

**Oxalic acid production by the ectomycorrhizal fungus  
Hebeloma westraliensis and its role in the nutrient acquisition and growth  
of Eucalyptus diversicolor in calcareous soil.**

by

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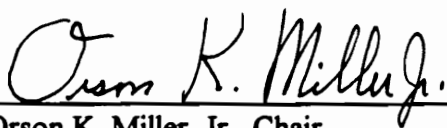
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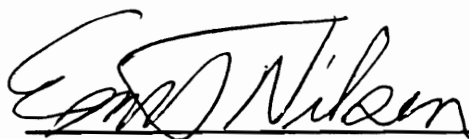
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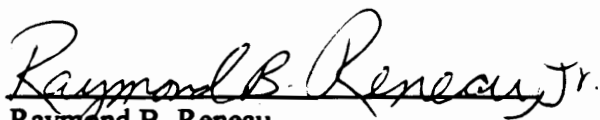
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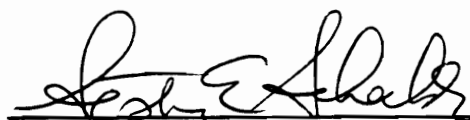
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**Abstract**

This work includes an introduction and literature review of mycorrhizal enhancement of the mineral nutrition of host plants. Particular attention was paid to the role of mycorrhizae in acquiring P from inorganic phosphates. Two experiments were designed to study a mechanism by which the ectomycorrhizal fungus Hebeloma westraliensis can enhance the availability and absorption of P from Ca phosphate by Eucalyptus diversicolor in calcareous soil. Chapter 2 reports on a study on the growth and oxalic acid production of H. westraliensis in calcareous and noncalcareous liquid media. Oxalic acid is thought to chelate cations from phosphate precipitates allowing the release of phosphate into solution. L-threonine was added as an additional treatment to assess the ability of this amino acid to inhibit oxalic acid production by the fungus. Growth and oxalic acid production of H. westraliensis were increased in the calcareous systems. L-threonine had little effect on dry weight of mycelium but substantially reduced oxalic acid production in

the calcareous media. Chapter 3 reports on a study of E. diversicolor X H. westraliensis mycorrhizal synthesis in model calcareous and noncalcareous systems. Oxalic acid production was measured in these systems as well as various measurements of solution and host plant nutrition. Solution P concentration decreased and solution Ca increased in the calcareous systems. Mycorrhization decreased the solution concentrations of both of these nutrient elements, due partly to sequestration of these elements in fungal and plant tissues. Plant tissue P concentration was decreased and tissue Ca concentration increased in the calcareous systems. Mycorrhization increased the concentrations of both of these elements in plant tissues. Mycorrhization also increased the height and dry weight of seedlings at harvest and ameliorated symptoms of nutrient deficiencies seen in nonmycorrhizal plants in the calcareous system. Oxalic acid production by the fungus and by the plant were increased in the calcareous system. Attempts to correlate oxalic acid production with solution and plant nutrition were unsuccessful. The relationships of these results to the mycorrhizal enhancement of plant nutrition in calcareous soil is discussed.

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# Chapter 1.

## Introduction and Literature Review.

The mycorrhizal relationship is a symbiosis between plant roots and soil fungi. It is described as a mutualism because both organisms realize some benefit to their growth and development from the association (Harley, 1969; Marks and Foster, 1973; Harley and Smith, 1983; Harley, 1989). The intimate connections between fungal hyphae and plant roots allow facilitated exchange of soil nutrients and water between extensive hyphal systems and the plant. The fungus associate is able to assimilate photosynthetic carbon resources diverted from plant metabolism (Melin and Nilsson, 1957; Ho and Trappe, 1973; Hackskaylo, 1973; Harley and Smith, 1983). The mutualistic nature of this symbiosis is strikingly evidenced by increased above and below ground growth of both symbionts. These benefits are most clearly realized in enhanced competitive vigor of plants and fungi in natural ecosystems where availability of soil nutrients is limited and competition for resources is intense (Bowen, 1973; Harley and Smith, 1983; Allen, 1991; Allen and Allen, 1986). In most cases the fungus does not survive apart from its host due to poor abilities as a saprophyte on complex carbon compounds. Throughout their natural ranges, neither associate survives to reproduce apart from the other (Harley, 1969; Harley and Smith, 1983).

Mycorrhizae may exhibit several morphological forms. Distinguishing among these forms has resulted in a classification of mycorrhizae that enables one to delimit the symbiont taxa involved and the ecological roles played by each (Lewis, 1973; Harley and Smith, 1983; Read, 1983). Vesicular-arbuscular mycorrhizae (VAM) are characterized by hyphal penetration of plant root epidermal and cortex cell walls. Some forms exhibit a beaded appearance but in general there is little outward modification of root morphology. Anatomical

differentiation of hyphae form specialized structures within the cortical cells of the root. Swollen hyphal cells called vesicles form within root cells and serve as organs for storage of transferable nutrients and metabolites. Arbuscules are minutely branched hyphal cells that serve to facilitate exchange of ions and compounds between fungus and plant host cells (Harley and Smith, 1983). This type of mycorrhizal relationship is the primary one in temperate and tropical grasslands and other natural and agricultural ecosystems where herbaceous and, in particular, graminaceous plants predominate.

The fungal species in VAM relationships are limited to three families of the Zygomycete order Glomales (Morton and Benny, 1990), but are distributed worldwide and have broad host ranges with many families of Angiosperms (Harley and Smith, 1983). Other unique endosymbiotic relationships are found among plants in the Orchidaceae and Ericaceae. Though the importance of these mycorrhizae to natural and agricultural ecosystems is tremendous, experimental studies have been faced with difficulties over the years. Most endomycorrhizal fungi have not been isolated axenically on synthetic media to any degree and exhibit little or no growth apart from their plant host (Harley and Smith, 1983; Allen, 1991). Physiological studies have therefore been problematic in controlled laboratory studies.

A wide array of ectomycorrhizal higher fungi in the Ascomycetes and Basidiomycetes are associated with a relatively small number of temperate woody plants (Miller, 1982). This relationship is characterized by formation of a sheath of fungal hyphae around the lateral and secondary short roots of trees. Hyphae extend from the sheath between the root epidermis and cortex cells without cell wall penetration. An extensive mycelium and rhizomorph system radiates from the sheath through the surrounding soil. The sheath of hyphae around the root takes on various forms and thicknesses and is termed the mantle, while the intracellular growth of hyphae between the root cells is called the Hartig net. In this symbiosis plant root hairs are inhibited and branch roots proliferate with many thick, short, often bifurcating rootlets in which epidermal or outer layer cortical cells can be obliquely radially elongated (Harley and Smith, 1983; Kottke and Oberwinkler, 1987). These morphological changes are thought to be hormonally controlled as the fungus synthesizes and exudes IAA and NAA which have been

variously shown to produce these effects in exogenous application under controlled conditions (Chilvers and Prior, 1965; Slankis, 1973). Cortical cell elongation may be the result of a combination of factors including fungal hormonal exudates, the retaining of an embryonic columnar trait in root cells in mycorrhizae (Chilvers, 1974) and biomechanical forces of internal root pressure and soil pressure (Clowes, 1981).

Many ectomycorrhizal fungi can be cultured axenically in nutrient agar or liquid media and can be manipulated in physiological studies apart from their host (Molina and Palmer, 1982). They also form distinctive, easily recognizable mycorrhizae when grown in association with the roots of a compatible host plant. This allows relatively easy observation of ectomycorrhizal synthesis in controlled laboratory settings. Study can be made of the physiology and growth of both symbionts, either separately or in synthesis, and differentiation between the effects of either is possible (Harley and Smith, 1983).

A remarkable aspect of mycorrhizal symbiosis is the nutritional relationship between the fungus and plant associates. Radioisotopic tracing of elements from substrate through hyphae to plant xylem and above ground organs have revealed much about the translocation and utilization of soil nutrients in mycorrhizal plants. Labeled N, P, K, Ca, Na, Zn and Rb have been traced from substrate compounds to the plant shoot (Bowen, 1973; Harley and Smith, 1983). Likewise,  $^{14}\text{C}$  from  $^{14}\text{CO}_2$  has been traced from ambient air, through photosynthesis and translocation to extramatrical hyphae of fungus associates and even between adjacent plant symbionts through apparent underground mycelial connections (Melin and Nilsson, 1957; Finlay and Read, 1986a, 1986b Newman, 1988). The mechanisms of absorption, metabolism, storage and translocation and reutilization of these compounds are numerous and complex and are the sources of considerable research work (reviewed extensively in: Bowen, 1973; Tinker, 1978; Smith, 1980; Harley and Smith, 1983).

Much of the study of mycorrhizal nutrition has focused on the plant symbiont. This has resulted partly from the greater economic concerns with plant nutrition particularly in agricultural or forest systems. Difficulties in manipulating microscopic fungi in the lab under

controlled conditions, especially those that remotely resemble natural situations, have complicated observations of fungal nutrition. Mycorrhizal fungi have a generally poor ability to metabolize complex carbon compounds (Palmer and Hackskaylo, 1970), hence their need in natural systems to assimilate carbon fixed in photosynthesis. In the laboratory this results in the need to amend growth media of these fungi with simple carbohydrates in order to grow them apart from their host (Molina and Palmer, 1982). Consequently, studies on the ability of mycorrhizal fungi to utilize soil nutrients necessitates the use of carbohydrate sources that are seldom encountered in a natural situation. Never-the-less some evidence has emerged about the processes of the mycobiont especially relating to methods of nutrient acquisition. These will be considered as they relate to nutrient transfer to the plant host.

Several methods of enhancement of plant nutrient acquisition have been proposed for the mycorrhizal relationship. These include: 1) a greater ability for physical exploration of the soil by fungal hyphae over plant roots, 2) a higher affinity in hyphae for certain soil mineral elements and 3) the ability of fungal hyphae to absorb and translocate phases of nutrient elements that would otherwise be unavailable to plant roots (Bolan, 1991). All of these mechanisms probably operate together with each becoming more important in particular environments. Though these main modes of nutrient enhancement may seem apparent, the latter two: that hyphae have a greater affinity for some mineral elements than plant roots, and that nutrient pools that are unavailable to plants may be available to mycorrhizal mycelium, have been the subject of considerable controversy (Mosse et al, 1981).

The physical exploration of soil is no doubt enhanced in the mycorrhizal system. Fungal hyphae range from 1 to 10  $\mu\text{m}$  in diameter compared to plant root hairs of 20+  $\mu\text{m}$ . This allows hyphae to explore micropores of soils, grow between the layers of weathering soil particles, and come into much closer contact with cation exchange surfaces. Hyphae are able to do this with more surface area than non-mycorrhizal roots (Hayman and Mosse, 1972). Given that mycelium can extend several centimeters from root and mantle and several meters away through secondary morphogenesis of rhizomorph systems, and that hyphae grow much faster than roots, this spatial advantage alone can considerably enhance soil nutrient availability to

plants (Barber, 1980).

It is proposed that mycorrhizal hyphae have a higher affinity or lower diffusion threshold for some soil nutrients. This is based on the observations that extra-matrical hyphae can continue to absorb nutrient ions even when the diffusion gradient for these is steep. This occurs when a plant root draws nutrient reserves from adjacent soil solution creating a depletion zone for that element around the root (Bolan, 1991). It has been argued that the mycorrhizal amelioration of this situation is related more to the ability of hyphae to explore soil regions beyond the depletion zone than to any increased affinity for nutrient ions (Sanders and Tinker, 1971; Barber, 1980; Safir, 1987). It has been shown though through isotopic labeling of P and N that hyphae are able to continue absorbing these nutrients, even from within their own zone of depletion, when ion concentration has dropped below the level necessary for plant root uptake (Owusu-Bennoah and Wild, 1979, 1980; Harley and Smith, 1983).

The hypothesis that mycorrhizae have access to nutrient pools unavailable to non-mycotrophic plants has been tested numerous times. This was demonstrated with Ericoid mycorrhizae and N bound in organic material of heathlands (Read, 1983; Read et al, 1985; Abuzinadeh and Read, 1986). Mycorrhizal fungi have a strong capacity to produce protease and exude this enzyme into the surrounding soil (Allen, 1991). Previously unavailable N bound in organic matter is released by this enzyme system in a form that is plant available. This effect on organic N has particular significance in acid, nutrient poor soils of alpine and tundra habitats where Ericaceous plants may predominate. It has been argued that organic N will become slowly available through the work of other soil microorganisms without the presence of mycorrhizae (Mosse et al, 1981). While this is no doubt the case, the mycorrhizal protease enzyme system has been shown in controlled laboratory experiments to be a legitimate means by which some nutrients are available only to plants with mycorrhizae (Read et al, 1985).

Organic forms of P are likewise made available through mycorrhizal action. As much as 50% of the total P in temperate forest soils can be tied up in organic matter (Häussling and Marschner, 1989). The ability of mycorrhizae to acquire P from organic matter is related to

the mechanisms of decomposer fungi (Hodgkinson, 1977). Fungi have a high growth demand for P and are uniquely adapted to enhance the solubility of organic phosphates through direct enzymatic action. The production and exudation of phosphatases has been demonstrated numerous times with VAM (Allen et al, 1981; Dodd et al, 1987) and with ectomycorrhizae (Antibus et al, 1981,1986; Kroehler et al, 1988). Bartlett and Lewis (1973) and Alexander and Hardy (1981) found that ectomycorrhizae produce several phosphatases that are active at different pH optima and hydrolyse a range of organic materials to release inorganic phosphate into the soil solution. Both acid and alkaline phosphatase activity has also been demonstrated in VAM (Gianinazzi-Pearson and Gianinazzi, 1983).

In addition to direct enzymatic action, soil fungi produce and exude a range of organic acids that enhance soil weathering through the dissociation of H<sup>+</sup> ions (Graustein et al, 1977). These effects in the degradation of organic matter are no doubt important to overall soil fertility, but phosphate in the soil solution is ephemeral and quickly precipitated by cations or fixed by other components in the rhizosphere. Since mycorrhizal hyphae are in close proximity to sites of phosphate release by acid weathering, absorption into plant roots is increased.

More work has been done with the effect of mycorrhizae on unavailable inorganic P. Duce (1987, in: Allen, 1991) studied the effects of mycorrhization on organic, labile and bound inorganic P pools. He found that of the total soil P, the fraction that showed the most effect from mycorrhizal action was the bound inorganic P pool. Labeled P in inorganic phosphates has been traced from soil, through ectomycorrhizal hyphae to associate plant roots and then into shoots (Bolan et al, 1984). In temperate forests with mineral soils, bound inorganic phosphates represent the largest fraction of soil P. Phosphate in mineral soils is tied up in poorly soluble inorganic precipitates with Al and Fe in acid soils and with Ca and Mg in alkaline soils. These sources of P are of limited availability to plants (Mattingly, 1975; Bolan, 1991). This is also the environment where ectomycorrhizae have evolved as the dominant form of this symbiosis. The mechanism of phosphatase on organic P sources also seems to be in effect on bound inorganic P (Bartlett and Lewis, 1973). Activity of this enzyme system is increased when inorganic phosphate in soil solution is low (Alexander and Hardy, 1981).

While the action of secreted organic acids by mycorrhizal fungi on P cycling was once considered minimal (Bowen and Theodorou, 1967), this mechanism is now being focused on as a primary method by which mycorrhizae enhance the nutrient turnover in soils and the nutrient acquisition of plant associates (Allen, 1991).

A major component of the fungal organic acid suite is oxalic acid (Hodgkinson, 1977). Researchers have shown that oxalic acid and Ca oxalate are important components in the infection process of the parasitic fungi *Sclerotium rolfsii* (Kritzman et al, 1977) and *Fomes annosus* (Hutterman et al, 1980). This led to the hypothesis that oxalate enhanced the decomposition of middle lamellae and cell walls in infected host plants by binding the calcium from host tissues. These investigations made progress in elucidating the oxalic acid pathway in fungi. In this process it was discovered that L-threonine added to substrate media inhibited the production of oxalic acid by reducing the activity of glyoxalate dehydrogenase, an enzyme that catalyzes the formation of oxalate from glyoxalate (Kritzman et al, 1977). The research on plant parasites raises questions about the infection process of mycorrhizal fungi and the possible role of oxalic acid in this regard. Oxalic acid may also act to increase P availability in soil not only through acid weathering of the substrate but also through unique abilities as a complexant (Graustein et al, 1977; Cromack et al, 1979; Malajczuk and Cromack, 1982; Lapeyrie et al, 1987). As soil inorganic phosphates are precipitated with soil cations, the ability of oxalate to complex with these cations is recently being viewed as extremely important to mycorrhizal P nutrition (Bolan, 1991).

The formation of calcium oxalate crystals in plants has long been considered an end product of Ca and oxalic acid metabolism. Both of these metabolites are often in supra-optimum concentrations in plant tissues and both can be detrimental or toxic in large quantities. The formation of Ca oxalate crystals was thought to insolubilize and compartmentalize both in a metabolically inactive form (Francheschi and Horner, 1980). Much has come to light regarding the active role of Ca oxalate crystals in plant systems, and their formation in fungi has special significance in mycorrhizal symbiosis.

In mycorrhizal research, work by Graustein et al (1977), Cromack et al (1979), Lapeyrie et al (1983, 1986, 1987, 1989) and others has led to the current hypothesis that oxalic acid produced by mycorrhizal fungi chelates Fe, Al and Ca in soil phosphates to release phosphate into the soil solution and prevent its reprecipitation in these tightly bound forms. Lapeyrie et al (1983, 1990), and Malajczuk and Cromack (1982), observed Ca oxalate crystals on mycorrhizal fungi in culture and on mantles of mycorrhizal Eucalyptus sp. and began intensive work into this phenomenon. They hypothesized that the fungal production of oxalic acid and the crystallization of Ca oxalate had particular relevance to mycorrhizal plants growing in calcareous soil (see also: Froidevaux and Kalin, 1981). According to this model, oxalic acid produced by fungi binds soil Ca, and opens up many cation exchange sites for other nutrients. Since the major P components of these soils are poorly soluble forms of Ca phosphate, P is made available in this system when oxalic acid preferentially binds and precipitates Ca, releasing phosphate into solution. Fe and Al phosphates are likewise complexed in this manner. Lapeyrie et al (1987) also showed that increasing  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and  $\text{NO}_3^-$ , all typical ions of calcareous soil, caused an increase in oxalic acid production by the mycorrhizal fungus Paxillus involutus. Recent evidence suggests that mycorrhizal fungi may also ameliorate conditions caused by toxic levels of  $\text{Ca}^{2+}$  in calcareous soil by limiting the uptake of  $\text{Ca}^{2+}$  by calcifuge (or acidophilic) plants (Azcon and Barea, 1992).

The use of Eucalyptus sp. in these experiments could have tangible economic benefits. Of the temperate forest woody plants that form ectomycorrhizae, those that constitute important timber species have received the most research attention. Knowledge of mycorrhizae in the Pinaceae, for example, has been easily applied to timber management situations in the Northern Hemisphere, and has allowed their establishment as fast growing exotic timber trees elsewhere in the world. Eucalyptus sp. (Myrtaceae) are important timber trees in the Southern Hemisphere and have become increasingly popular as a widely planted exotic especially adapted to warmer and drier climates worldwide (Pryor, 1976). Eucalyptus is a taxon of over 850 species native to the continent of Australia. As a genus they are considered fast growing and adaptable to a wide range of soil and climatic conditions, but they generally perform poorly

in managed forest stands when not in association with a compatible fungus symbiont (Chilvers and Pryor, 1965; Pryor, 1976). Eucalyptus mycorrhizae has been a focus of Australian botanical research for several decades. These studies have been absolutely crucial to the successful establishment of exotic plantations of these important timber trees.

The adaptability of eucalypts to soil conditions has been found to be limited in some situations outside of its range (Lapeyrie and Chilvers, 1985). In the dry climates to which many Eucalyptus species are adapted calcareous soil is a common. This phenomenon is rare in Australia due to the scarcity of limestone parent materials. Consequently, eucalypts have not evolved adaptations for the particular cultural rigors that calcareous soils place on the inhabiting plant communities. These soils are highly variable in structure and nutrient status. Still, several characteristics are consistent in their classification. In calcareous soils the predominant mineral is  $\text{CaCO}_3$  or  $\text{MgCO}_3$ . Calcium ion supply is abundant in solution and  $\text{HCO}_3^-$  is always present. These soils are strongly buffered to near neutral or alkaline pH (6.5-8.3). The active  $\text{Fe}^{3+}$  is minimal at high pH and  $\text{Al}^{3+}$  is never present in toxic concentrations. Other physical properties that are often associated with calcareous soils include unrestricted permeability to water resulting in often warm, dry surface layers, reduced availability of P, K and N, and a cation exchange capacity (CEC) that is saturated with  $\text{Ca}^{2+}$  (Etherington, 1982; Rorison and Robinson, 1984).

The occurrence of neutral to high pH calcareous soils is more common in arid, or seasonally arid, environments. Rainwater is inherently slightly acid from  $\text{HCO}_3^-$ , or other acids near industrial centers. Areas with relatively high annual rainfall are often leached of excess  $\text{Ca}^{2+}$  while acidified by  $\text{HCO}_3^-$ . Arid environments, on the other hand, may accumulate  $\text{Ca}^{2+}$  and other cations in the surface layers from soil moisture evaporation and are less influenced by acidification from rain (Kinzel, 1983).

The common occurrence of calcareous soil in dry climates has led to the evolution of communities of calcicole plants. The term calcicole refers to plants found predominantly in calcareous soils. While the ecological foundation for this classification may be secure, the

physiological adaptations exhibited by these plants suggest that they are more often acidiphobic than calcicolic. In fact they may be more resistant to the adverse conditions of calcareous soil than other plants but do not require a high pH or high Ca in the soil. The term calcifuge literally means to "flee calcium" and in this case, though Ca may not be the primary factor in limiting the growth of these plants, the term is more accurate (Jeffrey, 1987). Calcifuge plants seem unable to tolerate the limitations of increased pH in calcareous soils, and are adversely affected by the nutrient limitations and excesses imposed in this system.

Calcicole and calcifuge were proposed as ecological terms and as such represent two extremes of a continuum of adaptation to  $\text{CaCO}_3$  concentration in soils. In an effort to more clearly represent the physiology of these plant species, Kinzel (1983) reviewed what is known of the metabolic processes involved and suggested that plants may also be classified as calciotrophic, whereby they maintain appreciable amounts of dissolved calcium in plant sap, and calciophobic plants, those that precipitate incoming calcium in compounds such as Ca oxalate in vacuoles. Eucalyptus sp. are considered to be calcifuge plants. It appears that mycorrhizal fungi may contribute a secondary calcicole nature to compatible host plants and evidence for this has been found in Eucalyptus (Lapeyrie et al, 1983).

In considering the mycorrhizal amelioration of environmental limitations to plant growth, the importance of ecological and physiological traits of specific fungal partners must be taken into account. In natural situations mycorrhizal partners live to compete for resources successfully and reproduce or they die. Natural selection takes care of problems between incompatible partners and of problems of compatibility between these symbionts and their environment. The challenge lies in the ability of researchers to understand what occurs in nature and to apply this knowledge to a wide variety of situations.

The establishment of mycorrhizal plants outside of their natural range is often difficult, as exemplified by the failure of early exotic Eucalyptus plantations. A first consideration is

whether or not target plants are able to form mycorrhizae with fungi occurring in regions where planting is attempted. In VAM associations in which a relative few species of fungi form mycorrhizae with the majority of the world's plants, specificity may not be such a large concern. It is becoming clear lately however, that a good deal of difference exists between partnerships in this association. Even within one VAM species strains and ecotypes may behave very differently in another environmental situation.

In the ectomycorrhizal symbiosis the problem of specificity is compounded. Many thousands of species of fungi are ectomycorrhizal which may have very broad or narrow host ranges. The genus Eucalyptus may have the richest genus-specific fungal flora in the world. This may have evolved through the limited native geographic range of these plants, the huge number of species in the genus and the dominance of these plants in their native habitats (Molina et al, 1992). The fungus associates of Eucalyptus are not specifically limited to those native to Australia (Malajczuk et al, 1982, 1984). Still, the specificity of Eucalyptus to fungus associates was a factor in early exotic plantation failures. Since then the establishment of ectomycorrhizal fungus associates with Eucalyptus plantings has been integral to successful plantation establishment (Pryor, 1976).

The limitations imposed by calcareous soils in regions of the world where eucalypts would otherwise be superb candidate species for reforestation dictate that the fungus associates chosen for plantation establishment be ecologically and physiologically suited for these conditions. Numerous fungi have been tested in this regard; some meeting with more success than others. Considerable research has been done in southern France where calcareous soils predominate in a seasonally dry Mediterranean climate that would be a harsh environment for most timber species. Lapeyrie was a principle investigator both in Australia and in France of mycorrhizal amelioration of Eucalyptus stress in calcareous soils. Through the French governmental agency, AFOCEL he helped launch a major research effort aimed at the establishment of eucalypts in southern France (Marquestaut et al, 1977). The field application of this work was eventually scaled down as it became clear that low Winter temperatures in this region prevented perennial plantation growth of the fast growing species suitable for

timber. A good deal of knowledge about Eucalyptus mycorrhizae in calcareous soil came from this work however and eucalypts continue to be an excellent model organism for laboratory study of this phenomenon (Lapeyrie, personal communication).

