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Canada

VESICULAR-ARBUSCULAR MYCORRHIZAE AND BASE CATION FERTILIZATION IN SUGAR MAPLE (ACER SACCHARUM MARSH L.)

by

Margaret Anne Cooke

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

in the
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Canada

Short Title

Vesicular-Arbuscular Mycorrhizae of Sugar Maple

PREFACE

This thesis consists of three major sections which have been or will be published elsewhere and which are co-authored by the two Supervisors of the candidate, and a general introduction reviewing the literature, and an overall conclusion section.

This dissertation is in accordance with the Guidelines Concerning Thesis Preparation as published by the Faculty of Graduate Studies and Research of McGill University which states:

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All the work involved in the present thesis was the responsibility of the candidate. Paul Widden and Ivan O'Halloran were thesis supervisors, consequently they served as co-authors on the manuscripts presented in this document.

ABSTRACT

Under field conditions, vesicles were the most frequently observed mycorrhizal structures in sugar maple, while greenhouse grown seedlings formed more arbuscules. Seasonal fluctuations of vesicular-arbuscular mycorrhizae existed. Mycorrhizal associations formed within 30 days in the greenhouse. Arbuscules were usually formed from hyphal coils and occasionally from linear hyphae spreading from cell to cell. Degenerating arbuscules were not observed. The addition of basic cations increased the number of vesicles formed and decreased the overall infection rates and seedling growth. The uptake of calcium, magnesium, and nitrogen decreased, and potassium uptake increased as fertilization rates increased. Positive correlations existed between the incidence of arbuscules and plant growth and health and between the incidence of arbuscules and the uptake of calcium, magnesium, nitrogen and phosphorus, and with the uptake ratios of these elements with potassium. This suggests that vesicular-arbuscular mycorrhizae may in some way be regulating ionic balance in these seedlings.

RESUME

En habitat naturel, les vésicules ont été les structures endomycorhiziennes les plus fréquemment observées en association avec l'érable à sucre, alors que les jeunes plants cultivés en serre ont formé plus d'arbuscules. Des fluctuations saisonnières étaient présentes pour les endomycorhizes à vésicules et arbuscules. Dans la serre, les endomycorhizes se sont développées dans un délais de 30 jours. Les arbuscules ont été généralement formés à l'extrémité d'un hyphe spiralé, et occasionnellement à partir d'un hyphe linéaire qui se propageait de cellule en cellule. Aucune dégénérescence arbusculaire n'a été observée. L'addition de cations basiques a stimulée la formation de vésicules et a diminuée le taux d'infection total ainsi que le développement des jeunes plants. L'augmentation des taux de fertilisation a provoquée une diminution du contenu en calcium, en magnésium et en azote, alors que le contenu en potassium a augmenté. Il y avait une corrélation positive entre la fréquence des arbuscules et: le taux de croissance des plantes, ainsi que l'absorption de calcium, de magnésium, d'azote et de phosphore. De même, il y avait une corrélation positive entre la fréquence des arbuscules et le taux d'assimilation de ces éléments minéraux en rapport avec celui du potassium. Ceci suggère que les endomycorhizes à vésicules et arbuscules contribuent en quelque sorte à l'équilibre nutritionel de l'érable à sucre.

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CHAPTER 1

GENERAL INTRODUCTION

Increasing attention is being given to the effects of acid inputs to ecosystems caused by atmospheric pollution. While precipitation is normally acidic, concern about acid precipitation focuses on the increased quantities of sulfur and nitrogen oxides, stemming mainly from industrial emissions and fuel combustion, entering ecosystems (Fritze, 1991). Regions of southeastern Canada and northeastern United States are at particular risk since the flow of air steming from the industrial southern U.S. follows the Mississippi Valley northward, and is deposited in these regions of the continent (Krebs, 1988). These air masses carry with them heavy loads of sulfuric and nitric acids (Krebs, 1988). The issues surrounding acid precipitation range from concerns over human health, lake acidification, soil depletion and forest decline.

1. Forest Decline

Much consideration has been given to the problem of forest decline or die-back in the past several decades, in Europe and North America, in both coniferous and deciduous forests. The characteristic symptoms of decline include canopy thinning due to decreased foliage in the tree crown, decreased leaf size, death of old leaves and needles, discoloration of foliage, and early leaf abscision (Hendershot and Jones, 1989). In Europe coniferous trees such as Norway spruce (*Picea abies* (L.) Karst.), silver fir (*Abies alba* Mill.), and the broadleafed European beech (*Fagus sylvatica* L.) have been most affected, while in Canada concern is focused on forest decline of fir (*Abies* spp.), red spruce (*Picea rubens* Sarg.) and sugar maple (*Acer saccharum* Marsh.) (Jones, 1990).

1.1. Soil Acidification

A number of hypotheses have been proposed as to the causes of forest decline, and while none have been totally accepted, the acid deposition-soil acidification hypothesis is being given increasing attention. A common factor seen in declining forests is the presence of nutrient deficiencies or imbalances in forest soils, which is reflected in the tree foliage (Spankie, 1991). With an increase in acid deposition, accelerated leaching of soil nutrients, which includes cations such as calcium (Ca), magnesium (Mg) and potassium (K) occurs in the uppermost soil horizons as a result of displacement by hydrogen ions. This leads to an increased mobilization of potentially toxic cations such as aluminum (Al), iron (Fe) and manganese (Mn), which are solubilized by the higher hydrogen ion concentrations (Matzner *et al*, 1983; Fritze, 1991). While this process occurs naturally, the concern is over the increased rates of cation loss compared to the rates of cation input from such sources such as litter decomposition and deposition (i.e. rain and dust) (Fritze, 1991). This discrepancy causes a net loss of base cations from the soil, thus limiting essential nutrient availability to the tree.

1.2. Sugar Maple Decline

As one of the most important hardwood species in northeastern North America, sugar maple is used for lumber as well as maple syrup production. In many regions, these sugar maple forests are in a state of decline (Hendershot and Jones, 1989), and this decline has been linked to acid deposition which has resulted in poor mineral nutrition (Bernier and Brazeau, 1988a, 1988b, 1988c; Hendershot and Jones, 1989; Paré and Bernier, 1989a).

1.2.1. Québec Appalachians

Several authors have established a direct link between sugar maple decline and nutrient deficiencies. Bernier and Brazeau (1988a, 1988b) examined the nutrient status of trees in 45 healthy and declining sugar maple stands in the Appalachian Mountains in southeastern Québec, where they found high negative correlations between stand decline and foliar K. Potassium deficiency was sometimes concomitant with acute phosphorus deficiencies. In some individual sites sugar maple decline was additionally associated with foliar deficiencies of nitrogen (N), P, or Ca. (Bernier and Brazeau, 1988a, 1988b). Paré and Bernier (1989a) determined that the acute P deficiencies observed by Bernier and Brazeau (1988a, 1988b) occurred in soils of moderate acidity with a mull humus, while adequate P nutrition was observed in mor-type soils which were more acidic. The results of Bernier and Brazeau (1988a, 1988b) are further substantiated by Hendershot (1991) who examined different declining maple stands in the same general area of southeastern Quebec (Beauce-Megantic). Out of nine sites studied, foliar K was deficient in all, Ca was deficient in two and Mg was deficient in only one site (Hendershot, 1991).

1.2.2. Québec Lower Laurentians

In contrast to the Appalachian sites, Mg deficiencies were observed in declining sugar maple forests in the Lower Laurentian Mountains northwest of Québec City (Bernier and Brazeau, 1988c). Trees in this area exhibited visual symptoms of Mg-deficiency and observations showed that soil Mg was also low. In some sites, foliar K, Ca and sometimes N were found to be at low concentrations but not enough for the trees to show visual symptoms of deficiency. Potassium in these sites was consistently high.

In the Lower Laurentian Mountains northwest of Montréal, Hendershot and Lalonde (1988) and Spankie (1991) showed foliar K, Mg and Ca deficiencies in sugar maple stands exhibiting moderate decline symptoms. Nutrient imbalances involving Ca and K, Al and Ca, and Al and Mg were also reported in these studies. Close to their northern limit (Hosie, 1973), sugar maple forests here receive precipitation with an average pH of 4.3 (Svatek, 1988).

Differences in deficiencies between the Appalachian (K) and Laurentian (Mg, Ca, K) sites reflect differences in the bedrock from which the soils were derived (Bernier and Brazeau, 1988c). Laurentian soils are derived mainly from granite and syenite which contain relatively little Mg, while forests in the Appalachians are derived from sandstones, shales, slates, and mafic rock, which are rich in Mg but relatively poor in K (Bernier and Brazeau, 1988c). Thus, nutrient deficiencies in declining forests seem to vary with location and soil type, and there is an indication from these studies that nutrient deficiencies or imbalances exist prior to the onset of the most severe symptoms of dieback.

2. Forest Fertilization

In both Europe and North America attempts at fertilization of declining forests with the nutrient(s) found to be deficient have led to mixed results.

2.1. Europe

In a beech and spruce stand in Germany, Matzner et al, (1983) found mixed results fertilizing with N and K and with liming. Although an increased Ca/Al ratio in the soil solution was obtained, soil nitrate concentration increased. In a beech stand in the Black Forest of Germany, trees exhibiting severe Mg deficiency were fertilized with $MgSO_4$ (Ende and Zöttl, 1991). Within nine weeks, foliar Mg content increased 300%. Phosphorus and K concentrations were found to decrease but not to the point of becoming deficient, and foliar sulfate (obtained from fertilizer) was found to increase (Ende and Zöttl, 1991). Experiments in a pine forest in Norway revealed that 'acid rain' treatments decreased the foliar concentrations of Ca, Mg and Mn, while K concentrations increased. Liming was found to increase foliar concentrations of Ca, Mg, and Mn and to decrease the concentration of K. The positive effects of the liming treatments were observed after nine years (Tveite et al, 1991). Long term liming experiments on pine and spruce forests in Finland resulted in increased organic matter accumulation in the soil and a sustained decrease in soil acidity, but a substantial accumulation of boron (B) and manganese (Mn) in the humus layer (Derome, 1991). Over a four-year period, where liming and K and Mg fertilization was done in a pine stand in Germany, no improvement in tree health or productivity resulted and negative effects were observed, including elevated soil Al, and increased nitrate mobilization (Marschner et al, 1991). Similar negative results of liming were obtained by Marschner and Wilczynski (1991) in a pine plantation in urban Berlin, Germany. In this study, substantial losses of organic matter and an increase in heavy metals in the organic matter were observed. The effects of varying liming and fertilization treatments (N, Mg, K, dolomite) on the fine-root growth of spruce and pine in Sweden were examined by Persson and Ahlstrom (1991). While N fertilization reduced fine root growth in some instances, it increased fine root growth in other cases. Results indicated that liming, especially in combination with high N deposition, had a negative effect on the growth of fine roots, but that growth responses of fine roots appeared to be dependent upon the tree species, age and specific soil conditions (Persson and Ahlstrom, 1991).

While some fertilization schemes in Europe have therefore proven successful, negative results such as increases in soil nitrate and accumulation of potentially toxic elements have also been reported. Zoettl et al (1989) make a point of stating that 'appropriate' application of fertilizers results in improved decline symptoms associated with nutritional deficiencies. Evers and Hüttl (1991) have discussed new fertilization strategies for declining forests and indicate that, as we have seen, certain risks and constraints exist which are dependent upon specific site conditions. They contend that fertilization treatments must be adapted to the site, requiring specific treatments as indicated by "soil and foliar analysis, humus form, hydrologic parameters and atmospheric deposition rates".

2.2. North America

Experiments with forest fertilization in Canada have been concerned mainly with declining sugar maple stands, and have initially relied upon the European experience (Hendershot, 1991). However, one study by Janicki and Jones (1991), examined fertilization effects on spruce stands located in southwestern

Québec. Preliminary analysis indicated low foliar N and K, and border-line Mg concentrations, and when soil was amended with these nutrients, increases were detected in foliage and visual symptoms improved (Janicki and Jones, 1991). Twenty-one sugar maple stands in Massachusetts, showing symptoms of decline and deficient N levels in soil and foliage, were shown to improve after N-fertilization (Mader and Thompson, 1969). In a sugar maple stand in the Quebec Appalachians (Beauce-Megantic), Hendershot (1991) examined fertilization effects on sugar maples exhibiting foliar deficiencies of P, K, Ca and Mg. Fertilization treatments containing Ca, Mg and K resulted in increased levels of these elements in soil and foliage, and visual decline symptoms improved (Hendershot, 1991). In a similar experiment located in sugar maple stands in the Lower Laurentians northwest of Montréal, Spankie (1991) has shown that foliar concentrations of Ca, Mg, K and P were deficient or near deficiency levels in moderately declining stands. Fertilization with these elements resulted in increased concentrations of the deficient elements in both soil and foliage analysis (Spankie, 1991). Meyer and Camiré (1990) examined the destination of applied P, K and Ca in a sugar maple forest in St. Norbert, Québec. The application of varying levels of P and K resulted in increased concentrations of these nutrients in foliage, while foliar concentration of Ca did not change even when applied at 1,000 kg/ha as $Ca(OH)_2$ (Meyer and Camiré, 1990). Soil monitoring revealed that K additions resulted in greater leaching of Ca, Mg and SO_4, and Ca and Mg were dominant cation leachates in all treatments.

