry weight accumulation inhibited about 10% (data not shown). The primary root was not colonized with ectomycorrhiza. Longitudinal sections of the primary root tip from seedlings exposed to 1.18 mmol L^{-1} Al^{+3} activity were examined using lumogallion (Fig 3). A differential interference contrast (DIC) image of the longitudinal section shows the cellular structure of root tip cells (Fig 3A). The root cap structure was somewhat different than that in solution culture, as there were 8 to 10 root cap cell layers between the exterior surface of the root and the meristematic region compared to about 20 root cap cell layers in solution culture where the tip was more elongated (cf. Fig 2A). Staining the sections with DAPI indicated the location of densely packed cells in the meristematic region (Fig 3B). Lumogallion staining showed that Al accumulated mostly in cells forward of and to the side of the meristem (Fig 3C). In those areas, some fluorescence signal could be seen inside

cells, but most appeared to be located at the cell wall/plasma membrane interface. Analysis of the primary root tip indicated that the Al concentration was about 35 μmol g^{-1}. Some fluorescence signal was detectable in meristematic cells, but the amount of Al was low.

Lateral roots were extensively colonized by the ectomycorrhizal fungus *P. tinctorius* (Fig 4). The blue-white fluorescence resulting from UV illumination (Fig 4A) indicates the location of the ectomycorrhizal mantle (EM), a colonized lateral tip (MT) and extraradical hyphae (MB). The Hartig net, stained black by the fungal specific stain Chlorazol Black E, can be easily seen between the individual cortical cells of a longitudinally sectioned, colonized lateral root tip under dark field microscopy (4B). Examinations of many roots failed to reveal any differences in the extent of colonization in the presence of Al compared to roots not exposed to Al. We did not find ectomycorrhizal colonization of primary roots in any of these experiments, a pattern that evidently is common (cf. Chapter 6 in Smith and Read 1997).

A longitudinal section of a lateral root exposed to Al shows the extensive development of the ectomycorrhizal fungus, establishing the mantle outside of the root cells and the Hartig net at the periphery of cells at the epidermis and cortex (Fig 5A). Lumogallion staining produced an intense Al-fluorescence signal in the mantle and mycelia surrounding the lateral root tip (Fig 5B). High levels of Al also were present at the Hartig net. There was little Al- fluorescence signal in the symplasm of lateral root cells.

More detailed images were taken at higher magnification and laser strength to assess the degree of Al accumulation by extraradical hyphae. The images revealed a strong lumogallion fluorescence signal and substantial Al binding at mycelial strands (Fig 6B). Individual hyphae were examined under higher magnification. Images with DIC (Fig 6C)

and lumogallion/DAPI fluorescence superimposed, showed a strong Al-fluorescence signal coming from the hyphal interior (Fig 6D). Intracellular Al evidently was present throughout the fungal symplasm (Fig 6D).

DISCUSSION

The results of our experiments clearly demonstrated that the primary root of pine is extremely tolerant to Al, separate from any influence of ectomycorrhizae. In solution culture with Al^{+3} activities below 40 μmol L^{-1}, inhibition of root extension could not be detected, and inhibition was only 30% at 580 μmol L^{-1}. The magnitude of Al tolerance is brought into focus by the direct comparison with the crop species soybean, whose root extension was strongly suppressed at a solution activity of 5 μmol L^{-1} Al^{+3}. We have examined Al tolerance of a number of crop and native species in this solution culture system (Moyer-Henry et al. 2003), and the soybean sensitivity range is typical of that observed with most agronomic crop species (e.g. corn, sorghum, barley; and see Silva et al. 2001b for wheat). From an ecological perspective, it should not be surprising that pine has an exceptional degree of Al tolerance. In the southeastern U.S., its ecological niche is the later stages of vegetative succession (Keever 1950; Bormann 1953), when soils are characteristically acid with high levels of soluble Al (Richter & Markewitz 2001).

At least two general types of mechanisms apparently contributed to Al tolerance of the pine root. Most obvious is the ability to exclude Al from the root. Accumulation of Al in the root tip remained low and near background levels until external Al^{+3} activities were increased to about 40 μmol L^{-1}, and only 15 to 25 μmol g^{-1} accumulated in the tip when external Al^{+3} activity was in the 250 to 600 μmol L^{-1} range. This contrasted sharply with Al

accumulation of soybean, where a similar level of Al accumulation in the root tip occurred at external Al^{+3} activities ranging from 12 to 25 μmol L^{-1}.

A second type of Al tolerance mechanism appeared to be intercellular compartmentalization of absorbed Al. Lumogallion images of the root tip showed that most Al accumulated in outer cell layers of the root cap, with minimal Al penetrating into the meristematic cell area. The same localization pattern was present in root tips of plants grown in solution and sand culture conditions (Figs 2A and 3C). Evidence that Al compartmentalization might be important in avoiding Al toxicity again comes from comparisons with soybean. The sections from pine root tips in solution culture that were stained with lumogallion had an Al concentration of 18 μmol g^{-1} DW. At a similar Al level in the root tip of soybean, root extension was severely inhibited to less than 10% of the control (Figs 1A and B). It is known from previous research that such a severe growth inhibition in soybean is accompanied by extensive Al penetration into the meristem and binding to nuclei (Matsumoto 1991; Silva et al. 2000; Kataoka & Nakanishi 2001). The relatively small amounts of Al that did penetrate into the meristematic zone of the pine root tip accumulated at cell peripheries, with little Al detectable inside meristematic cells, and there was no indication of spherical accumulation in the location of nuclei (Fig 2D).

The mechanisms in pine that are responsible for Al exclusion from the root and compartmentalization in particular cell areas are unknown. In crop plants, Al tolerance has often been correlated with the ability of roots to excrete chelating substances, such as organic acids, that bind to Al in the rhizosphere or root apoplast and prevent it from entering root tip cells, or bind to Al inside cells (Kochian 1995; Ma 2000; Ryan, Delhaize & Jones 2001; Kochian et al. 2002; Silva et al. 2004). The detoxification effect might also

involve phosphates (Pellet et al. 1996) or phenolics (Heim et al. 2000). While it is uncertain what complexing agents might be involved, the efficiency of Al exclusion in pine implies substantial extracellular binding. Also, with the apparent compartmentalization of Al in the pine root tip, it is reasonable to think that synthesis of complexing molecules and the associated binding and detoxification of Al would be intense at the surface and inside of the root cap cells. Lumogallion fluorescence does not allow for determination of which Al species is accumulating or to what the Al is bound.

As mentioned previously, pine is the most Al tolerant plant species we have found after years of screening under these highly controlled experimental conditions. Pine thus may be an important source of Al tolerance genes that could be used in development of transgenic Al-tolerant crop plants. Essential next steps include defining the biochemical mechanisms involved in Al binding and their functional significance.

With pine seedlings colonized by the ectomycorrhizal fungus *P. tinctorius* and exposed to Al in sand, lumogallion images indicated that substantial Al accumulation occurred at extraradical hyphae and the mantle. Aluminum penetrating into cellular areas of the lateral roots appeared confined mainly to cell peripheries (i.e. walls) where the Hartig net is located, and there was no indication of large-scale penetration into the symplasts of root cells. Although our results do not provide definitive evidence, the Al accumulation pattern is consistent with the idea that mycorrhizal structures can limit Al penetration into the root symplasm (Cumming & Weinstein 1990; Jentschke & Godbold 2000).

While previous experiments using X-ray microanalysis have shown that Al accumulates in the walls of ectomycorrhizal structures (Wilkins & Hodson 1989; Godbold & Jentschke 1998; Brunner and Frey 2000), the technique has inherent spatial limitations (see Lazof et al. 1994) and the observations are fundamentally different from those in our

study. With the use of the citrate wash in these experiments with pine, it is likely that most of the readily exchangeable Al would have been removed from the fungal cell walls and the walls of root cortical cells, as occurs with crop plants (Zhang & Taylor 1990). That notion is supported by the absence of intense Al accumulation at hyphal cell peripheries and the uniformity of lumogallion fluorescence within hyphae. The images strongly suggest that substantial amounts of Al were present inside fungal cells. Because function of ectomycorrhizae did not appear to be compromised, i.e. colonization was similar to controls, efficient detoxification of internal Al is implied. Other experiments *in vitro* have indicated that some ectomycorrhizal species are highly tolerant of Al (Thompson & Medve 1984; Tam 1995), however, mechanisms of Al resistance have not been resolved. Complexing of Al to non-toxic forms by organic acids (Hamel, Levasseur & Appanna 1999) or phosphates (Martin et al. 1994) seem to be possibilities for Al detoxification, but available evidence is somewhat inconsistent. Polyphosphate granules were found in the hyphae of *P. tinctorius*, for example, but Al-polyphosphate complexes could not be detected in highly Al-tolerant ectomycorrhizal species (Tam 1995).

Our experiments were not intended to be an evaluation of the ability of ectomycorrhizae to alleviate Al toxicity. An Al concentration range was selected that allowed examination of Al accumulation patterns without severe growth effects. While ectomycorrhizae did not alleviate the small Al toxicity effect with additions of 1.18 mmol L^{-1} Al^{+3} activity in sand, it is conceivable that some protection might have occurred at higher Al treatments or with a longer period of Al exposure. And, it should be pointed out that beneficial effects of ectomycorrhizal colonization have not always been obvious (Wilkins & Hodson 1989; Jentschke, Schlegel & Godbold 1991; Meharg & Cairney 2000).

In summary, it would appear that the pine and ectomycorrhizae association has a number of characteristics that could contribute to pine functioning well in acid, high Al soil conditions. Ectomycorrhizae would offer the first line of defense, perhaps by producing or stimulating the root to secrete substances such as oxalic acid (Ahonen-Jonnarth et al. 2000) that bind Al and prevent its absorption. Aluminum in close proximity to the fungal sheath could become adsorbed to negative charges on the fungal cell wall or membrane (Hodson & Wilkins 1991), or it could be absorbed into fungal cells and be bound and detoxified in the fungal symplasm. Aluminum reaching the root surface still could face intercellular fungal structures of the Hartig net, as well as negative charges on pine root cell walls. Mycorrhizal roots seem to be particularly hydrophobic and the fungal network acts as a barrier to apoplastic diffusion, limiting cation movement toward inner root tissues (Bucking et al. 2002). Lastly, even if absorbed into pine root cells, Al could be bound by organic or phosphate compounds, preventing movement into meristematic and vascular tissues.

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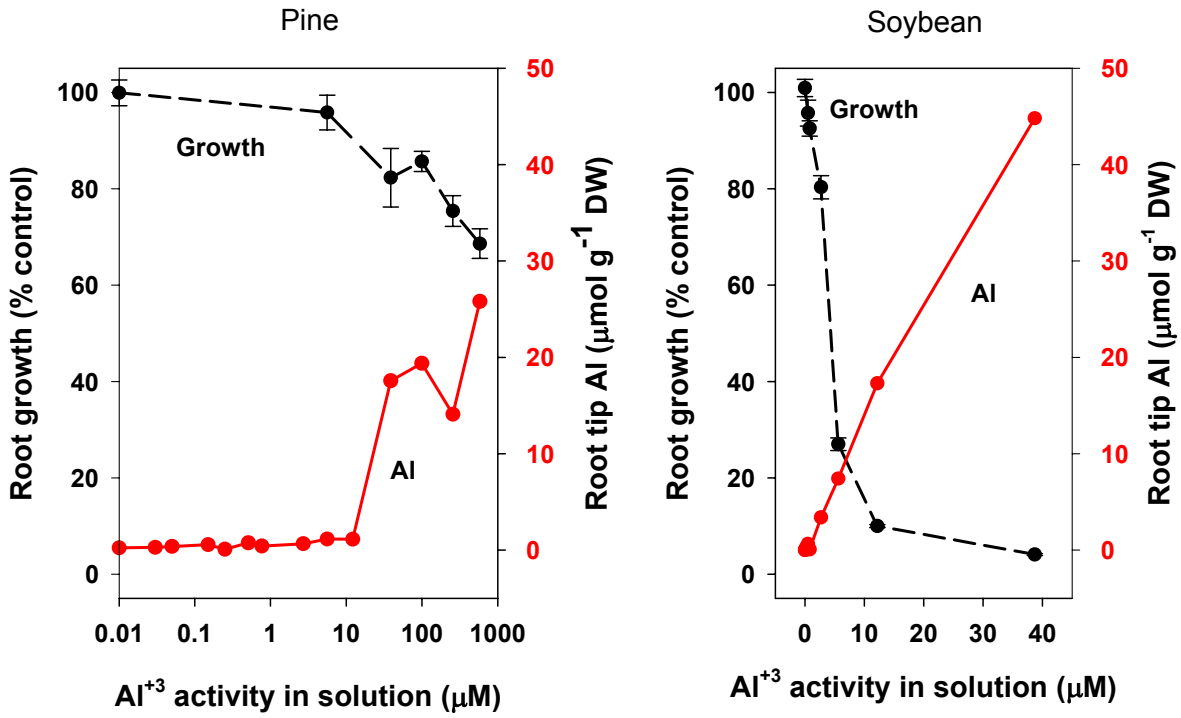

Figure 1. Extension of the primary root (left axis) and root tip Al (right axis) of pine (a) and soybean (b) as a function of Al^{+3} activity in solution. Primary roots of 4-week-old pine seedlings exposed to an Al^{+3} activity series in solution were measured prior to and just following 14 d of Al^{+3} exposure. Root extension is presented as a percent of the 0 Al control plant growth. Symbols: closed circle, root growth; open circle, root tip Al. Note the log scale on the pine x-axis. Each data point indicates the mean of 20 measurements, and error bars are standard errors of the mean.