The cosmopolitan mycobionts Paxillus involutus, Hebeloma sp., Cenococcum graniforme and Pisolithus tinctorius were tested to determine differential response to calcareous situations (Lapeyrie and Bruchet, 1982). Each fungus was grown in artificial media with and without the presence of CaCO<sub>3</sub>. Cenococcum graniforme produced the most growth as measured by dry weight of mycelium in culture whether CaCO<sub>3</sub> was present or not. Hebeloma sp. was the only fungus to show an increase in growth when CaCO<sub>3</sub> was present. This is supported by the findings of Hung and Trappe (1983) who found in a test of 10 species and numerous isolates of mycorrhizal fungi that Hebeloma crustuliniforme was the only one to exhibit an increase in growth with increased pH up to pH 7. They also reviewed related research on 31 other mycorrhizal species, none of which had an optimum pH for growth above 6.5.

In subsequent work with Eucalyptus and its fungus associates, Lapeyrie et al (Lapeyrie and Bruchet, 1982, 1986; Lapeyrie et al, 1983, 1987, 1990; Lapeyrie, 1988) chose P. involutus as a test associate in studies of reactions in calcareous conditions. They found that two isolates of this fungus, one isolated from calcareous soil and one from a more acidic soil, behaved very differently in relation to ion concentrations likely to be encountered in calcareous soil. They considered that the isolate from the calcareous soil reacted similarly to a calcicole species and that the other was calcifuge (Lapeyrie and Bruchet, 1986). These results support the notion that ecotypes within a species can be a significant consideration in choosing fungus associates for plantation trees.

Several studies have focused on the ability of mycorrhizae to enhance the nutrition of associate plants in calcareous soil. Azcon and Barea (1992) working with VAM fungi and the legume Medicago sativa found that VAM inoculation increased the levels of P, Mg, K and N in plant tissues when available P in the soil was low. The effect of mycorrhization was diminished

when additional P was supplemented to the growth medium. Perhaps of equal importance, mycorrhization decreased the concentration of Ca in plant tissue grown in calcareous soil. This they viewed as a principle benefit of mycorrhizae in this situation. Medicago sativa is classified as calcifuge and VAM inoculation allowed a secondary calcicole habit of these plants. Similar results were had with VAM inoculation of Helianthemum chamaecistus in calcareous soils (Kianmehr, 1978). Previous work with ectomycorrhizal trees showed a similar response of mycorrhization. Clement et al (1977) and Le Tacon (1978) found this to be the case with Austrian pine (Pinus nigra ssp. nigricans). This tree is inherently intolerant of calcareous soil and in these studies ectomycorrhizae of Tuber melanosporum, Boletus (syn. Suillus) granulatus and unidentified fungi allowed increased growth and reduced symptoms of nutrient deficiency in these soils.

More recently in Australia, Lapeyrie and Chilvers (1985) observed a VAM-ectomycorrhizae succession on Eucalyptus dumosa that enhanced the nutrition of this tree when growing in calcareous soil. They had located specimens of this tree growing in a localized patch of calcareous soil in New South Wales and collected seeds for greenhouse experiments. It was discovered that without mycorrhizal inoculum new seedlings were intolerant of the soils in which the parent was found. Upon addition of unsterilized native soil to the containers of these seedlings, mycorrhizae were formed in a succession of early colonizing VAM and later developing unidentified ectomycorrhizae. This mycorrhization ameliorated the nutrient deficiencies that were observed when no inoculum was present.

The pH response of Hebeloma crustuliniforme presented an interesting opportunity for the study of the reactions of mycotrophic trees associated with this fungus to calcareous soils. Froidevaux and Kalin (1981) recovered mycorrhizae from this fungus under Fagus sp and Quercus sp. in France and observed Ca oxalate crystals on the mycorrhizae and extra-matricular hyphae. The beech forest was located in a region of calcareous soil and they hypothesized that the formation of these crystals was important to the nutrition of the ectomycotrophic trees. H. crustuliniforme is known to occur in Australia but is thought to be an introduced species as it is associated with introduced Pinus and Populus and is not known to

form mycorrhizae with Eucalyptus. Only one other in this genus, H. aminophilum has been recorded from Australia in the literature, and its mycorrhizal status is unknown (Miller and Hilton, 1986).

A recent discovery and description by Bougher et al. (1991) of a new fungus in the genus from Australia (Hebeloma westraliensis), resulted in the inoculation with seedlings of five species of Eucalyptus under controlled conditions. This fungus established mycorrhizae with each species. These researchers concluded that H. westraliensis had significant potential for use in field inoculations of Eucalyptus in plantation settings due to remarkably rapid growth in culture, rapid colonization of soil and rapid colonization of roots of associate trees. The lack of specificity that was suggested by H. westraliensis in this experiment also could prove useful in future reforestation efforts.

Preliminary work with H. westraliensis in our laboratory suggested that this fungus achieved increased growth in media supplemented with CaCO<sub>3</sub>. In fact, additions of up to 0.5 g CaCO<sub>3</sub> per 85 ml of modified Hagem's liquid medium, achieving pH up to pH 8.0, still did not reach the apparent optimum for the growth of this fungus. The soil characteristics in which H. westraliensis was collected was not provided in the original description (Bougher et al, 1991), but these pH results are not contradicted by previous growth studies of other Hebeloma spp. (Hung and Trappe, 1983). These were exciting results and warranted a closer look at the ability of this fungus to tolerate calcareous conditions and to perhaps impart a tolerance of these conditions to a calcifuge Eucalyptus associate.

Several of the species of Eucalyptus with which H. westraliensis formed mycorrhizae are fast growing trees valuable as timber species. One of these, E. diversicolor or karri, was experimented with in our laboratory and proved to be an excellent model organism for growth chamber studies utilizing 24 X 3.5 cm glass synthesis tubes for growth and mycorrhizal synthesis observations. This tree grew quickly as a seedling in artificial media on peat and vermiculite and on perlite substrates. It has small leaves in the juvenile stage that conformed well to the limited area for growth that these tubes provide. Karri also exhibited a calcifuge

tendency in preliminary experiments, growing poorly on CaCO<sub>3</sub> amended media and showing severe symptoms of nutrient deficiencies that were not evident when CaCO<sub>3</sub> was not present. The speed with which E. diversicolor and H. westraliensis formed mycorrhizae under these conditions and the outward indications of a mycorrhizal amelioration of karri's intolerance to CaCO<sub>3</sub>, suggested that this was an excellent association with which to study responses to calcareous soils.

In summary, the planting of Eucalyptus trees offer an attractive solution to the afforestation problems of arid and semi-arid regions. Their adaptability to soil and moisture regimes, their rapid rate of growth and their economic value as timber species makes them ideally suited for this purpose. The limitations imposed on eucalypt growth by the common occurrence of calcareous soils in these environments may be ameliorated by the physiological actions of ectomycorrhizal fungi. In searching for appropriate mycorrhizal fungus associates with which to test this hypothesis one would hope to find a tolerance for CaCO<sub>3</sub> and the ability to form mycorrhizae quickly. In addition, with good colonization of host roots, to be able to alleviate the nutrient deficiencies and perhaps toxicities that limit eucalypt growth in these soils. As suggested by the previous work of other investigators, a primary means by which mycorrhizal fungi ameliorate nutrient deficiencies in calcareous soils is by the production of oxalic acid. It would be highly desirable to find a fungus symbiont that produces this acid efficiently. Care was taken in choosing the organisms for this study to test these phenomena. It is hypothesized that the oxalic acid produced by the mycorrhizal fungus Hebeloma westraliensis increases the tolerance of Eucalyptus diversicolor to calcareous soils. It does so by increasing P availability in these soils which allows increased P absorption, and by limiting the Ca in solution and the Ca absorption by these trees.

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## Chapter 2.

### **The growth and oxalate production of Hebeloma westraliensis in calcareous and noncalcareous liquid media with and without 1 mM L-threonine.**

#### **Abstract.**

The ectomycorrhizal fungus Hebeloma westraliensis was grown in a nitrate-modified liquid Hagem's medium supplemented with varying amounts of CaCO<sub>3</sub> and the presence or absence of 1 mM L-threonine. Treatments and controls included 0, 0.25 and 0.5 g CaCO<sub>3</sub> in 75 ml of liquid medium in 225 ml flasks. Each CaCO<sub>3</sub> level was duplicated with the addition of 1 mM L-threonine to the medium. These conditions were replicated 5 times and flasks were inoculated with 10 ml of mycelial homogenate of H. westraliensis. Inoculum was allowed to grow in the dark at room temperature for 50 days. At harvest total biomass was measured by dry weight and oxalate production was assayed by KMnO<sub>4</sub> titration. Growth of H. westraliensis and oxalate production were both enhanced by the addition of 0.25 g CaCO<sub>3</sub>. At the 0.5 g level growth was again increased while oxalate production leveled off. L-threonine had little effect on fungal growth but caused a significant reduction in oxalate production at the 0.25 g and 0.5 g levels of CaCO<sub>3</sub>. Oxalate produced per gram dry weight of mycelium decreased with increased CaCO<sub>3</sub>. L-threonine caused a further reduction in

this measure. Crystals were found growing on the hyphae of samples in the  $\text{CaCO}_3$  treatments and these were histochemically identified as calcium oxalate. The relationship of these results to ectomycorrhizal fungus nutrition in calcareous media is discussed as well as the possible significance to plant symbiont nutrition in calcareous soils.

## Introduction

Oxalic acid is a major component of the suite of organic acids produced by fungi (Cochrane, 1958). From as early as 1877 to 1907, the crystals of Ca oxalate had been described from cultures of Agaricus, Lactarius, Russula, Boletus and Polyporus in the Basidiomycetes (Hodgkinson, 1977). By the 1920s it had been determined that the largest producers of this acid in the fungi were the Deuteromycetes Penicillium and Aspergillus and certain plant pathogens in the genus Sclerotium (reviewed in Martin, 1960). These same fungal taxa are important model organisms for recent oxalic acid research. Of particular note is that several of the first Basidiomycete taxa identified as producing Ca oxalate crystals are mycorrhizal fungi and it is in this group that the most recent work on the ecology of oxalic acid production is focused (Allen, 1991).

The role of oxalic acid in fungal metabolism is apparently many-fold but as yet undefined. The crystals of Ca oxalate found early on have been the subject of recent efforts to determine the physiological and ecological significance of their localization and morphology. In a recent treatise on the formation of many types of calcium crystals, Kazmierczak et al. (1985) have proposed that biomineralization of  $\text{Ca}^{2+}$  is a cellular response evolved since organisms were faced with the gradual rise of  $\text{Ca}^{2+}$  concentrations in ancient oceans. A premise for this model is that  $\text{Ca}^{2+}$  is a potent toxin in cells when in excess and that  $\text{Ca}^{2+}$  concentrations are closely regulated in cell sap (Simkiss, 1977). The focus of much research on Ca oxalate crystals in plants, and lately with fungi, comes from a similar perspective. In their extensive review of Ca oxalate crystals in plants Francheschi and Horner (1980) outline many possible functions of these crystals. These include a method of storing  $\text{Ca}^{2+}$  for use early in the next growing season in perennial plants, an end product of oxalate and Ca metabolism in which both of these potentially toxic metabolites are rendered insoluble, a substance produced as a deterrent to herbivory and a product of maturation and senescence which can rupture cells of tissues that dehisce. All but the first

mentioned possible function have been attributed to Ca oxalate crystals in fungi. Except in the case of perennial polypores, few fungi produce perennial structures and these would likely not be stressed for calcium in any season since the substrates on which they grow are a ready source.

The role of Ca oxalate crystals in the dehiscence of fungal plant tissues was suggested by Whitney and Arnott (1986a). Crystals identified as such were found on the endoperidium of Geastrum saccatum an earth star (Gasteromycetes) and they attributed these as aiding in the dehiscence of the exoperidium. The crystals found on the endoperidium of G. minus were also attributed to exoperidium dehiscence (Horner et al., 1985). A role in tissue dehiscence in plants has been suggested often. Their presence in anther tissue of Capsicum annuum likely aids in pollen release (Horner and Wagner, 1992) and may also contribute to seed coat separation in Phaseolus vulgaris (Barnabas and Arnott, 1990) and in Simmondsia chinensis and Eucalyptus erythrocorys (Buttrose and Lott, 1977)

There have been numerous previous reports of Ca oxalate crystals in Geastrum. Krisai and Mrazek (1986) published an extensive list of Geastrum species that they identified as having Ca oxalate crystals. Their report, and most of those published earlier, attributed Ca oxalate as functioning in nutrient cycling of litter as these fungi are litter decomposers. This function has been focused on most recently by many authors studying many groups of fungi, though the role of crystal formation in dehiscence is not discounted.

Ca oxalate crystals may contribute to defense against herbivores in fungi similar to the reputed role of these crystals in some plants (Francheschi and Horner, 1980). Crystals found on the sporangiophores and zygothecia of Mucor mucedo, a Zygomycete (Urbanus and van den Ende, 1978), on cystidia in the hymenium of Inocybe spp. (Waterkeyn et al., 1991) and on the hyphae of aerial mycelium of Agaricus bisporus (Whitney and Arnott, 1987) were possibly functioning in this way. Whitney and Arnott also suggest that the

crystals may provide a hydrophobic coating to aerial mycelium that prevents hyphae from becoming sodden from high humidity.

Whitney and Arnott (1986, 1988) discuss the possible role of Ca oxalate crystals as a means by which fungi regulate cellular  $\text{Ca}^{2+}$ . They suggest that  $\text{Ca}^{2+}$  excess in the microenvironment may reduce the effectiveness of the fungal enzymes necessary for infectivity by Gilbertella persicaria, a soft rot of peaches. The excess  $\text{Ca}^{2+}$  would likely be released from degraded peach tissues and bound by oxalate into insoluble crystals. This function with certain variations seems to be at work in a number of plant pathogenic fungi. In other cases, pathogenic fungi release oxalic acid into host tissues. This has been demonstrated with Sclerotium cepivorum (Bateman and Beer, 1965), S. rolfsii (Punja and Jenkins, 1984a, 1984b; Stone and Armentrout, 1985), Sclerotinia sclerotiorum (Godoy et al., 1990), Leucostoma cincta and L. persoonii (Traquair, 1987) and Fomes annosus (Hutterman et al., 1980). The common thread throughout much of this work is that oxalic acid produced by these fungi is exuded into host tissue. There it may aid in the general dissociated acid attack on host tissues and specifically bind with  $\text{Ca}^{2+}$  in pectic substances in walls and middle lamella of host cells. Cell wall and membrane integrity are therefore disrupted facilitating growth of infective hyphae.

Godoy et al. (1990) demonstrated the necessity of oxalic acid production to pathogenicity in Sclerotinia sclerotiorum with the use of oxalic acid minus mutants. Kritzman et al. (1977) working on Sclerotium rolfsii and Hutterman et al. (1980) with Fomes annosus found that the addition of 1 mM L-threonine to culture media of these fungi significantly reduced their production of oxalic acid and effectively eliminated their infectivity in host tissue. L-threonine is an apparent inhibitor of glyoxalate dehydrogenase, an enzyme that converts glyoxalate to oxalate. It was suggested that this pathway was the major source of oxalic acid in Basidiomycetes. Alternate pathways apparently exist in Aspergillus niger (Hayaishi et al., 1956) in which oxalate can be converted directly from oxaloacetate. Lapeyrie (1988) suggested recently that the mycorrhizal fungus Paxillus

involutus may synthesize oxalic acid from soil bicarbonate. The importance of this work as it relates to this study will be discussed.

A role of major ecological significance of fungal oxalic acid is its importance in soil nutrient cycling. Of the low molecular weight organic acids assayed in several southeastern USA soils, oxalic acid had the highest concentration (25 to 1000  $\mu\text{M}$ ) and was present in all samples (Fox and Comerford, 1990). They did not determine what portion of these acids was produced by fungi. Graustein et al. (1977) observed Ca oxalate crystals in the litter layers of several different soils. They discussed the implications for oxalic acid on Fe and Al solubility and the importance of these crystals in binding  $\text{Ca}^{2+}$  in the vicinity of plant roots. They suggested that, as plants have a high demand for  $\text{Ca}^{2+}$ , this would allow plant roots easier access to this element. This could take place if  $\text{Ca}^{2+}$  activity declined in soil solution to levels undersaturated with respect to Ca oxalate. They also made note of chemical equilibrium models of oxalate and some phases of soil Al, Fe and Ca and suggested that oxalic acid in soils would increase the solubility of phosphates. This model was supported by Fox and Comerford (1992). Mats of soil fungi were also found at these sites and mention was made of these as likely producers of much of the oxalic acid.

Cromack et al. (1979) also studied the Ca oxalate crystals of fungal mats in forest soils and identified the fungus as the ectomycorrhizal symbiont Hysterangium crassum. It was assumed that most of the oxalate found there was produced by this fungus. In these soils, more Ca remained in the upper horizons in mats compared to uncolonized soils nearby. Observations with SEM revealed extensive weathering of the soil particles near fungal mats. They also made mention of the abilities of oxalate to increase the solution concentrations of P, though no other nutrients besides Ca were measured. Entry et al. (1992) also studied H. crassum in a more in-depth look at nutrient turnover. They measured the macro- and micronutrient concentrations in mats + residual soil, residual soil with hyphae removed and in non-colonized soil nearby. They found that the concentrations of all elements in mats were much higher but the largest difference was in

P. The concentration of  $\text{Ca}^{2+}$  in mat soil with hyphae removed was significantly lower than in uncolonized soil. They explained this as an instance of fungal sequestering of Ca in the mats, depleting the soil of this nutrient.

The soils in karri (Eucalyptus diversicolor) forests in Australia have also been found to have high levels of Ca oxalate (O'Connell et al., 1983). Much of this was in the form of whewellite ( $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ ) and was apparently formed within karri leaf tissue prior to senescence. This monohydrate form has been associated previously with plant tissues by Francheschi and Horner (1980). Additional amounts of weddelite ( $\text{CaC}_2\text{CO}_4 \cdot 2\text{H}_2\text{O}$ ) were found in fine decomposing litter. This was suggested as having formed from the oxalate metabolism of soil microorganisms and subsequent chelation with soil Ca. The dihydrate form of Ca oxalate has been associated with fungal metabolism in numerous reports (Whitney and Arnott, 1986a, 1986b, 1987).

High amounts of Ca were found in E. diversicolor forest soils in comparison to other eucalypt forests in Australia (O'Connell et al., 1982). Carbonates were not in abundance and these soils would not have been classified as calcareous. The high levels of Ca were proposed to have been released in regenerative burning that is common practice in these forests. The leaf litter of many understory species is high in Ca in these systems (Ashton, 1975) and E. diversicolor leaves, especially in the lower canopy, contain substantial Ca (Hingston et al., 1979). Regenerative burning would have allowed the release of this Ca from the leaf litter pool most effectively.

The total oxalates of karri forest were also high in comparison to other eucalypt forests and it was suggested that oxalate production by E. diversicolor and by soil microorganisms was increased due to high soil Ca (O'Connell et al., 1983). The effect of solution Ca on oxalate production in fungi has been supported by Lapeyrie and Bruchet (1986), and in plants by Francheschi and Horner (1980). In spite of the Ca present in natural karri forest soils, this tree is still considered a calcifuge species and will not tolerate calcareous conditions without a symbiotic fungus associate (Lapeyrie et al., 1990).

Extensive work has been done on the oxalate effects in calcareous soils in the western USA. In a series of articles from Utah State University, Jurinak and his colleagues described several studies aimed at the mechanisms of P availability in the soils of VAM prairie grasses. Jurinak et al. (1986) developed a model to describe the thermodynamics of oxalate effects on solution P. Experimental testing of the model on VAM Pascopyrum smitthii mycorrhizae, hyphae and soil revealed that oxalate produced by the fungus acted as an efficient scavenger of  $\text{Ca}^{2+}$  in these soils, allowing greater solubilization of orthophosphate from Ca apatite.

The work by Knight et al. (1989, 1992) explored both the oxalate effects and the respiration  $\text{CO}_2$  effects on solution P and VA mycorrhizal plant tissue P in calcareous soils. Their results were mixed and did not show consistent effects of VAM inoculation on either oxalate production or plant tissue P concentration. The amount of P in solution could be predicted based on either oxalate in the system or the partial pressure of  $\text{CO}_2$ , though these effects could not be attributed to the presence or absence of the fungus. In their studies, as in many others, the increased root biomass of inoculated plants complicated efforts to separate effects of fungus biomass increase from the effects of increased plant biomass.

The work with ectomycorrhizal fungi has yielded promising results in the area of oxalate metabolism in fungi. In their work with Paxillus involutus Lapeyrie and his colleagues (1990, 1988, 1987) measured the oxalate production of this fungus under various cultural conditions. They determined that oxalate production increased considerably when the culture medium contained nitrate N, high levels of  $\text{HCO}_3^-$  and high levels of  $\text{Ca}^{2+}$ . All of these ions are common components of a calcareous soil system. Lapeyrie also suggested that soil  $\text{HCO}_3^-$  can act as a carbon source for oxalate production in P. involutus (Lapeyrie, 1988) and this may partially explain the increased production of oxalic acid in calcareous soils. The effect of soil or medium pH on oxalic acid production is also at work in these systems. High pH was recognized as early as 1948 as a primary factor in fungal oxalate production by Cochrane (1958). Shimazano (1955) theorized that

the enzyme oxalate decarboxylase, which converts oxalate to formate and CO<sub>2</sub>, is activated at low pH. The effects of pH alone in recent studies have been difficult to separate from other ion effects as the levels of one effect the others.

There have been several reports of oxalic acid production by ectomycorrhizal fungi (Lapeyrie, 1987, 1988) and of Ca oxalate deposition in or on mycorrhizal sheaths and extra-matrical hyphae (Cromack et al, 1977; Froidevaux and Kalin, 1981; Malajczuk and Cromack, 1982; Lapeyrie et al, 1983, 1990; Snetselaar and Whitney, 1990). Only one of these studies dealt with the genus Hebeloma. Froidevaux and Kalin (1981) observed Ca oxalate crystals on the hyphae of Hebeloma crustuliniforme in association with Fagus sylvatica. This study focused on the description of the crystals and did not investigate total oxalates present in soil nor analyze soil or plant associate nutrient status. Hebeloma crustuliniforme has been reported to prefer high pH conditions for growth and this is unusual amongst ectomycorrhizal fungi (Hung and Trappe, 1983). These are important ectomycorrhizal fungi that hold promise as inoculum in forest plantation plantings. More work needs to be done in this genus on intra- and interspecific reactions to calcareous media. The work of Lapeyrie and Bruchet (1986) on calcicole and calcifuge ecotypes of Paxillus involutus is an example of this type of study.

Reports of ectomycorrhizal enhancement of host plant nutrition in calcareous soils were presented for Pinus nigra ssp. nigricans (Clement et al., 1977) and for Eucalyptus dumosa (Lapeyrie and Chilvers, 1985). These articles suggest that oxalic acid production and Ca oxalate crystal formation are important events in host plant nutrition in calcareous soil but these phenomena were not directly observed in these studies. Their conclusions were based on previous reports of related fungi and plant associates and the soil equilibrium models developed in association with VAM studies that helped to explain the mechanisms at work (Jurinak et al., 1986).

There have so far been no studies to look at oxalic acid production by ectomycorrhizal fungi in synthesis with a host plant. Neither are there studies that

measure ectomycorrhizal host plant nutritional changes and oxalic acid production at the same time. As part of a larger effort to address some of these shortcomings, this experiment will study the production of oxalic acid in calcareous and non-calcareous media by the ectomycorrhizal fungus Hebeloma westraliensis. This is a recently described fungus associate of several Eucalyptus spp. in Australia (Bougher et al., 1991) that may hold promise as plantation planting inoculum, especially in calcareous or other high pH soils. In addition, the effect of 1 mM L-threonine, reported as an effective metabolic block of fungal oxalic acid production (Huttermann et al., 1980; Kritzman et al., 1977) is evaluated for H. westraliensis. The process by which oxalic acid production can be blocked without significantly affecting fungus growth could be an important tool in assessing the effect of fungal oxalic acid in natural systems. L-threonine has been shown to significantly reduce oxalic acid production by disrupting the activity of glyoxalate dehydrogenase. This enzyme catalyzes the formation of oxalate from glyoxalate. The effect of this amino acid on oxalate biosynthesis has not been determined in any mycorrhizal fungi.