3. Effects of Forest Decline and Fertilization on Soil Biology

Although knowledge is fairly extensive regarding the effects of increased acidic deposition and/or ameliorative fertilizer additions upon soil chemistry and resultant foliar responses in forests, less is known about their effects upon soil microbial populations. Since soil microbes are responsible for decomposition of organic matter as well as nitrification processes, they form an integral part of the soil system and play a pivotal role in nutrient cycling in forest soils (Fritze, 1991). Thus, factors altering microbial processes in the soil may profoundly affect nutrient availability and ultimately the health of the forest trees. It has been established that the majority of the microbial biomass of normally acid forest soils is made up of a fungal component, and that biological activity decreases with increasing soil acidity (as reviewed by Fritze, 1991). Additionally, a shift (change) in fungal species populations has been observed in response to acid soil additions (Fritze, 1991).

3.1. Mycorrhizae

Some of the most important fungal components in both coniferous and deciduous forests are those that form mycorrhizal associations with the roots of the trees. These symbiotic associations are essential in the acquisition of nutrients by the tree, as the external mycelium of the fungus may explore a greater volume of soil than roots alone, and thus greatly increase the surface area for nutrient absorption. Coniferous trees and broadleaf trees such as beech, birch, and oak generally form ectomycorrhizal associations, while deciduous trees such as sugar maple, red maple, ash, sycamore, and walnut form endomycorrhizae. Despite their position at the interface between soil nutrients and the uptake of these nutrients by the tree, only limited research has been directed to the effects of acid precipitation and/or ameliorative cation additions on mycorrhizal associations, and of the studies done in relation to forest mycorrhizae most have been restricted to the coniferous or beech ectomycorrhizae.

3.1.1. Ectomycorrhizae

Forest decline in Europe has frequently been found to be associated with decreased ectomycorrhizal associations as well as a decrease in the ectomycorrhizal species diversity (Arnolds, 1991). This has been attributed to direct effects of air pollutants and soil acidification and subsequent leaching of essential nutrients. It has been suggested that ameliorative soil treatments will probably not restore species diversity or spore production and that these treatments may even have deleterious effects (Arnolds, 1991). Kowalski (as cited in Allen, 1991) demonstrated that in Polish forests subjected to heavy industrial pollution, there was a significant decrease in the diversity of ectomycorrhizae-forming fungi compared to forests subjected to less pollution. A high correlation between beech decline and reduction of both mycorrhizae and the number of feeder roots in topsoil was seen in mature beech forests in Germany suffering from decline (Vincent, 1989). In a laboratory experiment, Danielson and Visser (1989) demonstrated that pine ectomycorrhizae did not survive below pH 3.3 in forest soil that was artificially acidified, and the pine seedlings did not grow below pH 2.8. Adams and O'Neill (1991) reported that ectomycorrhizal infection of Loblolly pine decreased with increasing ozone treatments, but that acid rain treatments (pH 5.2, 4.5 and 3.3) had no effect on the levels of infection. Dehydrogenase and acid phosphatase activities in the rhizosphere of Loblolly pine have been found to be inhibited with increasing acidity (pH 5.3, 4.3, 3.3) (Reddy et al, 1991), suggesting that profound changes may also occur at the root-soil interface on a microsite basis.

In a declining spruce stand in Germany showing symptoms of Mg deficiency, Haug and Feger (1991) determined that fertilization with N (as ammonium sulfate) caused a decrease in the incidence of ectomycorrhizal infection, while the number of uninfected feeder roots increased. Plots fertilized with $MgSO_4$ exhibited no change in rates of ectomycorrhizal association (Haug and Feger, 1991). In an artificially established forest plot in England, fertilization of oak and birch seedlings with N, P and K fertilizers resulted in decreased ectomycorrhizal infection and a decrease in ectomycorrhizal species diversity (Newton and Pigott, 1991). After amelioration of a pine forest soil in the Netherlands with P and Ca, as well as lime, the number of ectomycorrhizal species was found to significantly decrease (Kuyper and de Vries, 1990; Kuyper, 1989). Soil analysis indicated an increased nitrate availability after P, Ca and lime additions, and since it is known that N fertilization leads to an increase in fungal species able to utilize both nitrate and ammonium (Kuyper and de Vries, 1990), this may explain the decrease in species diversity. Conversely, the addition of ameliorative calcium carbonate treatments to declining spruce soils in France resulted in increased ectomycorrhizal associations and improved tree health (Estivalet et al, 1990).

3.1.2. Vesicular-Arbuscular mycorrhizae

In severely declining spruce (ectomycorrhizal) forests of Germany, establishment of grass cover has been observed to occur following tree defoliation. An examination of the vesicular-arbuscular mycorrhizae (VAM) populations concomitant with grass invasion indicated that VAM infection of grass was positively correlated with areas subjected to the greatest pollution (Vosatka et al, 1991). In a coniferous (ectomycorrhizal) forest soil that was artificially acidified, Timothy grass did not grow below pH 3.3, and the endomycorrhizal VAM associated with timothy were not observed below pH 4.0 (Danielson and Visser, 1989). Experiments with brachiaria grass in Brazil indicate that liming additions to acid soils negatively affects VAM spore production and species composition of the soil and may cause profound shifts in VAM populations (Siqueira _et al_, 1990).

There have been few examinations of endomycorrhizal VAM associations in temperate forest trees. Sugar maples are one of the few dominant trees in the northern temperate zone of Canada known to form endomycorrhizae, despite the estimate that 90% of all vascular plants form endomycorrhizas (Kendrick and Berch, 1985). Thus the majority of research in relation to VAM has been with annual crop plants grown in (artificial) agricultural conditions, and the goal of most of these studies has been towards improved crop production.

3.1.2.1. Agricultural Systems

A number of experiments have demonstrated that crop plants with VAM associations are more efficient in acquiring P, often limiting in agricultural soils, than non-VAM plants (Furlan and Bernier-Cardou, 1989; Abbott and Robson, 1986; Schultz and Kormanik, 1982). VAM associations have also been implicated in the more efficient uptake of other nutrients such as N (Ames _et al_, 1983), Ca, Mg, K (Ingham and Molina, 1991), and zinc (Zn) (Manjunath and Habte, 1988; Sharma and Srivastava, 1991), while reduced VAM colonization has been observed in the presence of heavy metals (Gildon and Tinker, 1983). The presence of VAM associations has also been observed to enhance drought resistance in some plants (Bethlenfalvay _et al_, 1988).

Studies of the morphology of VAM associations have revealed that (in annual crop plants) the fungal infection begins as an appressorium forms on the surface of a young rootlet. Penetration of the outer cortical layer follows, with fungal hyphae extending longitudinally from cell to cell, or between cells. Hyphal constrictions are often evident as the fungus penetrates the cell wall of adjacent cortical cells. The fungus may or may not form coils intracellularly. Arbuscules are formed in the inner cortical layers as hyphae penetrate cells and give rise to a dichotomously branching structure consisting of progressively finer hyphal branches. The arbuscule is considered to be the site of metabolic exchange between host and fungal symbiont, as the fungus transfers essential water and nutrients to the plant and obtains photosynthate in return. As a final structure, vesicles may be formed throughout the cortical layers. These structures are globose, lipid containing bodies that are viewed as storage organs, and are often referred to as resting spores. The VAM fungal symbiont has not been observed to penetrate the vascular cylinder of the root, nor to be present in regions of the root where secondary growth has occurred (Bonfante-Fasolo, 1986; Brown and King, 1982).

3.1.2.2. Morphology in Sugar Maples

Very little work has been directed toward the examination of the VAM associations of perennial temperate forest trees, specifically sugar maple, despite early studies (McDougall and Liebtag, 1928) demonstrating the abundance of VAM fungi in sugar maple and other plants in a deciduous forest. Research in this area remained dormant until 1966, when Kessler provided the first in-depth description of the VAM associations of sugar maple in a Michigan forest (Kessler, 1966). Here, the VAM associations were shown to begin with an appressorium and infection proceeded with inter- and intracellular hyphal penetration, formation of hyphal coils, and formation of arbuscules and vesicles (Kessler, 1966), similar to the anatomical descriptions of VAM in annuals. It was not until 1990, that the morphological study of VAM in sugar maple continued. Yawney and Schultz (1990) examined the association with scanning electron microscopy, after inoculating sugar maple seedlings with the VAM fungus Glomus etunicatum. Intracellular hyphae, hyphal coils and arbuscules that appeared as 'arbusculate coils' were observed, but vesicles were absent in this system (Yawney and Schultz, 1990). In a survey of VAM associations of plants in a sugar maple forest in Ontario, Brundrett and Kendrick (1988) determined that wide seasonal fluctuations in the incidence of VAM infections occur in sugar maple.

The species of VAM fungi that naturally form associations with sugar maple is unknown. Yawney and Schultz (1990) report success in VAM fungus (Glomus etunicatum) inoculation of sugar maple seedlings under artificial greenhouse conditions. Berch (1988) quotes Clark (1969) as successfully infecting sugar maples with spores of Gigaspora gigantea, but Clark did not in fact show this. Schultz and Kormanik (1982) successfully infected sugar maple seedlings in pot culture with Glomus fasiculatus, a mixed culture of Glomus mosseae and Glomus etunicatus and a mixed culture of Glomus and Gigaspora species, but this inoculum consisted of root fragments from pot cultures of sorghum, and the purity of inoculum was not established in this study.

3.1.2.3. Sugar Maple Decline

Because the importance of VAM in nutrient uptake had been duly noted, recent studies have examined the relationship between mycorrhizal infection, nutrient status of sugar maples, and levels of decline in maples. Examination of 44 sugar maples exhibiting varying symptoms of decline through exposure to deicing salts along a roadside in Connecticut revealed a significant correlation between degree of decline and decrease in VAM infection (88% of sampled roots contained VAM in slightly declined trees, compared to 62% in severely declined trees) (Guttay, 1976). The author also noted correlations between degree of decline and increased rooting depth, and increased root content of Na and Cl (Guttay, 1976). In a similar experiment, Spitko et al (1978) examined the relationship between degree of sugar maple decline and incidence of VAM on 12 sugar maples growing on a university campus in Massachusetts. No relationship was seen by these authors between degree of crown decline and either soil pH or soil nutrient status, but negative correlations were obtained between degree of decline and level of

VAM infection of feeder roots. More than 90 cortical cells per cm of feeder root were found to be mycorrhizal in healthy sugar maples, while less than 5 cells per cm of feeder root were mycorrhizal in severely declining trees (Spitko *et al*, 1978). Healthy trees were observed to have VAM associations consisting mainly of arbuscules, while in moderate to severely declining trees, vesicle formation predominated (Spitko *et al*, 1978).

Relationships have been observed between the nutrient status of sugar maple seedlings and the degree of mycorrhizal infection. Fertilization of fumigated soil with varying levels of N, P and K, and inoculation with a mixture of *Glomus mosseae* and *Glomus etunicatum*, resulted in increased seedling growth and VAM infection (from 41% of sampled root being mycorrhizal to 69%) with increasing fertilizer (Schultz *et al*, 1981). An increase in the incidence of vesicle formation (from 6% to 21%) with increasing N, P and K additions was observed (Schultz *et al*, 1981). In a similar experiment, Schultz and Kormanik (1982) inoculated sugar maple seedlings with a *Glomus* species mixture and fertilized with varying levels of N, P and K. Seedling growth was observed to increase with fertilization, as did the degree of VAM infection (from 41% to 69%). Nutrient uptake of N, P, and Ca was greater in the mycorrhizal seedlings as compared to non-mycorrhizal seedlings, while K concentrations were higher in the non-mycorrhizal seedlings (Schultz and Kormanik, 1982).