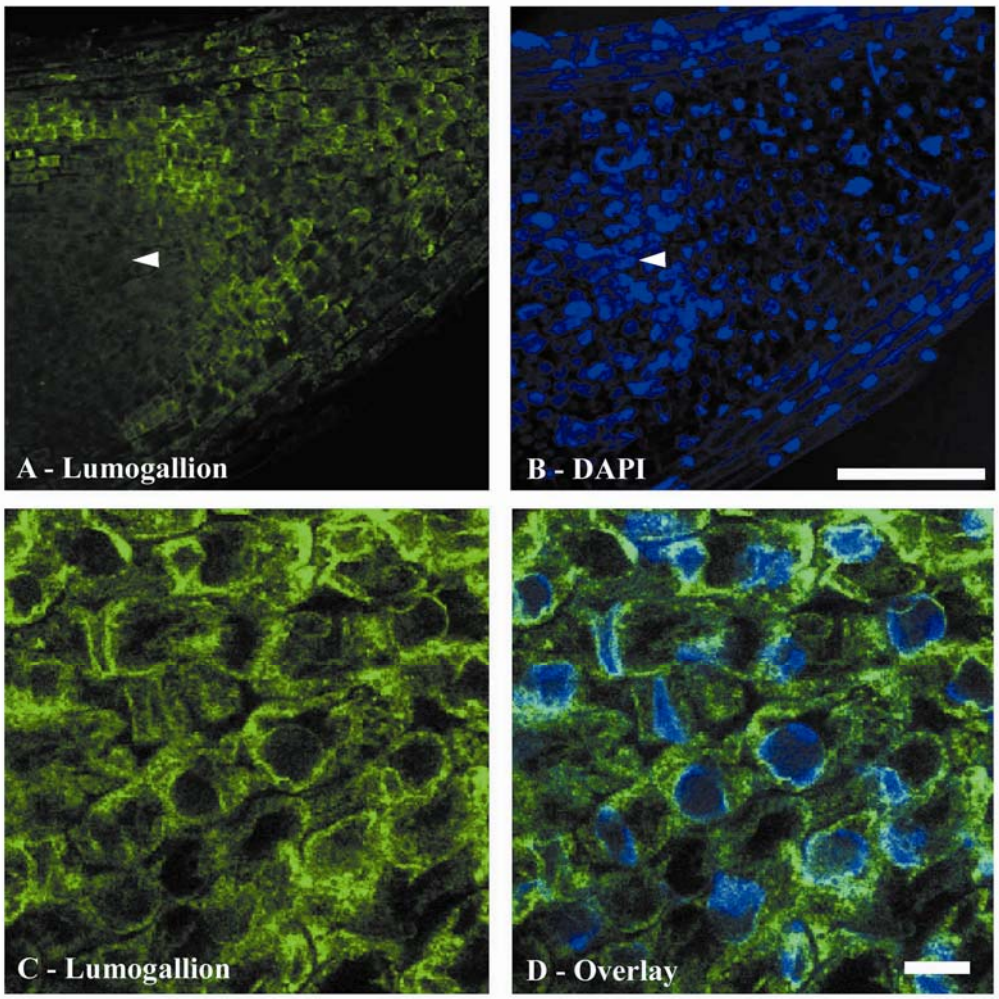

Figure 2. Confocal scanning microscopy images of primary root tip of pine exposed to 40 µmol L^{-1} Al^{+3} activity in solution. After 14 d, tips were collected, sectioned longitudinally, and stained with lumogallion and the nuclear stain DAPI. Lumogallion, (a); DAPI, (b); and lumogallion (c) and lumogallion and DAPI overlay (d) of meristematic cells at greater magnification. Arrows in (a) and (b) indicate areas of meristem that were magnified. Scale bar = 100 µm (b); scale bar = 10 µm (d).

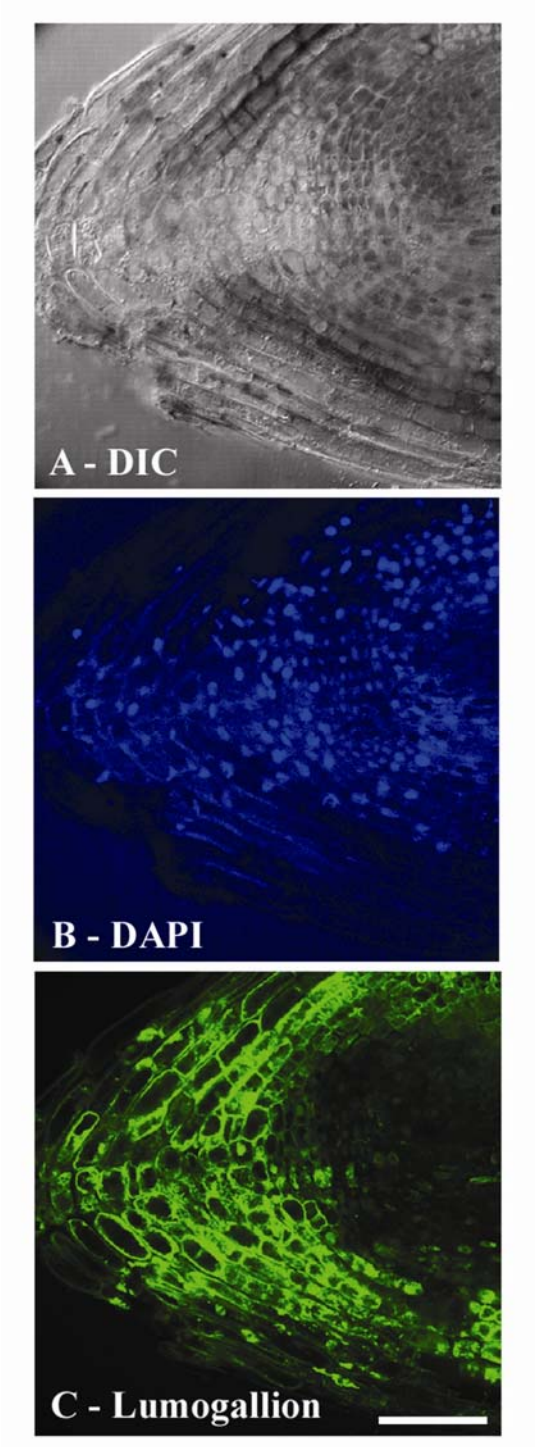

Figure 3. Confocal scanning microscopy images of primary root tip from pine seedlings exposed to 1.18 mmol L^{-1} Al^{+3} activity in sand culture for 8 weeks. The pine seedlings were inoculated with *P. tinctorius* and exposed to Al^{+3}. Tips were longitudinally sectioned, stained with lumogallion and DAPI. DIC (a); DAPI (b); and lumogallion (c). Scale bar = 100 μm.

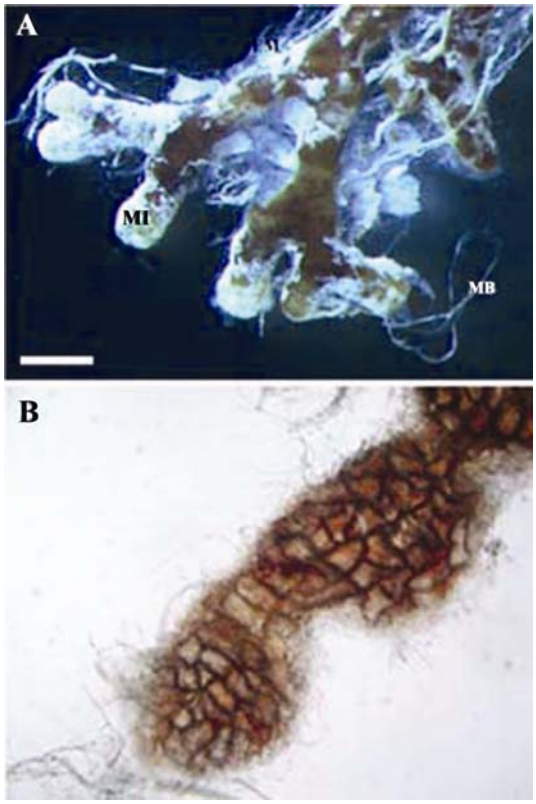

Figure 4. Micrograph of lateral root tips colonized by *P. tinctorius* using dark field microscopy with simultaneous UV illumination on a dissecting scope (a). Longitudinal section of lateral root tip stained with fungal specific Chlorazol Black E and visualized using dark field microscopy (b). Scale bar = 100 μm.

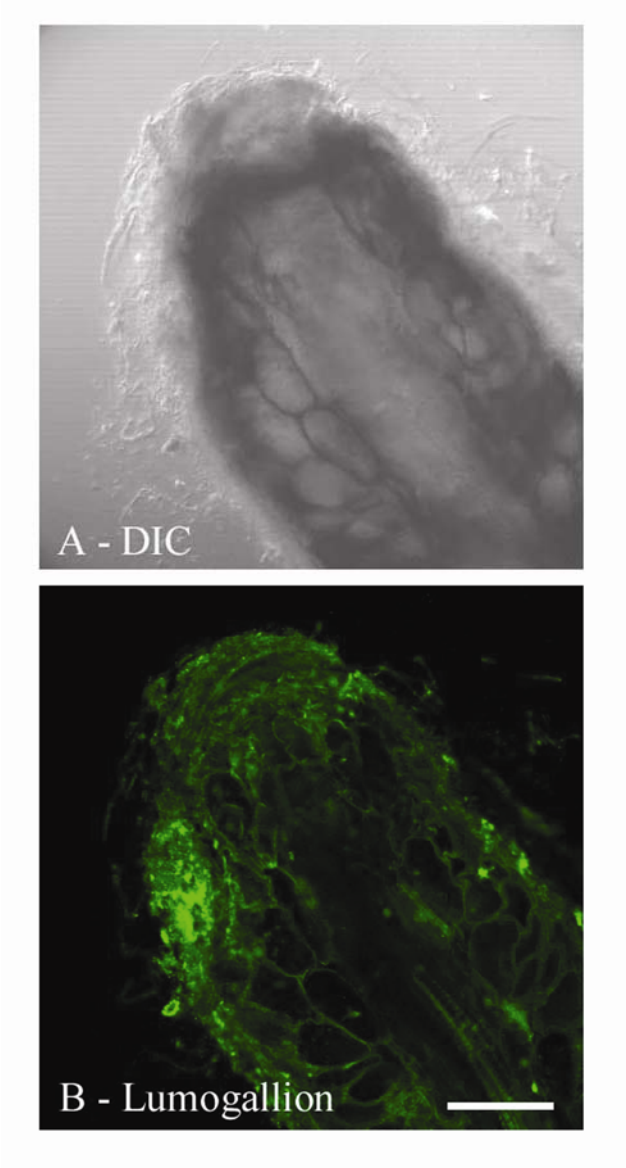

Figure 5. Confocal scanning microscopy images of a lateral root tip of pine colonized with *P. tinctorius* and exposed to 1.18 mmol L^{-1} Al^{+3} activity in sand culture, showing the presence of Al in fungal structures. DIC (a); lumogallion (b). Scale bar = 50 µm.

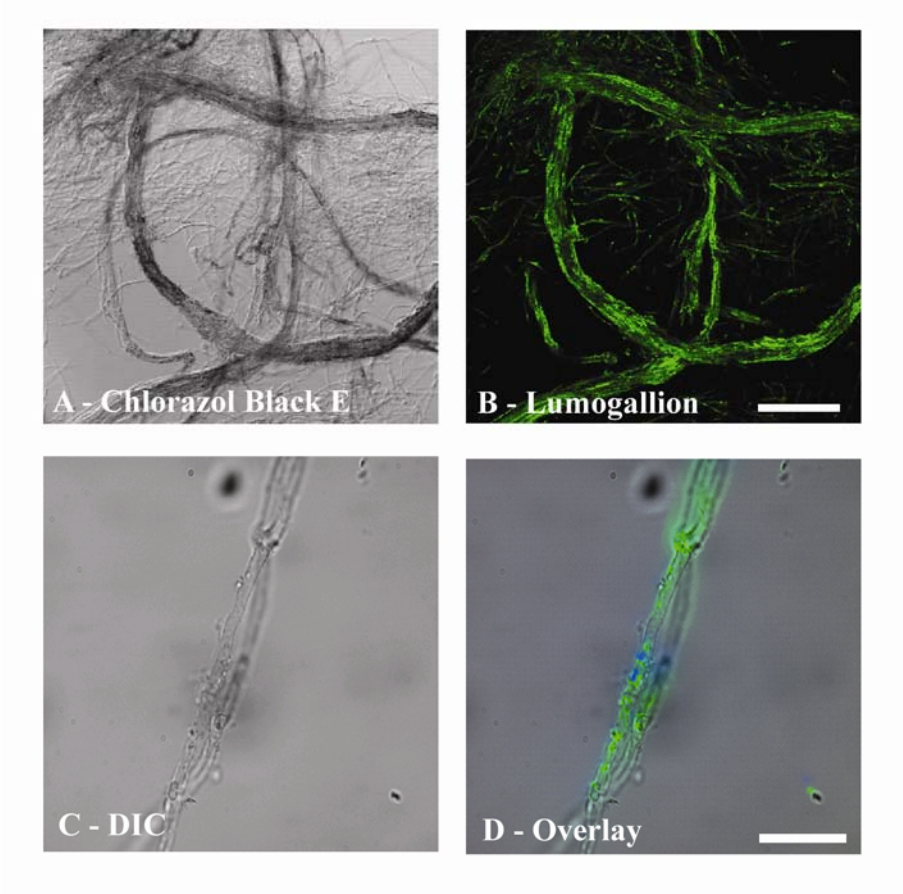

Figure 6. Micrographs of extraradical hyphae of *P. tinctorius* stained with lumogallion demonstrate the presence of Al. Chlorazol Black E (a), lumogallion (b), DIC (c) and lumogallion/DAPI overlay (d). Scale bar = 50 µm.

CHAPTER IV

ALUMINUM EFFECTS ON LATERAL ROOT DEVELOPMENT IN LOBLOLLY PINE

ABSTRACT

Past experiments have shown that the primary root of loblolly pine has a high degree of tolerance to rhizosphere aluminum. This study was designed to investigate Al tolerance in lateral roots, which comprise the majority of the total root system. The experiments included an examination of Al accumulation in lateral root tips before emergence through the primary root surface and assessment of whether organic acids were involved in the Al tolerance of the whole root system. The results clearly showed that lateral root extension was similar in tolerance to Al as the primary root. Both were inhibited to 65 to 70% of the control at an Al^{+3} activity of 581 μmol L^{-1}. This contrasted with the response of the Al-sensitive 'check' species soybean, whose lateral roots were significantly more sensitive to Al than primary roots at Al^{+3} activities less than 3 μmol L^{-1}. Tolerance was associated with exclusion of Al from the lateral root tip, and micro-analytical examination of the tissue using the fluorescing stain lumogallion showed that Al which was absorbed accumulated in peripheral cells, away from the meristem. In longitudinal sections of developing lateral roots, which had yet to break through the epidermis of the primary root, lumogallion fluorescence indicated substantial Al was present in the penetrating root tip, yet the rate of emergence through the primary root surface was not inhibited in either. The results suggested that the Al accumulation was spatially separate from the main site of cell division driving root penetration through the primary root

cortex and/or Al in the root interior was in a non-toxic form. Organic acid efflux (citrate and malate) could be detected at a low external Al activity, but not at >500 μmol L^{-1}, which implied that organic acids were not a major factor in the Al tolerance of pine.

INTRODUCTION

Aluminum toxicity in acid soils is recognized as one of the biggest problems for agriculture in many parts of the world (Von Uexkull and Mutert 1995, Eswaran et al. 1997). As a result, research over the past 20 years has focused on the mechanisms responsible for the Al toxicity effect and Al tolerance. Although much is known, understanding of fundamental processes remains elusive.

There are points of consistency in the Al toxicity literature. Most research has examined the response of primary roots of crop plants. Evidence indicates that Al entry into the primary root leads to disruption of any number of cell regulatory processes; for example, calcium homeostasis (Zhang and Rengel 1999), enzyme activity and ion transport (Huang et al. 1992a, 1992b), and DNA replication (Haug and Vitorello 1997). Structurally, toxicity mechanisms have been associated with the root apoplast (Clarkson 1967, Rengel 1996) and symplasm (Tice et al. 1992, Lazof et al. 1994a, 1994b, Jones and Kochian 1995, Jones et al. 1998), the plasma membrane (Huang et al. 1992a, 1992b, Barcelo et al. 1996), and the cell wall/plasma membrane/cytoskeleton continuum (Sivaguru et al. 1999).