## Methods and Materials

A nitrate modified Hagem's medium was used in this study consisting of the following nutrients per liter: 4 g malt extract, 1 g yeast extract, 5 g D-glucose, 0.25 g CaCl<sub>2</sub>, 0.05 g KH<sub>2</sub>PO<sub>4</sub>, 1.42 g KNO<sub>3</sub>, 0.5 g MgSO<sub>4</sub>\*H<sub>2</sub>O, 0.5 ml FeCl<sub>3</sub> (1% aqueous solution), 100 µl biotin (50 µg/ml solution), 100 µl Thiamine (1 mg/ml aqueous solution), and ddH<sub>2</sub>O balance. A total of 75 ml of this medium (Appendix A) was added to each of 60, 250 ml Erlenmeyer flasks. Additional phosphorus in the form of CaH<sub>4</sub>(PO<sub>4</sub>)<sub>2</sub>\*H<sub>2</sub>O was supplied by dissolving 1.5 g in 50 ml ddH<sub>2</sub>O and pipetting 1 ml of this solution into each flask resulting in 7.38 mg P in this form in each flask. Total P in each flask was 8.41 mg. Twenty flasks were supplied with 0.25 g of CaCO<sub>3</sub> and 20 others with 0.5 g to comprise the calcareous treatments. Twenty flasks were without CaCO<sub>3</sub> as controls. L-threonine was added to 10 flasks of each treatment by dissolving 506 mg L-threonine in 50 ml ddH<sub>2</sub>O and pipetting 1 ml into each flask. This resulted in an initial concentration of 1 mM L-threonine.

All flasks were autoclaved and placed in the dark at room temperature (ca. 22C) for four weeks to equilibrate and to allow any contamination to become evident. pH was measured of representative media from each of these treatments before and after equilibration. After four weeks observations were made for contamination and excessive evaporation of medium from the flasks and 5 flasks of each level of CaCO<sub>3</sub> and L-threonine treatment were chosen for the experiment. Two flasks of each treatment were reserved for inoculation that would not be included in growth and oxalate production analysis, but would be used for periodic microscopic checks for presence of calcium oxalate crystals.

Inoculum was taken from a pure culture of Hebeloma westraliensis (VT 1970) stored in the Virginia Tech Culture Collection and transferred to petri plates containing

nitrate modified Hagem's agar medium. Plates were incubated at 24 C for 10 days after which mycelium had grown to cover about 2/3 of the agar surface. Mycelium was then macerated with a scalpel and axenically transferred to 1 liter of nitrate-modified liquid Hagem's medium in a 2500 ml Erlenmeyer flask fitted with a sterilized stopper apparatus for bubble culture (Plate 1, p. 42). Mycelium was allowed to grow in bubble culture for 2 weeks at which time pieces of macerated mycelium had more than doubled in size.

Actively growing mycelium in liquid medium (which had evaporated to considerably less than 1 liter by this time) was transferred to an autoclaved 1 liter Waring blender canister and blended for 8 seconds at speed 5 to produce a homogenous liquid inoculum. Subsamples of this mycelial homogenate were plated on sterile Hagem's medium and incubated to establish continued purity of culture. Ten ml of mycelial homogenate was pipetted into each flask using sterile technique with an autoclaved pipette, the tip of which had been bored and sanded to accept the viscous homogenate. All flasks were placed in the dark at room temperature (21 - 23 C) to incubate for 50 days (Plate 2, p. 42). The flasks were arranged in a random array and shaken by hand once every day to ensure even distribution of nutrients and adequate aeration.

Small samples of mycelium and media were removed periodically using sterile technique from flasks not included in growth and oxalate analysis. These samples were placed on slides and examined microscopically for crystal formation. They were then allowed to dry to fix them somewhat to the slides (heat fixation distorted the mycelium excessively and did not enhance fixation). These samples were prepared by the method of Yasue (1969) for differential staining and histochemical recognition of calcium oxalate crystals. Slides were immersed in 5% acetic acid for 30 minutes to dissolve calcium salts other than calcium oxalate and gently rinsed in ddH<sub>2</sub>O. Slides were immersed in 5% silver nitrate for 15 minutes and again rinsed thoroughly but gently in ddH<sub>2</sub>O. Samples were then stained with ammoniacal dithiooximide (rubeanic acid) in 70% ethanol for 1 minute

and observed. Using this method, calcium oxalate crystals would stain brown to black and localization would be maintained.

Several crystals from these fixed samples were viewed with a Scanning Electron Microscope for photography and microanalysis by Electron-probe. X-ray Diffraction Spectrophotometry was also attempted on these crystals for comparison identification with results of histochemical method.

Standards for oxalate titration were prepared by duplicating the 0 g and 0.5 g  $\text{CaCO}_3$  conditions in liquid medium as were imposed in the experiment. Oxalate was provided in known quantities as 10%  $(\text{COOH})_2 \cdot 2\text{H}_2\text{O}$  and as  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ . The 10% oxalic acid dihydrate was pipetted directly into autoclaved media using sterile microliter pipette tips. Calcium oxalate hydrate was supplied by dissolving 0.25 g  $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$  in 50 ml of 1N HCl and asexenically pipetting this solution into autoclaved media. These flasks were left uncovered under UV light for 24 hours to reduce contamination. The oxalate concentrations used in standards were: 2, 10, 15, 20, 30, 50, 75, 100 and 150  $\mu\text{g ml}^{-1}$ . These were formulated to facilitate titration with  $\text{KMnO}_4$  delivered in 20  $\mu\text{l}$  increments. All flasks of standard solutions were incubated with the experimental flasks to equilibrate and to maintain uniformity of conditions.

After 4 weeks of incubation, oxalate standard solutions were removed for extraction and titration. Two subsamples of 5 ml were withdrawn from each flask and extracted according to the method of Bateman and Beer (1965) for the quantitation of oxalate in media. In order to increase the sensitivity of titration, 0.01N (0.002M)  $\text{KMnO}_4$  was used as the reactant.

Extraction and titration procedure is as follows (reagent preparations are included in Appendix, p.108): 5 ml subsamples of standard solutions were pipetted into 20 ml plastic centrifuge tubes. 5 ml of  $\text{CaCl}_2$  acetate reagent was added to each. Tubes were shaken and refrigerated overnight (ca. 17 hours) at 5C. Tubes were then centrifuged for 15 minutes at 5000 rpm and the supernatant discarded. Sediments were washed with 5 ml

acetic acid/calcium oxalate wash. Tube contents were thoroughly mixed, centrifuged again and the supernatant discarded. Precipitates were dissolved in 5 ml of 4N H<sub>2</sub>SO<sub>4</sub>, transferred to glass test tubes and heated in a water bath at 90C for 5 minutes before titration. Standards were titrated while hot with 0.01N KMnO<sub>4</sub> using a microliter pipette to deliver 20 µl KMnO<sub>4</sub> at a time. Samples were titrated to an end point of faint pink coloration that remained after mixing for 1 minute. Volume of KMnO<sub>4</sub> delivered was recorded in µl and oxalate present was computed as 1 µl, 0.01N KMnO<sub>4</sub> = 0.45 µg oxalate. From these data a standard curve for oxalate titration was obtained to use in comparing accuracy of experimental observations.

At 50 days flasks of actively growing mycelium of Hebeloma westraliensis were harvested. By this time mycelium of the most vigorous growth had spread through more than 1/2 of the liquid media volume. Each flask was shaken by hand and poured through a nylon stocking filter and the solution funneled to a clean 125 ml Erlenmeyer flask. Each sample of mycelium retained was rinsed briefly in running distilled water and gently drained of excess water. The samples were placed in pre-weighed aluminum weighing pans and oven-dried at 60C for 24 hours.

Samples of remaining media solutions were hand shaken and 5 ml subsamples were removed for oxalate extraction and titration as per Bateman and Beer (1965), described above. Samples of oven dried mycelium were weighed, pulverized with mortar and pestle and extracted for oxalate titration by the Cromack et al. (1979) modification of Baker's (1952) procedure for quantitation of oxalates from mycelium. Pulverized mycelial samples were placed in 80 ml beakers with 25 ml 1M HCl and boiled for 15 minutes. Each extract was filtered through Whatman #1 paper into a 50 ml screw-cap centrifuge tube and allowed to cool at room temperature overnight. After ca. 16 hours, 5 ml phosphoric tungstate reagent was added to each tube, shaken and allowed to sit for 5 hours. These were then centrifuged at 5000 rpm for 15 minutes and 20 ml of the clear supernatant was transferred to clean tubes. NH<sub>4</sub>OH was added dropwise until alkaline as determined by

pH meter and the formation of a persistent white precipitate of phosphotungstate. 5 ml of  $\text{CaCl}_2$  acetate reagent was added and tubes were shaken vigorously and allowed to sit overnight refrigerated at 5C. The remainder of the extraction and titration procedure followed that described for oxalate extraction from media samples and oxalate present was similarly determined. Dry weights of mycelial samples, total oxalate produced by each sample and the concentration of oxalate produced per gram dry weight was recorded.

All data on gram dry weights of mycelium samples, amount of oxalate produced per sample and oxalate produced per gram dry weight were screened for normality using the D'Agostino-Pearson Omnibus  $K^2$  Normality Test (D'Agostino, 1990). Homogeneity of variance across treatment groups was tested by equality of covariance and the Bartlett-Box Homogeneity Test (Bartlett, 1950). Differences between treatment means were analyzed using Fisher's Least Significance Difference Test (Ott, 1977). A Multiple Analysis of Variance (MANOVA) (Winer, 1971) was used to determine the relationships of mycelial dry weight, amount of oxalate produced and oxalate produced per gram dry weight to level of  $\text{CaCO}_3$  and the presence or absence of L-threonine. Significance levels were determined at  $\alpha = 0.05$ . These tests were performed with the aid of Number Cruncher Statistical System (NCSS) (Hintze, 1992).

## Results

Microscopic examination of mycelium and media samples revealed numerous polyhedral and amorphous crystals. Dissolution with effervescence in 5% acetic acid suggested that many of the polyhedral crystals were  $\text{CaCO}_3$ . Amorphous crystals remained on the mycelium after this treatment and the Yasue method of histochemical recognition of calcium oxalate (Yasue, 1969) confirmed that these were of that form. No calcium oxalate crystals were found free in the media. Plate 3 (p. 42) is a micrograph of Ca oxalate crystals stained by this method attached to clamped hyphae of H. westraliensis.

Plate 4 (p. 42) is a Scanning Electron Micrograph of one of these crystals attached to a hyphal fragment of H. westraliensis. Electron-probe microanalysis identified this crystal as calcium-rich. Analysis by X-ray Diffraction Spectrophotometry for more specific chemical identification was inconclusive.

For all data, normal distributions could not be rejected based on the D'Agostino-Pearson Omnibus  $K^2$  Normality Test (D'Agostino, 1992). Test of homogeneity of variance between treatment groups revealed no departures from homoscedacity. Therefore, parametric statistical tests for differences in treatment means and MANOVA analysis of response variable relationships were considered valid. Within-cell correlations analysis revealed no problems with covariance or collinearity of response variables. For this reason, the two-way Analysis of Variance (ANOVA) reports within the MANOVA analysis are effective determinates of significance of these data.

Data on pH of the medium formulations used in this study before inoculation and after harvest are presented in Figure 1, p. 41. The pH of media before inoculation was increased by the addition of  $\text{CaCO}_3$ . These solution pH values were not affected by the

presence of L-threonine. After 50 days growth of the fungus pH of all treatments increased. The presence of L-threonine allowed slightly less pH increase during growth.

Data on the accumulated biomass of *H. westraliensis* in all treatments, including the results of Fisher's Least Significant Difference Test for differences between the means, are summarized in Figure 2, p. 41. Growth of *H. westraliensis* was enhanced by the addition of CaCO<sub>3</sub> to the media. In the control group with 0 g added CaCO<sub>3</sub>, the mean gram dry weight of 10 replicates (including the L-threonine treated group) was 0.212 g (SE = 0.00157). The addition of 0.25 g CaCO<sub>3</sub> per flask increased dry weight over controls (mean = 0.409 g). At 0.5 g CaCO<sub>3</sub> per flask dry weight was again increased (mean = 0.512 g). The addition of L-threonine had no significant effect on growth of the fungus during incubation and only a slight effect on dry weight at harvest when all levels of CaCO<sub>3</sub> were taken together. When no L-threonine was added to the growth medium, the mean dry weight across all CaCO<sub>3</sub> levels was 0.394 g (n = 15, SE = 0.013). The mean dry weight across all CaCO<sub>3</sub> levels was 0.361 g with the addition of L-threonine.

The relationship between level of CaCO<sub>3</sub>, the presence or absence of L-threonine and the gram dry weight of mycelium samples was analyzed by two-way ANOVA within the MANOVA analysis with a balanced complete factorial design and 5 observations per treatment (Winer, 1971). There were no significant interactions between level of CaCO<sub>3</sub> and addition of L-threonine on dry weight (P = 0.544). The main effects of level of CaCO<sub>3</sub> were highly significant (P < 0.001) and explained 87% of the variation in dry weight. The main effects of presence or absence of L-threonine were not significant (P = 0.0776) and explained a little over 1.5% of the variation in dry weight. Slightly over 11% of the variation in dry weight was not explained by the model.

The results of oxalate standard extraction and titration revealed that for concentrations of oxalate above 10 µg ml<sup>-1</sup> recovery of spiked samples averaged 85 - 90% with little variation. A standard curve with percent of titratable oxalate recovered from samples of known oxalate concentration is represented in Figure 17, p. 107. Quantities of

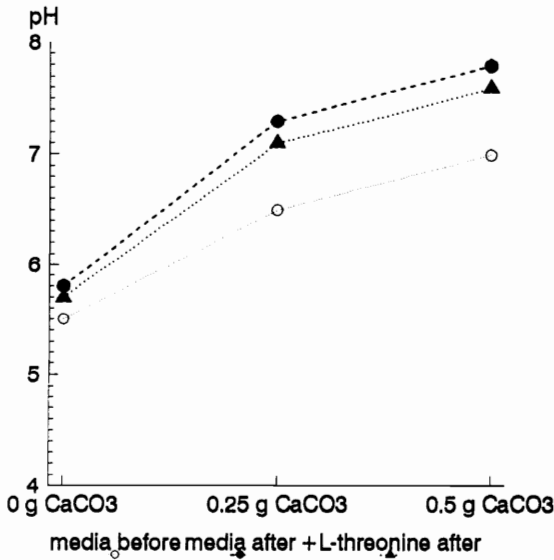
oxalate determined in this study are not to be taken as absolute values but can be used as relative concentrations. Extraction and titration of water soluble oxalate from media samples were below the limits of confident assay by this method.

The amount of oxalate produced by this fungus was highly dependent on both the level of  $\text{CaCO}_3$  and the presence or absence of L-threonine. Oxalate was produced in higher quantities with the addition of  $\text{CaCO}_3$  to the growth medium. When L-threonine was added oxalate production was reduced across all levels of  $\text{CaCO}_3$ . The means and standard deviations for oxalate production across all treatments, including the results of Fisher's LSD test for differences between the means are summarized in Figure 3.

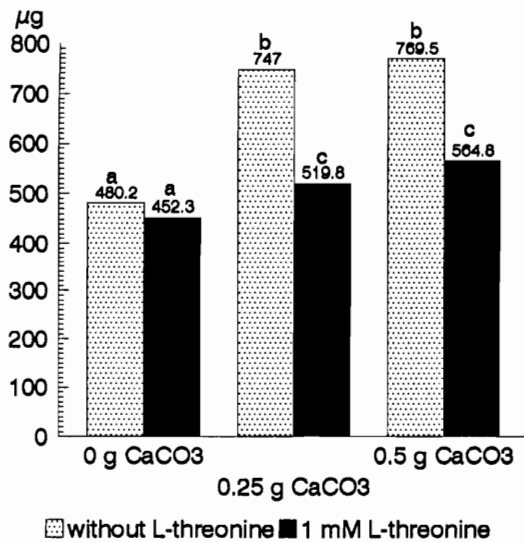
MANOVA analysis of the relationship between level of  $\text{CaCO}_3$ , presence or absence of L-threonine and oxalate produced by *H. westraliensis* revealed highly significant interactions between level of  $\text{CaCO}_3$  and the presence or absence of L-threonine ( $P < 0.001$ ). These interactions explained 12% of the total variation in amount of oxalate produced. The main effects of level of  $\text{CaCO}_3$  were also highly significant ( $P < 0.001$ ) and explained 45% of the total variation. The effect of presence or absence of L-threonine was highly significant ( $P < 0.001$ ) and explained 34% of the variation in oxalate produced by this fungus. About 9% of the variation in the amount of oxalate produced was not explained by this model.

The amount of oxalate produced per gram dry weight of mycelium decreased with added  $\text{CaCO}_3$  to the growth medium. The presence of L-threonine in the medium also decreased this ratio over and above that attributed to increased level of  $\text{CaCO}_3$  (Figure 4). MANOVA tests of the relationship between level of  $\text{CaCO}_3$ , L-threonine and amount of oxalate produced per gram dry weight of mycelium revealed no significant interactions between level of  $\text{CaCO}_3$  and presence or absence of L-threonine ( $P = 0.3676$ ). This relationship explained only 1.5% of the total variation of this ratio. The main effects of  $\text{CaCO}_3$  level in the growth medium were highly significant ( $P < 0.001$ ) and explained 74% of the total variation. The main effects of presence or absence of L-threonine were

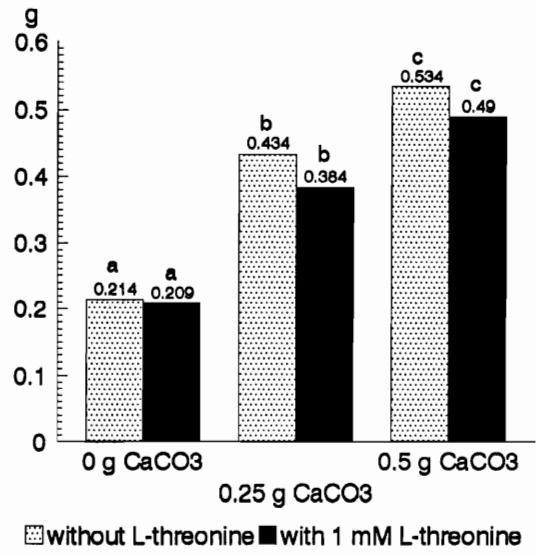
significant ( $P = 0.0047$ ) and this explained 7% of the total variation in oxalate per gram dry weight.



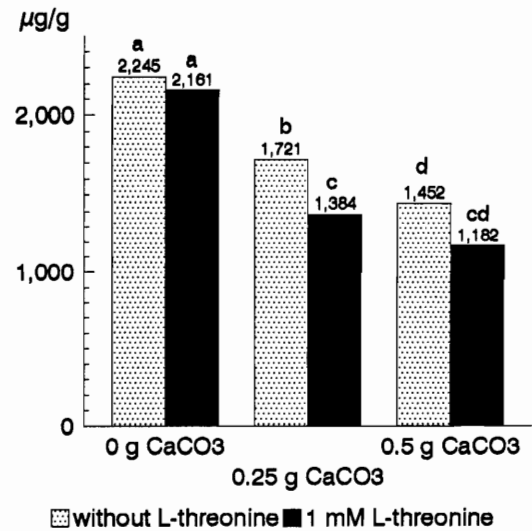
**Figure 1.**  
pH of liquid media before inoculation and after harvest of *H. westraliensis* grown in the presence or absence of L-threonine across 3 CaCO<sub>3</sub> levels.



**Figure 3.**  
Mean oxalate produced per replicate sample of *H. westraliensis* grown with 3 levels of CaCO<sub>3</sub> and with or without L-threonine. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



**Figure 2.**  
Mean dry weight of *Hebeloma westraliensis* grown in liquid media with and without CaCO<sub>3</sub> and with and without 1 mM L-threonine. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



**Figure 4.**  
Mean oxalate produced per gram dry weight of *H. westraliensis* mycelium grown with 3 levels of CaCO<sub>3</sub> and with or without L-threonine. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.

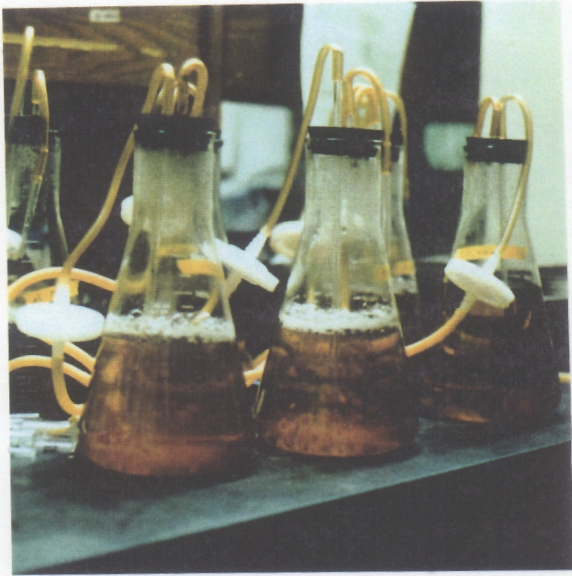


Plate 1.  
Bubble culture apparatus for bulking mycelium. (Photo by Jack Murphy).



Plate 2.  
Incubation of treatment flasks of H. westraliensis.

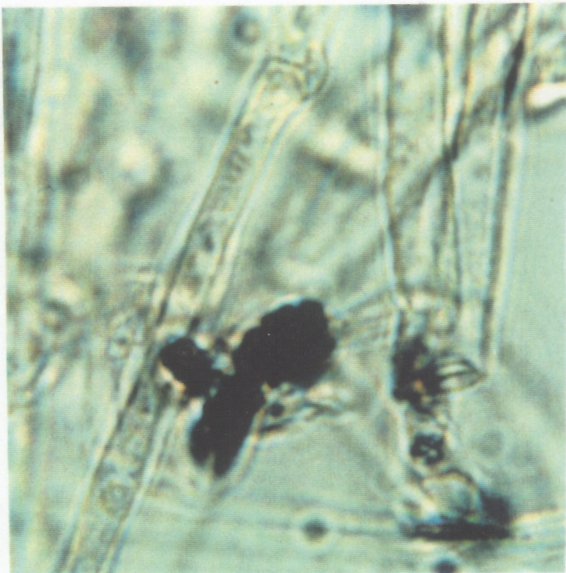


Plate 3.  
Ca oxalate crystals, stained dark brown, on the surface of H. westraliensis hyphae.

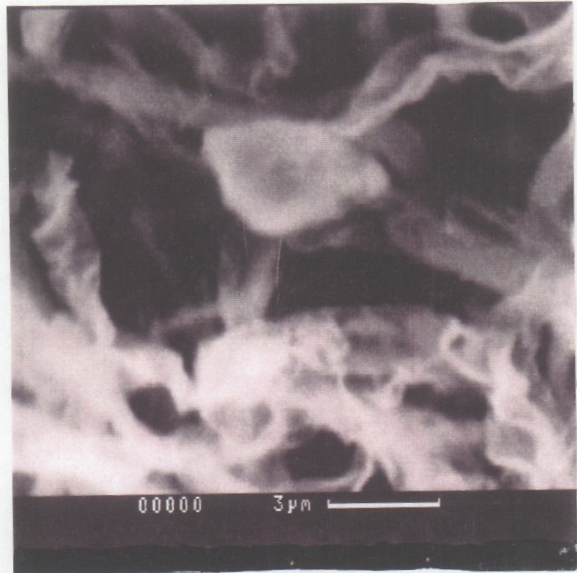


Plate 4.  
SEM of crystal on H. westraliensis hypha. Electron-probe microanalysis indicated this crystal was Ca-rich.

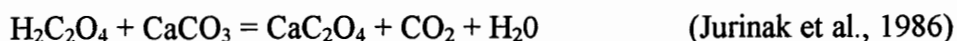
## Discussion

The growth and oxalate production of Hebeloma westraliensis in nitrate-modified liquid Hagem's medium were enhanced by the addition of CaCO<sub>3</sub>. The addition of L-threonine had little effect on growth but a substantial effect on oxalate production. The responses of the fungus in this study are affected not only by the independent variables in the model but also by the concomitant effects of these variables on system chemistry.

The addition of CaCO<sub>3</sub> to the medium increases pH and introduces a powerful alkaline buffering capacity to the system. In approaching equilibrium before inoculation CaCO<sub>3</sub> dissociates to some degree, releasing Ca<sup>2+</sup> and carbonate. Carbonate quickly bonds to H<sup>+</sup> to produce HCO<sub>3</sub><sup>-</sup>. The HCO<sub>3</sub><sup>-</sup> ion imparts particularly strong influence on media pH by acting as a proton scavenger (Bohn et al., 1985).

Several mechanisms are likely at work in this system to raise pH during growth of H. westraliensis. The combination of nitrate based media, CaCO<sub>3</sub> treatments and the chemical action of fungus exudates make determinations of cause and effect difficult. Nitrate metabolism of fungi causes an increase in substrate pH (Cochrane, 1956). This holds true for ectomycorrhizal fungi as well (Harley and Smith, 1983). Protons are consumed in the process of nitrate reduction to nitrite and ammonium and the concentration of H<sup>+</sup> in solution decreases.

Given the N and CaCO<sub>3</sub> conditions imposed in this study, the predominant organic acid produced by the fungus is likely to be oxalic acid (Cochrane, 1956). Oxalic acid has unique complexing abilities with divalent cations. As the oxalate produced in this system chelates Ca<sup>2+</sup>, the equilibrium reaction of CaCO<sub>3</sub> is pushed toward dissolution and CO<sub>2</sub> is released.