4. Conclusions

Research on VAM associations of declining sugar maple in a natural sugar maple forest are virtually non-existent, despite the evidence that VAM fungi may be playing a pivotal role in this phenomenon.

Experiments with ectomycorrhizal associations in declining forests in Europe indicate that these relationships are important in conferring a greater resistance toward soil acidification and drought, and definitely play a role in decline. However, these associations are fundamentally and structurally different from the VAM associations of trees such as sugar maple, and as such, we cannot conclude that the effects of VAM associations are the same as those involving ectomycorrhizae. Similarly, the trees involved grow in different conditions, with conifers generally growing on more acidic and homogenous sites than sugar maples. Decomposition (and thus nutrient cycling) may be more crucial in a sugar maple forest due to the loss of nutrients through leaf fall at the end of each growing season.

It is unreasonable to assume that the VAM associations of annual crop plants are the same as those of perennial woody plants such as sugar maple. As well as the fact that research with crop plants has been directed towards increased crop production, physiological factors such as root growth, nutrient acquisition and availability are all fundamentally different in an agricultural system than in a mature forest ecosystem.

This, then, points us to a large gap in scientific knowledge in terms of naturally occurring VAM associations of sugar maple. Research is needed to determine the following:-

1. What is the ecology of VAM in temperate hardwood forests, particularly sugar maple forests? What is the incidence of VAM in sugar maple and does this frequency change over the growing season? How does VAM develop, and what is the morphology of this association in natural sugar maple forests? What species of VAM fungus naturally infect sugar maples and does this change throughout the growing season? How many species of VAM fungus exist in a natural sugar maple forest and what are their relative incidences?

2. What is the effect of acid precipitation upon the natural VAM associations of sugar maple, and how is this related to sugar maple decline? What is the effect of ameliorative base cation additions to the forest soil upon the natural VAM associations of sugar maple?

3. What effect do natural VAM associations have on tree health and nutrient uptake in situations of poor nutrient availability, plant stress (as in declining maples) and increased availability of base cations (as in forest fertilization)?

This thesis examines the ecology of VAM in a natural sugar maple forest in the Lower Laurentians of Québec suffering from moderate decline by providing information on the incidence and morphology of VAM in sugar maple in this system over several growing seasons. We have also examined the effects of base cation fertilization on the VAM associations in this field site.

This thesis also investigates, under more controlled greenhouse conditions, using natural sugar maple forest soil (in order to provide natural substrate and inoculum), how base cation fertilization affects VAM colonization of sugar maple seedlings, seedling growth and nutrient uptake and whether the presence of VAM fungi affects either nutrient uptake or seedling health. In this system the time course of VAM development, its morphological characteristics, effects of fertilization on VAM morphology, and whether the health of sugar maple seedlings is related to the state of the VAM infection, was also examined.

CHAPTER 2

MORPHOLOGY, INCIDENCE AND FERTILIZATION EFFECTS ON THE VESICULAR-ARBUSCULAR MYCORRHIZAE OF ACER SACCHARUM IN A QUEBEC HARDWOOD FOREST[1]

1. Introduction

Sugar maple (*Acer saccharum* Marsh.) is one of the most important hardwoods in Eastern Canada and the Northeastern United States, where it is used for maple syrup production as well as lumber. In many regions the sugar maple is in a state of decline which has been linked by some to acid deposition (Bernier and Brazeau, 1988a; 1988b, Hendershot and Jones, 1989; Paré and Bernier, 1989a; 1989b). Nutrient deficiencies or imbalances resulting from acidic soil inputs have been suggested as contributing to the decline (Hendershot and Jones, 1989). In the Lower Laurentians of Quebec, maple stands, which are close to their northern limit (Hosie, 1973) receive precipitation with an average pH of 4.3 (Svatek, 1988). These trees show foliar potassium (K), magnesium (Mg) and calcium (Ca) deficiencies, and nutrient imbalances involving Ca and K, Al and Ca, and Al and Mg (Hendershot and Lalonde, 1988; Spankie, 1991).

Sugar maples are one of the few dominant trees of the northern

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[1] Cooke *et al*, 1992a.

temperate forest zone known to form endomycorrhizae, although it has been estimated that associations involving vesicular-arbuscular mycorrhizae (VAM) occur on 90% of all vascular plants (Kendrick and Berch, 1985). Consequently, while there has been extensive VAM research on temperate crop plants, little is known of VAM in temperate forest systems. Numerous experiments have demonstrated that plants having vesicular-arbuscular mycorrhizal (VAM) associations are more efficient in acquiring phosphorus than non-mycorrhizal plants (Furlan and Bernier-Cardou, 1989; Schultz and Kormanik, 1982). It has also been suggested that VAM associations may aid in the acquisition of other nutrients (Manjunath and Habte, 1988; Stribley, 1987; Ames _et al_, 1983; Gildon and Tinker, 1983; Cooper and Tinker, 1978), and may also play a role in drought resistance (Bethlenfalvay _et al_, 1988). However, most of this information comes from research involving crop plants, not forest trees.

In spite of early studies (McDougall and Liebtag, 1928) which demonstrated the abundance of VAM fungi in sugar maple and other plants in a deciduous forest, but questioned their function, there have been few investigations regarding the VAM associations in sugar maple. Recent studies have, however, revealed the following. 1) The VAM associations in sugar maple roots under both natural and greenhouse conditions proceed in the classical fashion, with penetration of the outer layer of young feeder roots followed by longitudinal advancement of the intra- and intercellular hyphae, hyphal coiling, and the formation of arbuscules and vesicles. (Kessler, 1966; Yawney and Schultz, 1990). 2) Wide seasonal fluctuations in the incidence of VAM infection occur in natural sugar maple ecosystems (Brundrett and Kendrick, 1988).

Because of the importance of VAM in nutrient uptake, recent studies have examined the relationship between mycorrhizal status, nutrient status of the trees, and levels of maple decline. Negative correlations have been reported between the degree of maple crown decline and the degree of mycorrhizal infection (Guttay, 1976; Spitko <u>et al</u>, 1978), as well as between degree of crown decline and tree nutrient status (Bernier and Brazeau, 1988a; 1988b). Tree nutrient status and degree of mycorrhizal infection have been found to be positively correlated (Schultz <u>et al</u>, 1981). The degree of decline has also been shown to be related to the condition of VAM (Spitko <u>et al</u>, 1978).

Since so little is known of the ecology of VAM fungi in temperate hardwood forests, this study was undertaken primarily to examine the morphology of VAM and their incidence in a natural sugar maple ecosystem in the Lower Laurentians of Québec. Additionally, since VAM fungi play a vital role at the interface between soil nutrients and the uptake of these nutrients by the tree, this study was designed to determine the effects of base cation forest fertilization on the VAM infection rates of maple roots.

2. Materials and Methods

2.1 Study site

The research site is located northwest of Montreal, at an altitude of 380m, in the Lower Laurentian Mountains (Lat. 46°N, Long. 74°W), at St. Hippolyte, Québec, Canada (University of Montreal, Biological Station). Six plots were established early in the summer of 1988 for a larger study involving the examination of soil nutrients, tree nutrient status and soil fertilization in relation to maple decline. The plots (40 X 40m) are located in a 60-80 year-old stand of sugar maple mixed with white birch (Betula papyrifera Marsh.) and striped maple (Acer pensylvanicum L.). The sugar maples show moderate symptoms of dieback (<25% defoliation). The soil is an Orthic Ferro-Humic Podzol (Humic Haplorthod). On the basis of preliminary studies on the nutrient status in these plots which showed that the major deficiencies were in the alkaline metals, three plots were randomly selected and fertilized on June 9, 1989 with a base cation mixture composed of 500 kg/ha K_2SO_4, 250 kg/ha $CaCO_3$ and 250 kg/ha $CaMg(CO_3)_2$.

2.2. Sampling of roots

Within each of the six plots, five trees (ca. 40 cm dbh) were selected arbitrarily (total of 30 trees) from which samples were to be collected. At each tree, three samples were collected at randomized compass points at a distance of 5 m from the base of the tree (total of 90 samples per sampling period). Each sample consisted of a 5.5 x 10 cm (deep) core, since preliminary investigations showed most of the feeder root growth to be in this region. Samples were taken prior to fertilization on July 15, 1988 and May 9, 1989 and after fertilization on July 15 and Aug. 15, 1989, and May 22, July 10, and Sept. 25, 1990.

2.3. Observations of VAM

Maple feeder roots (identified by their characteristic beaded appearance) were washed from the soil samples, stored for at least 24 h in a formalin-acetic acid-alcohol (5ml formalin, 5ml acetic acid, 90ml 50% ethanol) solution, cleared by autoclaving for 8 min in a 10% KOH solution, bleached in 35% hydrogen peroxide for 1 h, acidified in 1% hydrogen chloride, and stained using the chlorazol black E method (Brundrett et al, 1984), modified by using a 0.01% solution of Chlorazol Black E (Allied Chemical). Mycorrhizal structures were examined and photographed using a Nikon Optiphot differential interference contrast microscope, at a magnification of either 200 x or 400 x.

2.4. VAM quantification

The quantification of VAM infection was performed using a Wild M3 dissecting microscope with a magnification of 32 x. The percent of infection for each sample was estimated using the grid-line intersect method (Giovannetti and Mosse, 1980), by evaluating 100 intersects for each sample. The thickness of the grid-lines was

0.5 mm. Two measures of infection were recorded: the number of intersects at which vesicles were seen, and the number of intersects at which internal mycelium, but not vesicles were seen. The number of intersects where either vesicles or internal mycelium were found was defined as total infection. No arbuscules were seen at the intersects, thus arbuscules are not included in the quantitative data.

2.5. Statistical analysis

Descriptive statistics were obtained using the SPSS-X computer program (Norusis, 1985). An analysis of variance (ANOVA) was performed to examine seasonal effects. Scheffé post-hoc comparisons (P=0.01, and P=0.05) were used to determine individual differences in sampling periods. A nested ANOVA was used to test for treatment effects. The design employed consisted of root samples nested within base points (trees), nested within plots, nested within treatments.

3. Results

There were no obvious differences in VAM structures observed with regard to plot, sampling season, or year. Coarse aseptate hyphal coils were often seen to stem from initial penetration points (appressoria) in the outer cortical layer (Plate 1.1), and were also seen in the central and inner cortex of the feeder roots. Fine, dense coiling was sometimes observed (Plate 1.2). Hyphal constrictions were seen as the fungus passed through cell walls of the root cortex (Plate 1.3). Few arbuscular formations were seen in these field samples, however those that were observed seemed to arise from coarse intramatrical hyphal coils, which had budded and then branched as infection proceeded from outer to inner cortical layers (Plate 1.3-6). Arbuscules never occurred alone, but occupied clusters of cells (infection foci) in a section of root, and were not restricted to the inner cortical layers. Elongate vesicles were formed terminally on inter-and intra-cellular hyphae, and sometimes occurred in groups (Plate 2.7-9). Spore-like vesicles occurred singly and terminally on irregularly septate hyphae (Plate 2.10-11). Structures which resembled previously described hyphal masses resulting from arbuscule degeneration (Yawney and Schultz, 1990), were seen often in these roots. Figures 12 and 13 (Plate 2) show that these structures arose from fine septate hyphae.

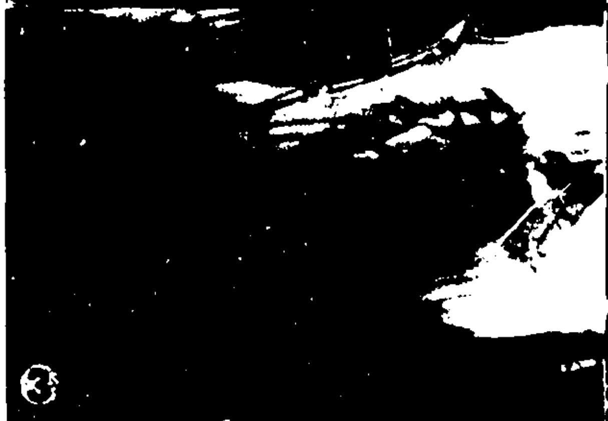
No differences in the degree or condition of VAM between control and fertilized plots were found at any sampling period. The mean total infection rates varied from 38% to 75% (Table 1). Differences in the indicence of VAM existed between the time of the year samples were taken, and also between years ($P<0.001$). Post-hoc comparisons confirmed that more vesicles (Fig.1), internal mycelium (Fig. 2), and higher total infection (Fig. 3) occurred in May of 1989 than in July 1989 ($P<0.01$). In 1990, more vesicles, but less internal mycelium was observed in May than in either July or September ($P<0.01$). No significant effects of season on total infection rates were found in 1990. Differences between years were observed for both vesicles and internal mycelium (Figs. 1 and 2) in spring (May) ($P<0.01$) and fall (August $P<0.01$ and September $P<0.05$). In July there were no differences in the percentage of vesicles for 2 of the 3 comparisons, but the samples from 1988 had a higher incidence than those from 1990. The incidence of internal mycelium consistently differed between years. Differences in total infection between 1989 and 1990 ($P<0.01$) were observed only in the May samples (Fig. 3). Total infection in July of 1988 was greater than in July of either 1989 or 1990 ($P<0.01$).