A crucial factor in the inhibition of primary root growth appears to be the extent that Al penetrates into cells of the root tip. Microanalytical techniques have established that degrees of root growth inhibition are related with Al accumulation in meristematic cells and binding to nuclei (Matsumoto 1991, Lazof et al. 1994a, Silva et al. 2000), which presumably leads to direct restriction of cell division and expansion processes (Clarkson 1965, Morimura et al. 1978, Ryan et al. 1993, Sivaguru and Horst 1998). It follows that

the Al tolerance of primary roots, at least in the short term, likely involves exclusion of Al from the growth centers.

Much Al research has led to the conclusion that organic acids were a central component to Al exclusion and tolerance. Because of the high binding affinity of organic acids to Al (Hue et al. 1986), enhanced cellular production, accumulation and efflux of organic acids lead to formation of Al-organic acid complexes that do not interfere with cellular processes (Kochian 1995, Ma 2000, Ryan et al. 2001, Kochian et al. 2002, Taylor 1991, Rengel 1997). Evidence for organic acid based Al tolerance generally has been gathered from experiments examining intra-specific differences in Al tolerance within crop species.

Recent experiments, however, have raised the issue of whether organic acids alone can be responsible for observed differences in Al tolerance. In experiments with soybean, for example, enhanced accumulation of citrate and malate was transient, lasting for several hours, while Al accumulation was lowered and greater Al tolerance occurred continuously for many days in tolerant genotypes (Ma et al. 1997, Zheng et al. 1998, Li et al. 2000, Silva et al. 2001a). Experiments with maize showed quite clearly that Al tolerance among a group of maize genotypes was not related with organic acid levels in the tissue or efflux (Pineros et al. 2005). Furthermore, organic acids did not appear to be responsible for Al tolerance differences in the grass species *Brachiaria,* as organic acid efflux was not positively correlated with the degree of tolerance in intra- or inter-species comparisons (Wenzl et al. 2001).

In research extending over several years, we have been screening for plant species that are highly tolerant of Al. In this paper, we continue to evaluate aluminum

tolerance of loblolly pine. Past experiments have revealed that primary root extension of pine is highly tolerant to Al compared to that of crop plants (Moyer-Henry et al. 2005). As a key successional plant in highly weathered, acid soils, pine experiences substantial inhibition of primary root growth at Al^{+3} activities greater than 500 μmol L^{-1}, while soybean and many other crop plants show severe inhibition at < 30 μmol L^{-1} Al^{+3} activity.

The present series of experiments addresses several important questions concerning Al tolerance of pine. First, does the extreme Al tolerance also apply to lateral roots? Lateral roots constitute the majority of the root system of dicotyledonous plants, up to 88% of the total root length for loblolly pine (McCrady and Comerford 1998), and thus provide the majority of the surface area for water and nutrient uptake. While a limited amount of evidence is available on Al toxicity in lateral roots, observations with crop species consistently show that lateral root growth can be much more sensitive to rhizosphere Al than is growth of the of the primary root (Silva et al. 2001a, Ferrufino et al. 2000, Sanzonowicz et al. 1998, Brady et al. 1993, Bushamuka and Zobel 1998). Second, using tissue Al measurements and the Al-specific stain lumogallion, we determine the extent of Al exclusion and/or intercellular compartmentation, as lateral roots push through the root cortex and extend from the root surface. Inter-cellular Al sequestration, preventing Al from accessing the meristematic region, appeared to occur in previous experiments with the primary root of pine (Moyer-Henry et al. 2005). And last, we address the question of whether organic acid accumulation and efflux are involved with Al tolerance of the pine root system.

MATERIALS AND METHODS

The experiments focused on loblolly pine (*Pinus taeda* L.), and soybean (*Glycine max* L. Merr.) cv. Young was included in some of the experiments to serve as an Al-sensitive check species. Seeds from one open-pollinated family of loblolly pine were stratified (moistened and refrigerated for 30 days at 4°C) and germinated in paper for seven days at 30°C. Seedlings were selected for uniformity and placed into 12 L hydroponics units with a flow rate of ~ 4 L per minute. Temperature was maintained at 25°C and pH controlled automatically at 4.3 ± 0.1. Seeds of soybean were germinated in paper rolls placed in a dark germination chamber at 25°C and 98% RH and kept moist by capillary action from a 0.1 mmol L^{-1} $CaSO_4$ solution. After 72 hours, seedlings were selected for uniformity (a primary root length of 8 to 12 cm) and transferred into the hydroponics units.

The hydroponics units were located in a growth room programmed for an 8/16h light/dark period. The aerial temperature at plant height was maintained at 32/24°C, and radiance was 1200 μmol m^{-2} sec^{-1}, provided by a combination of metal halide and incandescent lamps. The complete nutrient solution contained (in μmol L^{-1}): 800 $CaSO_4$, 300 $MgSO_4$, 600 KNO_3, 75 KH_2PO_4, 0.055 $ZnSO_4$, 0.06 $MnCl_2$, 0.3 $B(OH)_3$, 0.065 $CuSO_4$, and 25 $FeSO_4$. Aluminum was added from an acidified 1 mol L^{-1} $AlCl_3$ stock solution to establish activities specified in the Results section. The Al^{+3} activities, from here on noted as $\{Al^{+3}\}$, were calculated using Geochem-PC (Parker et al. 1995).

Root growth inhibition

For the slow growing pine, there was a 28-day adjustment period in solution culture before Al was added. At the end of the adjustment period, the primary roots were

8-12 cm in length. Extension was measured just prior to Al addition and 14 days later. Analysis of solutions at the end of the Al treatment periods indicated that total Al concentrations remained relatively stable. For soybean, seedlings were exposed to a 24 hr adjustment period, followed by Al exposure for 3 days, a protocol established previously (Silva et al. 2000; Silva et al. 2001b).

It was determined in preliminary experiments that lateral root growth was consistent among seedlings within a treatment condition. Thus, lateral root extension was estimated by a) harvesting 7 randomly selected seedlings at the beginning of the 14 day Al exposure, and measuring the length of 4 individual lateral roots that had recently emerged from the primary root, and b) harvesting 7 other plants at the end of 14 day Al exposure and measuring lateral root lengths at the same positions. For soybean, root extension was measured in the same manner, except at the beginning and end of the 3 d Al exposure.

In both pine and soybean experiments, each treatment consisted of 28 seedlings, so 21 separate measurements were made for primary root extension and 28 measurements for lateral roots (7 seedlings x 4 positions). Lateral root emergence from the 'parent' primary root was estimated from the number of laterals present per cm of new primary root produced during the Al exposure periods. All experiments were replicated over time.

Tissue aluminum

Total Al in tissues was measured in different root sections: primary root tips, the remaining primary root, lateral root tips, and the remaining lateral root material extending to the primary root epidermis. Seedlings were harvested over time to examine if increases occurred in Al accumulation as the Al exposure periods progressed. Material

was dissected (~5 mm in the case of root tips), weighed, placed in net well trays, and rinsed in ice-cold citric acid for 30 min. The citrate wash has become a standard procedure in Al experiments to remove loosely bound apoplastic Al and minimize metal redistribution (Zhang & Taylor 1990, Samuels et al. 1997, Silva et al. 2000, Watanabe et al. 2001). After the citrate wash, all samples were rinsed in de-ionized water, the trays placed in a 60°C drying oven for 72 hr, transferred to Teflon bottles, digested overnight in 1 mL Optima grade HNO_3, and then microwave-digested for 30 minutes under a steady stream of compressed N_2 gas. The root material and HNO_3 then were transferred into 15 mL tubes, taken to 5 mL with de-ionized H_2O, and total Al was determined by inductively coupled plasma (ICP) atomic emission spectrometry (Model 2000DV, Perkin-Elmer, Norwalk, CT).

Organic acids

Tissue organic acid levels were determined in root tips only. Primary and lateral root tips of seedlings were dissected as described above. The tips were weighed and ground in 10 ml glass grinders using approximately 4 mL of 80% ethanol. Once thoroughly ground, the ethanol portion of the samples was driven off in a vacuum evaporator overnight. Sample pellets were rehydrated in 0.6 mL de-ionized water, filtered with a 0.45 μm nylon syringe filter and analyzed by ion chromatography (HPLC) using a Dionex AS11 column (Dionex, Sunnyvale, CA).

Organic acid efflux from roots was measured after transferring 10 individual seedlings from the solution chambers to test tubes containing 15 mL of 800 μmol L^{-1} $CaSO_4$ plus $AlCl_3$ treatments. The solutions were aerated for a 24 hr collection period. After seedlings were removed, the solution samples were divided into 5 mL aliquots,

which were transferred to separate test tubes and taken to dryness by vacuum evaporation. The residue was dissolved in 1.0 mL of water and analyzed for organic acids as before.

Microscopy

The procedure for visualizing Al distribution among cells at the root tip using lumogallion staining and confocal microscopy has been detailed elsewhere (Kataoka et al. 1997, Kataoka and Nakanishi 2001, Silva et al. 2000). Briefly, plants were carefully removed from the hydroponics system at the end of the Al exposure period, and tips of primary and lateral roots (~1 cm) were excised and rinsed in ice-cold citric acid for 30 min. The tissue was then embedded in 7% (w:v) agarose and sliced in 100 μm-thick longitudinal sections using a vibratome (1000 Plus, The Vibratome Company, St. Louis, MO). Root sections containing pre-emerged lateral roots, ones that had not broken the primary root surface, were obtained by cross-sectioning primary root of pine and soybean through the zone of lateral root formation. Appropriate sections were identified by an exhaustive screening procedure using light microscopy. The sections were stained with the fluorescent Al-indicator lumogallion (12 mmol L^{-1}; TCI America, Inc., Portland, OR) and mounted on glass slides with a glycerol medium containing the nuclear stain DAPI (4',6-diamidino-2-phenylindole 2HCl; 1 μg mL^{-1}; Sigma, St. Louis, MO). The slides were examined using a Leica TCS-SP confocal system with an inverted microscope DMIRBE and a dry PL APO 20 x/0.60 Numerical Aperture (N.A.) objective (Leica, Wetzlar, Germany).

RESULTS

Aluminum inhibition of primary and lateral root growth

When pine was grown under a range of external $\{Al^{+3}\}$ in these experimental conditions, extension of the primary root was not affected until activities approach 500 μmol L^{-1} (Moyer-Henry et al. 2005). As shown in Figure 1, exposure of pine seedlings to an $\{Al^{+3}\}$ of 581 μmol L^{-1} resulted in an inhibition of primary root extension to about 70% of the control. At that $\{Al^{+3}\}$ concentration, lateral root extension was restricted to 66% of the control. Thus, lateral roots were similar to the primary root in their tolerance to Al.

The slight difference in sensitivity observed with primary and lateral roots of pine differed from that with soybean. In the same experimental conditions but at a much lower $\{Al^{+3}\}$, 2.7 μmol L^{-1}, primary root extension of soybean was inhibited to ~ 62% of the control. The inhibition of lateral root extension was more severe, to ~ 40% of the controls.

With pine, lateral root emergence from the primary root was not significantly affected at the external $\{Al^{+3}\}$ of 581 μmol L^{-1} that caused the inhibition of root extension. On portions of the primary roots that developed during the 14 day experimental period, 2.5 ± 0.7 laterals were present per cm of primary root in control plants and 2.4 ± 0.4 were present in the Al treatment (data not shown). Lateral root development of soybean also was not noticeably affected (see also Ferrufino et al. 2000; Sanzonowicz et al. 1998).

Aluminum accumulation in roots

Some differences in Al concentration were apparent in primary and lateral root tips. At 581 μmol $L^{-1}\{Al^{+3}\}$, the internal Al concentration in primary root tips was approximately 38 μmol g^{-1} DW after 3 days of Al exposure, but decreased by almost 75% to 10 μmol g^{-1} DW after 8 days and remained low thereafter (Fig 2A). The decline in Al concentration indicates that, after the initial Al entry, Al accumulated at a slower rate than development of new root tip tissue. The concentration of Al in the older portion of the primary root increased from ~ 20 to 60 μmol g^{-1} DW after 12 to 14 days of exposure.

The concentration of Al in the lateral tip was more stable than in the primary root, remaining between 30-40 μmol g^{-1} DW throughout the experiment (Fig 2B). Thus, Al accumulation and growth were proceeding at about the same rate. The Al concentration in lateral root tissues behind the root tip, however, increased steadily during the experiment and reached 75 μmol g^{-1} DW after 12 to 14 days.

Although not shown here, previous experiments with soybean have demonstrated that the concentration of Al increases steadily when the plants are exposed to external Al concentrations in the low micromolar range (Silva et al. 2001b; Moyer-Henry et al. 2005). The response is much different than that of pine.

Aluminum localization

Aluminum accumulation in lateral roots of pine was examined microscopically at two developmental stages, before and after breaking through the primary root surface. A differential interference contrast (DIC) image shows a newly developing lateral root that is still in the cortex, pushing outward from its point of initiation in the pericycle (Fig. 3A). The green fluorescence of the Al-lumogallion stain is primarily localized in the

outer, ‘peripheral’ cell layers of the lateral root (Fig. 3B). The accompanying DAPI overlay image (Fig. 3C) shows the high density of nuclei just behind the lateral tip, which indicates the location of densely packed meristematic cells. Aluminum appears to be largely excluded from the meristematic cell region.

Tissue Al accumulation also was examined in pine lateral roots that had recently emerged from the primary root epidermal surface (Fig. 4). Lumogallion-Al fluorescence indicated that the most intense Al accumulation occurred just behind the tip. The strongest fluorescence intensity was localized in the cell wall/plasma membrane region of each cell with little signal originating from the cell interiors.

For comparative purposes, soybean sections were examined using the same microscopic techniques. A DIC image shows a newly formed soybean lateral root tip before emergence, inside the primary root cortex (Fig. 5A). Lumogallion fluorescence indicates intense signal in the central region of the lateral just back from the tip (Fig. 5B). The DAPI overlay (Fig. 5C) places the Al lumogallion fluorescence in the location of densely packed cells, and the fluorescence signal appears stronger than that in pine. Thus, even though Al was having little effect on lateral emergence through the root surface, substantial amounts were accumulating in the lateral tip cells. As with pine, a separate longitudinal section of a soybean lateral root was examined just after emergence from the epidermis. The lumogallion-Al fluorescence signal at the tip was extremely strong and close to saturating the detector (Fig. 6). The most intense lumogallion signal corresponded with highest signal from DAPI, indicating Al accumulation in the meristematic cell region.