The action of the product CO<sub>2</sub> and of respiration CO<sub>2</sub> also has an effect on system chemistry. In the presence of CaCO<sub>3</sub> the accumulation of CO<sub>2</sub> in the media will promote CaCO<sub>3</sub> dissolution and the release of HCO<sub>3</sub><sup>-</sup>.



As HCO<sub>3</sub><sup>-</sup> is a principal H<sup>+</sup> scavenger in this system, oxalate and CO<sub>2</sub> contribute to pH increase rather than acidification. This is likely given that water and CaCO<sub>3</sub> are in adequate supply as reactants. These reactions may be slowed somewhat by the presence of free Ca<sup>2+</sup> in solution. Respiration CO<sub>2</sub> was not measured in this study so its contribution to the chemistry of this system is unknown. The diffusion of CO<sub>2</sub> to the atmosphere in the flasks was not insured though flasks were swirled daily during growth.

The alkaline buffering capacity of CaCO<sub>3</sub> would likely allow pH to increase to about pH 8.3 (Bohn et al., 1985). This pH was approached but not reached in the 50 days of fungal growth in this experiment. Release of oxalate or other organic acids may have been a factor. It is not known if solution pH may have approached pH 8.3 if this system was equilibrated longer.

Hebeloma is among few ectomycorrhizal genera to be reported as having enhanced growth at alkaline pH. H. crustuliniforme was shown by Hung and Trappe (1983) to exhibit enhanced growth at higher pH. A similar case was observed in this study in which H. westraliensis had not shown a maximum growth at the highest levels of CaCO<sub>3</sub> imposed. The genera of ectomycorrhizal fungi that can tolerate the high pH, nitrate and bicarbonate of calcareous soils are not known. A calcicole ecotype of Paxillus involutus showed the ability to tolerate components of these soils (Lapeyrie et al., 1987). Fungi with this ability are expected be few, however. Optimum growth of most ectomycorrhizal fungi *in vitro* is achieved at considerably lower pH. Many Basidiomycetes are also unable to utilize nitrate as the sole source of N (Cochrane, 1956). This holds true for many ectomycorrhizal fungi as well (Harley and Smith, 1983). The fungal taxa that show

tolerance to nitrate N and high pH prominent in calcareous mineral soils will prove invaluable as inoculum in these regions.

As far as is known, Hebeloma westraliensis is an Australian endemic. This fungus was first isolated from litter beneath Eucalyptus spp. and forbs described as sand plain species (Bougher et al., 1992). Soil characteristics of the collection location including nutrient and other chemical analyses were not stated. The isolate used in this study was collected beneath Eucalyptus diversicolor (OKM 24003). Soil characteristics of this collection location were also not known. Soils with pH as high as those imposed in this study are not common in Australia (Pryor, 1976). The high optimum pH for growth of this fungus is as yet unexplained by native habitat conditions. In preliminary studies H. westraliensis grew well using ammonium-based medium at lower pH with no added CaCO<sub>3</sub>. The calcareous media is certainly not required by this fungus for growth. In fact, the wide range of pH and nutrient balance conditions that this fungus tolerates is additional cause for optimism in its utility as plantation inoculum.

The oxalate extracted and titrated from the media solutions was negligible and below the limits of confident quantitation by the KMnO<sub>4</sub> method. The presence of Ca in the medium would likely have chelated with oxalate produced by the fungus on hyphal strands. This would have allowed little soluble oxalate to remain in solution. This was shown to be the case with P. involutus (Lapeyrie et al., 1987) in which a low concentration of Ca<sup>2+</sup> (10 meq L<sup>-1</sup>) drastically reduced free oxalates in the medium and also increased total oxalate and oxalates found in the mycelium. The 4.5 meq L<sup>-1</sup> of Ca<sup>2+</sup> provided from CaCl<sub>2</sub> in this study may have been sufficient to achieve similar results. Certainly in the calcareous treatments Ca<sup>2+</sup> would have been plentiful.

The oxalate associated with the mycelium of H. westraliensis increased with increasing CaCO<sub>3</sub> in the medium. Cochrane (1956) noted that oxalic acid production increased as a result of increased pH. Increased HCO<sub>3</sub><sup>-</sup> in the calcareous system may also initiate an increase in oxalate production (Lapeyrie et al., 1987). Lapeyrie (1986) found

that *P. involutus* could use  $\text{HCO}_3^-$  as a carbon source for oxalic acid production. He traced labeled  $^{14}\text{C}$  from  $\text{NaH}^{14}\text{CO}_3$  to oxalates that the fungus produced. It is not known to what extent  $\text{HCO}_3^-$  may be utilized in this regard but apparently the fungus could metabolize this simple carbon source in some way. This has been supported in other mycorrhizal fungi (France and Reid, 1983). It was suggested by Lapeyrie (1986) that bicarbonate may enter the oxalate conversion pathway prior to oxaloacetate formation. From there it could be incorporated into the precursors of oxalate formation through either of the two oxalate biosynthetic pathways known from fungi (Lapeyrie, 1986). This was conjecture based on known oxalate biochemistry.

The effects of  $\text{CaCO}_3$  as an independent variable in this study can not be delimited by the individual effects of  $\text{Ca}^{2+}$  or  $\text{HCO}_3^-$ . Both are likely at work in the calcareous formulations used. Nitrate N is also known to allow increased oxalate production both in plants (Dijkshoorn, 1962; Raven and Smith, 1976) and in fungi (Punja and Jenkins, 1984b; Lapeyrie, 1987).

The addition of 1 mM L-threonine significantly reduced oxalate production when  $\text{CaCO}_3$  was present. The inhibitory effect of L-threonine on oxalate production has been previously shown with *Sclerotium rolfsii* in the Deuteromycetes (Kritzman et al., 1977) and with *Fomes annosus* in the Basidiomycetes (Huttermann, 1980), but has not been previously demonstrated with ectomycorrhizal fungi. The degree to which oxalate production was inhibited in this study was not as great as seen previously with other fungi. Punja and Jenkins (1984b) found that not only is L-threonine not inhibitory to oxalate production in *Sclerotium rolfsii*, but it may be used as a sole source of nitrogen by this fungus. A suggested explanation by Punja and Jenkins for this apparent contradiction between their results and those of Kritzman et al. (1977) was that the length of experiment was possibly longer in their study. They suggested that an initial inhibition by L-threonine on oxalate production may have been overcome in this fungus over time. The length of

the experiment in the study by Kritzman et al. was not stated. The fate of L-threonine in the system in this study was not determined.

The formation of oxalate from soil  $\text{HCO}_3^-$  as reported by Lapeyrie (1986) may explain the continued production of this acid under L-threonine treatments. The point at which  $\text{HCO}_3^-$  enters the biochemical pathway of oxalate has not been demonstrated. It may be that the metabolism of  $\text{HCO}_3^-$  into oxalic acid bypasses the primary oxalate biosynthetic pathway as outlined by Maxwell and Bateman (1968) and Huttermann et al. (1980). If *H. westraliensis* were able to utilize this as yet unknown pathway, the action of L-threonine on oxaloacetate decarboxylase would only partially limit total oxalate production.

The quantity of oxalates produced by various fungi as reported in the literature is highly variable. Comparison of these values is made difficult due to a wide variety of extraction and quantitation techniques. The extraction and titration methods used in this experiment were similar to previously published methods used on Deuteromycetes, Ascomycetes and Basidiomycetes. While GLC, IC and HPLC quantitation methods may be more sensitive, the reports of the method used here are numerous and reproducible. The use of oxalate standards allowed reasonable confidence that this method was accurate in determining relative oxalate production.

In the Deuteromycetes, *Sclerotium rolfsii* was reported as producing from 70 to 250 mg oxalate per gram dry weight of mycelium (Bateman and Beer, 1965; Kritzman et al., 1977; Punja and Jenkins, 1984b). On the other hand, Stone and Armentrout (1985) reported in a similar study on pathogenicity that *S. cepivorum* produced only 1.3 to 3.3 mg oxalate per gram dry weight of infected onion tissue! In the Ascomycetes, Godoy et al. (1990) reported that *Sclerotinia sclerotiorum* produced from 0.02 to 24 mg oxalate per gram dry weight of mycelium. Pierson and Rhodes (1992) reported that *S. trifoliorum* produced 360 mg to nearly 3 grams of oxalate per gram dry weight!

In the Basidiomycetes Hüttermann et al. (1980) measured oxalate produced by Fomes annosus but did not record the amount of mycelium that produced it. Lapeyrie et al. (1987) in perhaps the most relevant report to this study, measured oxalic acid produced by the ectomycorrhizal fungus Paxillus involutus between 1.8 and 81 mg per gram dry weight of mycelium. The isolate used in their study was previously classified as a calcifuge ecotype. This isolate accumulated Ca in much higher concentrations than a calcicole isolate of the same fungus (Lapeyrie and Bruchet, 1986). In the present study the values for oxalate produced by H. westraliensis were between 1.1 and 2.2 mg oxalate per gram dry weight of mycelium. These values are on the low end considering that the media formulation used in this study would likely be conducive to oxalate formation. The genus Hebeloma has not been measured for oxalate production previously though Ca oxalate crystals have been described from mycorrhizae of H. crustuliniforme (Froidevaux and Kalin, 1981). These results on quantitation of oxalate production are perhaps not unusual for this genus.

Total oxalate produced increased with increase in CaCO<sub>3</sub> but the ratio of oxalate produced per gram dry weight of mycelium decreased. Given that H. westraliensis exhibited increased growth at higher concentrations, the results of this ratio are likely due to a dilution effect (Jarrell and Beverly, 1981). The length of this experiment may have allowed considerable dead or metabolically inactive hyphae to accumulate. This would have resulted in a contribution to dry weight without oxalate production. It could be supposed that relatively insoluble Ca oxalate crystals once formed would have remained even on dead hyphae. If this was the case oxalate concentration may have increased along with dry weight and total oxalate produced. Several researchers have found that Ca oxalate in soils is not long lasting in spite of its limited solubility (Graustein et al., 1979; O'Connell et al., 1983). It was suggested that microorganisms or plant roots metabolized soil oxalate. It is not known to what extent Ca oxalate is stable in the system of this study. Perhaps the low levels of oxalate found were a result of reassimilation and further

metabolism of Ca oxalate. The 50 days allowed for growth was longer term than previous studies of this kind.

Another facet of the dilution effect in this experiment may have been the effect of CaCO<sub>3</sub> on the rate of growth of this fungus. It could be safely assumed that, in order to produce greater biomass throughout this experiment in the presence of CaCO<sub>3</sub>, that H. westraliensis grew faster under these conditions. As hyphal strands lengthened in growth, oxalate metabolism may not have kept pace. Similarly, greater expansion of hyphal length may have allowed more space between sites of crystal formation.

The hypothesis that oxalate metabolism in fungi is a means by which Ca<sup>2+</sup> concentrations in cell sap are kept in balance seems a viable premise. Ca<sup>2+</sup> in hyphal cytoplasm is closely regulated and can be toxic in excess (Pitt and Ugalde, 1984). Excess Ca<sup>2+</sup> may inhibit important enzyme systems (Whitney and Arnott 1986, 1987). Fungi, like plants, apparently have an active Ca<sup>2+</sup> efflux mechanism to help regulate Ca concentration in cells. Ca oxalate precipitation may be another regulating mechanism.

Phosphorus nutrition may also have been affected by oxalate production in this fungus. There were 2 forms of phosphorus provided in the medium in this study. The more plentiful calcium phosphate would be moderately available at the lower pH of the noncalcareous treatment. In the calcareous treatments equilibrium would have found most of the phosphate tightly bound and precipitated by Ca<sup>2+</sup>. Other studies with this system in our laboratory indicated that P in solution is considerably reduced when CaCO<sub>3</sub> is present (see Chapter 3). Fungi are reported to have a high demand for phosphate (Cochrane, 1958) and ectomycorrhizal fungi have been shown to store large amounts of phosphate in polyphosphate granules within hyphal vacuoles (Harley, 1983). The increased growth of the fungus in CaCO<sub>3</sub> treatments suggests that bound P is made available by some fungal mechanism. That this fungus grows so well in calcareous media may be explained by other mechanisms than oxalate action on the substrate. Analysis of Ca accumulation in the mycelium in calcareous and noncalcareous media may provide insight on the calcicole or

calcifuge nature of this fungus. The mechanism of oxalate action on inorganic phosphates (Jurinak et al., 1986) has been often alluded to and is thermodynamically sound but has not yet been convincingly demonstrated. Nutrient analyses were not made of the fungus or the solution in this study.

In future studies, fungus nutrient concentrations and changes in media nutrient concentrations should be measured in relation to oxalate produced. A correlation could be made between oxalate produced and oxalate action on the substrate. This would also provide more direct evidence of the accuracy of the hypothesized model of Jurinak et al. (1986) of oxalate effects on nutrition in the mycorrhizal symbiosis. The mechanism by which L-threonine inhibits oxalic acid biosynthesis may be a helpful tool in future efforts to demonstrate the effects of fungal oxalates on nutrient availability in soils. It would be interesting to see if L-threonine would be stable, and its effects persistent, in an ectomycorrhizal synthesis system with the presence of a plant associate. Perhaps by inhibiting oxalate production in an otherwise normal mycorrhizal symbiosis, the effects of fungal oxalic acid production on plant symbiont nutrition could be separated from the other mechanisms of growth enhancement attributed to mycorrhizae.

Some of the shortcomings of this study will be addressed in Chapter 3 of this volume. In that study mycorrhizae of H. westraliensis is synthesized with a eucalypt symbiont. Solution P and Ca and plant P and Ca nutrition is analyzed along with oxalate produced in a model calcareous soil system. These results should provide insight into the mechanisms of mycorrhizal enhancement of plant growth and nutrition in a calcareous soil.

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## Chapter 3.

### **The growth and P and Ca nutrition of Eucalyptus diversicolor in model calcareous and noncalcareous systems, with and without an ectomycorrhizal fungus associate.**

#### **Abstract**

The influence of mycorrhization in calcareous and noncalcareous systems on the growth and nutrition of Eucalyptus diversicolor seedlings were studied in this experiment. Seedlings were grown with or without the ectomycorrhizal fungus symbiont Hebeloma westraliensis in calcareous and noncalcareous media in synthesis tubes in a climate-controlled growth chamber. Solution pH, Ca and P concentrations and rhizosphere oxalates were measured at harvest. Total Ca and P in shoots and Ca and P concentrations in shoots at harvest were also measured. Seedling growth was measured by height and dry weight at harvest. The solution pH and Ca concentration was increased and solution P concentration decreased in the calcareous system. Mycorrhization further increased solution pH and decreased the solution concentration of Ca and P. Oxalic acid production was increased in the calcareous system and increased further with mycorrhization. Total Ca and Ca concentrations in shoots were increased by mycorrhization and, with the fungus associate present, were increased in the calcareous system. Total P and P concentrations in shoots were decreased in the calcareous system and increased by mycorrhization. Heights and dry weights of shoots showed little effect from calcareousness of media but were both increased by

mycorrhization. Macroscopic symptoms of nutrient deficiency, seen in nonmycorrhizal seedlings in the calcareous system were not apparent in the mycorrhizal seedlings. Efforts to correlate oxalic acid production in the rhizosphere to solution and shoot tissue Ca and P concentrations were unsuccessful. The importance of these results to mycorrhizal enhancement of E. diversicolor nutrition and growth in calcareous soils are discussed.

## Introduction

Improvement of host nutrition by mycorrhizae has been studied extensively. The role of mycorrhizae in plant uptake of macronutrients was reviewed in a number of volumes (Harley, 1969; Marks and Kozlowski, 1977; Harley and Smith, 1983; Allen, 1992). Principal benefits to host nutrition are increased N and P uptake by mycorrhizal fungi. Low availability of these elements in soils of natural ecosystems is considered a limiting factor to plant growth. Increased growth of mycorrhizal plants can be attributed to increased absorption of these nutrients (Harley and Smith, 1983).

There are numerous hypothesized mechanisms involved in mycorrhizal nutrient enhancement. The increase in absorptive surface area of mycorrhizal roots and increased physical exploration of the substrate by extra-matrical mycelium are central benefits to host plant nutrient acquisition (Harley and Smith, 1983; Allen, 1991). In addition, several mechanisms by which the fungus increases the availability and enhances the absorption of plant unavailable nutrients have been proposed.

Enzymes produced by mycorrhizae act on complex phases of organic nutrient compounds. This is thought to be of particular importance in organic soils and in the litter layers of mineral soils where mycorrhizae may proliferate. Proteases and phosphatases produced by the fungus, and exuded into the substrate, catalyze the mineralization of organic compounds. This process facilitates the release of plant available forms of N and P (Read et al., 1985; Dodd et al., 1987; Allen, 1992). These enzymes have been shown to be many times more active in mycorrhizal than non-mycorrhizal plants (Bartlett and Lewis, 1973; Mitchell and Read, 1981; Harley, 1989).

In temperate forests where inorganic minerals may be the principal source of plant nutrients, the availability of P from inorganic phosphates may be most limiting to plant growth (Harley and Smith, 1983). Mycorrhizal enhancement of inorganic P availability

has been studied in depth. Rosendahl (1942) found that mycorrhizae enhanced the availability and absorption of P from rock phosphate in soils by Ulmus americana. He suggested this was due largely to the production of organic acids by the fungus and the action of those acids on fixed phosphate. Studies by Stone (1950) and by Bowen and Theodorou (1967) suggested that while mycorrhizal fungi were apparently able to solubilize inorganic P, their ability to do this was no greater than other soil microorganisms. The mycorrhizal fungi in these studies had no appreciable enhancement effect on the growth of Pinus radiata over uninoculated (and unsterilized) soil. Bowen and Theodorou cautioned against the presumption that mycorrhizal organic acids had much relevance in natural soils. They argued that *in vitro* organic acid production by fungi was enhanced considerably by the addition of large quantities of carbohydrates in laboratory growth media. It is now thought that organic acid production by soil fungi has considerable significance in soil mineral weathering and the release of P fixed in inorganic precipitates and layer silicate surfaces (Allen, 1991; Bolan 1991). Studies by Graustein et al. (1977), Parfitt (1979) and Knight et al. (1992) have revealed that organic acid production may be a principal method by which mycorrhizae make inorganic P more plant available.

A continual dispute exists over the claim that mycorrhizal fungi increase the availability of phosphates that are bound in plant-unavailable forms. Opponents of this view stress that insoluble forms of phosphate are in equilibrium with the soil solution. As solution P is absorbed by the plant, whether mycorrhizal or not, concentration of this element in solution decreases. The resulting concentration gradient then forces equilibrium dissolution of P from insoluble forms. The assertion is that observations of increases in P uptake by mycorrhizal plants from plant unavailable phosphates may be due merely to the facilitated absorption of P from solution (Hayman and Mosse, 1972; Mosse et al., 1981; Barber, 1984). The alternative argument suggests that some nutrient compounds are chemically unavailable to the plant as opposed to positionally unavailable. Mechanisms present only in the mycorrhizal plant may chemically alter these compounds

into available nutrient forms (Barrow et al., 1977). This later hypothesis has yet to be convincingly demonstrated.

Barrow et al. (1977) found that by incubating a soil at high temperature with added soluble Ca phosphate the phosphate would quickly become fixed into forms unavailable to plants. This soil was compared to a soil with freshly added phosphate of the same form to determine mycorrhizal effectiveness in absorbing this P. The action of VAM with clover and onion in this study released fixed P into solution and allowed increased P absorption in the plant. They did not suggest a mechanism by which this may occur. Kothari et al. (1990) suggested that plant unavailable mineralized P was directly made available to *Zea mays* by VAM fungi. They suggested that VAM organic acids acted on phosphate minerals to release this P. In long term plant growth experiments, P from similar inorganic phosphates may eventually be released through slow equilibrium reactions with the soil solution. It is clear, however, that plant unavailable forms of P are more quickly made available in the mycorrhizal symbiosis. The fact that it may be only a temporal matter does not reduce the importance of enhanced nutrition to the plant.

Oxalic acid is often the predominant organic acid extracted from mineral soils (Fox and Comerford, 1990, 1992). It is likely that a large percentage of total soil oxalate is produced by fungi (Cochrane, 1956; Hodgkinson, 1978). Much soil oxalate is found as precipitate crystals and usually very little is extractable from the water column (L. M. Dudley, personal communication). Fe, Al, Ca and Mg oxalates have been described in soils and these have generally been correlated with abundance of the free cation in soil solution (Treeby et al., 1989; Fox and Comerford, 1990, 1992). In acid to nearly neutral soils, Al and Fe can be abundant and found with more prevalence on cation exchange surfaces. In calcareous soils these are replaced by Ca and Mg (Bohn et al., 1985). The common occurrence of Ca oxalate crystals in litter layers (Graustein et al., 1977; Cromack et al., 1979; O'Connell et al., 1983) suggests that this most abundant ion in soils is readily chelated by oxalate even in slightly acid soils. In calcareous soils Ca saturates

the CEC and the precipitation of Ca oxalate may reduce the concentration of Ca in solution and temporarily free cation exchange sites for the retention of other nutrients.

An additional benefit of oxalic acid on soil fertility is its proposed ability to aid in the solubilization of fixed soil phosphates. As early as 1938 the ability of organic acids to form complexes with Ca was known (Greenwald, 1938). In the 1950s Johnson worked on organic acid reactions with fixed phosphates. His work demonstrated that oxalic acid was most efficient at chelating Ca from tricalcium phosphate and resulted in the most insoluble Ca crystals (Johnston, 1956, 1959). This work apparently escaped the interest of those concerned with plant nutrition until a good while later. Graustein et al. (1977) and Cromack et al. (1979) reevaluated the role of Ca oxalate crystals in soils. It was their contention that these crystals represented the by-products of unique soil weathering reactions. They were biomineralized by the action of fungal produced oxalic acid on soil substrates rich in Ca. The abundance of these crystals in hyphal mats of an ectomycorrhizal fungus in these studies suggested that organic acid production by mycorrhizal fungi was more important to soil weathering and nutrient release than previously believed.

In calcareous soils it is thought that solution P is limited by the solubility of Ca phosphate minerals (Sample et al., 1980). Jurinak et al. (1986) developed a chemical equilibrium model for the effects of soil oxalates on solution P in calcareous soils. In this model the commonly occurring hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , was used as the source of solution P.  $\text{Ca}^{2+}$  concentration was controlled for by the solubility of  $\text{CaCO}_3$ . The results of their study suggested that solution P is effectively controlled by the formation of oxalates in calcareous soils. The oxalate chelates  $\text{Ca}^{2+}$  from  $\text{CaCO}_3$  or Ca phosphate minerals, reducing the  $\text{Ca}^{2+}$  activity in solution and forcing the reaction of hydroxyapatite toward dissolution and the release of phosphate.

Knight et al. (1992) studied the effects of soil oxalates on solution P in calcareous soils. In this study fumigated and nonfumigated native calcareous soils were used to grow

inoculated and noninoculated VAM wheatgrass. HCl extractable oxalates from these soils at harvest and solution pH were compared to solution orthophosphate saturation extracted periodically throughout the experiment. They found that oxalate and pH were effective predictors of solution P based on the equilibrium model of oxalate action on hydroxyapatite in calcareous soils developed by Jurinak et al. (1986). In this study there were no consistent treatment differences in extractable oxalate and they could neither attribute oxalate as fungus produced nor eliminate residual oxalates from the native soil at collection from the total.

The focus of these studies is related to the difficulties researchers have had in explaining the calcicole and calcifuge reactions in plants to calcareous soils. In arid and semi-arid regions of the world afforestation is a major concern. It is thought that the important detriments to plant growth in the calcareous soils common to these regions include high pH, excess  $\text{Ca}^{2+}$  on the CEC, high levels of  $\text{HCO}_3^-$  and the severe limitation of available P (Kinsel, 1983; Rorison and Robinson, 1984).

The term calcicole refers to plants (and fungi) that are found in calcareous soils. Calcifuge is used to describe those that are intolerant of these conditions. The relationship of these plants to their environment was one of the earliest areas of research in plant ecology (Salisbury, 1920). A great deal more than intolerance to  $\text{Ca}^{2+}$  characterizes the reactions of calcifuges in calcareous soil. The difficultly transportable and relatively inactive trivalent Fe (ferric) iron is predominant in calcareous soils and contributes to the relative unavailability of this nutrient in these environments. Calcicole plants are considered Fe efficient as they have the capacity to induce biochemical reactions that make iron available in the active  $\text{Fe}^{2+}$  form. These reactions could involve the efflux of  $\text{H}^+$  ions in the rhizosphere, lowering the local pH to favor the active form (Brown, 1961, 1978), or formation of reducing compounds in root exudates (Brown and Ambler, 1970; Jeffrey, 1987).