Plate 1

Figs. 1-6. Development of arbuscules from hyphal coils. **Fig. 1.** Appressorium (arrow) on root surface (in root-hair zone), leading to coarse hyphal coils in the first cortical cell layer. (Mag. 200x). **Fig. 2.** Fine hyphae forming dense coils. (Mag. 200x). **Fig. 3.** Hyphal coil entering an adjacent root cell where it is beginning to branch (arrows). (Mag. 400 x). **Fig. 4.** Further branching of coils (arrow)as infection proceeds from cell to cell. (Mag. 400x). **Fig. 5.** Pre-arbuscule. Note original coil structure that has progressively branched to finer hyphae (arrow). A full arbuscule is seen in the adjacent cell. (Mag. 400x). **Fig. 6.** Arbuscule. Note typical fine branches, with coarse hyphal coil structure still visible (arrows). (Mag. 400x).

①

②



③

④

⑤

⑥

Plate 2

Figs. 7-13. Comparison of vesicle types formed in Sugar Maple feeder roots. **Fig. 7**. Intracellular vesicle associated with hyphal coils. (Mag. 200x). **Fig. 8**. Intracellular vesicles formed in groups. (Mag. 200x). **Fig. 9**. Intercellular vesicles. (Mag. 200x). **Fig. 10**. Spherical vesicle associated with septate hyphae (arrows). (Mag. 200x). **Fig. 11**. Spherical vesicle in cuboidal root cell. (Mag. 400x). **Figs. 12-13**. Structures, resembling hyphal masses, often seen in sugar maple feeder roots. A septate hypha is seen in Fig. 12 (arrows). (Fig. 12, Mag. 200x; Fig. 13, Mag. 400x).


8
12
13

FIG. 1. Seasonal changes in the presence of vesicles in sugar maple feeder roots. Each data point represents the mean percentage of intersects positive for vesicles. Error bars represent 95% confidence limits. N=90, except for July 1989, where N=88.

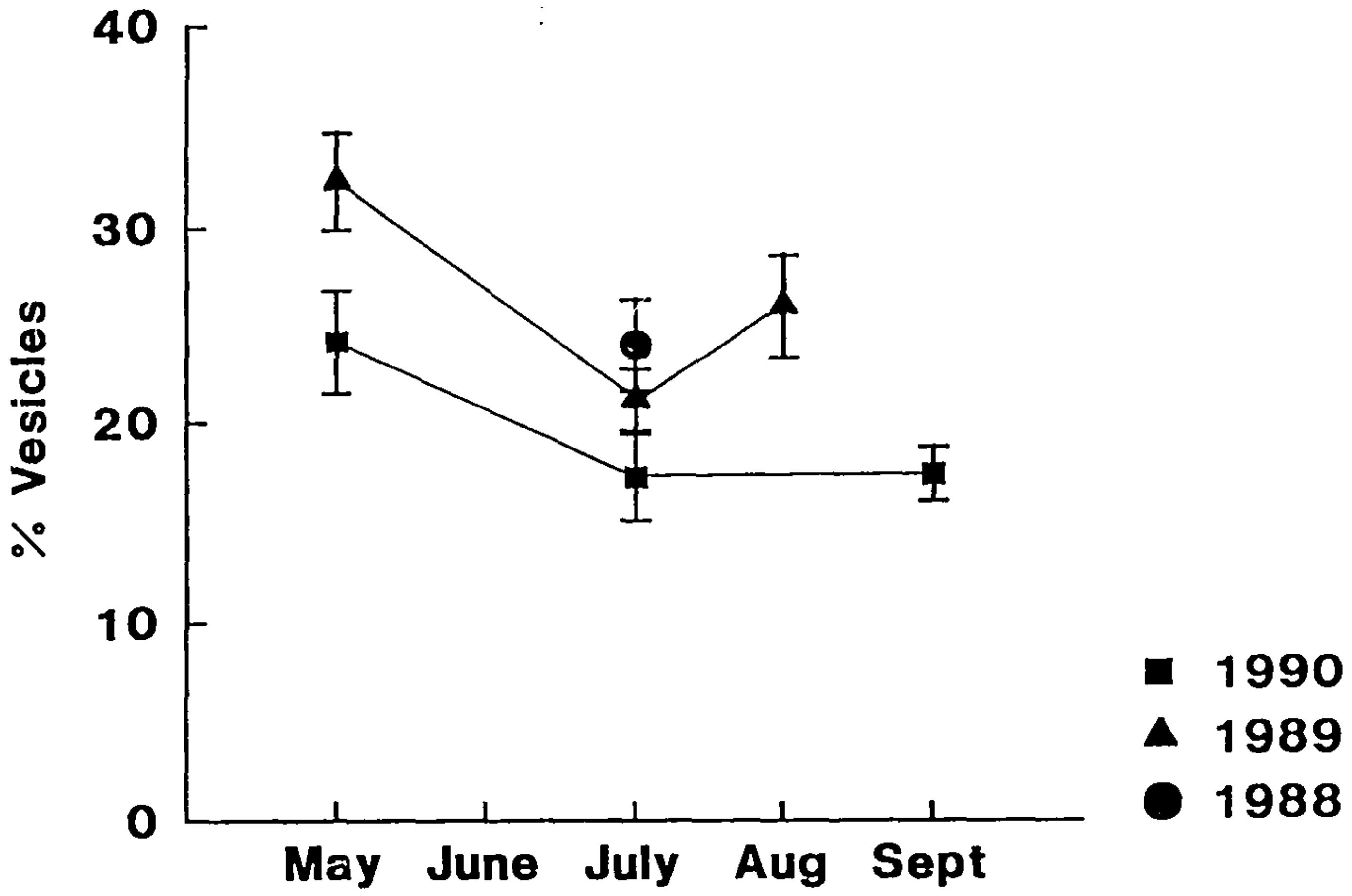
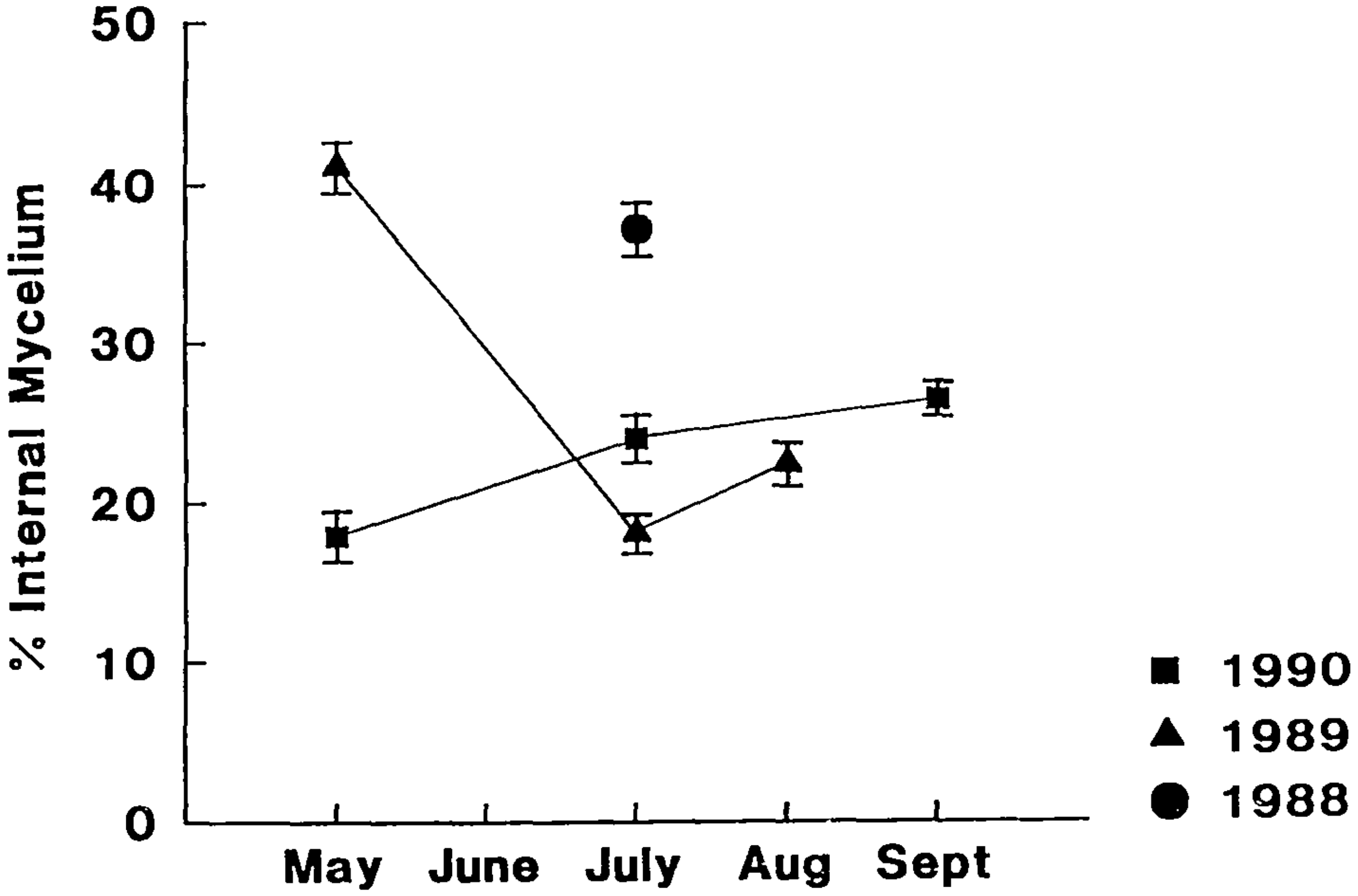

40
30
20
10
0
% Vesicles
May
June
July
Aug
Sept
■ 1990
▲ 1989
● 1988

FIG. 2. Seasonal changes in the presence of internal mycelium in sugar maple feeder roots. Each data point represents the mean percentage of intersects positive for internal mycelium. Error bars represent 95% confidence limits. N=90, except for July 1989, where N=88.



% Internal Mycelium
50
40
30
20
10
0
May
June
July
Aug
Sept
■ 1990
▲ 1989
● 1988

FIG. 3. Seasonal changes in total VAM infection in sugar maple feeder roots. Each data point represents the mean percentage of intersects positive for either vesicles or internal mycelium. Error bars represent 95% confidence limits. N=90, except for July 1989, where N=88.