Organic acid accumulation in roots and efflux

Once it became evident that both primary and lateral roots of pine had a high degree of Al tolerance, additional hydroponics experiments were conducted to investigate the potential role of organic acids as an Al tolerance mechanism. Analyses of primary and lateral root tip tissues indicated that malate and citrate were the dominant organic acids present (Fig. 7). In control plants grown without Al, malate was present in the largest quantities; ~ 8000 nmol g^{-1} FW compared to ~ 2000-2500 nmol g^{-1} FW citrate. When Al was present at $\{Al^{+3}\}$ of 581 μmol L^{-1}, malate and citrate concentrations both decreased markedly. In primary root tips, malate was decreased to about 2500 nmol g^{-1} FW and citrate to about 800 nmol g^{-1} FW, and concentrations in lateral root tips were decreased to a somewhat greater extent. A lower Al treatment was included, 38.7 μmol L^{-1} $\{Al^{+3}\}$. While this level of external Al has no impact on growth of the primary or lateral roots (Fig. 1), it decreased the concentrations of malate and citrate.

Organic acid efflux also was examined at the different $\{Al^{+3}\}$ treatment levels. With control plants grown in the absence of Al, malate efflux was about 0.2 nmol g^{-1} FW h^{-1}, and no citrate efflux could be detected (data not shown). With the lower Al treatment, 38.7 μmol L^{-1}, malate efflux was ~ 0.3 nmol g^{-1} FW h^{-1} and citrate efflux 0.6 nmol g^{-1} FW h^{-1}. When Al was present at $\{Al^{+3}\}$ of 581 μmol L^{-1}, neither malate nor citrate could be detected in the collection solution.

DISCUSSION

In nature, lateral roots of pine can become colonized by ectomycorrhizal fungus early in development, and lateral root growth ceases soon afterward (Smith and Read 1997). In our previous paper, we examined Al accumulation in lateral roots colonized

with ectomycorrhizae (Moyer-Henry et al. 2005). Colonization, however, typically does not occur throughout the root system (Rosado et al. 1994, Schier and McQuattie 1995, 1996, Visser 1995). It has been estimated that as many as 50% of the lateral roots can remain non-colonized, depending on the fungal isolates present in the soil (Dixon et al. 1987), the tree species, root growth rate, and season of the year (Marschner 1995). Thus, the Al tolerance of non-mycorrhizal roots that come into direct contact with the soil environment is of considerable importance in field situations.

The results of these experiments indicate that non-colonized lateral roots of loblolly pine possess a high degree of Al tolerance, similar to the primary root. Little difference could be seen between the two responses, as extension of both was inhibited to about 65 to 70% of the controls when the pine root system was exposed to an $\{Al^{+3}\}$ greater than 500 μmol L^{-1}. The tolerance of pine contrasted with that of the check species soybean. Lateral extension of soybean was noticeably more sensitive to Al than the primary root, and a pronounced inhibition occurred at only 2.7 μmol L^{-1} (Fig. 1). The soybean responses are similar to previous observations in the literature (Silva et al. 2001a; Ferrufino et al. 2000; Sanzonowicz et al. 1998; Brady et al. 1993; Bushamuka and Zobel 1998), reassurring that the unusual tolerance of pine was not an artifact of this particular experimental system.

The Al tolerance of the pine root system seems anchored in an efficient Al exclusion mechanism. The amounts of Al accumulating in the pine root tissues at a solution $\{Al^{+3}\}$ of ~ 500 μmol L^{-1} are less than those in soybean tissues when solution Al is in the low micro-molar range (refer to Moyer-Henry et al. 2005). The implication is that Al binding molecules are synthesized within the root cells and released into the

surrounding media, minimizing Al absorption. It is interesting that a relatively high rate of Al accumulation, to about 30 to 40 μmol g^{-1} FW, occurred during the first several days of exposure. This could indicate that Al enters and saturates an Al pool that is not tightly regulated by an exclusion mechanism and not susceptible to removal by a citrate wash. Such a tightly bound cell wall fraction was identified in experiments with wheat (Zhang and Taylor 1990). Alternatively, the initial accumulation could indicate that a time-lapse is needed before the exclusion mechanism becomes functional. The observed steady decline in the primary tip Al concentration (Fig. 2A) would be consistent with an exclusion process becoming more active over time.

The Al concentration patterns (Fig. 2) indicated that Al entry into root tips was limited to a greater extent than into the root sections behind them. Differences in Al accumulation in root tips and older tissues have been seen before. With wheat, for example, greater Al tolerance among genotypes was associated with reduced Al accumulation in the root apex, but not in the mature root (Rincon and Gonzales 1992; Tice et al. 1992; Delhaize et al. 1993a). Such observations imply that Al exclusion mechanisms are localized, primarily, in the root region containing meristematic activity, i.e. the growth centers.

Once lateral roots emerge from the root surface and come into contact with Al in the soil solution, it would seem that they would be much more vulnerable to Al toxicity than the primary root. Newly emerged pine laterals appear to lack the distinctive root cap evident in primary roots (Kaska et al. 1999), with only a few cell layers separating the meristem from the surrounding environment. This contrasts with the anatomy of a primary root tip, where there can be as many as 10 to 12 cell layers between the meristem

and the root exterior (Moyer-Henry et al. 2005). One might expect that a more extensive series of parenchyma cells, and perhaps their exudations, would be a more effective barrier for Al entry into the meristematic tissue. Nonetheless, that did not appear to be the case.

Lateral root before emergence

One of the most interesting observations from our experiments was that much Al accumulated within the root cortex and within cells of the lateral root as it moved through the cortex, but that rates of lateral root emergence through the primary root surface were not restricted. This was true for pine and for the more Al sensitive soybean. Not a great deal has been published about lateral root development, but some insights can be taken from a conceptual model proposed for *Arabidopsis* (Celenza et al. 1995; Malamy and Benfey 1997). Evidence from the molecular studies suggested that the main site of cell division in a developing lateral root remains located at the base of the primordium, and cell expansion drives outward movement through the cortex. The meristem is formed, but does not become active until the lateral reaches the root surface. From this model, a couple of factors could explain the lack of an Al inhibition. One is that the majority of the Al inside the cortex is in a molecular form that is not toxic. As the primordium grows inside the cortex, many cortical cells collapse due to the pressure of the outwardly penetrating lateral root (McCully 1975; Laskowski et al. 1995). This, presumably, would lead to release of molecules from cortical cell protoplasts and walls. The resulting presence of organic molecules, negatively charged surfaces, and perhaps alkaline pH, would contribute to an apoplastic environment where Al would be bound in molecular complexes that are non- toxic. Further, if the main site of cell division is in the stele at the

root interior, then Al may not reach this far and thus may not be in position to exert a toxic effect. We do not currently have appropriate sections from pine stained with lumogallion that would help to evaluate this possibility. From previous experiments, we do know that Al seems to be present throughout the root cylinder (including the stele) in soybean (Silva et al. 2000), and lateral emergence is not inhibited. Thus, the most likely explanation appears to be that substantial amounts of Al are not present in a toxic form.

One of the questions to be addressed in these experiments was whether there was evidence that Al in lateral root tips was intercellularly compartmentalized, separate from the meristem, as was observed with primary roots (Moyer-Henry et al. 2005). The lumogallion images of the lateral root tip suggested that may have been the case, as most Al was present in peripheral cells and little Al was detected in the immediate meristematic cell area (Fig. 3). We have suggested that such sequestration evidently serves a protective function, because Al levels in the root tip tissue as a whole can be similar to those in soybean, where Al does accumulate in the meristematic cell area and lateral extension is restricted almost completely (Moyer-Henry et al. 2005). It would be reasonable to think that Al binding compounds responsible for Al exclusion from the root tip also might be involved in keeping Al in cells at the root tip periphery and away from the meristem.

A key element in this paper is the use of the fluorescent stain lumogallion to detect Al at a cell level. The methodology was first pioneered for plant analysis by Kataoka and colleagues (Kataoka et al. 1997). Up to this time, it has only been used in a small number of experiments (Kataoka et al. 1997, Kataoka and Nakanishi 2001, Silva et al. 2000, Silva et al. 2001c). The high degree of lumogallion specificity for Al and the

spatial resolution associated with a fluorescent probe allow localization of Al accumulation within root cells and tissues that cannot be attained with other technologies (with the possible exception of morin; Eticha et al, 2005).

Organic acids

The rationale for believing organic acids are involved in Al tolerance generally stems from several factors. First, organic anions have a high binding affinity for Al (Ma et al. 2001). Thus, internal or external binding could detoxify Al, preventing it from interfering with root function (Taylor 1991, Kochian 1995, Rengel 1997). A second factor is that organic acids in root tips and organic acid efflux respond positively to the presence of Al. In soybean, for example, the citrate concentration in root tips increased from 400 to 850 nmol g^{-1} FW and malate increased from 4000 to 5500 nmol g^{-1} FW when seedlings were exposed to 1.45 µmol L^{-1} $\{Al^{+3}\}$ (Silva et al. 2001c). Efflux of organic acids has been associated with opening of anion channels when roots are exposed to Al (Ryan et al. 1997b, Kollmeier et al. 2001, Pineros and Kochian 2001, Zhang et al. 2001, Yamaguchi et al. 2005). Whole root efflux rates (comparable to our experiments) have been extremely variable, ranging from 4 to almost 300 nmol g^{-1} FW h^{-1}among different species (refer to Table 2 in Ryan et al. 2001). Elevated organic acid levels in tissues and accelerated efflux, together, suggest enhanced organic acid synthesis occurred.

The evidence with pine does not, for the most part, fit the expected profile for organic acid involvement in Al tolerance. Root tip malate and citrate decreased when roots were exposed to Al. This was true for roots exposed to 38.7 µmol L^{-1} $\{Al^{+3}\}$ when growth was not affected, and at over 500 µmol L^{-1} $\{Al^{+3}\}$ when root growth was suppressed to ~70% of the control (Fig. 7). Some organic acid efflux was detected at

38.7 μmol L^{-1} $\{Al^{+3}\}$, which is within the range of Al toxicity responses with crop species. But at 500 μmol L^{-1}, organic acid efflux was absent and the growth restriction was to about 70% of control. If organic acids were involved in Al tolerance, their contribution would appear to be minimal.

In the absence of a prominent role for organic acids, it is unclear what mechanism(s) might be responsible for the high degree of Al tolerance in pine. To efficiently exclude Al from root tip tissues at the 500 μmol L^{-1} $\{Al^{+3}\}$ level, one would expect that molecules with a high affinity for Al or a physical barrier would be involved.

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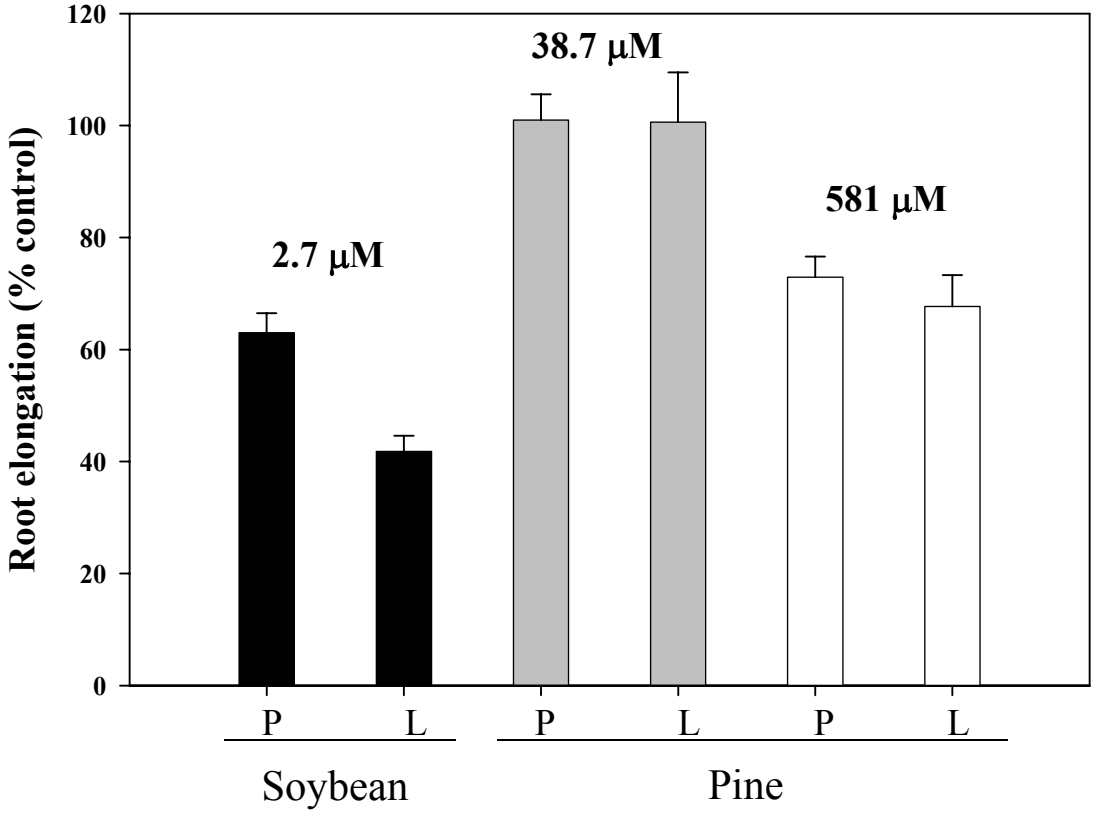

Figure 1. Elongation of primary and lateral roots of soybean and pine following exposure to solutions containing Al^{+3} activities of 2.7, 38.7, or 581 μmol L^{-1}, represented as a percent of controls. P = primary, L = lateral. Error bars represent standard error of the mean.