Calcifuge plants have not evolved this biochemical apparatus. In calcareous, Fe limited, conditions they often exhibit what has been termed lime-induced chlorosis due to degradation and slow regeneration of chlorophyll. It was once thought that this was an effect of simple iron deficiency. Work reviewed by Brown (1978) revealed that leaf iron concentrations of these chlorotic plants were adequate but the active divalent  $\text{Fe}^{2+}$  was limited and chlorophyll synthesis was inhibited. He noted that Wallace and Lunt (1960) proposed an induced Fe-inactivating compound was produced in the roots of calcifuges in the presence of  $\text{HCO}_3^-$  and P. Lime-induced chlorosis is most often observed in agricultural soils supplementally fertilized with P. It is unclear whether the low P availability in native calcareous soils contributes to the infrequency of this disorder seen in natural plant communities. The reducing capacity of calcicole plant roots and their general Fe efficiency is viewed as a key adaptation to all but the wettest calcareous soils (Jeffrey, 1987).

Aluminum is not a requirement in plant metabolism and so the lack of this element in calcareous soil does not in itself exclude calcifuges from these environments. It is usually present in appreciable amounts in siliceous soils however, and under extreme acid conditions is available in toxic concentrations. Aluminum toxicity disrupts membrane permeability and DNA synthesis and arrests cell division in root apex cells in the S phase of mitosis (Clarkson, 1984). Calcifuge plants have adapted means by which trivalent cations are bound in cytoplasmic exchange sites, chelated, or precipitated on cell walls where they can be harmlessly accumulated (Clarkson, 1969). Calcicole plants have evolved no such binding mechanism and  $\text{Al}^{3+}$  toxicity in acid environments is common and considered a strong limiting factor of calcicole growth.

It has been suggested by Grimes and Hodgson (1969) that the mechanism of complexing  $\text{Al}^{3+}$  by calcifuges in siliceous soil, while immobilizing  $\text{Al}^{3+}$  also binds nonspecifically with  $\text{Fe}^{3+}$ . This is not deleterious in acid environments as there is ready availability of the active  $\text{Fe}^{2+}$  ion. The same immobilizing mechanism may also be operating in calcifuge plants in calcareous soil. There, with little available Al, the

complexing compounds (perhaps the same "inactivating compounds" of Wallace and Lunt) precipitate  $\text{Fe}^{3+}$  and prevent its reduction to the active state. So, the adaptive mechanism of calcifuges to their high Al environment may work against them when Al is not available. According to Kinzel (1983), this "inactivating compound is as yet unknown, and so these suggestions, however plausible and elegant in their simplicity, are as yet unproven" (Kinzel, 1983).

Calcium is generally the most abundant soluble element in soils and is rarely limiting to plant growth (Bache, 1984). Its structural and metabolic functions in plants are better known than many elements and include stabilization of plasma membranes, Capectate bridges in cell walls, and activation of enzymes and growth regulators (Kirkby and Pilbeam, 1984). Calcium is absorbed passively in roots and is translocated by mass flow. Concentrations of  $\text{Ca}^{2+}$  in the cytoplasm are maintained at low levels by active ATP driven Ca efflux pumps in plasma membranes and tonoplasts (Kinzel, 1983).

In instances of Ca deficiency, the most immediate effect is destabilization of cell membranes and the leakage of important cytoplasmic ions, particularly  $\text{K}^+$ . Calcicole plants have evolved a high capacity for  $\text{Ca}^{2+}$  absorption and translocation. In addition, their demand for  $\text{Ca}^{2+}$  is high as evidenced by severely retarded growth when  $\text{Ca}^{2+}$  is limiting. Calcifuges have a low demand for  $\text{Ca}^{2+}$  and it is proposed that they have alternative membrane stabilization apparatus that reduce their minimum requirement for this element (Kinzel, 1983; Rorison and Robinson, 1984; Jeffrey, 1987).

Specific Ca toxicity is being recognized in calcifuge plants growing in calcareous soil. Portis et al. (1977) described an inhibition of photosynthesis enzyme activity and the derangement of chloroplasts by  $\text{Ca}^{2+}$  excess. Kinzel (1983) suggested that Ca efflux pumps that remove  $\text{Ca}^{2+}$  from cytoplasm can be overwhelmed in extreme calcareous situations. The function of  $\text{Ca}^{2+}$  in membrane stability can perhaps be disrupted if excess ions are bound to membrane lipids and proteins, as additional cross-linking becomes deleterious to membrane semi-permeability.

. In addition to calcicoly and calcifugy, Kinzel (1983) suggested that plants may also be classified as calciotrophic or calciophobic. Calciotrophs maintain appreciable amounts of dissolved  $\text{Ca}^{2+}$  in plant sap. Calciophobes precipitate incoming  $\text{Ca}^{2+}$  in compounds such as Ca oxalate in vacuoles. In a calcareous soil, with  $\text{Ca}^{2+}$  in excess, and water often limiting, calciotrophic plants use water soluble Ca in their tissues to increase cell osmotic pressure to avoid loss of turgor. Calciophobic plants that precipitate osmotically inactive Ca oxalate in their vacuoles cannot use this most abundant ion as osmoticum. They may adapt to water limitation by the production of sugars and related compounds to be used in osmotic adjustment, but at high metabolic cost (Kinzel, 1983; Rorison and Robinson, 1984).

Work on calcareous situations in France has focused on the dominant role of soil  $\text{Ca}^{2+}$  concentration on limiting plant distribution. Salsac (1973) and others (Lamant and Heller, 1975; Ghorbal and Salsac, 1979; Rorison, 1980) have suggested that calcicole plants may be able to limit  $\text{Ca}^{2+}$  absorption by altering root cell membrane structure, while calcifuge plants have no such ability and are faced with dealing metabolically with overwhelming concentrations of this element in their tissues. Particular attention has been focused on the role that mycorrhizae play in regulating  $\text{Ca}^{2+}$  absorption. This symbiosis may be a key adaptive feature for many plants in extreme calcareous soils (Lapeyrie and Bruchet, 1986; Azcon and Barea, 1992).

Several studies have focused on the ability of mycorrhizae to enhance the nutrition of associate plants in calcareous soil. Azcon and Barea (1992) working with VAM fungi and the legume Medicago sativa found that VAM inoculation increased the levels of P, Mg, K and N in plant tissues when available P in the soil was low. The effect of mycorrhization was diminished when additional P was supplemented to the growth medium. Perhaps of equal importance, mycorrhization decreased the concentration of  $\text{Ca}^{2+}$  in plant tissue grown in calcareous soil. This they viewed as a principal benefit of mycorrhizae in this situation. Medicago sativa is classified as calcifuge and VAM inoculation allowed a secondary calcicole habit of these plants. Similar results were had with VAM inoculation of Helianthemum chamaecistus in calcareous

soils (Kianmehr, 1978). Previous work with ectomycorrhizal trees showed a similar response of mycorrhization. Clement et al. (1977) and Le Tacon (1978) found this to be the case with Austrian pine (Pinus nigra ssp. nigricans). This tree is inherently intolerant of calcareous soil and in these studies ectomycorrhizae of Tuber melanosporum, Boletus (syn. Suillus) granulatus and unidentified fungi allowed increased growth and reduced symptoms of nutrient deficiency in these soils.

More recently in Australia, Lapeyrie and Chilvers (1985) observed a VAM-ectomycorrhizae succession on Eucalyptus dumosa that enhanced the nutrition of this tree when growing in calcareous soil. They had located specimens of this tree growing in a localized patch of calcareous soil in New South Wales and collected seeds for greenhouse experiments. It was discovered that without mycorrhizal inoculum new seedlings were intolerant of the soils in which the parent was found. Upon addition of unsterilized native soil to the containers of these seedlings, mycorrhizae were formed in a succession of early colonizing VAM and later developing unidentified ectomycorrhizae. This mycorrhization ameliorated the nutrient deficiencies observed when no inoculum was present.

Calcicole eucalypts are rare. The scarcity of limestone parent materials in Australia has placed no evolutionary burden on these trees to adapt to calcareous soils. Any of the aforementioned nutrient conditions of these soils can restrict the growth of Eucalyptus spp. (Pryor, 1976). The tolerances of many eucalypts to drought and high temperature, their rapid rate of growth and economically valuable wood make them attractive species for plantation establishment in seasonally dry regions. As calcareous soils are so common to these regions, the amelioration of the intolerance of Eucalyptus spp. to calcareous soils would advance plantation efforts.

The mechanisms by which mycorrhizae enhance host plant tolerance to calcareous soils is not well understood. The studies investigating this phenomenon suggest that fungal oxalic acid production may be a primary method of nutrition enhancement for calcifuges in these soils. Little is known about the differential abilities of fungi to produce

oxalate. It has been studied in many groups of plant pathogenic fungi and very little in mycorrhizal species (see Chapter 2). Growth studies involving ectomycorrhizal fungi under various nutrient regimes (reviewed in Chapter 2 of this volume, p. 27) suggest that the genus Hebeloma has members that will grow well in calcareous soil conditions. Results of experimentation reported in Chapter 2 revealed that H. westraliensis was so adapted and would form mycorrhizae quickly with a number of eucalypts. This fungus also produced oxalic acid and production was stimulated by calcareous conditions. These results suggest that this fungus may be a good source of inoculum in Eucalyptus plantations in calcareous soil. Hebeloma westraliensis was used in the present synthesis study.

There have so far been no studies to look at oxalic acid production by ectomycorrhizal fungi in synthesis with a host plant. Neither are there studies that measure ectomycorrhizal host plant nutritional changes and oxalic acid production at the same time. Through synthesis of mycorrhizae between H. westraliensis and E. diversicolor, this study will investigate a mechanisms by which mycorrhizae provide a tolerance to calcareous soils. A model calcareous system was created to study the effects of mycorrhizal oxalic acid on host nutrition. The incorporation of nitrate - N, Ca phosphate and  $\text{CaCO}_3$  into the growth medium for mycorrhizal synthesis approaches the ionic conditions likely to be encountered in calcareous soils. Changes in solution nutrient availability as a result of mycorrhizal action were observed, especially as they relate to the availability of P from plant unavailable forms of Ca phosphate. Plant P and Ca nutrition changes and other growth parameters as a result of mycorrhization were studied. The production of oxalic acid was measured in this system in order to observe patterns of correlation between oxalic acid production and soil and plant nutrient levels. It is hypothesized that oxalic acid produced in this system allows increased availability of P in the calcareous system and increased absorption of P by E. diversicolor. The significance of the results of this study to plantation establishment of Eucalyptus spp. in calcareous soils are discussed.

## Methods and Materials

Preparations for this experiment included bulking the mycelium of *Hebeloma westraliense* following much the same procedure as in Chapter 2. Inoculum of this fungus was grown in bubble culture for three weeks in a 2L Erlenmeyer flask with 1L nitrate-modified liquid Hagem's medium. Basal medium consisted of the following nutrient concentrations per liter: 4 g malt extract, 1 g yeast extract, 5 g D-glucose, 0.25 g CaCl<sub>2</sub>, 0.05 g KH<sub>2</sub>PO<sub>4</sub>, 1.42 g KNO<sub>3</sub>, 0.5 g MgSO<sub>4</sub>\*7H<sub>2</sub>O, 0.5 ml FeCl<sub>3</sub> (1% aqueous solution), 100 µl Biotin (50 µg/ml aqueous solution), 100 µl Thiamine (1 mg/ml aqueous solution), to 1000 ml ddH<sub>2</sub>O. Fifty, 1.5 X 13 cm screw-capped glass synthesis tubes were filled with 11.25 g of perlite. The perlite had been previously passed between 4 mm mesh and 2 mm mesh screens to retain particles between 2 and 4 mm in diameter. CaH<sub>4</sub>(PO<sub>4</sub>)<sub>2</sub>\*H<sub>2</sub>O was added to each tube by dissolving 1.25 g in 50 ml of ddH<sub>2</sub>O and pipetting 1 ml of this solution to each tube to provide 6.15 mg of P per tube in this form. Total P amounted to 6.72 mg in each tube. The calcareous treatment was provided by adding 0.5 g CaCO<sub>3</sub> to 25 of the tubes. Basal medium was added to each in 40 ml volume and autoclaved for 20 minutes at 270<sup>0</sup> F and 20 psi. Treatments and controls consisted of the following:

no plant, no fungus, 0 g CaCO <sub>3</sub> , 5 reps	no plant, + fungus, 0 g CaCO <sub>3</sub> , 2 reps	+ plant, no fungus, 0 g CaCO <sub>3</sub> , 7 reps	+ plant, + fungus, 0 g CaCO <sub>3</sub> , 10 reps
no plant, no fungus, 0.5 g CaCO <sub>3</sub> , 5 reps	no plant, + fungus, 0.5 g CaCO <sub>3</sub> , 2 reps	+ plant, no fungus, 0.5 g CaCO <sub>3</sub> , 7 reps	+ plant, + fungus, 0.5 g CaCO <sub>3</sub> , 10 reps

After inoculum had bulked sufficiently, the bubble culture was macerated in a sterile 1 L aluminum blender canister using a Waring blender for 5 seconds at speed 5. Mycelial homogenate was pipetted in 10 ml volumes into each tube with a wide-bore pipette providing the + fungus treatments. Ten ml of nitrate-modified liquid medium was

added to the non-fungus treatments. All tubes were placed in the dark at room temperature (ca. 22 C) to incubate for 3 weeks. After this period of time mycelium had spread throughout most of the perlite in the fungal treatment tubes and small subsamples were plated on Malt agar to check for contaminants.

Seeds of Eucalyptus diversicolor were sorted for uniformity of size and sterilized and germinated by a method modified from Bougher et al (1990). These seeds were immersed in sterile 0.625% NaClO and gently swirled by hand for 15 minutes. They were then transferred through three separate rinses of sterile ddH<sub>2</sub>O and plated out on nitrate-modified agar medium and placed in the dark to germinate and to check for contamination. After 2 days germination had occurred for 96% of the seeds. The plates were partially covered over the root zone with black plastic and placed upright in a lighted growth chamber to develop (Plate 5). After 2 more days radicals of germinated seedlings were more than 1.5 cm long and the first true leaves had emerged beyond the cotyledons. It was determined that this was adequate growth for transplanting the seedlings to synthesis tubes. Ten additional tubes with fungus and plant added were started at the same time, 5 with 0 g CaCO<sub>3</sub> and 5 with 0.5 g CaCO<sub>3</sub>. These were not included in growth, oxalate or nutrition analyses, but harvested sequentially and examined for oxalate crystals and mycorrhizae formation.

Seedlings in plates were again sorted for uniformity of development and one seedling was planted in each synthesis tube using a Spoonula (Fisher Scientific Inc., Pittsburgh, PA). All tubes were placed canted at a slight angle in a lighted growth chamber (Plate 6) set to provide 16 hours of light with incandescent and fluorescent bulbs and 8 hours of darkness. Temperature was maintained at a constant 23 C for both cycles. Incident light intensity was not measured in these experiments, but previous studies using this same growth chamber found it to be ca. 163  $\mu\text{E m}^{-2} \text{sec}^{-1}$  of photosynthetically active radiation (PAR) (Gruhn et al., 1992).

At time of seedling transfer to tubes, the comparison treatment tubes with no fungus and no plant were harvested and the solution extracted for assay of solution nutrient levels before any organismal action on substrate chemistry. Perlite and liquid media was removed from each tube and thoroughly mixed with 20 ml added sterile ddH<sub>2</sub>O. These were suction-filtered through a 0.45 µm millipore filter membrane and transferred to screw-capped test tubes. The pH of these solutions was measured with a glass electrode pH meter and they were frozen until assayed by Inductively Coupled Plasma (ICP) Emission Spectroscopy (see below).

Observations were made of seedling leaf coloration throughout the growth of these seedlings for any signs of nutrient deficiency (Plate 13). Seedlings were allowed to grow in these conditions for 50 days by which time the most vigorously growing seedlings had reached the limit of visual observation of height (synthesis tubes allowed about 10 cm of unobstructed growth in the tubes). Crystals found during sampling of extra tubes throughout the experiment were tested by Yasue's (1969) histochemical method for Ca oxalate identification. Mycorrhizae and uninfected roots were photographed fresh. They were then dehydrated and infiltrated by the method of Johansen (1940, in: Schenk, 1982). Fixed mycorrhizae were then sectioned on a microtome and stained in a series of ethanol, FAA, Safronin and Fast Green. They were then observed microscopically for the presence of a mantle and Hartig net. See Appendix, pp. 109,110 for a summary of dehydration, infiltration and staining procedures.

At 50 days the tubes were harvested. Seedling height was determined by measuring from the swelling at the root-shoot interface to the apical meristem (attachment of the newest expanding leaves). Small subsamples of perlite and growth medium were removed and plated on malt agar to test for contamination. Plant shoots were severed at the root-shoot interface, weighed and placed in an oven to dry at 60 C for 24 hours. Weight was recorded at 18 and 24 hours to assure constant dry weight. Dried plant tissue was ground with a mortar and pestel, transferred to 15 ml crucibles and dry ashed at 500

C for 2 hours, or until all tissue had been reduced to a clean white ash. The ash for each sample was dissolved in HCl, diluted to 1 M and filtered for analysis of tissue nutrients by Inductively Coupled Plasma (ICP) Emission Spectroscopy (Donohue and Aho, 1992). This analysis was done by the Virginia Tech Soil Testing and Plant Analysis Laboratory. Plant tissue P and Ca were expressed as  $\mu\text{g ml}^{-1}$  solution and these values were transformed to total  $\mu\text{g}$  quantities in each sample and to  $\mu\text{g}$  nutrient element per gram dry weight of plant tissue.

Perlite and liquid medium was removed from each tube and extracted by the Saturation Extract method (Kidder, 1992) for determining nutrients in solution. Twenty ml sterile ddH<sub>2</sub>O was added to each sample. They were thoroughly mixed and suction-filtered through a 0.45  $\mu\text{m}$  filter membrane. Solution pH was recorded with a glass electrode pH meter. Solutions were kept frozen until assayed for P and Ca by ICP for solution nutrients by the Virginia Tech Soil Testing and Plant Analysis Laboratory. Values for these nutrients were expressed as  $\mu\text{g ml}^{-1}$  solution.

Remaining perlite (and accompanying root and/or fungus tissue depending on treatment) was oven dried at 60 C for 36 hours until constant dry weight had been achieved. These samples were extracted for oxalate determination using a modification of the Cromack et al (1978) modification of Baker's (1952) procedure for quantitation of oxalates in mycelium. All oxalate present in precipitate form, whether attached to perlite particles or associated with root or fungus tissue, would be detected. Water soluble oxalates, if in solution, would not. Dried samples were boiled in 25 ml 1 M HCl for 15 minutes in 100 ml beakers fitted with watch glasses to reduce evaporation. They were filtered while hot through Whatman #2 paper into 35 ml plastic centrifuge tubes and left overnight to cool. Five ml of phosphoric tungstate reagent was added to each tube, mixed and allowed to sit for 5 hours at room temperature. Tubes were centrifuged at 5000 rpm for 15 minutes. Twenty ml of the resulting clear supernatant was pipetted into clean centrifuge tubes and adjusted to alkaline with the addition of NH<sub>4</sub>OH until a persistent

white precipitate of phosphotungstate was formed and pH was above 7 as measured by a pH meter with a glass electrode. Five ml of CaCl<sub>2</sub> acetate reagent was added to each, mixed thoroughly and allowed to sit overnight refrigerated to 5 C. Tubes were centrifuged again and the supernatant discarded. Precipitate was washed with 20 ml acetic acid saturated with Ca oxalate and mixed thoroughly to dissolve precipitate. These were again centrifuged at 5000 rpm and the supernatant discarded. Remaining precipitates were dissolved in 10% (4 N) sulfuric acid, heated in a water bath to 90 C and titrated while hot with 0.01 N (0.002 M) KMnO<sub>4</sub> delivered in 20 µl increments with a micropipette. Total oxalate was determined by the formula: 1 µl KMnO<sub>4</sub> = 0.45 µg oxalate. Comparisons of oxalate recovered from these samples were made to previous tests on oxalate extraction and recovery from standards (see Chapter 2). All reagent components are listed in Appendix 1.

In tubes reserved for Ca oxalate crystal observation mycorrhizae were removed and mounted whole on microscope slides. These were briefly examined under a light microscope and samples containing crystals were subjected to Yasue's (1969) histochemical method for Ca oxalate identification. Slides were immersed in 5% acetic acid for 30 minutes and rinsed gently with ddH<sub>2</sub>O. Slides were immersed in 5% aqueous AgNO<sub>3</sub> for 15 minutes and again thoroughly but gently rinsed. Samples were stained with dithiooxamide (rubeanic acid) in 70% ammoniacal ethanol for 1 minute and observed again under the light microscope for crystal staining. Those crystals staining dark brown or black and with amorphous or tetrahedral shape were scored as Ca oxalate.

Recordings were made of plant height, tissue fresh and dry weights, total P and Ca in plant samples and concentrations of P and Ca per gram dry weight of plant tissue. Soil solution P and Ca concentrations in µg ml<sup>-1</sup> were also recorded as well as total oxalate produced per sample. These data were analyzed for normality using the D'Agostino-Pearson Omnibus K<sup>2</sup> Normality test (D'Agostino, 1990). Groups not distributed normally were tested using probability plots of data points for each variable over the normal

distribution. Extreme outliers were evaluated as to their singularity in the data and removed from the statistical analysis. Homogeneity of variance between the treatment groups was tested using equality of covariance matrices and the Bartlett-Box Homogeneity Test (Bartlett, 1950). Differences between treatment means were analyzed using Fisher's Least Significant Difference Test (Ott, 1977). A Multiple Analysis of Variance (MANOVA) (Winer, 1971) was used to determine the relationships of these measures to presence or absence of fungus and to presence or absence of CaCO<sub>3</sub>. Significance levels were determined for  $\alpha = 0.05$ . Though statistical analyses of the groups: no plant, no fungus; and no plant, +fungus, were not done, these groups were used as comparison baseline measures. All statistical tests were performed with the aid of Number Cruncher Statistical System (NCSS) (Hintz, 1992).

## Results

During the macroscopic and microscopic inspections of synthesized mycorrhizae in this experiment, there were no noticeable effects of  $\text{CaCO}_3$  treatment on mycorrhizal formation or morphology. Eucalyptus diversicolor X Hebeloma westraliensis mycorrhizae were monopodially branched and covered by a sparse white mantle (Plate 7). These were compared to nonmycorrhizal roots of E. diversicolor (Plate 8). Mantle hyphae and extra-matrical hyphae had clamp connections. Fixed and stained cross sections revealed a continuous mantle 4-5 cells thick (Plate 9) and a Hartig net penetration between epidermal cells only (Plate 10). These results support the original description of this symbiosis (Bougher et al., 1991). Subsequent plating of substrate samples on nutrient media revealed no contamination of these tubes by other microorganisms.

Mycorrhizal seedlings of E. diversicolor that were harvested throughout the experiment had numerous crystals associated with the mycorrhizae and with the extra-matrical hyphae. Crystals were much more numerous in the calcareous group. Many of these were  $\text{CaCO}_3$  or Ca phosphate and dissolved with effervescence in 5% acetic acid. Electron probe microanalysis in conjunction with Scanning Electron Microscopy identified some of these crystals as rich in Ca and others as rich in Ca and P. Many more crystals remained after acetic acid treatment and these were stained with  $\text{AgNO}_3$  and rubeanic acid as per Yasue (1969). These crystals stained dark brown and were identified as calcium oxalate (Plates 11 and 12).

Eucalyptus diversicolor seedlings grew poorly when not in association with H. westraliensis. Differences in height and dry weight of shoots at harvest supported these observations. There were no obvious differences in growth rate between seedlings in the calcareous and noncalcareous groups. The groups with no fungus however, did exhibit differences in coloration between the two levels of  $\text{CaCO}_3$ . Those seedlings grown in the calcareous treatment without the fungus associate had red to purplish coloration in the

leaves (Plate 13) possibly indicating P deficiency (Taiz and Zeiger, 1991). There was also some necrosis of the leaf apices in this group that was not evidenced in the noncalcareous group, possibly an indication of Ca toxicity (Taiz and Zeiger, 1991). The seedlings grown without fungus in the noncalcareous treatment retained the dark green coloration typical of *E. diversicolor*. No macroscopic differences in growth or coloration were observed in either the calcareous or noncalcareous treatments when the fungus associate was present.

Four instances of non-normality were found among the treatment groups. Probability plots revealed one extreme outlier in each of these groups and they were removed from the data set. After removing those four data points normality was established in all treatment groups as measured by the D'Agostino-Pearson Omnibus  $K^2$  Normality Test (D'Agostino et al, 1990). Tests of homogeneity of variance revealed only slight departure from homoscedacity. Therefore, the assumptions of the MANOVA were met and the results of these parametric tests were considered valid.

Data on the pH of growth media including background pH of calcareous and noncalcareous systems are summarized in Figure 5. The addition of  $\text{CaCO}_3$  resulted in increased pH before inoculation with fungus or planting of seedlings. pH was also increased in both the calcareous and non-calcareous systems after the growth of plants and fungus. The increase in pH of the calcareous system was much more pronounced at harvest. pH data was not statistically analyzed.