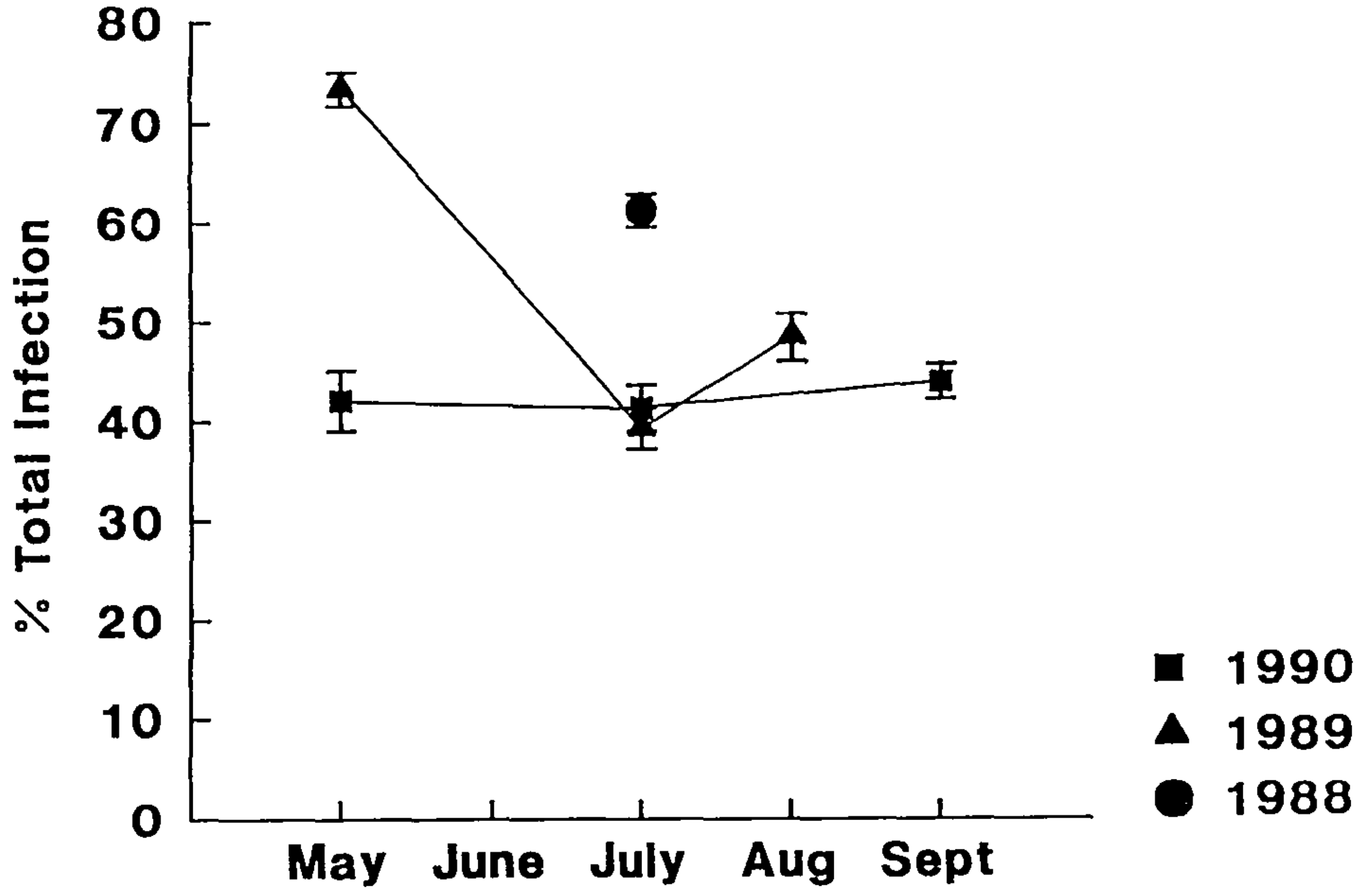

80
70
60
50
40
30
20
10
0
% Total Infection
May
June
July
Aug
Sept
■ 1990
▲ 1989
● 1988

TABLE 1

Mean values for the incidence of vesicles, internal mycelium (mycelium) and total infection (total) of control (cont) and fertilized (fert) plots.[a]

Sample Date	Vesicles		Mycelium		Total	
	Cont	Fert	Cont	Fert	Cont	Fert
July 1988	23.8	24.3	37.8	36.6	61.6	61.0
May 1989	34.1	30.6	40.7	41.4	74.8	72.1
July 1989	21.5	20.9	19.1	17.1	40.7	38.0
Aug 1989	26.7	25.4	21.0	23.8	47.7	49.2
May 1990	23.8	24.6	19.2	16.7	43.0	41.2
July 1990	18.4	16.2	25.8	22.2	44.2	38.5
Sept 1990	17.9	17.0	27.0	26.0	45.0	43.0

[a] N=45 for each treatment at each sampling period, except July 1989, where N=44. No significant treatment effects were found (P>0.05). The plots were fertilized in June 1989.

4. Discussion

In general, the anatomy of the mycorrhizal infection in these sugar maple feeder roots was similar to that previously described by Kessler (1966) and Yawney and Schultz (1990). The arbuscules that were observed in these roots clearly arose from hyphal coils (Plate 1.3-6). Yawney and Schultz (1990) showed photographs of this type of arbuscule in sugar maple roots, but made no comment on their development from coils. These arbuscules appear to be quite different from the classical arbuscules, which arise as dichotomously branching stuctures from a single, uncoiled hypha (Bonfante-Fasolo, 1986). The production of both coarse, loose coils and fine, dense coils (Plate 1.1-2), and of both elongate and spherical vesicles (Plate 2.7-10), may be characteristic of the VAM fungal species. There have, however, been no studies of the structure of VAM in sugar maples infected with known species of VAM fungi, nor are the VAM fungi infecting maples in natural conditions known. It is therefore not yet possible to relate the morphology of vesicles or arbuscules to specific VAM taxa.

The structures shown in Plate 2.12 and 2.13, which resemble the structures referred to as degenerating arbuscules by Yawney and Schultz (1990), were found frequently in this study. These structures were invariably borne on narrow, septate mycelium, which leads us to question whether they are in fact VAM structures, or whether they are formed by non-VAM fungi present in these roots.

Although little is known concerning the relationship between VAM and forest decline, there have been some studies in Europe which associate decreased numbers of ectomycorrhizae, especially in older conifers, with forest decline (Arnolds, 1991). Norway spruce (Picea abies) seedlings grown in soil collected from forest areas of the Vosges in France showed poor ectomycorrhizal development and nutrient status in soils of low pH (4.2, 3.6). The addition of a calcium carbonate fertilizer increased the ectomycorrhizae and restored growth and normal mineral nutrition (Estivalet et al, 1990).

The fact that few arbuscules, but numerous vesicles and internal hyphae were found in this sugar maple stand deserves mention. In an examination of the incidence and condition of the VAM of 12 declining sugar maples on the University of Massachusetts campus, Spitko et al (1978) noted that the degree and nature of mycorrhizal infection changed as crown dieback increased. The roots of healthy trees had few vesicles and extensive arbuscule development. As tree health declined fewer arbuscules were observed but vesicles increased in abundance.

We can state with confidence that a tree in a declining state is under stress. We also know that many fungi produce resting structures in response to stress, and that mycorrhizal vesicles have been regarded as resting spores (Bonfante-Fasolo, 1986). We may therefore expect an increase in the number of vesicles produced as tree decline increases (as Spitko et al, 1978 observed), or as stress increases. Although the maples at our site have been evaluated as being in a state of moderate decline, the abundance of vesicles and scarcity of arbuscules may be an indicator that the trees are stressed. If this is so, it may be worth investigating the use of vesicle formation as an indicator of the degree of stress of the system.

Despite the tendency of ecological data of this sort to have great variation, the variation in our data was quite manageable. Variation within groups was small enough to show differences in seasonal infection rates and differences between plots. Nonetheless, the nested ANOVA was unable to demonstrate treatment effects, although some effects of trees and plots were apparent (data not shown). Because of this, we may be fairly certain that fertilization has not had an effect upon VAM infection rates in this site.

Fluctuations in seasonal infection rates have been observed by Brundrett and Kendrick (1988), and despite differences in the method of quantification and their lack of detailed statistical analysis, the results of their evaluation and those of the present study are similar. Both studies have shown that VAM colonization in natural sugar maple stands fluctuates widely (up to 35%) over the growing season, and that colonization rates may vary substantially from year to year.

Because different VAM fungi have different periods of maximum sporulation (Gemma et al, 1989; Gemma and Koske, 1988), seasonal differences in the incidence of VAM may be a reflection of the seasonality of sporulation of the species of VAM fungus colonizing the sugar maples in this stand. Additionally, the rise and decline of VAM activity over the growing season may parallel the intermittent growth of the maple feeder roots. It has now been accepted that the characteristic beaded appearance of sugar maple feeder roots is the result of environmental as well as endogenous factors. In periods of drought, root growth is often interrupted, cells at the tip of the growing root become smaller and more densely arranged, and root growth is arrested. When sufficient moisture again becomes available, the root resumes growth and cells become larger and less densely packed (Kessler, 1966; Brundrett and Kendrick, 1988). If such intermittent root growth is characteristic of sugar maple it is possible that colonization by VAM fungi would similarly be affected. It is possible that a lag period between resumption of root growth and colonization by mycorrhizal fungi occurs, during which colonization rates would be lower. It is also known that the growth of VAM fungi is directly affected by moisture availability (Hetrick, 1986). Thus, to understand the seasonal dynamics of VAM fungi, detailed simultaneous studies of environmental conditions, infection rates, morphology and root dynamics are needed.

No fertilization effects were seen in the present study. Differences between plots in most samplings were evident both before and after fertilization. Some plots tended to exhibit a lower (or higher) level of colonization, which appeared fairly consistent across all sampling periods. Variation in colonization rates was therefore related more to seasonality and the heterogeniety of the plots than to the treatment.

The present study has shown that VAM colonization rates in this natural sugar maple stand vary considerably over a growing season, and may be quite different from one year to the next, probably due to local climatic variations. The addition of basic cations to the soil did not affect VAM infection rates, even one year after fertilization. The rarity with which arbuscules were observed in these samples may be a reflection of the condition of the trees. Examination of the VAM morphology in sugar maple stands exhibiting differing levels of decline, or controlled experiments on the interactions between VAM infections, soil conditions and maple decline, would help elucidate the relationships between VAM fungi and tree health.

5. Acknowledgments

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Preface to Chapter 3

In the previous study, fertilization of the forest site with base cations that were found to be deficient did not appear to affect either the condition or the frequency of VAM infection rates. However, because of the importance of VAM in nutrient uptake, and the potential for fertilization to influence these associations, a more controlled greenhouse study was devised. Soil gathered from the field site was used as a substrate and source of natural VAM fungal inoculum. Under these conditions it was possible to vary the rates of base cation fertilization, to determine it's influence on the frequency of VAM fungal infection of sugar maple seedlings, seedling growth and nutrient uptake, and whether VAM infection affected either seedling growth, health, or nutrient uptake.

CHAPTER 3

VAM INFECTION AND NUTRIENT UPTAKE IN RESPONSE TO BASE-CATION FERTILIZATION IN SUGAR MAPLE SEEDLINGS[2]

1. Introduction

In many regions of Eastern Canada and the Northeastern United States, sugar maple (Acer saccharum Marsh.) is in a state of decline. Nutrient deficiencies or imbalances resulting from acidic inputs may contribute to this decline (Hendershot and Jones, 1989). In the Lower Laurentian and the Appalachian regions of Québec, foliar potassium (K), magnesium (Mg), calcium (Ca) and phosphorus (P) deficiencies and/or imbalances have been found in sugar maples (Spankie, 1991; Paré and Bernier, 1989a, 1989b; Bernier and Brazeau, 1988a, 1988b, 1988c; Hendershot and Lalonde, 1988).

Attempts to ameliorate sugar maple decline symptoms in these regions by replacing basic cations found to be deficient have been encouraging. After fertilization with K_2SO_4, visual symptoms of decline such as leaf discoloration, transparency, and dwarf foliage improved (Hendershot et al, 1989). Spankie (1991) has shown that fertilization with differing combinations of K_2SO_4, $CaCO_3$, and $CaMg(CO_3)_2$ results in an increase in the base cation concentrations in both soil and foliage when those cations are supplied in

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[2] Cooke et al, 1992b.

sufficient quantity.

Sugar maples are one of the few abundant northern temperate zone forest trees to form vesicular-arbuscular mycorrhizae (VAM), although it has been estimated that this form of endomycorrhizal association occurs on 90% of all vascular plants (Kendrick and Berch, 1985). While there have been few investigations of VAM in sugar maples, experiments with crop plants have repeatedly shown that VAM improve growth and increase the efficiency of phosphorus uptake (Furlan and Bernier-Cardou, 1989; Schultz and Kormanik, 1982). VAM associations may also aid in the acquisition of other nutrients (Manjunath and Habte, 1988; Stribley, 1987; Ames et al, 1983; Gildon and Tinker, 1983; Cooper and Tinker, 1978), and play a role in drought resistance (Bethlenfalvay et al, 1988). However, it should not be assumed that the VAM associations of crop species and of forest trees are functionally identical.

Since VAM fungi may play a vital role in nutrient uptake by trees, there have been some recent studies of the relationship between nutrient status, mycorrhizal status, and levels of decline in maples. Although most studies were not performed in natural sugar maple forests, negative correlations between the degree of maple decline, the degree of mycorrhizal infection (Guttay, 1976; Spitko et al, 1978) and nutrient status (Bernier and Brazeau, 1988a; 1988b) have been found. The nutrient status of sugar maple seedlings and the degree of mycorrhizal infection in pot experiments (i.e. in boxes in the field) have been found to be positively correlated (Schultz et al, 1981). However, in a previous experiment, we found that base cation forest fertilization did not appear to affect either the condition or the incidence of VAM in a natural sugar maple stand (Chapter 1).