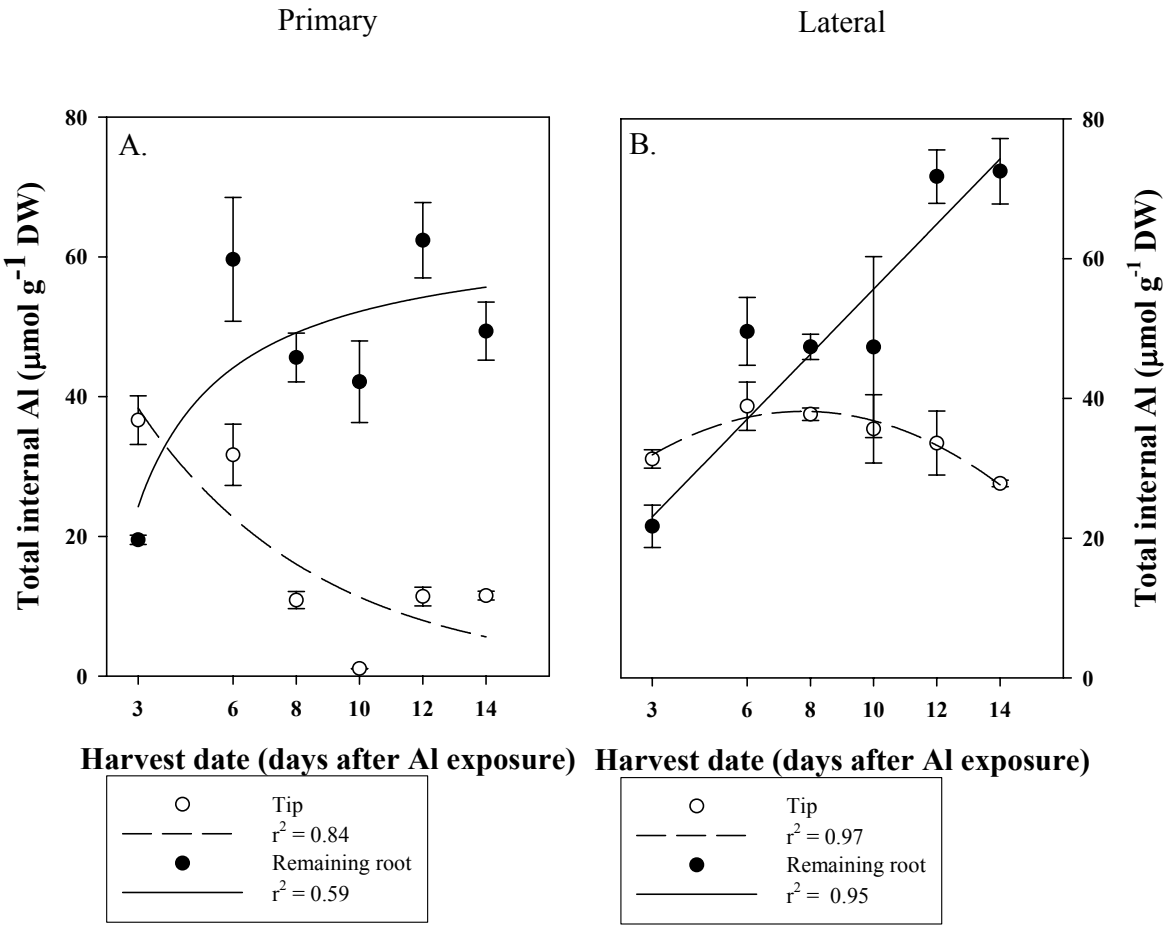

Figure 2. Total Al accumulation in pine roots exposed to 581 µmol L^{-1} Al^{+3} activity in solution. A) primary roots, B) lateral roots. Open circles = root tips (5 mm), closed circles = the remaining root tissue. Error bars represent standard error of the mean.

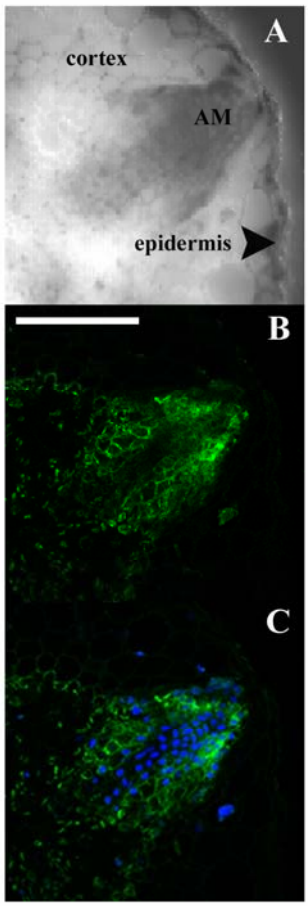

Figure 3. Images of transverse sections of pine primary roots and the associated sections of pine lateral roots penetrating through the primary root cortex. Roots were exposed to 581 μmol L^{-1} Al^{+3} activity. A) DIC, B) lumogallion, C) lumogallion/DAPI overlay. Scale bar = 100 μm.

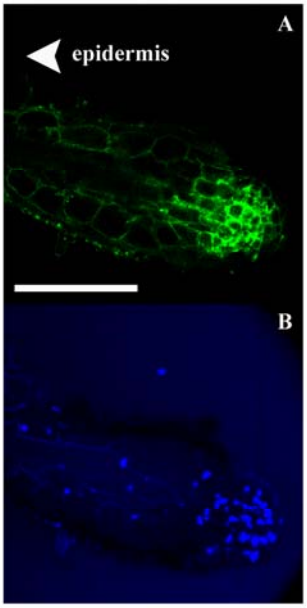

Figure 4. Images of pine lateral root tips longitudinally sectioned just after emergence from the primary root surface. The roots were exposed to 581 µmol L^{-1} Al^{+3} activity. A) lumogallion, B) DAPI. Scale bar = 100 µm.

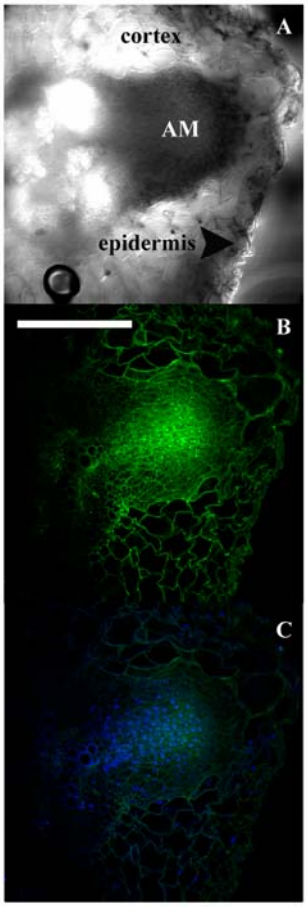

Figure 5. Images of transverse sections of soybean primary roots and the resulting longitudinal sections of lateral roots penetrating through the primary root cortex. Roots were exposed to 7.3µmol L^{-1} Al^{+3} activity. A) DIC, B) lumogallion, C) lumogallion/DAPI overlay. Scale bar = 100 µm.

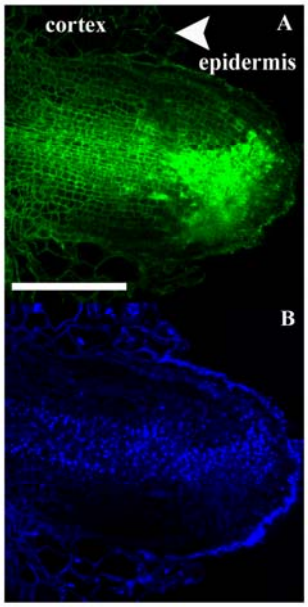

Figure 6. Images of soybean lateral root tips longitudinally sectioned just after emergence from the primary root surface. Roots were exposed to 7.3 µmol L^{-1} Al^{+3} activity. A) lumogallion, B) DAPI. Scale bar = 100 µm.

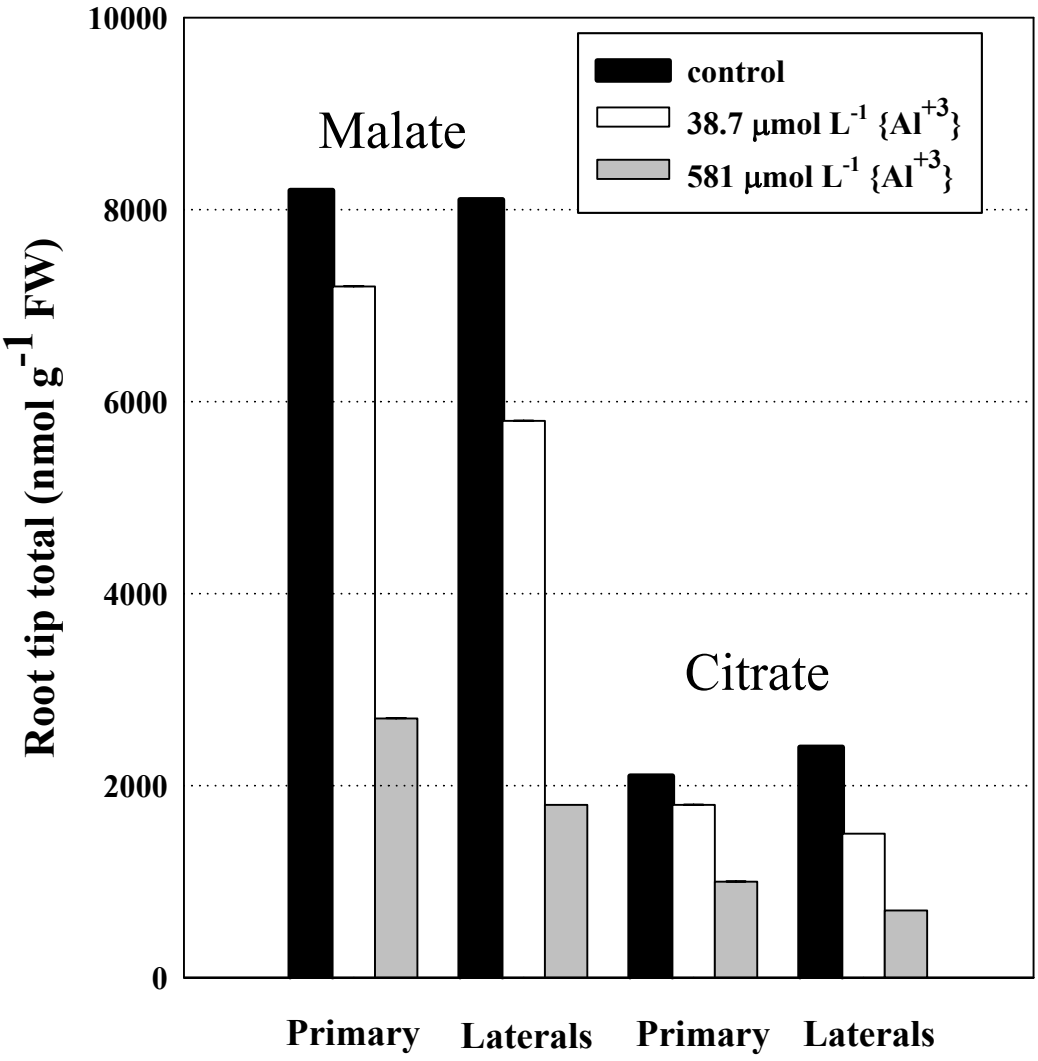

Figure 7. Malate and citrate concentrations in primary and lateral root tips. Roots were exposed to 38.7 or 581 μmol L^{-1} Al^{+3} activity. P = primary, L = lateral.

CHAPTER V

ALUMINUM ACCUMULATION IN ARBUSCULAR MYCORRHIZAL FUNGI AND INTERFERENCE WITH MYCORRHIZAL FUNCTION

ABSTRACT

A series of experiments examined aluminum affects on arbuscular mycorrhizal (AM) colonization and spore germination. Soybean was inoculated with *Glomus etunicatum* or a mixture of AM species and exposed to Al solutions for 4 weeks. Colonization per cm of roots was suppressed by Al, and the suppression was greater with increasing Al concentrations. Images generated by lumogallion staining and confocal laser microscopy showed that the decrease in colonization was associated with substantial accumulation of Al within the mycorrhizae, particularly in arbuscules and vesicles. Nonetheless, AM function did not appear to be impaired, as secondary colonization of maize occurred by hyphae extending through a 20 micron fabric barrier. The results suggested that AM are able to detoxify Al accumulating internally, a characteristic much like that being proposed for ectomycorrhizae. The results suggest that the main constraint for AM colonization of soybean was the Al toxicity effects exerted on physical properties or biochemical processes in the root. Exposure of *G. etunicatum* and *Gigaspora margarita* spores to Al resulted in decreased germination, but only at the highest Al treatment level. Lumogallion/Al staining showed that substantial amounts of Al accumulated in the spore walls, and Al accumulation could be detected inside spores of *G. etunicatum*. Because spore germination of *Gi. margarita* was more sensitive to Al, it appeared that Al inhibition of spore germination could occur from effects exerted at the spore wall.

INTRODUCTION

Low productivity of crop plants is common on acidic soils containing high levels of aluminum (Al) (Von Uexkull and Mutert 1995, Eswaran et al. 1997). Aluminum is extremely reactive in biological systems and has been found to disrupt many cellular processes in plant roots. Although the main toxicity effect appears to be direct interference with processes localized at growth centers at the root tip (Ryan et al. 1993, Horst et al. 1997, Samuels et al. 1997, Sivaguru and Horst 1998), many of the toxicity responses (e.g. altered transport and metabolism) are those that likely occur throughout the root system. Aluminum restrictions in the root indirectly influence growth of the whole plant in field environments by predisposing plants to drought stress and limiting the ability of the plant to acquire necessary nutrients.

Over 80% of all terrestrial plant root systems form root-fungus symbioses with arbuscular mycorrhizal (AM) fungi (Smith & Read 1997). The fungus enhances roots' ability to access previously unavailable soil resources, while the plant provides the fungus with carbon. Fungal hyphae serve as extensions of the plant's root system, increasing the absorptive surface area in the soil. Improved plant growth following colonization by AM fungi is often the result of the ability of the fungi to reach nutrients that are otherwise unavailable to the plant's root system, most notably the least soluble forms of phosphorus (Smith and Read 1997, Harrison 1999).

Numerous studies have indicated that AM can ameliorate the negative effects of Al toxicity (Koslowsky and Boerner 1989, Medeiros et al. 1994, Clark and Zeto 1996, Mendoza and Borie 1998, Borie and Rubio 1999, Clark et al. 1999, Rufyikiri et al. 2000, Cuenca et al. 2001, Lux and Cumming 2001, Cumming and Ning 2003, Rohyadi et al.

2004, Kelly et al. 2005). Generally, Al does not adversely affect AM colonization, although the amelioration of Al toxicity in plants has been observed even when Al suppresses AM colonization (Clark 1997, Koslowsky and Boerner 1989).

A role for arbuscular mycorrhizae in decreasing plant sensitivity to Al is similar to that proposed for ectomycorrhizal fungi (EM). Ectomycorrhizae provide plant protection against metal toxicity in soil (Hentschel et al. 1993, Godbold 1994, Schier and McQuattie 1995, 1996, Godbold et al. 1998). In recent experiments with loblolly pine seedlings colonized by the ectomycorrhizal fungus *P. tinctorius* and exposed to Al, images produced using the Al stain lumogallion revealed that substantial Al accumulation occurred at extra-radical hyphae and the fungal mantle (Moyer-Henry et al. 2005). Colonization was not depressed, so EM function did not seem to be disrupted. Because there was no indication of large-scale Al penetration into root cells, the results were consistent with the idea that EM acted as a barrier to Al penetration into the root symplasm (Cumming and Weinstein 1990, Jentschke and Godbold 2000).

Interactions between AM and Al may extend into different aspects of the mycorrhizal life cycle. Fungal spore germination, for example, was inhibited by the presence of Al (Bartolome-Esteban and Schenck 1994). The mechanisms responsible for this inhibition, and whether it is associated with Al entry into spores and disruption of internal germination processes, are unknown.

The purpose of this study was to further evaluate the relationship between Al accumulation and AM function in an experimental system using soybean. The initial focus was on whether mycorrhizal colonization was sustained in the presence of Al, and if not, whether Al accumulated in mycorrhizal structures. The results of a previous study

using the Al stain hematoxylin suggested that Al might accumulate within the mycorrhizae (Cuenca et al. 2001). If substantial Al accumulation occurred in AM, then it would be made to determine whether it was associated with decreased ability of AM to colonize other roots, which would indicate impaired function. Additional experiments focused on Al affects on AM spore germination and whether changes in spore germination were associated with Al accumulation at the surface or inside of spores.