Analysis of oxalate production amongst the treatment groups included extractable oxalate that was titratable with 0.01 N  $\text{KMnO}_4$ . Oxalates free in solution extracts were not measured. Results of oxalate standard extraction and titration revealed that for oxalate concentrations above  $10 \mu\text{g ml}^{-1}$  the recovery of oxalate from spike samples was consistently between 85% and 90% (Appendix, p. 108). The values obtained for oxalate concentrations in substrate samples should therefore not be taken as absolute concentrations. They can however be used as reliable values of relative oxalate production. Two replicates of comparison measures of fungal oxalate production in this

system without the presence of the plant associate were made. These values were not analyzed statistically but were included for comparison in Figure 6. Oxalate production increased with mycorrhization. This increase was most dramatic in the calcareous system. Oxalate production also increased in the calcareous system when there was no fungus present. Mean oxalate produced, standard deviations and results of Fisher's Least Significant Difference Test for differences between the means are summarized in Figure 6.

The effects of interaction between fungus treatment and  $\text{CaCO}_3$  treatment were not significant according to individual ANOVA within the MANOVA procedure ( $F=2.51$ ,  $P=0.1253$ ) and explained only 2.4% of the total variation. The effects of fungus treatment across  $\text{CaCO}_3$  levels were highly significant ( $F=39.98$ ,  $P<0.0001$ ) explaining 38% of the total variation. Mean oxalate for the no fungus groups was  $156.15 \mu\text{g}$  ( $n=12$ ,  $\text{SE}=18.96$ ) and  $312.25 \mu\text{g}$  ( $n=18$ ,  $\text{SE}=15.48$ ) in the presence of the fungus. The effects of  $\text{CaCO}_3$  level across fungus treatments were also highly significant ( $F=26.22$ ,  $P<0.001$ ). The mean oxalate at  $0 \text{ g CaCO}_3$  was  $171.0 \mu\text{g}$  ( $n=16$ ,  $\text{SE}=16.42$ ) and at  $0.5 \text{ CaCO}_3$  level,  $297.4 \mu\text{g}$  ( $n=14$ ,  $\text{SE}=17.55$ ). These effects explained 25% of the total variation in titratable oxalate. 25% of the total variation was not explained by the model.

The analysis of soil solution nutrient concentrations by ICP from solution extracts included  $\mu\text{g ml}^{-1}$  measurements of Ca and P. Solution Ca increased in the calcareous treatments and decreased with mycorrhization. Mean solution Ca, standard deviations and results of Fisher's LSD test for differences between the means are shown in Figure 7. Comparison values of background solution Ca and after treatment with fungus alone are included in Figure 8. In the analysis of solution Ca there were significant effects of interactions between fungal treatment and  $\text{CaCO}_3$  treatment ( $F=11.42$ ,  $P=0.0023$ ) that explained a little over 4% of the total variation. There were highly significant effects on solution Ca from the presence or absence of fungus across  $\text{CaCO}_3$  levels ( $F=193.35$ ,  $P<0.0001$ ) that explained just under 70% of the total variation. With no fungus associate present the mean Ca in solution was  $112.4 \mu\text{g ml}^{-1}$  ( $n=12$ ,  $\text{SE}=3.62$ ). This mean was

reduced to  $62 \mu\text{g ml}^{-1}$  ( $n=18$ ,  $\text{SE}=2.96$ ) in the presence of the fungus. The main effects of level of  $\text{CaCO}_3$  were also highly significant ( $F=37.03$ ,  $P<0.0001$ ), explaining 13% of the total variation. At the 0 g  $\text{CaCO}_3$  level mean solution Ca concentration was  $80.5 \mu\text{g ml}^{-1}$  ( $n=16$ ,  $\text{SE}=3.14$ ) and  $109 \mu\text{g ml}^{-1}$  ( $n=14$ ,  $\text{SE}=3.35$ ) at the 0.5 g  $\text{CaCO}_3$  level. 9.4% of the total variation in solution Ca was not explained by the model.

Solution P concentration was considerably reduced in the calcareous treatments. Mycorrhization reduced solution P in the noncalcareous treatment but had little effect on these values in the calcareous treatment. Mean solution P in the various treatments, standard deviations and results of Fisher's LSD test are included in Figure 9. Comparison figures for background solution P and that which was found after fungus growth only are included in Figure 10. There were highly significant effects of the interaction between fungus treatment and  $\text{CaCO}_3$  treatment ( $F=43.18$ ,  $P<0.0001$ ) that explained 9% of the total variation. The main effects of presence or absence of fungus across  $\text{CaCO}_3$  levels on solution P were highly significant ( $F= 41.58$ ,  $P<0.0001$ ) and explained 9% of the total variation in solution P. The mean P concentration in the absence of fungus was  $54 \mu\text{g ml}^{-1}$  ( $n=12$ ,  $\text{SE}=2.76$ ) and  $31 \mu\text{g ml}^{-1}$  in the presence of the fungus ( $n=18$ ,  $\text{SE}=2.25$ ). The main effects of  $\text{CaCO}_3$  level across fungus treatments were also highly significant ( $F=358.5$ ,  $P<0.0001$ ), accounting for 77% of the total variation. The mean solution P concentration at the 0 g  $\text{CaCO}_3$  level was  $76 \mu\text{g ml}^{-1}$  ( $n=16$ ,  $\text{SE}=2.39$ ) and  $8 \mu\text{g ml}^{-1}$  ( $n=14$ ,  $\text{SE}=2.55$ ) at the 0.5 g  $\text{CaCO}_3$  level. 5.6% of the total variation in solution P concentration was not explained by the model.

Total Ca in shoots of *E. Diversicolor* was increased considerably by mycorrhization. When the fungus was present the calcareous system also allowed increased Ca accumulation in shoots. There was no significant difference in total Ca in shoots of the treatments with no fungus. Mean total Ca in shoots of all treatments, standard deviations and results of Fisher's LSD test for differences between the means are reported in Figure 11. There was a significant effect from interactions between fungus

association and level of  $\text{CaCO}_3$  on the total Ca in seedling shoots ( $F=13.22$ ,  $P=0.0012$ ) and this explained 5% of the variation. The effect of presence or absence of fungus on total plant Ca was highly significant ( $F=205.0$ ,  $P<0.0001$ ) accounting for 76% of the variation. In the absence of fungus the mean total Ca in shoots was 0.085 mg ( $n=12$ ,  $SE=0.049$ ). With the fungus associate present this mean increased to 0.994 mg ( $n=18$ ,  $SE=0.04$ ). The level of  $\text{CaCO}_3$  had a significant effect ( $F=13.61$ ,  $P=0.0010$ ) and explained 5% of the total variation in Ca in plant tissue. With no  $\text{CaCO}_3$  added the mean total Ca in shoots across both fungus treatments was 0.42 mg ( $n=16$ ,  $SE=0.042$ ). In the calcareous treatment this mean increased to 0.66 mg ( $n=18$ ,  $SE=0.045$ ). A total of 8% of the variation was not explained by the model.

The Ca concentration in shoots also increased with mycorrhization. This increase was most evident in the calcareous treatment. There was no significant difference in the Ca concentration in seedlings with no fungus associate. The means Ca concentrations for all treatments, standard deviations and results of Fisher's LSD test for differences between the means are recorded in Figure 13. In the measurements of mg Ca per gram dry weight of plant shoot there was a highly significant effect of interaction between fungus association and level of  $\text{CaCO}_3$  ( $F=22.17$ ,  $P=0.0001$ ) that explained 16.5% of the total variation. The main effects of presence or absence of fungus were also highly significant ( $F=60.36$ ,  $P<0.0001$ ) and explained 45% of the total variation in tissue Ca concentration. The mean across  $\text{CaCO}_3$  levels for the no fungus treatment was 5.5  $\text{mg g}^{-1}$  Ca ( $n=12$ ,  $SE=0.5$ ). This mean in the presence of fungus was 10.7  $\text{mg g}^{-1}$  ( $n=18$ ,  $SE=0.4$ ). The main effects of  $\text{CaCO}_3$  level were highly significant on Ca concentration ( $F=15.82$ ,  $P=0.0005$ ) and explained 12% of the total variation. At the 0 g  $\text{CaCO}_3$  level across fungus treatments the mean Ca concentration in shoot tissue was 6.8  $\text{mg g}^{-1}$  ( $n=16$ ,  $SE=0.45$ ). At the 0.5 g  $\text{CaCO}_3$  level this mean increased to 9.5  $\text{mg g}^{-1}$  ( $n=14$ ,  $SE=0.47$ ).

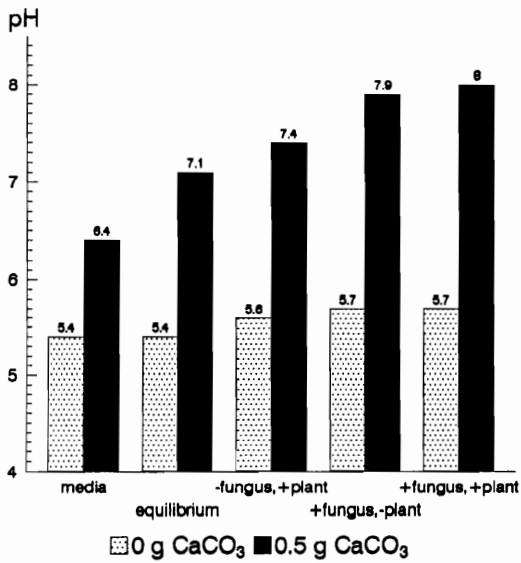
Total seedling P in shoots of *E. diversicolor* were increased dramatically by mycorrhization. In the presence of the fungus associate, total P in shoots was decreased in the calcareous system. There was no significant difference in this measure in the groups

with no fungus associate. Mean total P in shoots among all treatments, standard deviations and results of Fisher's LSD test for differences between the means are represented in Figure 12. The effects of interaction between fungus association and level of CaCO<sub>3</sub> on total seedling P were highly significant (F=15.21, P=0.0006), explaining 8.4% of the total variation. The main effects of presence or absence of fungus across both levels of CaCO<sub>3</sub> on total seedling P were highly significant (F=108.90, P<0.0001) and explained 60% of the total variation. The mean total shoot P in the absence of fungus was 0.05 mg (n=12, SE=0.04) and 0.6 mg in the presence of fungus associate (n=18, SE=0.035). The main effects of level of CaCO<sub>3</sub> across fungal treatments on total shoot P was also highly significant (F=19.87, P=0.0001). The mean total P in seedlings at 0 g CaCO<sub>3</sub> was 0.47 mg (n=16, SE=0.037) and with 0.5 g added CaCO<sub>3</sub>, 0.22 mg (n=14, SE=0.04). This factor explained 11% of the total variation. 14.4% of the total variation was not explained by the model.

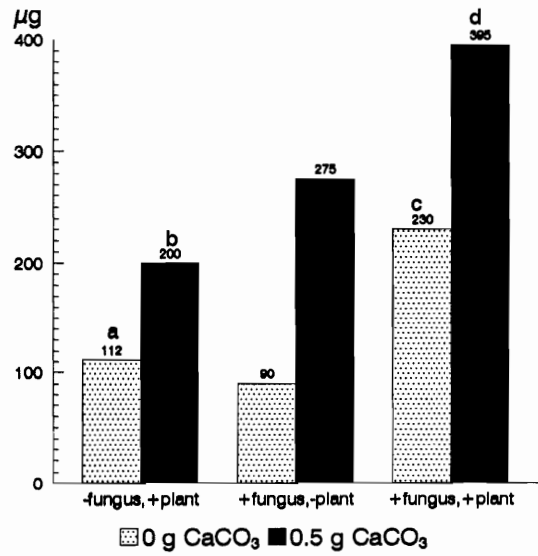
The concentration of P in shoots were significantly reduced in the calcareous systems and increased in both systems by mycorrhization. Mean P concentrations in shoots, standard deviations and results of Fisher's LSD test are shown in Figure 14. There were slightly significant effects from the interaction of fungus treatment and CaCO<sub>3</sub> treatment in the concentration of P in seedling shoot tissue (F=4.11, P=0.0530). These effects explained 3% of the total variation. There were highly significant effects on seedling P concentration from the presence or absence of fungus (F=43.38, P<0.0001) that explained 32% of the total variation. Mean P concentration in the mycorrhizal association across CaCO<sub>3</sub> levels was 6.7 mg g<sup>-1</sup> (n=18, SE=0.28). In the absence of fungus associate this mean was reduced to 3.7 mg g<sup>-1</sup> (n=12, SE=0.35). The effects of level of CaCO<sub>3</sub> across fungus treatments were also highly significant (F=58.03, P<0.0001) and explained 43% of the total variation. Mean P concentration at 0 g CaCO<sub>3</sub> was 6.9 mg g<sup>-1</sup> (n=16, SE=0.3) and 3.5 mg g<sup>-1</sup> at the 0.5 g CaCO<sub>3</sub> level (n=14, SE=0.3). 19% of the total variation was not explained by the model.

Height of seedlings was increased with mycorrhization. This increase was greater in the calcareous system (Figure 15). With no fungus added the mean height of all seedlings was 3.32 cm (n=12, SE=0.25). The fungus association increased this to a mean of 8.56 cm (n=18, SE=0.21). The difference was highly significant (F=252.62, P<0.0001). The presence or absence of fungus explained 88% of the variation in height. The addition of CaCO<sub>3</sub> had no significant effect on seedling height when this variable was considered by itself (F=1.25, P=0.2747) and explained only 0.4% of the variation. Mean seedling height with 0 g CaCO<sub>3</sub> was 5.75 cm (n=16, SE=0.22) and 6.12 cm with 0.5 g CaCO<sub>3</sub> per tube (n=14, SE=.23). There was no significant interaction between the factors on variation in height (F=1.55, P=0.2239). About 9% of the variation was not explained by the model.

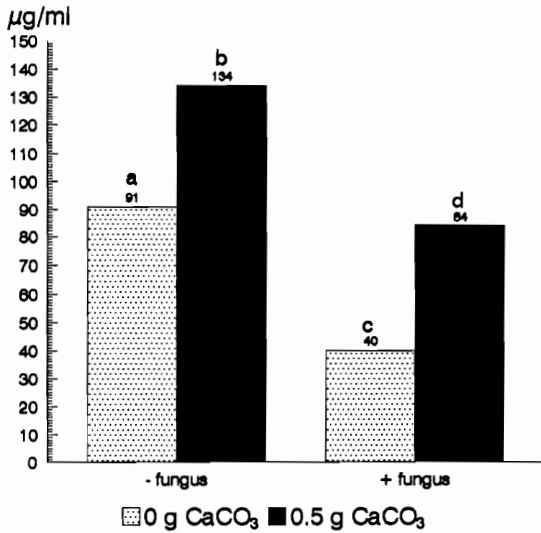
Shoot dry weights were increased dramatically with mycorrhization. There were no significant differences in dry weight across CaCO<sub>3</sub> treatments. Mean shoot dry weights at harvest, standard deviations and results of Fisher's LSD test for differences between the means are shown in Figure 16. MANOVA analysis revealed a significant effect from fungus association on seedling dry weight at harvest across both levels of CaCO<sub>3</sub> (F=634.71, P<0.0001). Mean dry weight for all seedlings without the fungus association was 0.015 g (n=12, SE=0.0024) and 0.093 g with the addition of the fungus. The mycorrhizal association explained 93% of the variation. There was no significant effect of level of CaCO<sub>3</sub> on dry weight (F=0.67, P=0.4198). The effect on dry weight of the interaction between fungus treatment and CaCO<sub>3</sub> treatment was also not significant (F=3.31, P=0.0804). Taken together, the effect of CaCO<sub>3</sub> level and the interaction between CaCO<sub>3</sub> and fungus treatment explained less than 0.6% of the variation in dry weight. A total of 4% of the variation was not explained by the model.



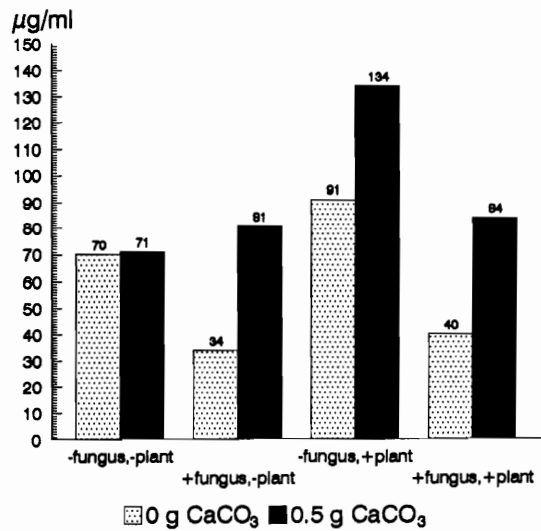
**Figure 5.** Solution pH prior to organismal action and after growth of combinations of *E. diversicolor* and fungal associate at 0 and 0.5 g CaCO<sub>3</sub>.



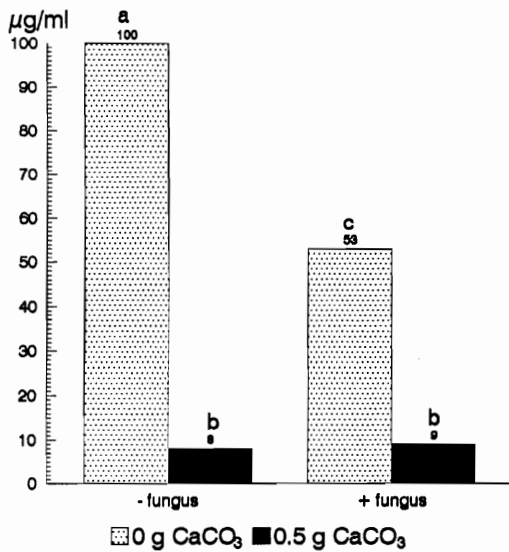
**Figure 6.** Mean oxalate extracted from substrate of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>, including values for action of fungus alone (center bars). Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



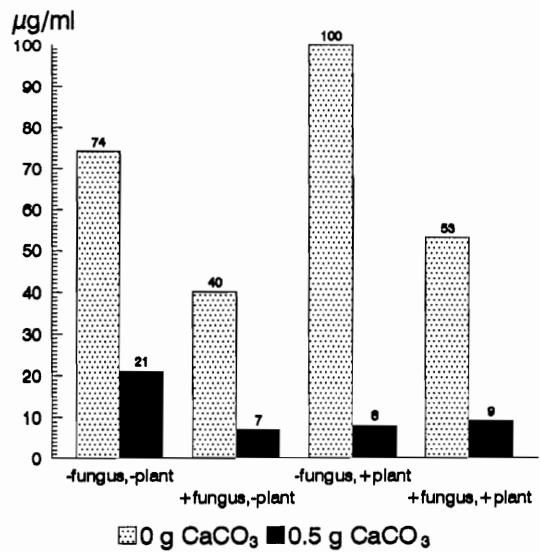
**Figure 7.** Mean solution Ca concentration in media samples of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



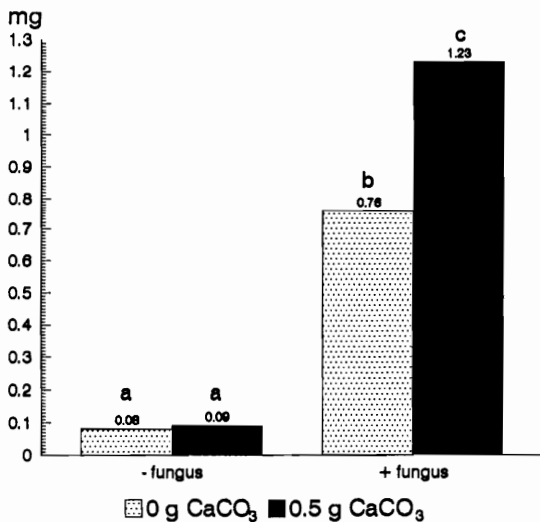
**Figure 8.** Mean solution Ca concentration including initial values prior to inoculation and after fungus action with no plant at 0 and 0.5 g CaCO<sub>3</sub>. See text for discussion.



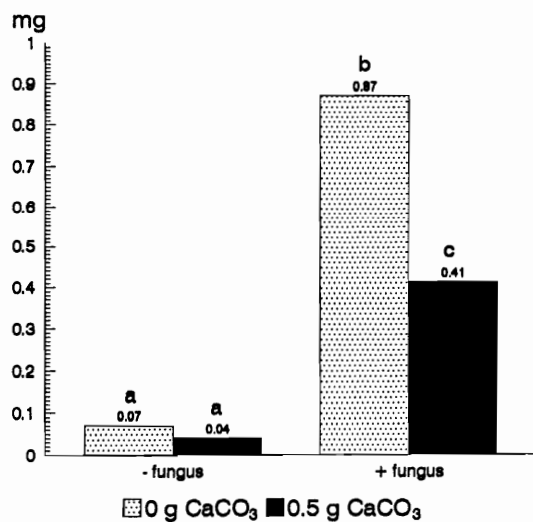
**Figure 9.** Mean solution P concentration in media samples of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



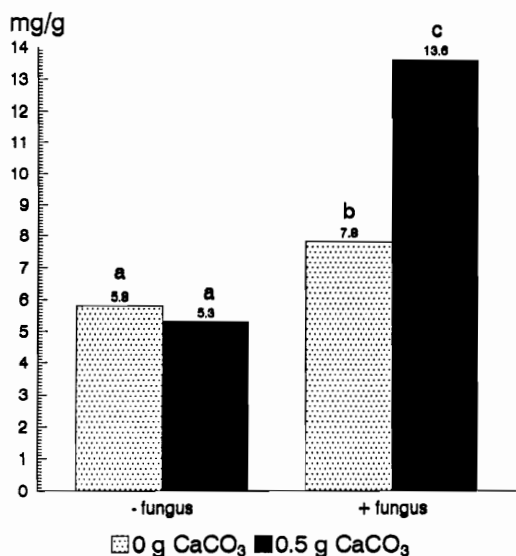
**Figure 10.** Mean solution P concentration including initial media values prior to fungal inoculation and planting of *E. diversicolor* at 0 and 0.5 g CaCO<sub>3</sub>. See text for discussion.



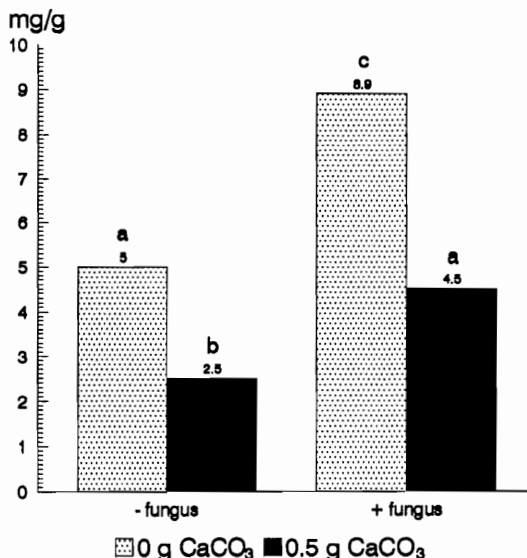
**Figure 11.** Mean total Ca in shoots of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



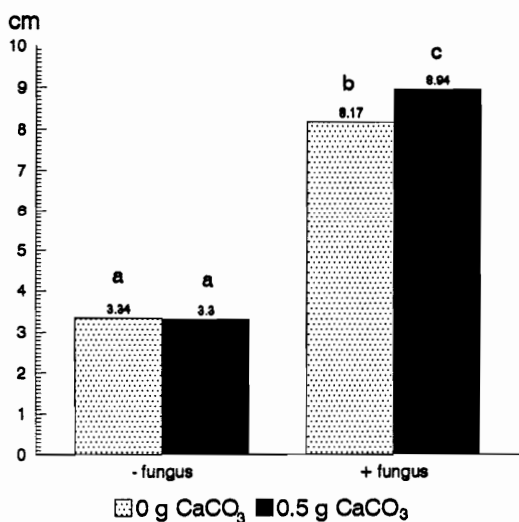
**Figure 12.** Mean total P of *Eucalyptus diversicolor* shoots with and without fungus associate *Hebeloma westraliensis* at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



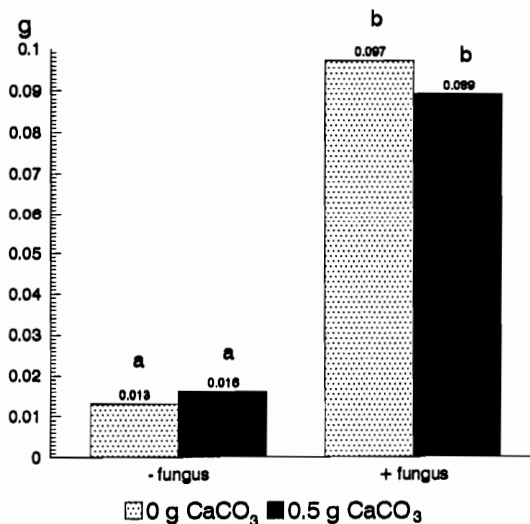
**Figure 13.** Mean Ca concentration in shoots of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



**Figure 14.** Mean P concentration in shoots of *E. diversicolor* with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different at  $\alpha=.05$  using Fisher's LSD test.