Because of the importance of VAM in nutrient uptake, and because our field results were inconclusive, we undertook this study under controlled greenhouse conditions. We wished to determine how base cation fertilization affected VAM colonization of maple seedling roots, seedling growth and nutrient uptake and whether VAM infection affected either nutrient uptake or seedling health.

2. Materials and Methods

2.1. Seedling establishment

Sugar maple seeds from a single maple tree in the Niagara Peninsula Conservation Authority Property (Comfort Maple Location), were obtained from the Petawawa National Forestry Institute, Chalk River, Ontario (seed lot # 8830273). The seeds were processed in batches of 144 seeds. They were soaked in distilled water overnight, surface sterilized in 35% hydrogen peroxide for one-half hour, rinsed 3 times in sterile distilled water, placed singly in test tubes on 1% agar/water slants, and stored at 5°C in the dark until germination (about 3 months). The seedlings were removed from the test tubes at the primary leafing stage, planted in 3" plastic pots and watered with sterile distilled water (about 10ml per day) to maintain moisture levels. To minimize attrition, two seedlings were planted in each pot and allowed to grow for 15 days, at which point the less vigorous plant in each pot was removed.

2.2. Soil

Soil (0-10cm) from a sugar maple forest described previously (Chapter 2) was collected at St. Hippolyte, Québec, Canada (University of Montréal, Biological Research Station) and sterilized by gamma irradiation for 24h. Where needed, non-sterile soil from the same site was used as a natural source of VAM inoculum.

2.3. Experimental design

In experiment 1 the treatments consisted of 3 fertilizer levels and 3 types of inoculum, each replicated 20 times. The fertilizer levels were, control, (0 fertilizer); level 1, 0.45g per pot (equivalent to 1000kg/ha), and level 2, 0.90g per pot (equivalent to 2000kg/ha) of a base cation mixture composed of 2 parts (by weight) of fertilizer grade K_2SO_4, 1 part $CaCO_3$, and 1 part $Ca,Mg(CO_3)_2$. This mixture contained a final analysis of 10% Ca, 3% Mg and 22.7% K. Inoculum treatments consisted of control (sterile soil), natural inoculum (5g unsterile soil mixed with 45g sterile soil), and a pure spore inoculum of Sclerocystis rubiformis spores (approx. 500 spores/pot) added to 50g sterile soil. The

Sclerocystis spores had been recovered from the soil at Ste. Hippolyte by wet sieving followed by density gradient centrifugation.

Because the levels of infection by VAM in experiment 1 were low, the second experiment, using increased levels of inoculum was conducted. In experiment 2, four levels of fertilizer were used, the control, level 1 and level 2 described in experiment 1, plus level 3, 1.35g per pot (equivalent to 3000kg/ha). Natural inoculum was provided to all treatments in this experiment by mixing sterile soil with an equal weight of the non-sterile soil. Each treatment was replicated 6 times.

2.4. Soil and plant analyses

At the end of 120 days, the plants were removed from the greenhouse. The number of leaves on each plant was recorded, the stem diameter of each plant was measured using callipers, and plant condition was rated using a subjective scale (1=dead or dying; 2=poor health; 3=good health). The plants were then removed from the pots and the shoots and roots were cut apart using scissors. The roots were carefully separated from the soil by washing in tap-water, the excess water was removed by gently pressing the roots between two pieces of absorbent paper, and the fresh weight recorded. A weighed portion of the root was then separately placed in formalin-acetic acid-alcohol(FAA) for subsequent analysis of VAM structures. The remaining portion of the root was bagged for further analysis, as was the shoot. The shoots and roots were oven-dried at 65^{0}C for 48h to determine dry weight.

Root and shoot tissue were digested in conc. sulphuric acid/hydrogen peroxide (Thomas et al., 1967). Nitrogen and P concentrations were determined colorimetrically using a Technicon autoanalyser. Concentrations of Ca, Mg and K were determined spectrophotometrically using an air/acetylene gas mixture. The pH of the soil remaining in the pots was measured in water using a glass combination electrode.

2.5. VAM quantification

The root sub-samples were kept in FAA for at least 24h. They were then cleared by autoclaving for 8min at 15psi in a 10% KOH solution, bleached in 35% hydrogen peroxide for 25min, acidified in 1% hydrochloric acid, and stained using the chlorazol black E method (Brundrett et al, 1984) modified by using 0.01% Chlorazol Black E (Allied Chemical) instead of a 0.1% solution.

The percent of VAM infection for each seedling was estimated using the grid-line intersect method (Giovannetti and Mosse, 1980), by evaluating 100 intersects for each root system using a Wild M3 dissecting microscope (mag. 32x). The thickness of the grid-lines was 0.5mm. The measures of infection recorded were the number of intersects at which 1) only internal mycelium, 2) vesicles, or 3) arbuscules were seen. Percent incidence was calculated as the mean percent positive intersects for all plants in a treatment.

2.6. Statistical Analysis

All statistical analyses were performed using the SPSS-X (4.0) computer program (Norusis, 1985). ANOVA was performed to assess effects of fertilizer level and inoculum treatment. Where main effects were significant, post-hoc Tukey tests ($P=0.05$) were performed to determine group differences. Correlation analyses were performed on dependent variables to examine trends in the data not revealed through mean-variance analysis (ANOVA).

3. Results

3.1. VAM infection rates

In experiment 1 the infection rates were very low in all treatments, with only 17 of 180 plants showing evidence of VAM formation. There was no infection in either the control plants or those infected with the spores of S. rubiformis. Of the plants inoculated with 5g of unsterile soil 11/20, 4/20 and 2/20 plants were infected at levels of fertilization of 0, 1 and 2 respectively. The unfertilized plants also tended to have heavier infections than the fertilized plants, though this was not statistically significant ($P>0.05$). The higher inoculum level used in experiment 2 resulted in more successful VAM establishment, all plants being infected. The data from experiment 2 confirm the trends of experiment 1: infection rates were higher at low levels of fertilization, and the infections tended to show more arbuscules than vesicles (Fig. 1).

Arbuscules were more prevalent than vesicles at all levels of fertilization and tended to be most abundant in the unfertilized treatment, while the greatest incidence of vesicles occurred in the level 1 fertilizer treatment (Fig. 1). Although the mean-variance analysis (ANOVA) post-hoc tests failed to reveal differences in the incidence of arbuscules with regard to fertilizer treatments, correlation analysis revealed a significant ($P<0.001$) negative correlation between arbuscules and fertilizer treatment ($r=-0.5743$) (Table 1). Significant correlations did not exist between vesicles and fertilizer level ($r=-0.2232$, $P>0.05$).

3.2. Plant Health and Growth

Maple seedlings grown in the soil inoculum treatment (expt. 1) exhibited the greatest increase in biomass over the 4 month growth period as evidenced by their greater shoot, root, and total dry weights ($P<0.05$) (Fig. 2). In both experiments plant health declined with increasing levels of fertilizer ($P<0.05$) and in experiment 2, plant biomass and stem diameter decreased with increasing fertilizer ($P<0.05$) (Fig. 3).

3.3. Nutrient Uptake

Inoculum treatment had significant effects on nutrient uptake (Fig. 4). The uptake of Ca, K and N was highest in the soil inoculum treatment ($P<0.05$), while no significant differences were seen for Mg and P. The concentrations of Ca, Mg, K and P were substantially greater in shoot tissue whereas the concentration of N was greater in root tissue, and tended to be less in the roots of soil inoculum seedlings (Fig. 4). Although not statistically significant, the concentration of shoot Mg and P tended to be less in the soil inoculum treatment (Fig. 4).

Despite differences in the inoculum treatments between the two experiments, the effects of fertilization on nutrient concentration and uptake followed similar patterns in both experiments (Fig. 5). In both experiments, Ca concentration in the shoots decreased with increasing fertilizer (up to 2000 kg/ha) ($P<0.05$), whereas K and N concentrations increased as fertilizer was added ($P<0.05$). In experiment 2, Mg concentration in the shoots increased with fertilization ($P<0.05$). In both experiments concentrations of K in the roots were found to increase with increasing fertilizer ($P<0.05$). The same was true for concentrations of Mg in the roots in experiment 1 ($P<0.05$). Total plant uptake of Ca decreased with increasing fertilizer in both experiments ($P<0.05$). Total plant uptake of K was found to increase with increasing fertilizer in experiment 1, and was greatest at levels 1 and 2 in experiment 2 ($P<0.05$). Total uptake of Mg and N by the plants decreased as fertilizer level increased in experiment 2 ($P<0.05$).

Thus, increasing levels of cation fertilization tended to decrease both concentrations and uptake of Ca, decrease total uptake of Mg and N, and increase concentrations and uptake of K. Fertilization appeared to have no effect on either the concentration or the uptake of P in these seedlings.

3.4. Fertilizer/Inoculum Interactions

Significant interactions ($P<0.05$) between fertilizer treatment and inoculum treatment were seen for soil pH (Fig. 6). Where no fertilizer was added, the pH of the soil inoculum was lower than that of the control or spore inoculum treatments. As fertilizer was added, the pH of the control and spore inoculum treatments increased very little, while that of the soil inoculum increased dramatically from 4.7 to 5.5.

3.5. Interactions between VAM infection, plant health, and nutrients

Across the data set, both arbuscules, and to a lesser extent, vesicles were significantly correlated with seedling growth (Table 1). Plant health was positively correlated with plant weight and the presence of arbuscules and negatively related to fertilizer level and soil pH ($P<0.01$). The incidence of arbuscules was positively correlated with the uptake of all nutrients tested ($P<0.01$) except K, while the incidence of vesicles was positively correlated with Mg ($P<0.05$), N and P ($P<0.01$) uptake. Arbuscules were also highly positively correlated with the ratio of plant uptake of Ca/K, Mg/K, N/K, and P/K ($P<0.01$). (Table 1).

Fig. 1. Means for the percent incidence of arbuscules and vesicles at different levels of cation fertilization in experiment 2. Error bars represent 95% confidence limits. (n=6).

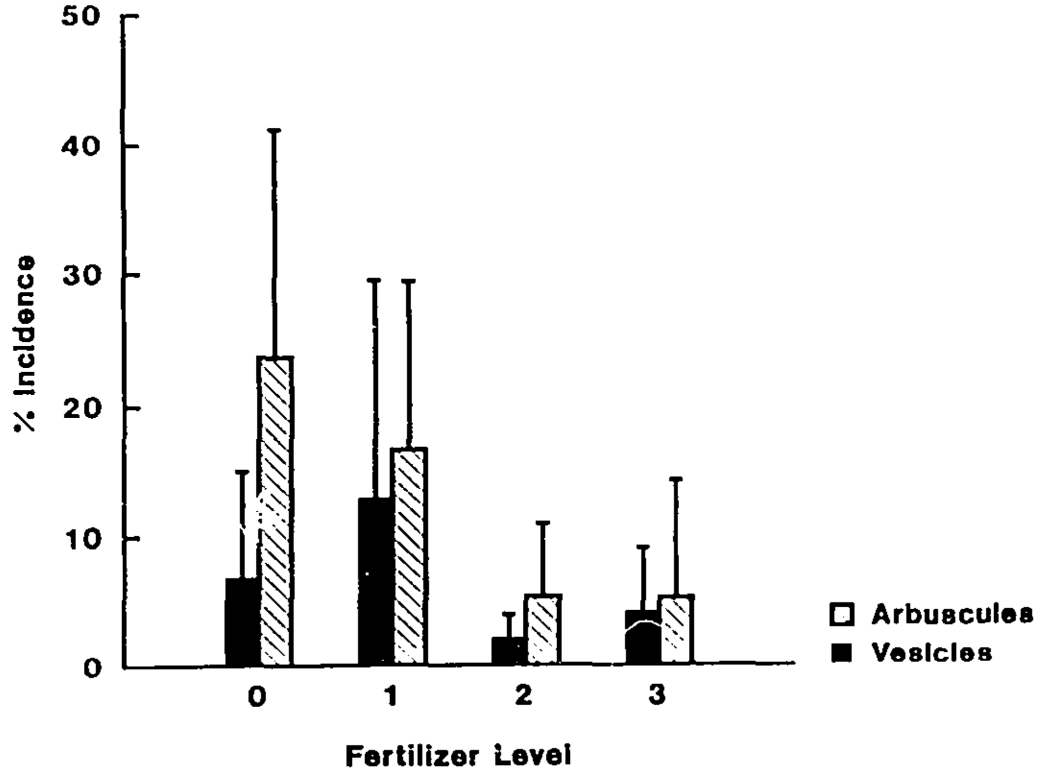
50
40
30
20
10
0
% Incidence
0
1
2
3
Fertilizer Level
Arbuscules
Vesicles

Fig. 2. Means for root, shoot and total plant dry weights (g) as affected by inoculum treatment in Experiment 1. Error bars represent 95% confidence limits. (n=60).