Our experiments make extensive use of the Al-specific stain lumogallion. Many aspects of Al toxicity in plants remain obscure, and one of the most imposing experimental difficulties has been localization of Al. The lumogallion method, which utilizes fluorescence and confocal laser microscopy, allows a high degree of sensitivity and spatial resolution in the detection of Al at cellular and sub-cellular levels.

MATERIALS AND METHODS

Colonization

Experiments were conducted in a greenhouse at the Southeastern Plant Environment Laboratory located on the campus of North Carolina State University, Raleigh, NC. The day/night temperatures in the greenhouse were 26/22°C on a 9/15 h cycle with a radiant flux density inside the greenhouse of ~80 to 88% natural light. A 3 hr dark period interruption from incandescent filament lamps at a photosynthetic photon flux (PPF) of 11-12 μmol s^{-1} m^{-2} was used to simulate a long-day photoperiod and suppress flowering (Thomas et al. 2004).

Seeds of soybean (*Glycine max)* cv. Young were germinated in 25 cm x 6.4 cm deepots (Stuewe and Sons, Corvallis, OR) containing ~450g of a 2:1 pasteurized

sand:soil mixture. Mycorrhizal treatments consisted of a mixed mycorrhizal spore population originating from a trap culture using field soil from Goldsboro, N.C. (Table 1), a single species isolate of *Glomus* aff. *etunicatum* Becker & Gerdemann (*G. etunicatum*) from a field soil obtained at the Central Crops Research Station at Clayton, N.C., and a non-mycorrhizal control.

Both mycorrhizal treatments and a soil filtrate were prepared using the wet-sieving method (Morton et al. 1993). Mycorrhizal inoculum was prepared by washing ~ 115 g of soil in 500 ml of tap water and stirring thoroughly to separate AM spores and microbes from the soil. The heavier soil particles settled and the water was poured through 500 and 75 µm sieves. Material collected on the 500 µm was sieve was discarded. Material on the 75 µm sieve served as the AM fungal inoculum. It was added to ~ 1000 g of a 2:1 sterile sand:soil mixture and placed in deepots. The original filtrate solution was passed through a Whatman #1 filter to insure removal of all mycorrhizal spores, and then used as a microbial background solution. For mycorrhizal treatments, each deepot received 10 ml of the microbial solution, and for non-mycorrhizal each received 20 ml.

Fungi were propagated on sudan grass (*Sorghum sudanense*) according to Morton et al. (1993). For development of the *G. etunicatum* single species culture, sudan grass was planted in 24 in. clay pots and grown in a greenhouse for 2 months. After 2 months, plant shoots were removed and the soil left dormant for 4 months to allow for AM spore formation. Following dormancy, AM spores were isolated as above, identified and collected using a dissecting microscope. About 100 spores were added to a 24 in clay pot containing pasteurized soil and placed in the greenhouse. Sudan grass was again planted

in the pots and grown for another 2 months, followed by a 4 month dormancy as before. Then, the soil containing root fragments and *G. etunicatum* spores was ready to be sieved or used directly as inoculum.

Seedlings growing in the deepots received water daily as needed until emergence of the first trifoliolate leaf, at which point 150 ml of Al treatment solution was added 3 times a week for 4 weeks. Aluminum treatments were 0, 2, 4, or 8 mmol L^{-1} $AlCl_3$ at pH 4.3, assigned in a randomized complete block design with 4 replications. Aluminum solutions were prepared freshly before each application. For sampling procedures used at the end of 4 weeks, see details below.

Infection potential

Experimental units consisted of 2 connected plexiglass chambers measuring 12 x 13 x16 cm^3 each (designated A and B), separated by a 20 μm mesh fabric panel (Tetko/Sefar mesh, Sefar America, NY) (Fig. 1). The fabric panel blocked growth of roots between the chambers, but allowed passage of mycorrhizal hyphae (refer to Hodge et al. 2001).

One half of each system (chamber A) was filled with a 1:1 sand:soil mixture. The soil mixture included 100 g of soil from the single isolate culture, which served as an inoculum. Seeds of sudan grass were planted in one chamber to promote mycorrhizal colonization. The sudan grass was allowed to grow for about 2 weeks. Seeds of soybean cv. Young were germinated in paper for 3 days at 30°C and then transplanted into the chamber occupied by the sorghum sudan grass. After 2 additional weeks, the shoots of the grass were removed. The soybean plants then grew until the first trifoliolate leaf stage. At that time, the other side of the system, was filled with a sterilized 1:1 sand:soil

mixture. Maize (*Zea maize,* hybrid sweet, extra early sweet 82) seedlings, which had been germinated in paper at 30°C, were transplanted into chamber B, opposite the soybean seedlings.

Soybean and maize received water 3 times weekly for 4 weeks at which time Al applications to the soybean chamber (A) began. The Al treatments were 0, 2, 4, or 8 mmol L^{-1}. A volume of 150 ml was applied to soybean 3 times weekly for 4 weeks. Aluminum treatments were assigned in a randomized complete block design with 3 replications. Maize seedlings received water on days when Al was applied to soybean. At the end of Al applications, maize and soybean roots were harvested for assessment of root colonization.

Spore germination

Spores were extracted from field soil taken from the Clayton site. Spores were extracted as previously described for the colonization experiment. In this case, the material collected by the 75 μm sieve was transferred to a 150 ml test tube. Ten ml of 10% sucrose solution was added, and the tubes centrifuged at 75 rpm for ~5 min. Sucrose centrifugation was modified from Daniels and Skipper (1982) such that mycorrhizal spores migrate to the top of the sucrose fraction during centrifugation. The mycorrhizal fraction was collected and washed through a 20 μm sieve. The spores were then transferred to a Petri dish, examined under a dissecting microscope, and *G. etunicatum* and *G. margarita* spores were selected.

Ten spores of *G. etunicatum* or *Gi. margarita* were placed into individual embedding cassettes (Fisher Scientific, Suwanee, GA) containing pasteurized field soil. Four cassettes with the same species were inserted into a 12 in. pot containing a 1:1

sterilized sand:soil mixture. Four pots per species were prepared. Two maize seedlings were planted into each pot. Each pot received 150 ml of solutions with 0, 2, 4, or 8 mmol L^{-1} $AlCl_3$ at pH 4.3 three times weekly. Following four weeks of Al treatment, soil was removed from each embedding cassette, spores isolated as before, and germination determined under a dissecting microscope. Spores of *G. etunicatum* were considered germinated when a germ tube had extended beyond the end of the subtending hypha (Maia and Kimbrough 1998). Spores of *Gi. margarita* were considered germinated when one or more germ tubes had extended beyond the spore surface (http://invam.caf.wvu.edu/fungi/taxonomy/Gigasporaceae/Gigaspora/margarita/margarita.htm).

Mycorrhiza-Al staining and microscopy

Root samples were stained and evaluated for mycorrhizal colonization. Harvested root systems were dried at 60 C for 48 h, rehydrated for 2 days, cut into 1" sections, and then stained with ink and vinegar (Vierheilig et al. 1998). The sections were cleared of pigment by boiling in a 10% (w/v) KOH solution for 5 minutes, and rinsed several times with tap water. Next, the root sections were boiled in a white vinegar solution (5% acetic acid) containing 5% Parker blue-black ink for 3 minutes to stain the mycorrhizal structures. Roots were cleared of excess stain by rinsing in tap water acidified with a few drops of vinegar. The grid-line intersect method was used with a dissecting microscope to estimate root length and the extent of mycorrhizal colonization (Giovannetti and Mosse 1980).

The ink stained root segments were used for Al detection using lumogallion staining. The lumogallion procedure for visualizing Al in mycorrhizal fungal hyphae has

been detailed elsewhere (Moyer-Henry et al. 2005). A difference in methodology was that these soybean roots did not need to be sectioned for visualization of Al in mycorrhizae. The root segments were rinsed in ice-cold citrate for 30 min. The citrate wash has become a standard procedure in Al experiments to remove loosely bound apoplastic Al and minimize metal redistribution (Zhang and Taylor 1990, Samuels et al. 1997, Silva et al. 2000, Watanabe et al. 2001), and it had no impact on the ink stain. The root segments were then stained with the fluorescent Al-indicator lumogallion (12 mmol L^{-1}; TCI America, Inc., Portland, OR) and mounted on glass slides. The slides were examined using a Leica TCS-SP confocal system with an inverted microscope DMIRBE and either a dry PL APO 20 x/0.60 Numerical Aperture (N.A.) or a 40 x/1.25 N.A. oil immersion objective (Leica, Wetzlar, Germany). Mycorrhizal colonization and fungal structures stained with ink were imaged using a Hamamatsu cool CCD color camera mounted on the confocal microscope (Model C5810; Hamamatsu Bridgewater, NJ). The lumogallion and ink stains did not interfere with one another.

For visualization of Al associated with mycorrhizal spores, the fungal spores were rinsed in ice-cold citric acid (BP399, Fisher Scientific) for 30 min. Spores were then stained with lumogallion, and examined for the incorporation of Al during formation using confocal microscopy as described for the root segments.

RESULTS

Colonization potential

Initial experiments examined Al effects on root growth and AM colonization. Root extension was restricted over the range of Al treatment concentrations (Fig. 2).

The extent of the restriction was variable in two experiments with a maximal decrease of ~ 35% occurring in experiment 1 at 8 mmol L^{-1} Al and 85% in experiment 2. Because root growth of mycorrhizal and non-mycorrhizal seedlings decreased similarly, and could not be separated statistically, single curves were used to describe the responses. Root fresh weights also were inhibited to a greater extent by Al in experiment 2, and the mycorrhizal treatments could not be separated (data not shown).

Colonization of the soybean roots by mycorrhizae was inhibited by Al to a greater extent than root extension (Fig. 3). In the 0 Al control, the % of the root length colonized by *G. etunicatum* was 63 and 32%, and the % colonization decreased to minimal levels at 4 and 8 mmol L^{-1} Al (Fig. 3A). Colonization by the mixed mycorrhizal species also decreased with Al exposure, but was affected less severely than that with *G. etunicatum*.

Lower Al sensitivity of colonization by the mixed mycorrhizal species becomes more evident when colonization in the two experiments is plotted together and expressed as a % of the 0 Al controls (Fig. 4). At the intermediate Al concentrations, 2 and 4 mmol L^{-1}, mixed mycorrhizal colonization was consistently inhibited less than *G. etunicatum* in both experiments.

Aluminum accumulation

The Al-specific stain lumogallion was used to determine if the colonization changes were associated with Al accumulation within mycorrhizal structures. Colonization of roots by *G. etunicatum* was associated with extensive intracellular hyphae, prominent arbuscules and vesicles (Fig. 5A). When the soybean seedlings were exposed to Al, in this case 8 mmol L^{-1} Al, extensive Al accumulation occurred in areas occupied by the hyphae and arbuscules (Fig. 5C). The fluorescence signals from

lumogallion also indicated that Al accumulated inside vesicles. This can be seen more clearly in roots colonized by the mixed mycorrhizal inoculum, which typically had larger numbers of vesicles compared to the *G. etunicatum* (Fig. 6A). At higher magnification, lumogallion staining indicated that Al had accumulated in the vesicle wall and the vesicle interior (Fig. 6B and C). In all of our microscopic examinations, spanning many experiments with Al, extensive Al accumulation was present in mycorrhizal structures, even at intermediate and high Al treatment levels.

Infection Potential

To investigate whether Al altered mycorrhizal function, a bioassay experiment was set up to study the infection potential of established *G. etunicatum* mycorrhizae that had colonized soybean. In this experimental system with separate root chambers, *G. etunicatum* colonization of soybean was decreased by Al treatments as before. (refer to Fig. 7). Less *G. etunicatum* colonization of soybean led to lower colonization of maize roots, but the decreases in maize colonization were noticeably less that of soybean colonization. At 2 mmol L^{-1} Al, for example, while soybean colonization was 43% of the 0 Al control, maize colonization was 76% (Fig. 7A). The soybean *G. etunicatum* was clearly capable of colonizing the maize root system through the fabric barrier. Maize root extension was not consistently affected by the Al treatment applied to the soybean side of the barrier (Fig. 7B).

Spore germination

Additional experiments examined Al effects on mycorrhizal spore germination. The spores were from *G. etunicatum* and also *Gigaspora margarita*, a species found in

the mycorrhizal mixture used in previous experiments. The presence of Al had a negative impact on spore germination, but only at the highest Al treatment level (Fig. 8). *Glomus etunicatum* germination was somewhat less sensitive than *Gi. margarita*; about 38% germination occurred at 8 mmol L^{-1} Al compared to 10% for *Gi. margarita.*.

Lumogallion staining and sequential scanning with the confocal laser microscope allowed for examination of Al in the spore wall and the spore interior. Lumogallion images revealed that increasing amounts of Al accumulated in the spore wall as the external Al concentration increased (Fig. 9), as can be seen in successive images of Fig. 9B, C, and D. Increasing amounts of Al could also be detected inside the spores at 4 and 8 mmol L^{-1} Al (Figs. 9C and D).

Aluminum accumulation was different in spores of *Gi. margarita*. Levels of Al at the spore periphery increased only slightly with increasing Al, and Al could not be detected in the spore interior (Figs. 9F,G, and H). Even though no Al accumulation was confined to the wall, the increase in external Al from 4 to 8 mmol L^{-1} resulted in a drop of germination from 50% to 10% (refer to Fig. 8).

DISCUSSION

The results of these experiments with AM and soybean provide an example of a system where Al had an adverse affect on AM colonization. The consistent decline in the percent of root length colonized as the Al concentration in treatment solutions increased indicates that colonization was affected more than root growth. As mentioned in the introduction section, we are aware of only a few previous cases where this has occurred (Clark 1997, Koslowsky and Boerner 1989). In our experiments, the suppression of AM

colonization may have contributed to the lack of amelioration of Al toxicity effects on root extension. But, because the experiments ran for a relatively short period compared to most others, it seems just as likely that ameliorative effects would have become apparent with a longer treatment period or had there been water or nutrient stresses which would increase the importance of hyphal absorption surface.

A main objective of these experiments was to determine whether Al accumulated in the AM structures and, if it did, whether there were indications that internal Al interfered with AM function. The lumogallion images show that substantial Al was present inside mycorrhizal hyphae. Indeed, the Al fluorescence signal was particularly intense in areas occupied by arbuscules, which are thought to be involved in carbon exchange with the plant root cells and sites of high metabolic activity, and inside vesicles. Confocal microscopy involves sequential detection of image layers within a sample. So, it is relatively certain that Al resided in the hyphal interiors and not confined to hyphal walls. Over the past year, many root systems colonized by AM and treated with Al have been examined using these techniques, and large amounts of Al always have been found in mycorrhizal structures. It is difficult to find and image hyphae extending from the root surface, but we assume the pattern of Al accumulation seen in hyphae associated with the root was typical of that occurring throughout the hyphal network. It should be mentioned that Al accumulation in AM fungal walls and an association with vesicles was observed previously using hematoxylin (Cuenca et al. 2001).