**Figure 15.** Mean height of *E. diversicolor* seedlings with and without the fungus associate *Hebeloma westraliensis* at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different using Fisher's LSD test.



**Figure 16.** Mean dry weight of *E. diversicolor* shoots with and without fungus associate at 0 and 0.5 g CaCO<sub>3</sub>. Bar values accompanied by the same letter are not significantly different using Fisher's LSD test.

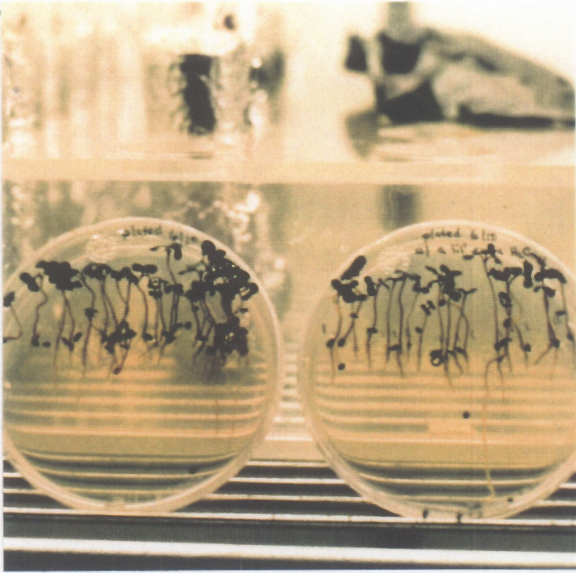


Plate 5.  
Axenic germination of E. diversicolor seeds.

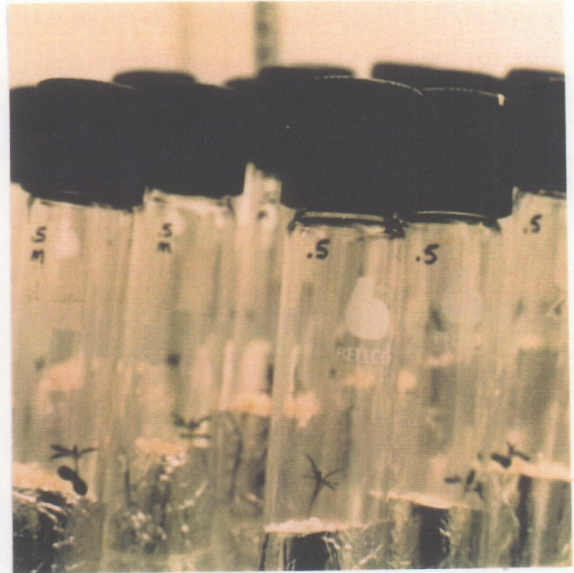


Plate 6.  
Growth chamber with synthesis tubes and transplanted E. diversicolor seedlings.

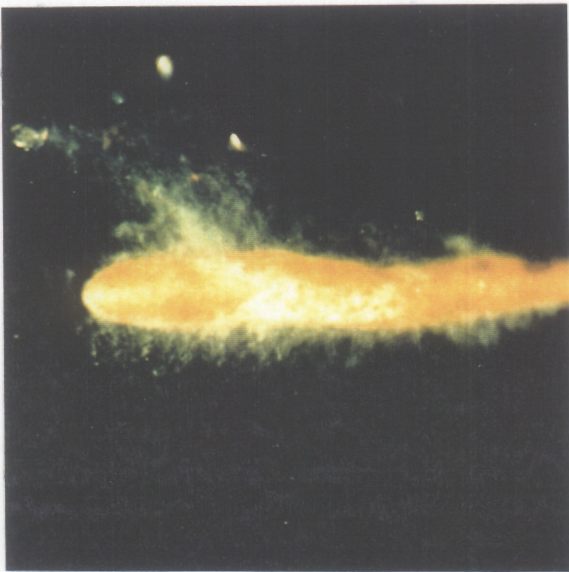


Plate 7.  
Mycorrhiza of E. diversicolor X H. westraliensis. Note emanating extra-matrical hyphae and lack of root hairs.

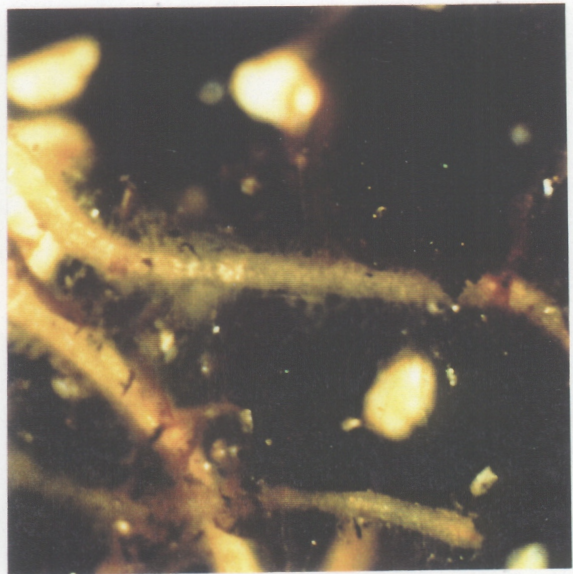


Plate 8.  
Nonmycorrhizal roots of E. diversicolor.

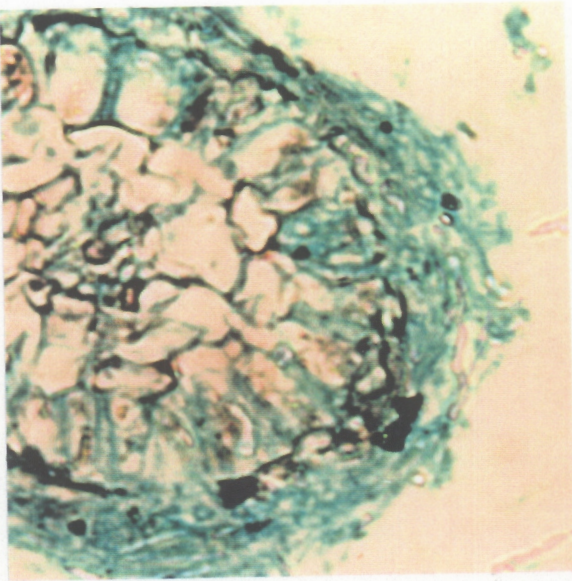


Plate 9.  
 Cross section of E. diversicolor X H. westraliensis mycorrhizal root. Mantle and Hartig net stained with Fast Green.

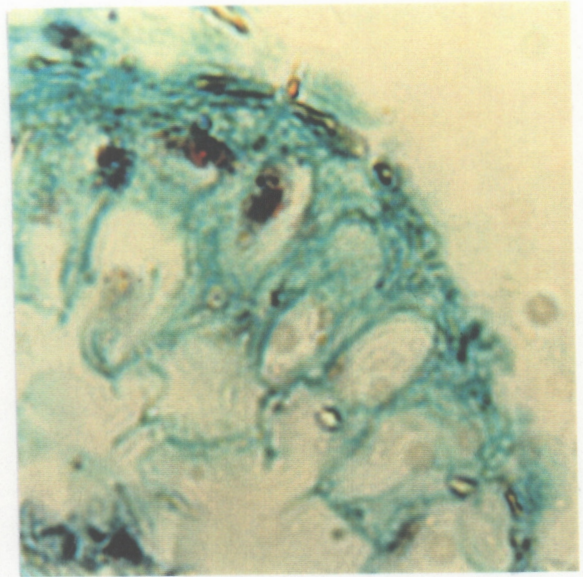


Plate 10.  
 Close up of cross section in Plate 9 showing Hartig net extending only between epidermal cells. Mantle and Hartig net stained with Fast Green.

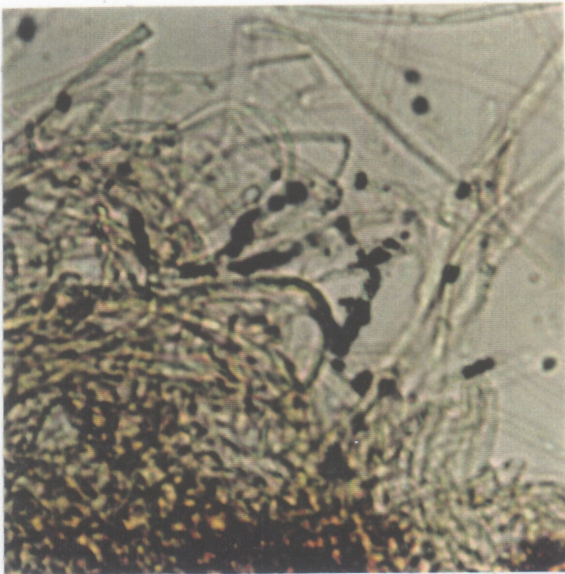


Plate 11.  
 Extra-matrical mycelium of E. diversicolor X H. westraliensis mycorrhiza. Ca oxalate crystals stained dark brown.

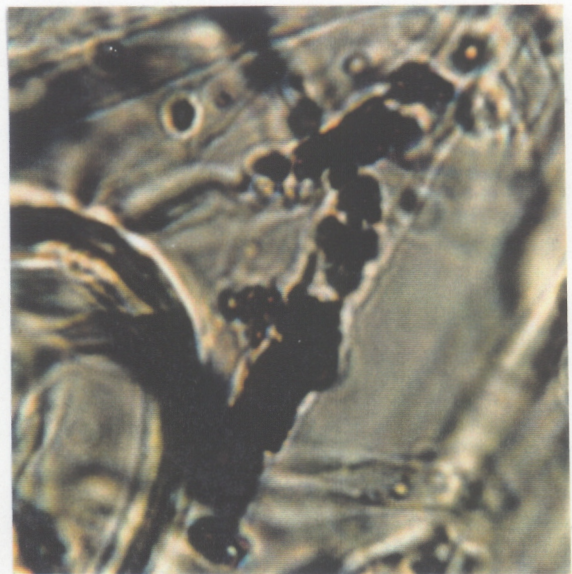


Plate 12.  
 Close up of extra-matrical hyphae in Plate 11 showing Ca oxalate crystals stained dark brown.

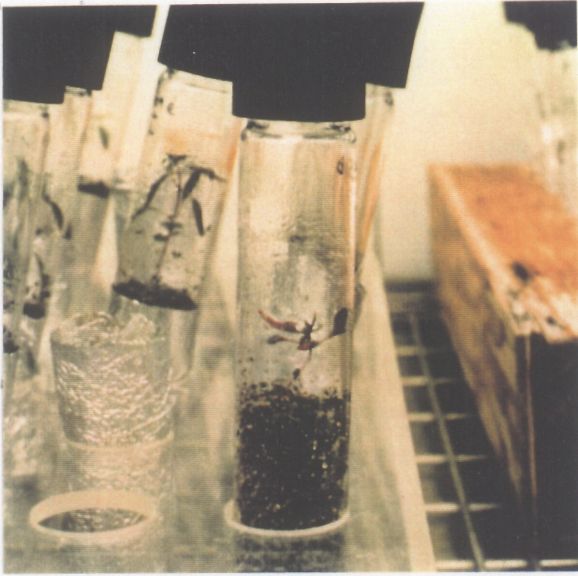


Plate 13.  
***E. diversicolor*** seedling without fungus associate in calcareous system. Note symptom of nutrient deficiency in leaf coloration.



Plate 14.  
***Eucalyptus*** sp. in plantation setting in Ecuador. Successful plantation establishment with ectomycorrhizal inoculated trees. (Photo by Duncan M. Porter).

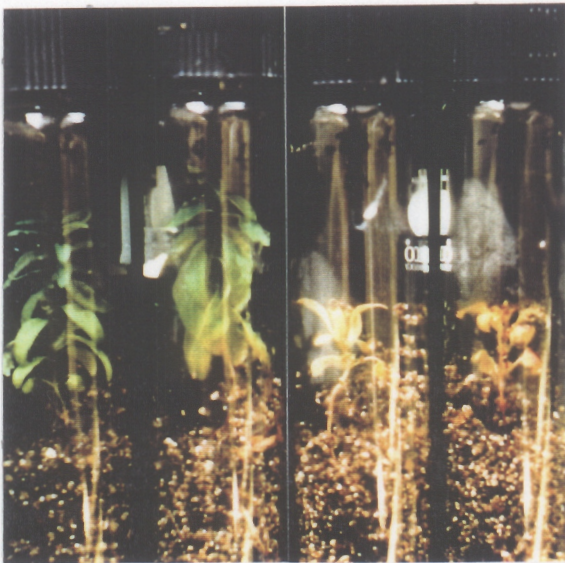


Plate 15.  
***E. diversicolor*** with no fungus associate in increasingly calcareous substrates. 0 g  $\text{CaCO}_3$  added on the left to 0.45 g  $\text{CaCO}_3$  added on the right.

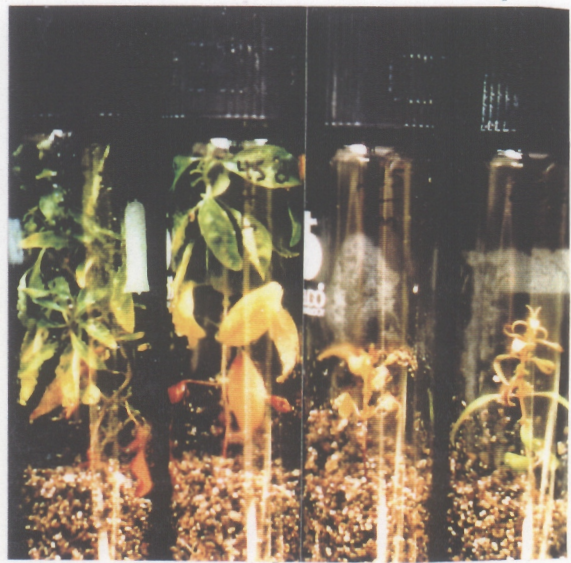
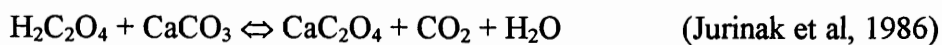


Plate 16.  
***E. diversicolor*** in calcareous system with fungus associate in two tubes on the left, without fungus associate in 3 tubes on the right.

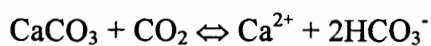
## Discussion

The current model of oxalate action in soil allows prediction of a number of plant and fungus responses. This model is thermodynamically sound and can help predict soil chemical equilibria reactions in which oxalate is involved. So long as adequate controls over soil solution nutrition are in place and solution nutrients are accurately measured, oxalate effects on the rhizosphere system can be predicted (Jurinak et al, 1986). One parameter that must be known in order to predict results based on this model is system pH. In most instances pH measurements reflect the  $H^+$  activity in the bulk solution and may not accurately represent the microenvironment of root or mycorrhizal surfaces. Confident pH measurements are difficult to obtain. Every effort was taken to insure accuracy in the solution pH reported in this study. Never-the-less, there is little doubt that the reproducibility of these results in absolute terms would be unlikely. This is unfortunate given the importance of pH as a driving force in chemical equilibrium models.

The increase in pH of nutrient solutions after growth of organisms in this study is likely due to the combination of a number of mechanisms. The oxalates produced and possibly exuded into the substrate can chelate  $Ca^{2+}$  from  $CaCO_3$ . This reaction forms crystals of  $CaC_2O_4$  and releases  $CO_2$ .

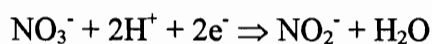


The product  $CO_2$  along with  $CO_2$  produced from fungus or plant respiration in the substrate can in turn act on  $CaCO_3$  in aqueous solutions to produce  $HCO_3^-$ , a very efficient proton scavenger (Bohn et al, 1985).



In this way, the production of oxalates and  $CO_2$  in the calcareous system actually contribute to a high equilibrium pH rather than to acidification.  $CaCO_3$  and  $HCO_3^-$  also act to buffer the system against further increase in pH. In addition, the nitrate metabolism

of plants and fungi consumes protons in nitrate and nitrite reduction. This action reduces the  $H^+$  activity in solution and pH rises.

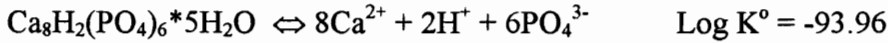
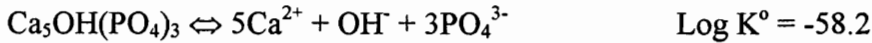
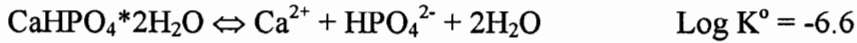


This process occurs in the cytosol but can have significant effects in the solution environment outside the root or hypha. The products of nitrate metabolism were not studied here. Their quantitative effects on pH in this system are not known.  $CO_2$  was also not measured in this system. While its effect in the closed glass synthesis tubes used in this experiment could be considerable, the magnitude of its effect is unknown. While pH measurements in the best of circumstances may be unreliable, the relative pH in the different treatment conditions in this study are helpful in explaining some of the results. The effects of pH on the variables studied in this experiment will be discussed as they pertain to each variable.

The effects of pH on soil solution P availability are strongly implied. At the moderate pH of the non-calcareous treatments  $Ca(H_2PO_4)_2 \cdot H_2O$  is water soluble. The defining reaction is:



The high P concentrations found in solution in this study in the non-calcareous system indicate that much of the added P was dissolved. At the pH of the calcareous treatments phosphorus released from this reaction would likely be precipitated from solution in any of several Ca phosphate phases. The more likely, in order of decreasing solubility are:  $CaHPO_4 \cdot 2H_2O$  (dicalcium phosphate),  $Ca_5OH(PO_4)_3$  (hydroxyapatite) and  $Ca_8H_2(PO_4)_6 \cdot 5H_2O$  (octacalcium phosphate). Over the short term, dicalcium phosphate and octacalcium phosphate can be adsorbed on the surfaces of  $CaCO_3$ . Over time all three would precipitate. The defining reactions are:



With free  $\text{Ca}^{2+}$  present in the calcareous system,  $\text{HPO}_4^{2-}$  maintained in solution and high pH, all of these reactions would be forced to the left and phosphate precipitated (Lindsay and Vlek, 1977; Sample et al, 1980). The reduced P in solution found in the calcareous treatments suggest much was bound in this way. Determinations of which Ca phosphate species were present and their relative concentrations were not made. But the assumption that in the calcareous system P is much less available is supported.

The difference in solution P measured in the non-calcareous treatment between the nonmycorrhizal and mycorrhizal treatments could be explained in part by the depletion of solution P by accumulated biomass. That which was assimilated by shoots was measured but much more was likely sequestered in below ground tissues. Mycorrhizae may accumulate significant P in the form of polyphosphate granules (Gerlitz and Werk, 1994). As below ground biomass was sacrificed for oxalate measurements the quantities of any nutrient sequestered there could not be measured.

The solution concentrations of P measured at harvest are all well above concentrations expected to be found in unfertilized mineral soil. Measured solution concentrations of P in the non-calcareous soil with no fungus present were nearly  $100 \mu\text{g ml}^{-1}$ . Even in the calcareous systems, representing the lowest P concentrations found at harvest, solution concentrations were above  $8 \mu\text{g ml}^{-1}$ . Soil solution P in natural soils will rarely get above  $0.3 \mu\text{g ml}^{-1}$  (Sample et al, 1980). The total P supplied by the readily soluble  $\text{KH}_2\text{PO}_4$  amounted to  $11.4 \mu\text{g ml}^{-1}$  in all treatments. Additional P supplied as  $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$  contributed  $133.9 \mu\text{g ml}^{-1}$ . ICP measures total P in the sample. It is possible that some of the P measured in these solutions was not dissolved and so not plant available. The filtration of these solutions may have allowed fine particulate (i.e.

precipitate) forms of P to remain in the samples. It can be assumed that, in relative terms, the higher levels of total P measured would also reflect more that was plant available. Since these levels are so high, relating the results of this study to natural situations is ill advised.

High levels of P fertility are often associated with reduction in the amount of mycorrhization between symbionts. It is thought that reduced P nutrition in the plant may contribute to carbohydrate leakage from the roots. This would in turn facilitate the growth of mycorrhizal fungi in the vicinity of roots and aid in infection (Harley and Smith, 1983). Mycorrhization as a percent of root tips was not measured directly in this study, but intact mycorrhizae were not lacking. In fact, the efficiency with which *H. westraliensis* formed mycorrhizae in this study was surprising. The first mycorrhizae were observed only 23 days after seedling transplantation and mycorrhizae observed macroscopically and sampled for Ca oxalate crystals were abundant throughout the experiment.

Another factor complicating solution P results is that solution concentrations of nutrients were taken as discreet measurements at the end of the experiment. Phosphate anions were being absorbed by the fungus and/or the plant throughout the experiment and concentrations were continually changing. Also, there is neither any indication of solution P concentration near the root or mycorrhiza surface, nor to what extent concentration gradients affected the osmotic pressures on fixed P. Given the reported ability of hyphae to increase P absorption rate (Bolan et al, 1987) and the limited extent to which nonmycorrhizal roots were able to explore the substrate volume, these unknowns are likely to be significant.

The hypothesis that mycorrhization allows increased availability and absorption of fixed soil P is supported here. An interesting observation was that in spite of levels of P higher than would be expected in soil solution, nonmycorrhizal seedlings exhibited poor growth and reduced concentrations of this element in shoots. It has been reported that a high Ca concentration in the solution around root absorptive surfaces decreases P activity

there. Hyphae from mycorrhizae are able to extend the absorptive surface area beyond the zone of Ca accumulation to areas where P is more available (Barrow, 1978). In addition, the increased absorption and utilization of Ca by mycorrhizae has been implicated in the increased solubility and absorption of P from Ca phosphate by the host plant (Ross, 1971). It is apparent from this study that mycorrhization provides facilitated absorption of phosphate both from fixed Ca phosphate and from that already available in solution.

Solution Ca concentrations were increased in the calcareous system and decreased by mycorrhizal action. Based on additive measurement of total plant shoot Ca and solution Ca concentration nearly all of the Ca present in the noncalcareous, nonmycorrhizal treatment was dissolved. This Ca was provided by  $\text{CaCl}_2$  and Ca phosphate monohydrate both of which are readily soluble at low pH. In the presence of the fungus, the decrease in Ca concentration in solution can partly be explained by sequestration of Ca in increased biomass. Ca accumulated in shoots was measured but that incorporated into below ground tissues was unaccounted for. As mycorrhizae can sequester large amounts of Ca in the form of Ca oxalate crystals (Graustein et al., 1977; Entry et al., 1992), the total Ca unaccounted for in or on tissues could be substantial. The soil  $\text{Ca}^{2+}$  unaccounted for could have been bound by  $\text{CaCO}_3$ , Ca phosphates or Ca oxalate.

The equilibrium dissolution of  $\text{CaCO}_3$  did not allow the maintenance of increased free  $\text{Ca}^{2+}$  in the nonmycorrhizal system than was provided by the  $\text{CaCl}_2$ . The equilibrium levels of  $\text{Ca}^{2+}$  were perhaps suppressed by precipitation with either phosphate or oxalate as both of these anions were increased in concentration in the same treatments. The action of the mycorrhizal fungus may have caused increased dissolution of  $\text{CaCO}_3$  in the mycorrhizal treatments. This is likely due to the action of oxalates, and perhaps other organic acids, on  $\text{CaCO}_3$  as they worked to dissolve this compound. In addition the increase in respiration  $\text{CO}_2$  in the mycorrhizal system likely worked to dissolve  $\text{CaCO}_3$  to produce  $\text{HCO}_3^-$  and  $\text{Ca}^{2+}$ . The likelihood of this mechanism is supported by the increased

pH of these treatments. Increases in Ca in solution of the mycorrhizal treatments also can be related to the increase in assimilation of this element in shoot biomass.

Oxalic acid extracted and titrated by  $\text{KMnO}_4$  increased in the calcareous system and increased further with symbiosis. The increase in production of oxalate by increased  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  and pH are well established both in plants (Francheschi and Horner, 1980) and in fungi (Cochrane, 1956; Hodgkinson, 1977). This holds true for ectomycorrhizal fungi as well (Lapeyrie and Bruchet, 1986). In this study oxalates were increased in below ground plant tissues in the calcareous system and also by the action of the mycorrhizal fungus. It is apparent that H. westraliensis is a much more active producer of oxalate than E. diversicolor. There is no evidence that the production of oxalate in symbiosis is anything but additive in this system. Efforts to correlate oxalate in the system to Ca and P in solution were complicated by nutrient unknowns in precipitates and below ground biomass. Regression analysis of all variables revealed a significant positive correlation between oxalate produced and plant height ( $R^2 = 0.43$ ,  $P < 0.0001$ ) and oxalate and plant shoot dry weight ( $R^2 = 0.43$ ,  $P < 0.0001$ ). These results while significant may not be important as they could not be substantiated by correlations with any other variable.