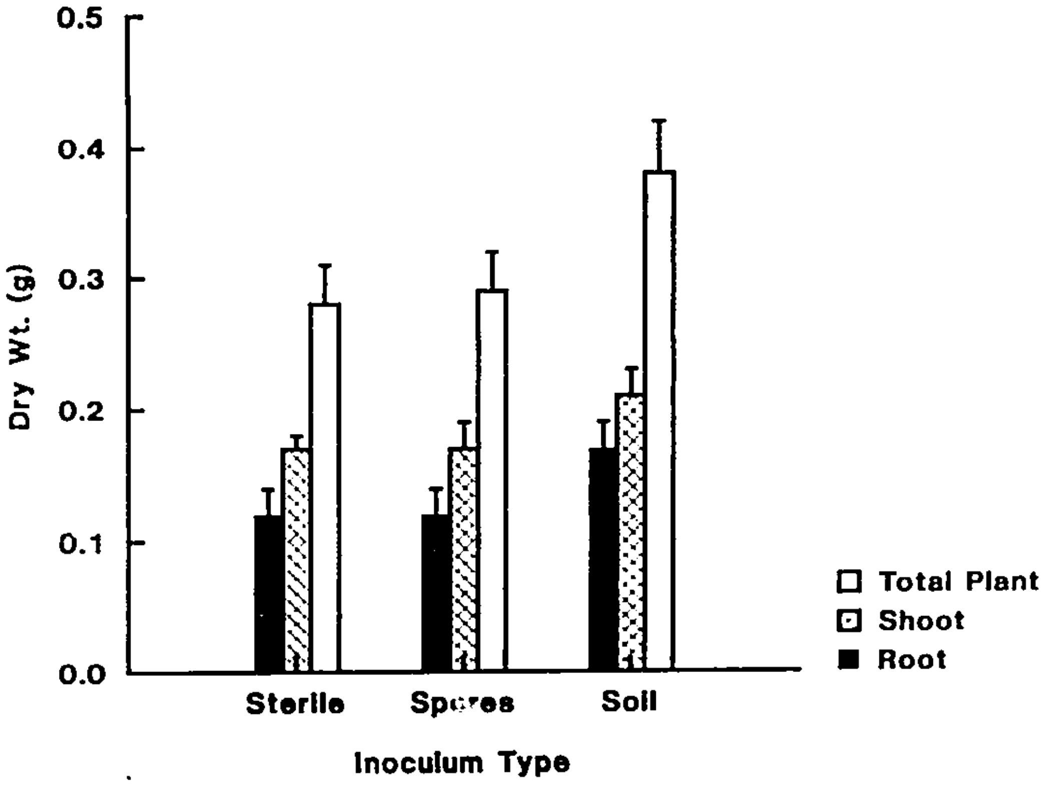
0.5
0.4
0.3
0.2
0.1
0.0
Dry Wt. (g)
Sterile
Spores
Soil
Inoculum Type
□ Total Plant
⊡ Shoot
■ Root

Fig. 3. The effect of fertilizer level on plant health, stem diameter and plant biomass (dry weight) for a) experiment 1 (n=60) and b) experiment 2 (n=6). Error bars represent 95% confidence limits.

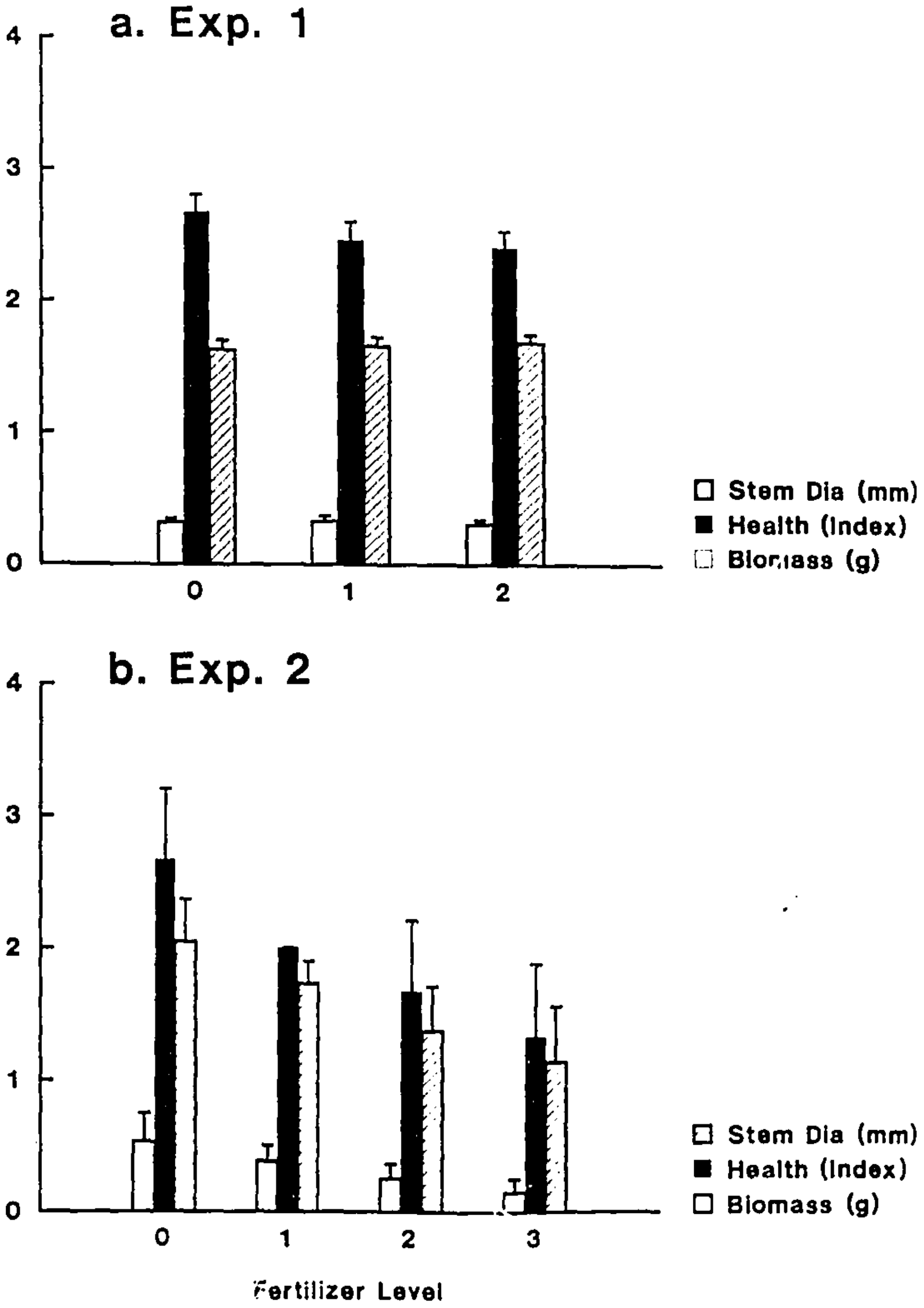
a. Exp. 1
4
3
2
1
0
0
1
2
Stem Dia (mm)
Health (Index)
Biomass (g)
b. Exp. 2
4
3
2
1
0
0
1
2
3
Stem Dia (mm)
Health (Index)
Biomass (g)
Fertilizer Level

Fig. 4. Means for element shoot and root concentrations (mg/g) and total plant uptake (mg) as affected by inoculum treatment in Experiment 1. Error bars represent 95% confidence limits. (n=60).

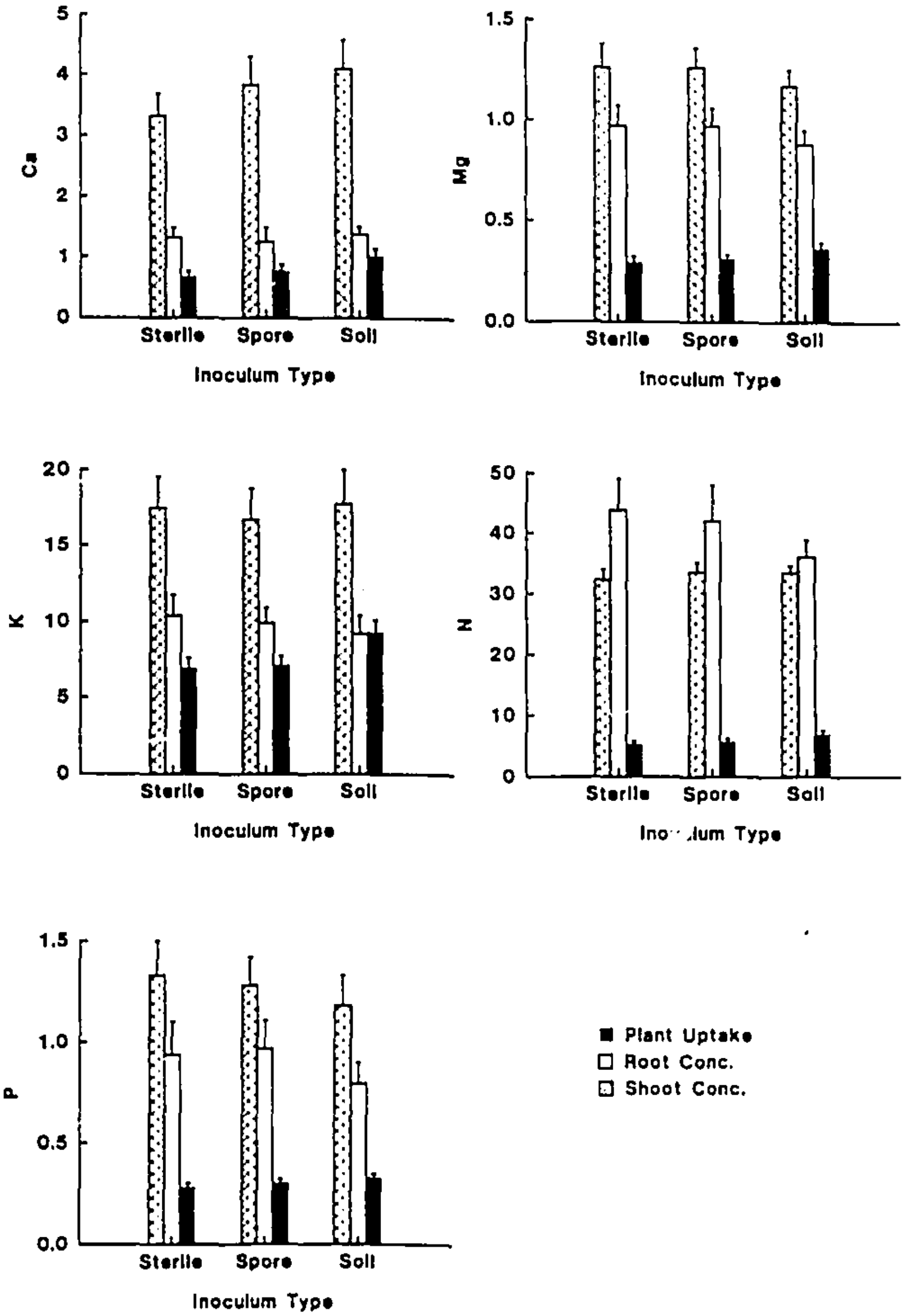
Ca
5
4
3
2
1
0
Sterile
Spore
Soil
Inoculum Type
Mg
1.5
1.0
0.5
0.0
Sterile
Spore
Soil
Inoculum Type
K
20
15
10
5
0
Sterile
Spore
Soil
Inoculum Type
N
50
40
30
20
10
0
Sterile
Spore
Soil
Inoculum Type
P
1.5
1.0
0.5
0.0
Sterile
Spore
Soil
Inoculum Type
■ Plant Uptake
☐ Root Conc.
▨ Shoot Conc.