Even though mycorrhizal colonization was reduced by Al and much Al accumulated within the mycorrhizae, the results suggest that the mycorrhizae were not functionally impaired. In the divided chamber experiments, secondary colonization of

maize decreased with increasing Al application to the soybean side of the divided chamber, but it was consistently less affected than soybean colonization (Fig. 7A). This indicates that the hyphae extending through the fabric barrier from the soybean chamber were capable of successfully colonizing the roots of maize. A concern in these types of experiments is that Al added to the soybean side may have moved through the barrier and suppressed maize root growth and AM colonization. Had this occurred, however, it would mean that the degree of maize root colonization observed was a minimal estimate of the infection potential of the soybean associated AM fungi.

We cannot know for certain how AM tolerates internal Al accumulation, but it is logical to assume that Al complexes are formed which result in Al 'detoxification'. Possible complexing agents include organic acids, or perhaps metal binding proteins and peptides (metallothioneins) that bind metal ions to cysteine thiolate groups (Hamer 1986). Fungal vacuoles can also function to regulate cytosolic concentrations of toxic metals (Gadd 1993). Inorganic phosphates inside vacuoles, for example, may transform metal ions to non-toxic forms.

Considering that Al inhibits root colonization but apparently did not impair mycorrhizal functioning, it seems that disruption of primary colonization must involve Al disruption of physical or metabolic processes in the root. The implication is that soybean sensitivity to Al may be responsible for the colonization decline. Soybean is relatively sensitive to Al compared to other crop species, and cv. Young has been shown to be an Al sensitive genotype (Silva et al. 2001a).

Certainly, Al alters a number of root functions in sensitive plants that could affect AM colonization. Aluminum binds to negative charges in root cell walls and at the

plasma membrane surface (Taylor 1991, Kinraide et al. 1992, 1994, Ryan et al 1997, Kindraide 1998, Rengel 1992, Silva et al. 2000). The membrane structural changes and Al disruptions of metabolism result in altered membrane permeability, so it is not surprising that many transport processes are altered (Gassman and Schroeder 1994, Ryan et al. 1995, Huang et al. 1994, Pineros and Tester 1995). All these types of effects could interfere with the process of colonization, e.g. signals involved in root recognition, appressorium formation, and hyphal penetration of the root (Harrison 1999).

A point of interest is that colonization by the mixed mycorrhizal species was less affected than that of *G. etunicatum* at the intermediate Al treatment levels. It has been shown previously that individual fungal species vary in their ability to adapt to environmental stresses, which results in changes in fungal population composition (Jakobsen et al. 1992, Leyval et al. 1995, Clark 1997, Siquiera and Moreira 1997, Cuenca et al. 2001, Kelly et al. 2005), and substantial differences have been seen in colonization and amelioration of Al toxicity with different mycorrhizal isolates (Kelly et al. 2005). The mixed mycorrhizal species inoculum and the *G. etunicatum* both originated from soil groups that are characteristically acid with high Al. Nonetheless, evidently mycorrhizal species present in the mixture had an advanced degree of Al tolerance, or at least greater tolerance than *G. etunicatum*.

The current experiments also investigated Al effects on spore germination. The results indicate that spore germination was somewhat resistant to Al compared to mycorrhizal colonization of roots, as germination of *G. etunicatum* and *Gi. margarita* was noticeably inhibited only at the highest Al treatment level (8 mmol L^{-1}; Fig. 8). Studies by Kelly et al. (2005) and Bartolome-Esteban and Schenck (1994) have shown

differential germination among fungal genus, including finding that *Gi. margarita* is more tolerant to Al than *G. etunicatum*, opposite to what we observed. Caution should be taken when comparing results of these studies directly since results can be affected by experimental conditions, species abundance and species origin (Porter et al. 1987a).

The spore wall is the spore's primary defense against environmental stress. It consists of sporopollenin, one of the most resistant polymers in nature (Furch and Gooday 1978), as well as chitin, the substance responsible for protective exoskeletons of insects (Maia and Kimbrough 1998). The spore wall of *G. etunicatum* actually consists of an outer and inner wall (see details in Maia and Kimbrough 1998). The outer wall is primarily constructed of parallel microfibrils. The inner wall may or may not be laminated and can vary in thickness from 2-12 μm. The matrix of the inner wall includes fibrils positioned in an arched or helicoidal organization and may incorporate spore cytoplasm into the structure. Bacteria are also present in the spore wall that may stimulate germination of AM fungi.

With both *G. etunicatum* and *Gi. margarita*, Al accumulated in the spore walls (Fig. 9). Prior to lumogallion staining, spores were rinsed in 10 mmol L^{-1} ice-cold citrate for 30 min to remove loosely bound Al. It has been established that the citrate wash is effective at removing apoplastic Al and Al loosely held on cell wall exchange sites in roots (Zhang and Taylor 1990). Thus, it is logical to assume that Al detected in the spore walls using lumogallion was tightly bound. The citrate wash is necessary because the intensity of the fluorescent signal from the cell wall area tends to saturate the confocal microscope detector, making it difficult to distinguish Al in separate cellular compartments.

In *G. etunicatum*, lumogallion staining and confocal microscopy revealed that Al also accumulated in the spore interior (Fig. 9B, C, D). The Al effects on *Gi. margarita* spore germination were consistently greater than those on *G. etunicatum* even though the *Gi. margarita* spore wall was an adequate barrier against Al (see Fig. 8 and 9F, G, H). The results therefore suggest that Al binding to the spore wall is the key event causing decreased germination over this Al treatment range.

Morphological characteristics of spore germination vary according to fungal species. Spores of *G. etunicatum* germinate by the extension of a germ tube through a subtending hypha (Pawlowski et al. 1999). Typically germ tubes will come in contact with host roots within 24 hr of germination. *Gi. margarita* spores germinate by the development of hyphal germ tubes emerging directly from the spore wall. We would assume that Al interference could occur at many steps in the spore germination process, including disruption of signal reception or development of the germ tube.

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Table 1. AMF species contained in the mixed inoculum

Acaulospora aff. *mellea* Spain & Schenck
Acaulospora aff. *scrobiculata* Trappe
Acaulospora sp. brown with a rough outer wall
Gigaspora aff. *margarita* Becker & Hall
Gigaspora aff. *rosea* Nicolson & Schenck
Glomus aff. *botryoides* Rothwell & Victor
Glomus aff. *clarum* Nicolson & Schenck
Glomus aff. *etunicatum* Becker & Gerdemann
Glomus aff. *intraradices* Schenck & Smith
Glomus aff. *mosseae* (Nicolson & Gerdemann) Gerdemann & Trappe
Paraglomus occultum (?) (Walker) Morton & Redecker
Scutellospora aff. *heterogama* (Nicolson & Gerdemann) Walker & Sanders
Scutellospora aff. *pellucida* (Nicolson & Schenck) Walker & Sanders
Scutellospora aff. *nigra* (Redhead) Walker & Sanders
Scutellospora sp. *heterogama* variant

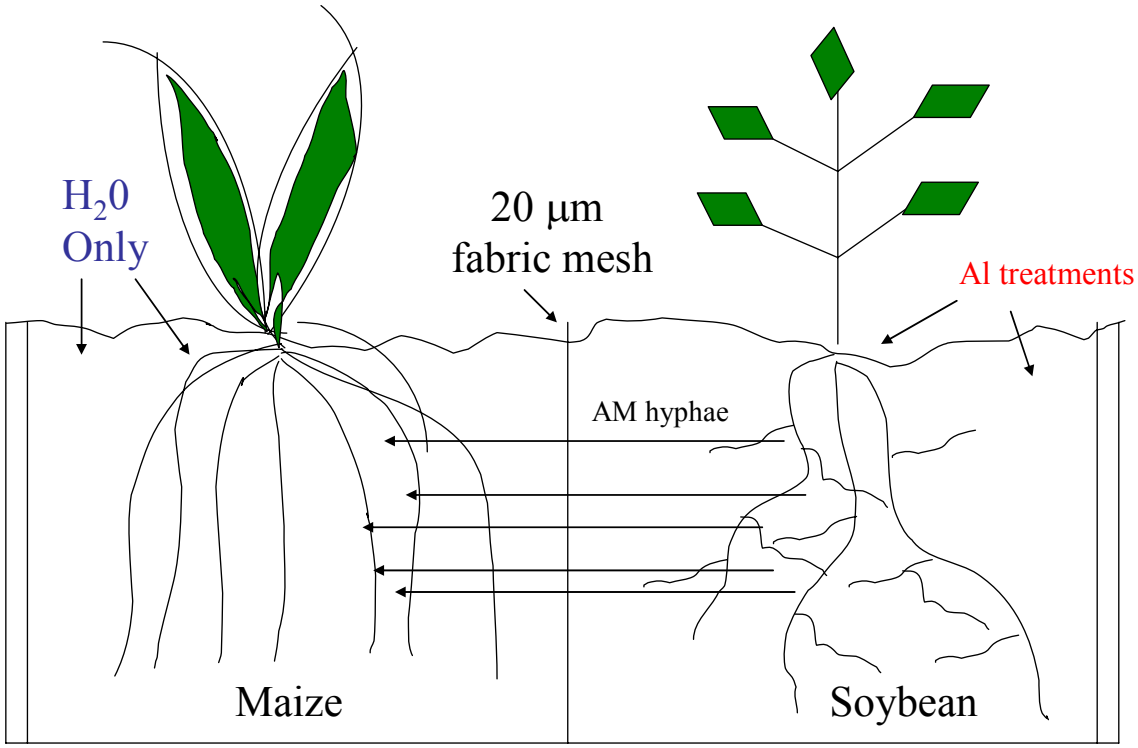

Figure 1. Diagram of chambers and experimental set-up used for the infection potential experiment.

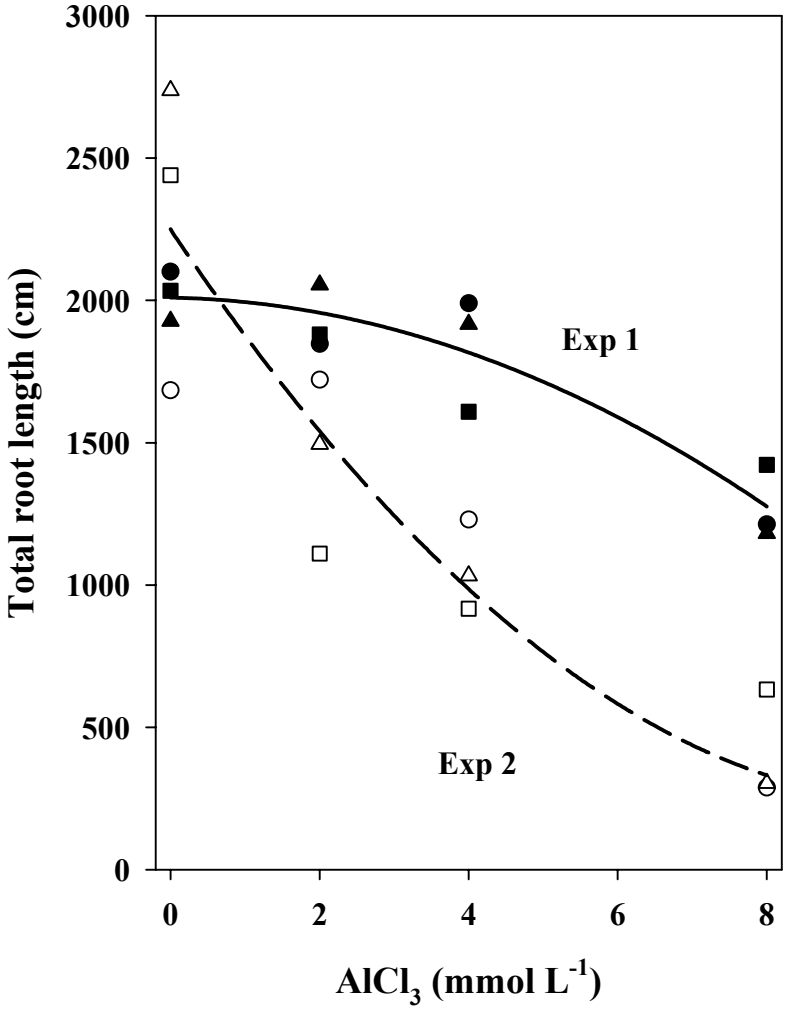

Figure 2. Total root length of soybean seedlings as a function of $AlCl_3$ concentration applied to soil. Solid symbols represent experiment one, open symbols represent experiment two. Triangles represent non-mycorrhizal plants, squares represent plant colonized by *G. etunicatum* inoculum, and circles represent plants colonized by the mixed inoculum. Data points are the average of 4 seedlings and lines represent the trend line for all data points for each experiment.

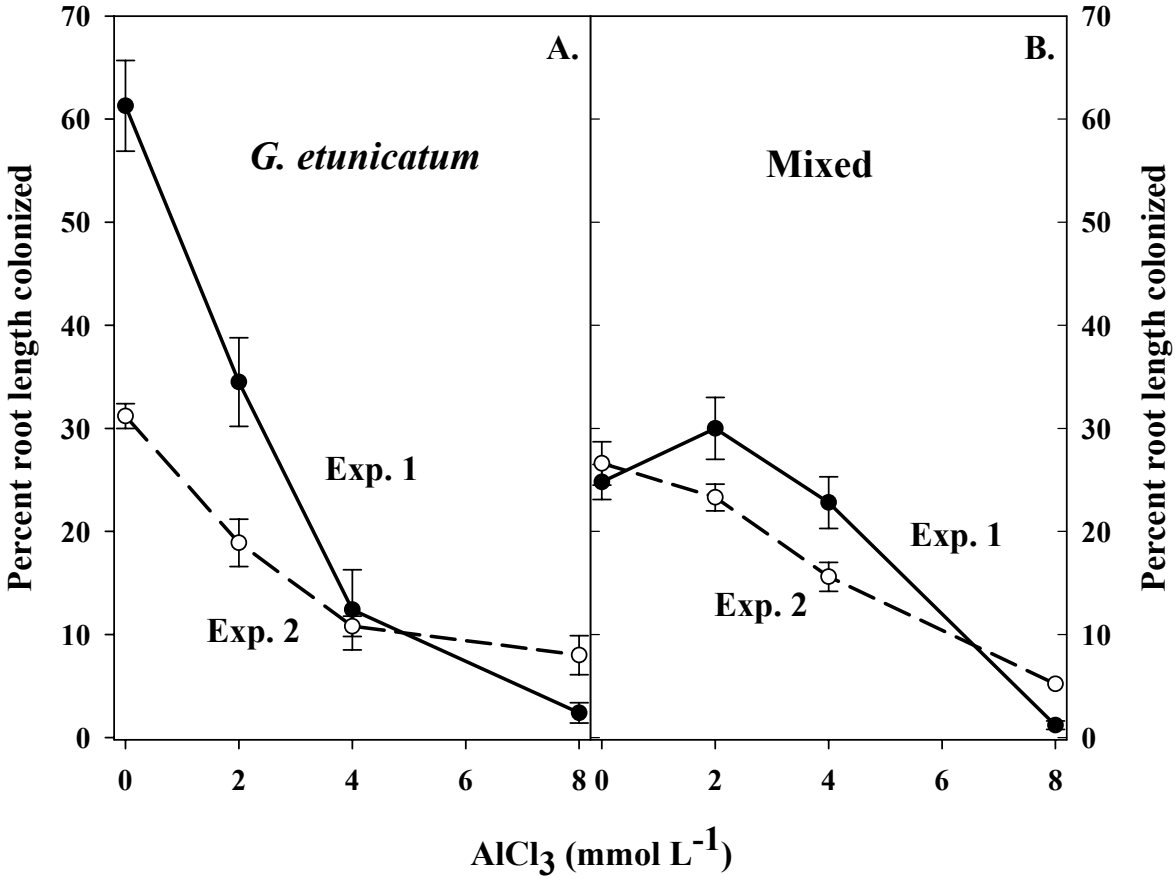

Figure 3. Percent colonization of seedling roots as a function of $AlCl_3$ applied to soil. A) roots colonized by *G. etunicatum* inoculum, B) roots colonized by the mixed inoculum. Data points are the average of 4 seedlings, bars = s.e.