The use of perlite in this study as the substrate on which to base the nutrient solution provided important benefits. Perlite has no appreciable cation exchange capacity (CEC). Therefore nutrients in the medium would have either remained in solution, be absorbed by fungus or plant, or be precipitated out of solution. In the calcareous treatments some adsorption of phosphates on  $\text{CaCO}_3$  surfaces may have occurred. In preliminary experiments, peat and vermiculite was used as the substrate. Difficulties arising from unknown CEC and its effect on nutrient availability in essentially a hydroponic system made this mix less desirable. The contributions of decomposition products of peat also complicated the system as these by and large were unknown. One of the drawbacks to perlite substrate is large particle size. This restricted capillary rise of nutrient solution in the synthesis tubes and was of some concern. In the mycorrhizal treatments this seemed not to be a problem as the mycelium acted to increase the

capillarity of the substrate. In the treatments without the fungus associate the roots of young seedlings may not have been as effective in contacting solution nutrients due to their reduced ability to explore the resources throughout the substrate volume.

In order to increase the chances that measurable oxalates would be extracted from these treatments the entire rhizosphere was used in the extraction procedure. This included perlite and residual nutrient solution left after saturation extraction, all root tissue and all fungus tissue. The destructive methods of sampling for oxalates restricted analyses of nutrient concentrations in below ground tissues. Consequently, any P or Ca that was absorbed from solution and not passed on to shoots was not measured. It is thought that the amounts of plant and fungal nutrients lost to analysis for this reason are substantial. In mycorrhizae especially, P and Ca can be sequestered in large amounts. The formation and retention of polyphosphate granules in mycorrhizae are common and have been viewed as important to the mineral reserves of both the fungus and the host (Martin et al, 1983; Gerlitz and Werk, 1994). In addition, large amounts of  $\text{Ca}^{2+}$  have been found associated with ectomycorrhizal Hysterangium crassum in the form of Ca oxalate crystals (Graustein et al, 1977; Entry et al, 1992). It was thought that shoot nutrient concentrations could give a reliable estimate of relative total plant nutrition. Given that oxalates extracted and titrated in this experiment were on the low end of values obtained in work with other fungi, the decision to sacrifice below ground nutrient analyses in favor of total oxalates may have been fortuitous. Never-the-less, the loss of below ground nutrient analyses contributed to the nutrients unaccounted for and to the degree of speculation necessary to extrapolate on the distribution of nutrients in this system.

The effects of  $\text{CaCO}_3$  and mycorrhization treatments on seedling height and dry weight illustrate clear benefits of the symbiosis to plant growth both in calcareous and noncalcareous systems. The height of mycorrhizal seedlings in calcareous medium was significantly increased over those in the noncalcareous medium (Figure 15, p.85). This may be explained by the increased growth of H. westraliensis in calcareous media and the benefits to the host of increased fungal action. While results of mycorrhizal enhancement

of seedling growth may not be surprising, it serves to establish the original assumptions of this model, i.e. that mycorrhization enhances the growth of E. diversicolor in calcareous soils (Plate 16, p. 85).

In preliminary experiments the calcifuge nature of E. diversicolor without a fungus associate was dramatically evident (Plate 15, p. 85). In these studies in ammonium based medium and peat / vermiculite substrate, nonmycorrhizal seedlings exhibited good growth in noncalcareous media and poor growth and symptoms of nutrient deficiency in the calcareous system. In the present study nonmycorrhizal seedlings grew equally poorly in either the calcareous or noncalcareous systems. This may be partly related to the relative inability of young seedlings to access the nutrient resources throughout the entire volume of the perlite substrate. One of the presumed benefits of mycorrhization is the ability to better explore the substrate for nutrients (Tinker, 1978) and this could not be controlled for in this experiment. It is hoped that the creation of essentially a hydroponic system with no CEC would have made solution nutrients equally available throughout the substrate. The creation of zones of depletion of solution nutrients in the limited substrate volume occupied by non-mycorrhizal roots cannot be discounted as a factor in the reduced growth of these seedlings (Owusu-Bennoah and Wild, 1980).

There are likely several mechanisms at work to explain the reduced growth of non-mycorrhizal seedlings. Any of the factors that would limit calcicole species in calcareous soil may be relevant here. Lapeyrie and Chilvers (1985) suggested that the calcifuge habit of E. dumosa may be related more to high pH than to  $\text{Ca}^{2+}$  concentration in calcareous soil. High pH would likely disrupt membrane electrochemical potentials and consequently the passive and facilitated movement of solution nutrients into roots. Such a pH effect could explain much of the differential nutrient concentrations in the calcareous treatments in which there was no fungus to provide a sheltering microenvironment for the roots.

The mycorrhizal association allowed increased absorption of P and Ca by the plant from both the calcareous and noncalcareous solutions. This is evidenced by the increase in

total P and Ca levels in mycorrhizal seedlings and by increased concentrations of these nutrients in seedlings at harvest. Dilution effects of gram dry weight on nutrient concentrations were not particularly evident in this study. Jarrell and Beverly (1981) suggest that definitive evidence that a factor is increasing the availability of a nutrient for plant uptake is not only increased nutrient levels in the plant but increased nutrient concentrations in tissues as well. Based on this assumption it is clear that mycorrhization in this study increased the availability of both P and Ca to E. diversicolor.

The seedling Ca concentrations of the nonmycorrhizal treatments were not significantly different in either the calcareous or noncalcareous systems. When these results are compared to solution Ca concentration in these treatments it appears that, in the calcareous medium, non-mycorrhizal seedlings were able to employ some mechanism to limit the absorption of Ca. The active Ca efflux mechanism often associated with plants grown in high Ca soil (Kinsel, 1983; Rorison and Robinson, 1984) may be operating here. One of the characteristics of calcicole plants is the ability to tolerate higher levels of Ca in their tissues. In calcifuge plants the ability to reduce tissue concentrations of this element by way of active efflux Ca pumps may be a mechanism by which these plants attempt to tolerate these conditions at some expense to their energy budget (Kinsel, 1983). Gigon and Rorison (1972) found that acidophilic plants growing in  $\text{NO}_3^-$  media absorbed increased amounts of Ca and had reduced growth at high pH. The relationship of these suggestions to the high levels of Ca found in the vigorous mycorrhizal plants is unclear.

Seedling Ca concentration increased with mycorrhization and did so dramatically in the calcareous system. These results appear related to the solution concentrations of Ca in these treatments. The action of the mycorrhizal fungus apparently enhanced the solubility of  $\text{CaCO}_3$  or Ca phosphate allowing increased absorption of Ca by the plant. This may be partly due to increased respiration  $\text{CO}_2$  in these treatments and the action of this on  $\text{CaCO}_3$  to release  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ . The portion of the model of oxalate action in soils that predicts increased precipitation of solution  $\text{Ca}^{2+}$  is not supported by these data.

The findings of Azcon and Barea (1992) on the ability of mycorrhizal oxalate to restrict the absorption of Ca by a calcifuge plant were based on observations of a VAM association. Evidence of this ability in VAM plants has been supported by Kothari et al. (1990) and in an endomycorrhizal-ectomycorrhizal succession in *E. dumosa* (Lapeyrie and Chilvers, 1985). The differential abilities of VAM fungi and ectomycorrhizal fungi to use this mechanism are not known. It is apparently not one of the mechanisms by which ectomycorrhizae enhanced plant tolerance to calcareous soil in this study. In fact, it does not appear that intolerance to calcareous conditions is due to excess  $\text{Ca}^{2+}$  in the solution. The suggestion of Lapeyrie and Chilvers (1985) that high pH and  $\text{HCO}_3^-$  concentrations, and limited P availability are the limiting factors to eucalypt growth in these soils is more likely applicable here. These limiting factors of calcifuge plant tolerance to calcareous soil are supported by Rorison (1980).

The development of more sensitive methods of oxalate quantitation will allow smaller homogenized subsamples reserved for this purpose and remaining material for below ground tissue nutrient analyses. The formation of Ca oxalate crystals on the hyphae or mantle of mycorrhizae suggests that these are substantial sites of oxalate accumulation. It is necessary to assay this biomass for oxalate in order to get reliable estimates of total oxalate in the system. Perhaps reserving residual soil and associated extra-matrical hyphae for oxalate analysis along with one half of the dry matter of roots and mycorrhizae (taking care to get representative tissue from young and old roots) and analyzing the other half of root systems separately for nutrients would alleviate some of the drawbacks to the method of oxalate analysis used in this study.

Oxalate in the system was not a good predictor of P solubility or of P uptake by the plant. Oxalate production was correlated with P availability in the calcareous system. While this may be important to the nutrition of mycorrhizal plants in calcareous soil, this evidence is too scanty to support the prediction model of oxalate effects on soil P. Oxalate was not negatively correlated with either solution Ca or plant uptake of Ca as

suggested by others (Jurinak et al, 1986; Knight et al, 1992). In fact higher oxalate levels were associated with higher free Ca in solution and in plant shoots.

The mycorrhizal enhancement of eucalypt growth in calcareous soil is supported by this study. This can be attributed in part to increased P absorption by the mycorrhizal plants. Also supported is the higher oxalate production in the calcareous systems by the fungus and the plant. The mycorrhizal mechanisms involved in the enhanced P nutrition in this study are not clear. While oxalate produced in the system may be a contributing factor, evidence of its primary role is not presented here. Experimental difficulties inherent in symbiosis studies have complicated efforts to draw conclusions about any of the effects of any particular hypothesized mechanism.

The addition of L-threonine to inhibit oxalic acid production by the fungus as demonstrated in Chapter 2, may be a means by which the effects of fungal oxalic acid production in symbiosis can be measured. It is important to separate the effects of fungal-produced oxalic acid on the rhizosphere and the symbiosis from other hypothesized mechanisms of mycorrhizal enhancement of plant nutrition. This control over mycorrhizal action has not been shown. Never-the-less, the ability of H. westraliensis to enhance the growth of E. diversicolor in calcareous soil, by whatever mechanisms, is certainly supported.

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# Appendix

**Table 1.**

**Mean dry weights, oxalate produced and oxalate produced per gram dry weight of mycelium samples of *Hebeloma westraliensis* grown in liquid media.<sup>1</sup>**

Treatment		pH <sup>2</sup>		Mean dry weight <sup>3</sup>		Mean oxalate per sample <sup>4</sup>		Mean oxalate per gram <sup>5</sup>	
CaCO <sub>3</sub> (g)	L-thr. <sup>6</sup>	I	II	g	s	µg	s	µg/g	s
0	-	5.5	5.8	0.214 <sub>a</sub>	0.009	480.20 <sub>a</sub>	53.13	2245.2 <sub>a</sub>	270.5
0	+	5.5	5.7	0.209 <sub>a</sub>	0.005	452.27 <sub>a</sub>	48.67	2161.1 <sub>a</sub>	211.8
0.25	-	6.5	7.3	0.434 <sub>b</sub>	0.016	747.02 <sub>b</sub>	39.43	1720.6 <sub>b</sub>	93.2
0.25	+	6.5	7.1	0.384 <sub>b</sub>	0.069	519.78 <sub>c</sub>	35.03	1384.4 <sub>c</sub>	206
0.5	-	7.0	7.8	0.534 <sub>c</sub>	0.055	769.46 <sub>b</sub>	51.36	1451.7 <sub>d</sub>	166.4
0.5	+	7.0	7.6	0.490 <sub>c</sub>	0.080	564.78 <sub>c</sub>	18.49	1181.7 <sub>cd</sub>	220.4

<sup>1</sup> values for means in each column followed by the same letter were not significantly different at  $\alpha=0.05$  according to Fisher's Least Significant Difference Test for differences between the means.

<sup>2</sup> Mean pH of liquid media before inoculation (I) and after harvest (II). See text for standard deviations and statistical analysis.

<sup>3</sup> mean dry weight of mycelium samples at harvest, s=standard deviation.

<sup>4</sup> mean oxalate extracted and titrated with KMnO<sub>4</sub>, s=standard deviation.

<sup>5</sup> mean oxalate in µg per gram dry weight of mycelium.

<sup>6</sup> absence (-) or presence (+) of 1 mM L-threonine in culture medium.

**Table 2.**

**Mean solution pH, Ca and P concentration and titratable oxalate extracted from substrate of E. diversicolor with and without the mycorrhizal fungus associate H. westraliensis.<sup>1</sup>**

Treatment		Solution pH <sup>3</sup>		Solution Ca (ppm)		Solution P (ppm)		Oxalate <sup>4</sup> (µg)	
CaCO <sub>3</sub>	Fungus <sup>2</sup>	I	II	Mean	s	Mean	s	Mean	s
0	-	5.4	5.6	107.8 <sub>a</sub>	5.0	99.68 <sub>a</sub>	9.48	112.5 <sub>a</sub>	17.0
0.5	-	7.1	7.4	134.0 <sub>b</sub>	19.17	8.06 <sub>b</sub>	1.05	199.8 <sub>b</sub>	46.3
0	+	5.4	5.7	39.76 <sub>c</sub>	3.44	52.91 <sub>c</sub>	8.92	229.5 <sub>c</sub>	10.03
0.5	+	7.1	8.0	84.38 <sub>d</sub>	9.35	8.51 <sub>b</sub>	0.96	395.0 <sub>d</sub>	83.7
the following treatments are with no seedling present and were not analyzed statistically: <sup>5</sup>									
0	-,-	5.4		69.96	5.9	73.54	6.1		
0.5	-,-	7.1		71.28	3.9	20.62	9.9		
0	+,-	5.4	5.7	34.25		40.25		90.0	
0.5	+,-	7.1	7.9	81.45		7.4		274.5	

<sup>1</sup> values for means in the same column followed by the same letter were not significantly different at  $\alpha=0.05$  according to Fisher's Least Significant Difference Test for differences between the means.

<sup>2</sup> absence(-) or presence (+) of the mycorrhizal fungus associate Hebeloma westraliensis.

<sup>3</sup> pH of nutrient solution before inoculation (I) and after harvest (II) of treatment organisms.

<sup>4</sup> oxalate in µg extracted from bulk substrate and titrated with KMnO<sub>4</sub>,

<sup>5</sup> as mention in the text, these data are for discussion purposes only; they were not analyzed statistically due to small sample size; (-, -)=no fungus, no plant; (+, -)=with fungus, no plant.

**Table 3.**

**Mean seedling height and dry weight of *Eucalyptus diversicolor* with and without the mycorrhizal fungus associate *Hebeloma westraliensis*.<sup>1</sup>**

Treatment		Seedling Height (cm)		Seedling Dry Weight (g)	
CaCO <sub>3</sub> (g)	Fungus <sup>2</sup>	Mean	s	Mean	s
0	-	3.34 <sub>a</sub>	0.73	0.013 <sub>a</sub>	0.005
0.5	-	3.30 <sub>a</sub>	0.48	0.016 <sub>a</sub>	0.004
0	+	8.17 <sub>b</sub>	0.83	0.097 <sub>b</sub>	0.012
0.5	+	8.94 <sub>c</sub>	1.03	0.089 <sub>b</sub>	0.007

<sup>1</sup> values for means in each column followed by the same letter were not significantly different at  $\alpha=0.05$  according to Fisher's Least Significant Difference Test for differences between the means.

<sup>2</sup> absence (-) or presence (+) of the mycorrhizal fungus associate *Hebeloma westraliensis*.

**Table 4.**

**Mean total seedling Ca and P and mean seedling Ca and P concentration in *E. diversicolor* with and without the mycorrhizal fungus associate *H. westraliensis*.<sup>1</sup>**

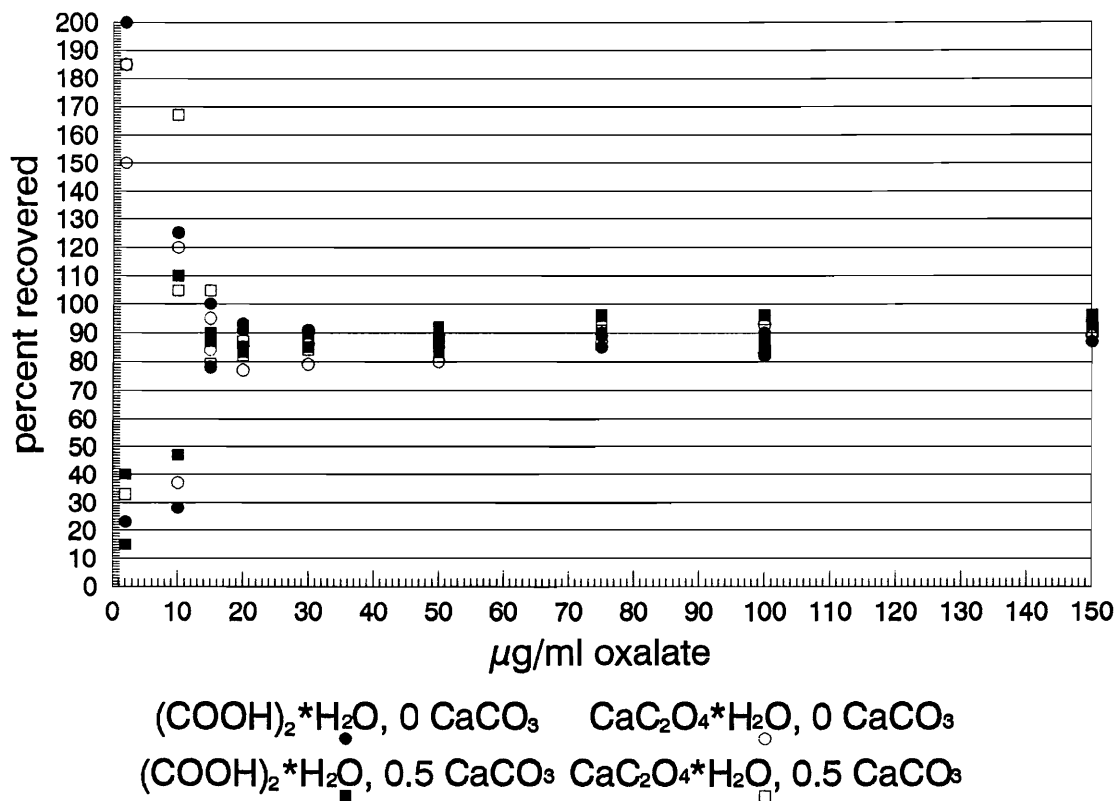
Treatment		Total Seedling Ca ( $\mu\text{g}$ )		Seedling Ca conc. <sup>3</sup> ( $\mu\text{g/g}$ )		Total Seedling P ( $\mu\text{g}$ )		Seedling P conc. <sup>4</sup> ( $\mu\text{g/g}$ )	
CaCO <sub>3</sub> (g)	Fungus <sup>2</sup>	Mean	s	Mean	s	Mean	s	Mean	s
0	-	83.40 <sub>a</sub>	23.78	5759.0 <sub>a</sub>	964.5	68.97 <sub>a</sub>	3.70	4992.7 <sub>a</sub>	550.6
0.5	-	86.80 <sub>a</sub>	14.82	5268.1 <sub>a</sub>	830.0	37.59 <sub>a</sub>	5.26	2467.7 <sub>b</sub>	601.5
0	+	761.22 <sub>b</sub>	105.0	7814.1 <sub>b</sub>	1052.3	874.89 <sub>b</sub>	157.16	8883.2 <sub>c</sub>	1241.3
0.5	+	1226.1 <sub>c</sub>	195.0	13,646 <sub>c</sub>	1728	405.01 <sub>c</sub>	119.36	4526.8 <sub>a</sub>	1189.5

<sup>1</sup> values for means in each column followed by the same letter were not significantly different at  $\alpha=0.05$  according to Fisher's Least Significant Difference Test for differences between the means.

<sup>2</sup> absence (-) and presence (+) of the mycorrhizal fungus associate *H. westraliensis*.

<sup>3</sup> mean Ca in  $\mu\text{g}$  per gram dry weight of shoot.

<sup>4</sup> mean P in  $\mu\text{g}$  per gram dry weight of shoot.



**Figure 17.**

**Standard curve of oxalate extraction and titration with  $\text{KMnO}_4$ .**

These values obtained by incubating known oxalate concentrations in calcareous and noncalcareous media. Standards were based on oxalate in the form of 10% oxalic acid dihydrate and Ca oxalate monohydrate added to liquid media. Extraction and titration method of Bateman and Beer (1965) was used.

## Reagents for oxalate extraction and titration

1. Phosphoric tungstate reagent: 24 g Na tungstate dissolved in 600 ml ddH<sub>2</sub>O. Add 40 ml concentrated phosphoric acid (specific gravity = 1.75), dilute to 1 L with ddH<sub>2</sub>O.
2. CaCl<sub>2</sub> reagent: dissolve 25 g anhydrous CaCl<sub>2</sub> in 500 ml of 50% (v/v) glacial acetic acid. Add this solution to a solution of 330 g of Na acetate in 500 ml ddH<sub>2</sub>O.
3. Wash solution: 5% (v/v) acetic acid kept over (saturated) Ca oxalate at room temperature for 24 hours. Shake periodically. Filter before use.
4. 4 N (ca. 10%) sulfuric acid: Add 111.1 ml concentrated H<sub>2</sub>SO<sub>4</sub> to 500 ml ddH<sub>2</sub>O, dilute to 1 L with ddH<sub>2</sub>O.
5. 1 M HCl: Add 82.64 ml concentrated HCl (12.1 N) to 500 ml ddH<sub>2</sub>O. Dilute to 1 L with ddH<sub>2</sub>O.
6. 0.01 N KMnO<sub>4</sub>: 10 ml, 0.1 N KMnO<sub>4</sub> solution. Dilute to 100 ml with ddH<sub>2</sub>O.

## Dehydration and infiltration procedure for mycorrhizae fixation

Tissues to be sectioned should be trimmed to the size desired prior to this sequence of steps. It is important to remove all traces of substrate, as even tiny pieces will nick the microtome knife. For this reason, samples from outside soils should be sectioned with a razor blade rather than the microtome.

<u>Solution</u>	<u>Concentration</u>	<u>Soaking time</u>
1. EtOH / H <sub>2</sub> O	30 % / 70%	2 hours
2. EtOH / H <sub>2</sub> O / TBA	45 / 50 / 5	2 hours
3. EtOH / H <sub>2</sub> O / TBA	50 / 30 / 20	2 hours
4. EtOH / H <sub>2</sub> O / TBA	50 / 20 / 30	2 hours
5. EtOH / H <sub>2</sub> O / TBA	50 / 10 / 40	2 hours
6. EtOH / TBA	50 / 50	2 hours
7. EtOH / TBA	25 / 75	16 hours
8. TBA	100	2 hours
9. TBA	100	16 hours
10. TBA	100	16 hours
11. TBA / Parafin oil	50 / 50	24 hours
12. Parafin oil	100	24 hours
13. Parafin oil / Paraplast	50 / 50	24 hours
14. Paraplast	100	24 hours
15. Paraplast	100	24 hours
16. Paraplast (optional)	100	24 hours

Embed in Paraplast.

TBA = Tert. Butyl Alcohol

Mycorrhizae stored in FAA can enter this sequence at step #4.

## Safranin-O / Fast Green Staining of fixed cross sections

After "staining schedule A" in O.K. Miller's lab procedure notebook.

Note: Xylol = Xylene (dimethyl-benzene, mixture of ortho, meta and para. It's probably carcinogenic).

	<u>Reagent</u>	<u>Soaking Time</u>
1.	Xylol	5 min.
2.	Xylol	5
3.	Xylol	5
4.	100% EtOH	5
5.	100% EtOH	5
6.	95% EtOH	5
7.	Safranin-O in 95% EtOH	30 min.
8.	95% EtOH	dip
9.	95% EtOH	dip
10.	Fast Green	10-15 seconds
11.	95% EtOH	dip and quick exam. More green or red if necessary.
12.	Clove oil / EtOH / Xylol (50 / 25 / 25)	5 min.
13.	Xylol / EtOH (50 / 50)	5 min
14.	Xylol	15 min
15.	Xylol	20 min
16.	Mount in permanent mount solution. Cover with cover slip and weight down overnight on level surface.	

# Vita

Gregory Kent Eaton was born October 22, 1959 in Winchester, Virginia. He is the oldest of three children of Doris Ray and Donald Starritt Eaton. He grew up in Giles and Montgomery Counties in southwestern Virginia where he was active in Boy Scouts of America and the high school Jazz Band as a guitarist.

Greg attended Virginia Tech as a Psychology major from 1978 to 1981 before resigning to travel and pursue musical interests. From 1983 to 1990 he was employed in various capacities in the Landscape Horticulture industry. It was in this vocation that a strong interest in plant science developed. From 1987 to 1990 he taught Landscape Horticulture to economically disadvantaged youth in the Job Corps program in Monroe, Virginia. In this job he discovered an aptitude and a joy in teaching.

He returned to Virginia Tech in 1990 to pursue a Bachelors degree in Horticulture. This was completed in 1992. He was married in May, 1992 to Suzanne Rozgonyi and the same year entered the Masters degree program in Biology. He worked in the Mycology laboratory of Dr. Orson K. Miller, Jr. studying the physiological ecology of mycorrhizal symbiosis.

His thesis is titled "Oxalic acid production by the ectomycorrhizal fungus Hebeloma westraliensis and its role in the nutrient acquisition and growth of Eucalyptus diversicolor in calcareous soil." He received his M.S. in Biology in January, 1995. Greg and his wife relocated to New England to continue graduate studies.