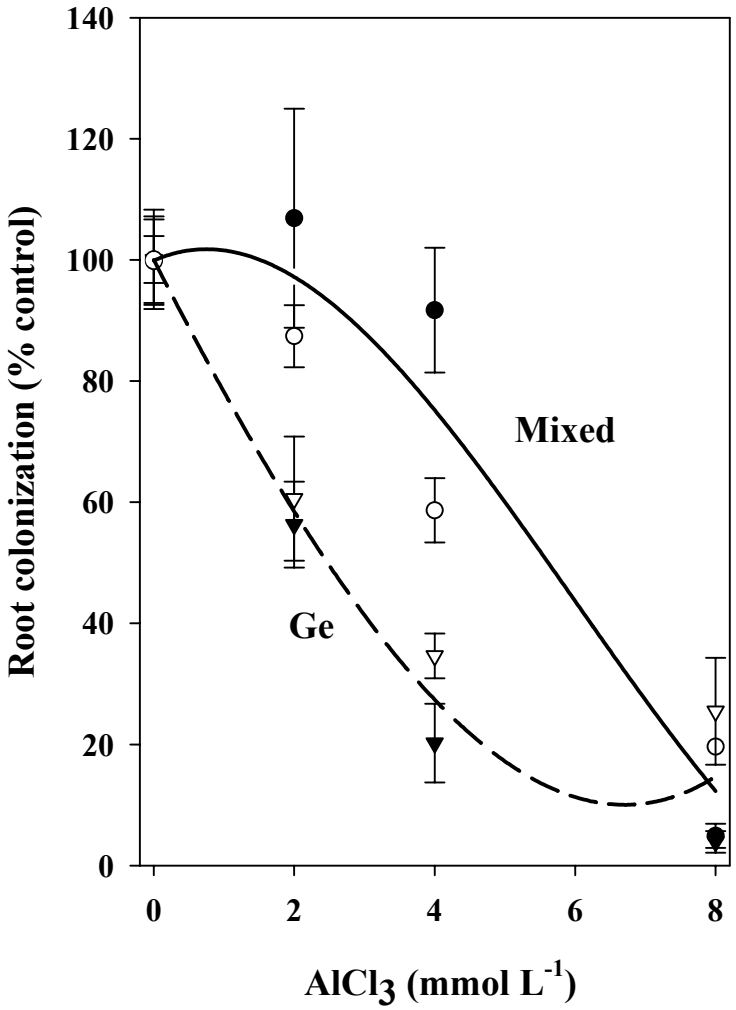

Figure 4. Root colonization as a percent of zero Al control plant colonization. Solid symbols represent experiment one, open symbols represent experiment two. Triangles represent seedlings colonized by *G. etunicatum* inoculum, circles represent seedlings colonized by mixed inoculum. Data points are the average of 4 seedlings and lines represent the trend line for all data for each inoculum. Bars = s.e.

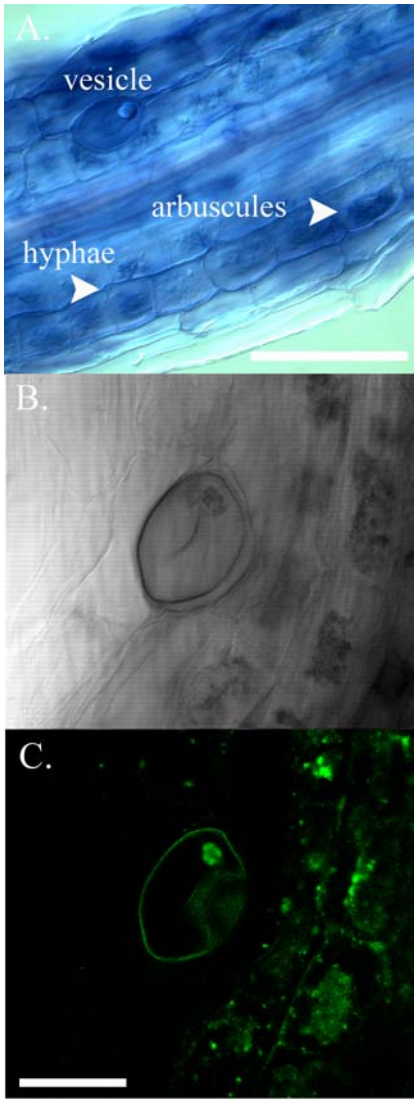

Figure 5. Soybean root colonization by *G. etunicatum*. A) colonization of control plant root stained with Parker ink, B) DIC image of vesicle and arbuscules in root of seedling exposed to 8 mmol L^{-1} $AlCl_3$, C) lumogallion image. Scale bar = A) 50 µm, C) 10 µm.

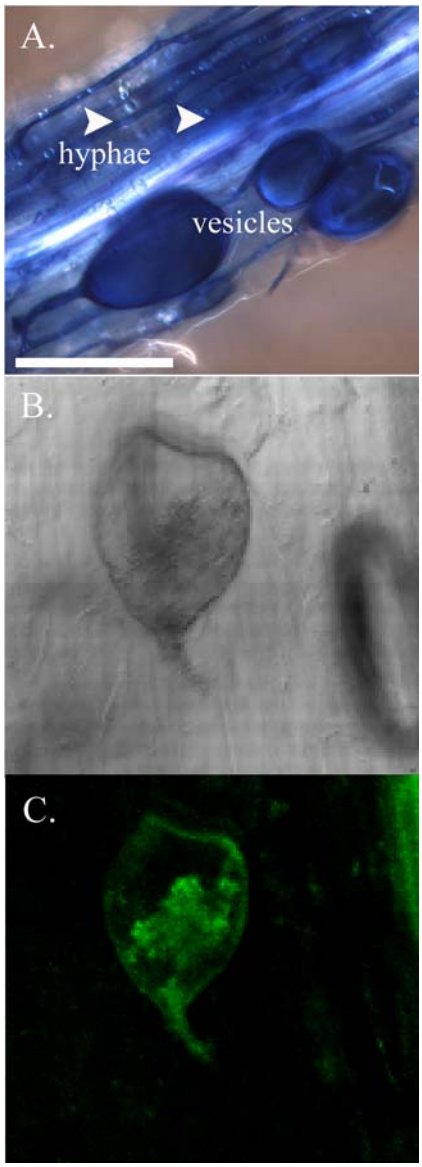

Figure 6. Soybean root colonization by mixed inoculum. A) Colonization of control plant root stained with Parker ink, B) DIC image of vesicle in root of seedling exposed to 8 mmol L^{-1} $AlCl_3$, C) lumogallion image. Scale bar = 50µm.

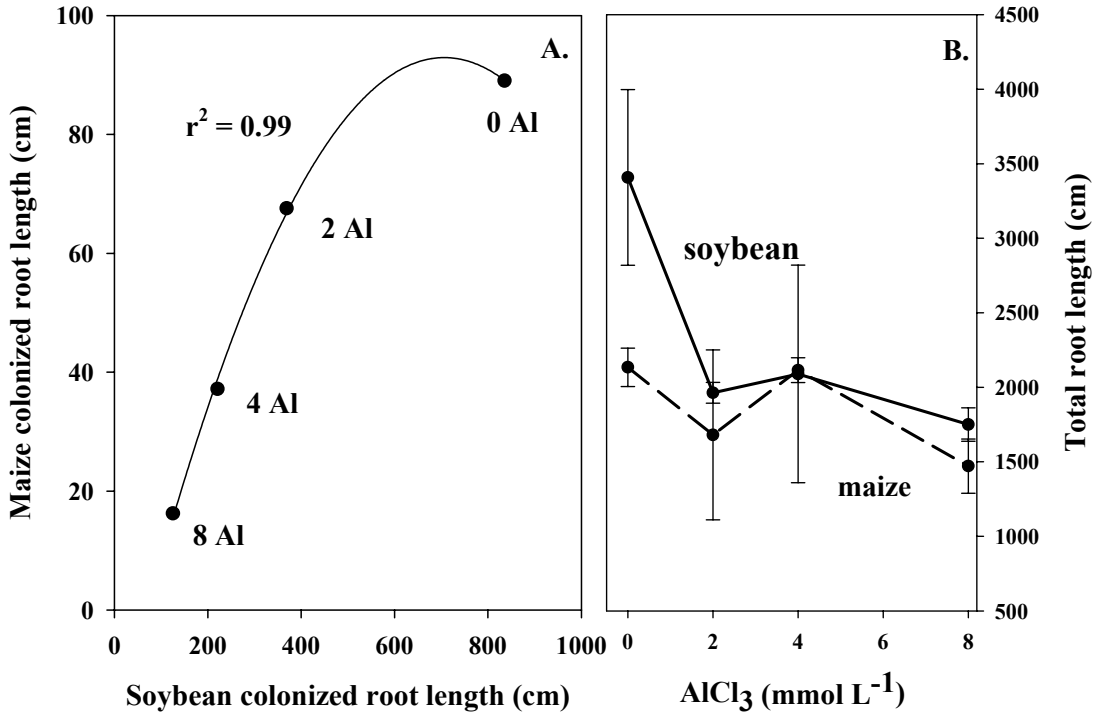

Figure 7. Infection potential of *G. etunicatum* as a function of $AlCl_3$ concentration. A) Colonized root length of maize as a function of colonized root length of soybean. Numbers next to data points represent the concentration of $AlCl_3$ applied to soybean. B) Total root length of seedlings as a function of $AlCl_3$ concentration applied to soybean. Data points are the average of 4 seedlings and bars = s.e.

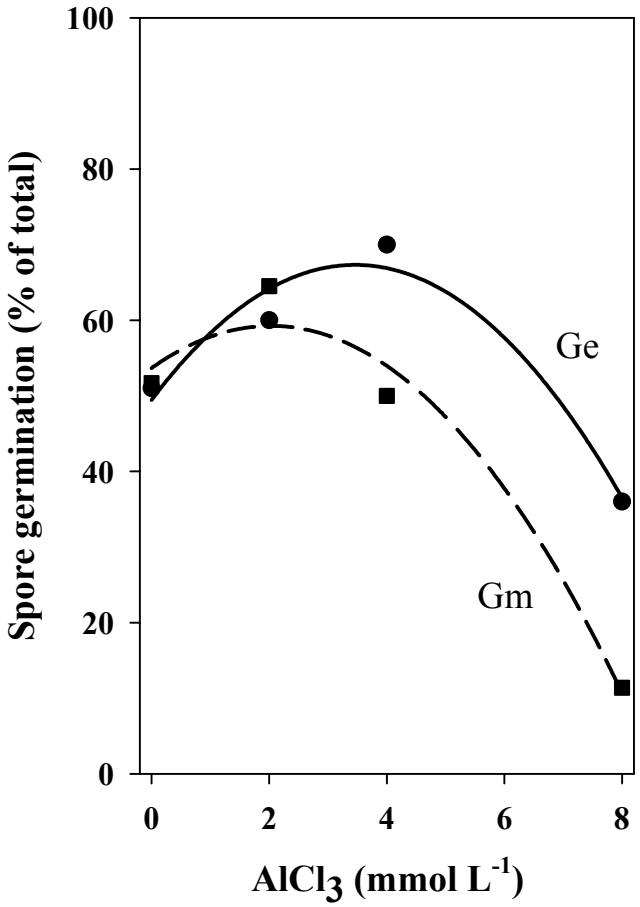

Figure 8. Spore germination as a function of $AlCl_3$ applied to soil. Circles represent *G. etunicatum* spores, squares represent *Gi. margarita* spores. Lines represent the trend line. Data points are the average of 4 replications of 10 spores each.

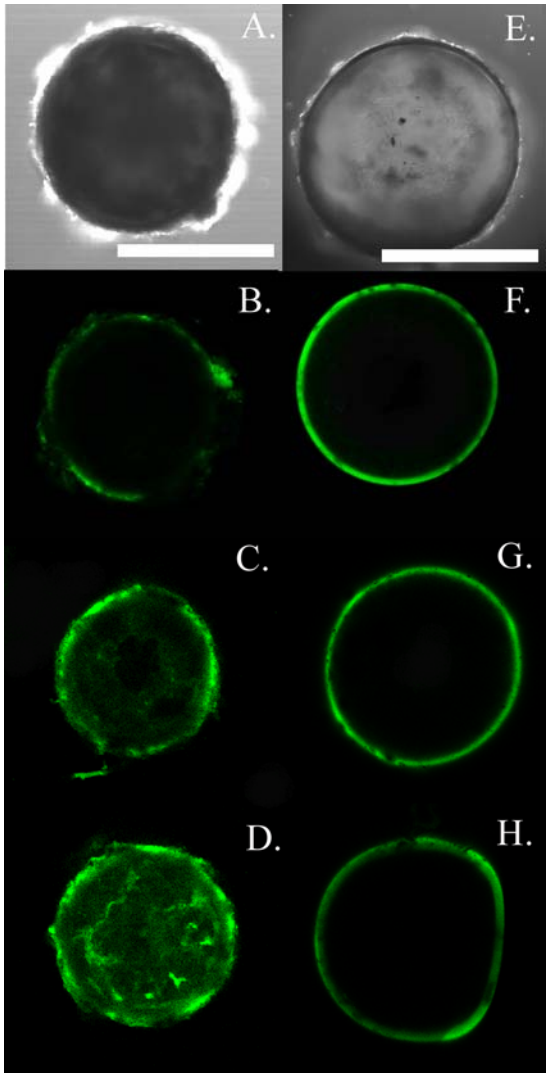

Figure 9. Confocal scanning laser microscopy images of *G. etunicatum* (A-D) and *Gi. margarita* (E-H) spores exposed to increasing concentrations of $AlCl_3$ in soil. DIC images of zero Al spores (A, E), lumogallion images of 2 mmol L-1 treatment spores (B, F), 4 mmol L-1 treatment spores (C, G), and 8 mmol L-1 treatment spores (D, H). Scale bar = 100 μm.