Fig. 5. Means for element shoot concentration (mg/g), root concentration (mg/g), and total plant uptake (mg) as affected by fertilizer level in experiments 1 and 2. Error bars represent 95% confidence limits. (experiment 1, n=60; experiment 2, n=6).

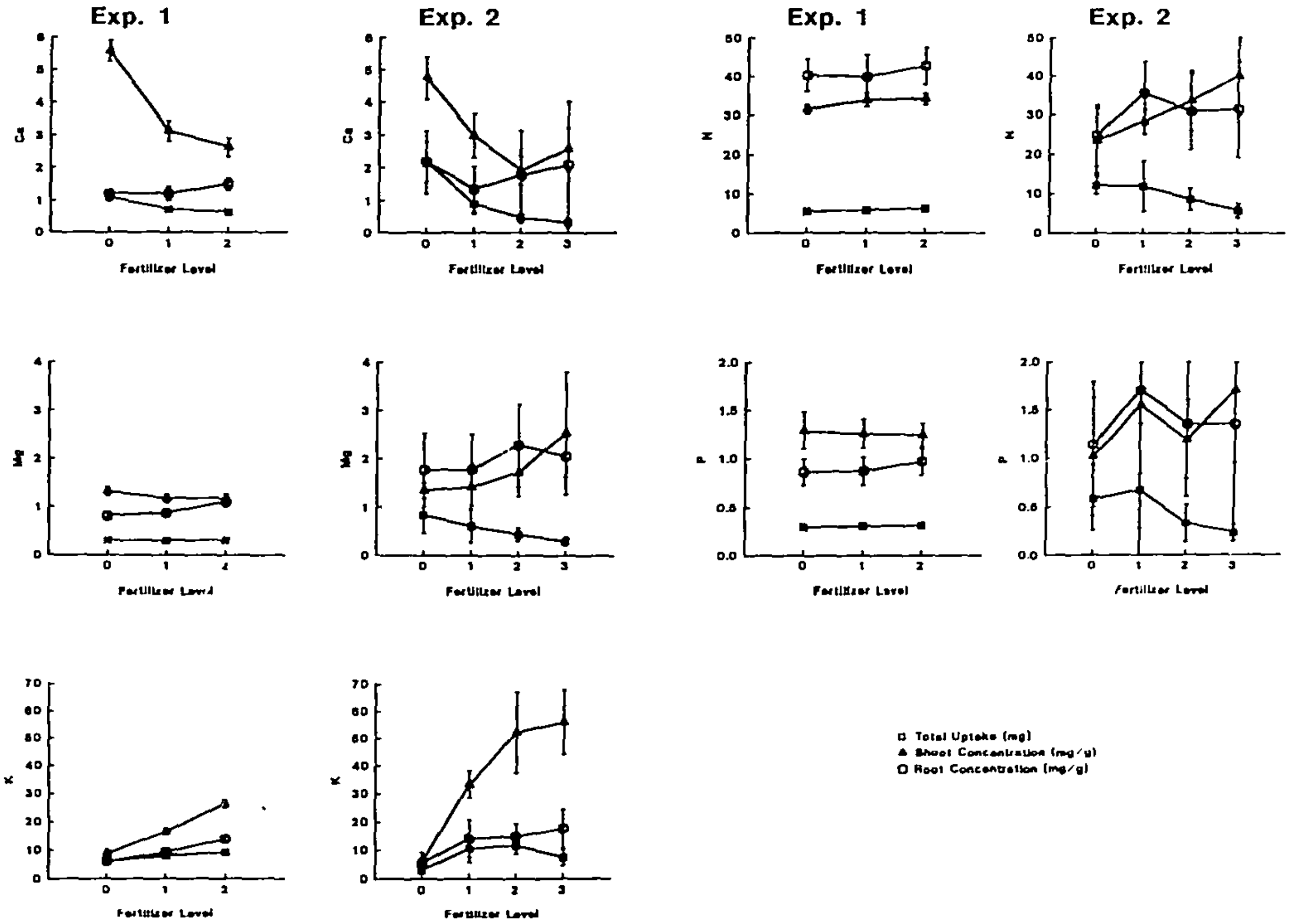
Exp. 1
Exp. 2
Exp. 1
Exp. 2
Ca
Mg
K
N
P
Fertilizer Level
0
1
2
3
□ Total Uptake (mg)
▲ Shoot Concentration (mg/g)
○ Root Concentration (mg/g)

Fig. 6. Interactions between fertilizer treatments and soil inoculum treatments for soil pH in experiment 1. Error bars represent 95% confidence limits. (n=20).

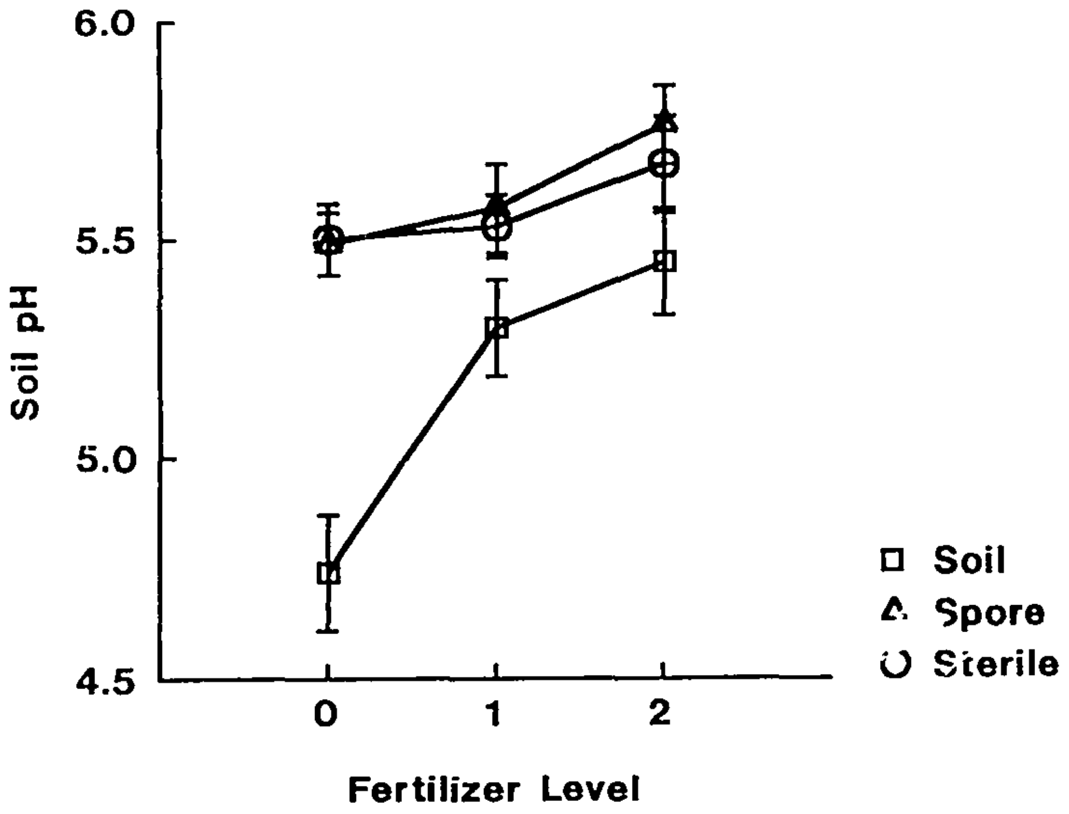
6.0
5.5
5.0
4.5
Soil pH
0
1
2
Fertilizer Level
□ Soil
△ Spore
○ Sterile

Table 1

Correlation coefficients (r) between the incidence of arbuscules, vesicles and plant health for measures of plant health and nutrient uptake in experiment 2. (n=24) (**=P<0.01; *=P<0.05; n.s.=not significant; -=not applicable).

	% Arbuscules	% Vesicles	Plant Health
Plant Health	.6857**	n.s.	-
Plant Dry Weight	.6846**	.4831*	.8054**
Fertilizer Level	-.5743**	n.s.	-.7569**
Soil pH	-.4730*	n.s.	-.6426**
Ca Uptake	.7109**	n.s.	.8284**
Mg Uptake	.7180**	.4776*	.7827**
N Uptake	.6126**	.6485**	.6078**
P Uptake	.6529**	.7126**	.4771*
K Uptake	n.s.	n.s.	n.s.
Ca/K Uptake	.6807**	n.s.	.7730**
Mg/K Uptake	.7130**	n.s.	.7684**
N/K Uptake	.5919**	n.s.	.6690**
P/K Uptake	.6813**	n.s.	.5328*

4. Discussion

There is little proof that any known VAM species infects sugar maples, although Yawney and Schultz (1990) did successfully infect sugar maple seedlings with *Glomus etunicatum* under artificial conditions. Berch (1988) quotes Clark (1969) as stating that *Gigaspora gigantea* infects maples, but Clark did not in fact show this. Schultz and Kormanik (1982) successfully infected sugar maple in pot culture with *Glomus fasiculatus*, a mixed culture of *Glomus mosseae* and *Glomus etunicatus* and a mixed culture of *Glomus* and *Gigaspora* species, but inoculum consisted of root fragments from pot cultures of sorghum, and the purity of cultures had not been established in this study.

Sclerocystis rubiformis spores were among the most abundant species found in the sugar maple stand at St. Hippolyte, Québec (unpublished data), but we were unsuccessful in our attempts at establishing infection with these spores. There is only one report of the successful inoculation of (crop) plants with the spores of *Sclerocystis rubiformis* in pot culture experiments (Gerdemann and Trappe, 1974). It is possible that *S. rubiformis* does not naturally infect sugar maples, despite their abundance in the predominately sugar maple forest, or that the amount (500 spores/pot) of inoculum used was insufficient to establish infection. The 5g soil inoculum used in experiment 1 was pretested and found to contain approximately 500 VAM fungal spores.

Where adequate inoculum was provided (experiment 2), arbuscule formation decreased as fertilizer increased, vesicles were most abundant at the first level of fertilizer, and then decreased as fertilizer increased. It is generally thought that as nutrients (especially P) become more available for plant use, mycorrhizal infection decreases. However, our data show that the health of the plants was negatively correlated with the level of fertilizer. The result is that there was a positive relationship between VAM infection rates and plant health, but it is not possible to determine from our data which is cause and which is effect. More vesicles were formed at the lower fertilizer level than in unfertilized plants. These plants were also less healthy than the unfertilized plants. It is therefore possible that the preponderance of vesicles in these plants was a response by the fungus to stress in the plant.

Despite the very low VAM infection rates in experiment 1, the soil inoculated seedlings were clearly more healthy than those in other treatments and also showed the greatest uptake of Ca, K, and N. Because the unsterile soil inoculum contains microorganisms other than VAM fungi, it is possible that the increased growth and nutrient uptake seen in this treatment resulted from interactions with rhizosphere microorganisms rather than VAM fungi. This would be in agreement with Kothari <u>et al</u> (1990) who reported that rhizosphere microorganisms increased seedling K and Ca concentrations in non-mycorrhizal maize.

In general, plant health and biomass appeared to decrease with increasing fertilizer. Statistically, plant health and biomass were greatest in the control and the level 1 fertilizer treatments. Thus base cation fertilization did not appear to have the beneficial effect expected. Although Ca and Mg were added in substantial amounts in the fertilizer, the uptake of Ca was seen to decrease with increasing fertilization in both experiments, while that of Mg as well as N decreased with fertilization in experiment 2. Potassium was the only element supplied with the fertilizer for which uptake increased with supply (up to level 2). Since K application has been shown to decrease foliar P concentrations in ectomycorrhizal oak and birch seedlings (Newton and Pigott, 1991), it may also affect the acquisition of other nutrients, such as Ca and Mg. In the present experiment, increasing shoot concentrations but decreasing root concentrations of K were evident as fertilizer level increased. Preferential K partitioning towards the shoot has also been seen in endomycorrhizal bahiagrass (Douds and Schenck, 1990). In our experiments, shoot Ca concentrations decreased, while root concentrations appeared to increase with increasing fertilizer level (Fig. 5). Similar results were reported by Azcón *et al* (1991) who showed that, in a soil rich in Ca and Mg, mycorrhizal inoculation of *Medicago sativa* lowered shoot concentrations of both Ca and Mg but did not affect K concentration.

High positive correlations were seen between the incidence of arbuscules, plant biomass, and plant health. The incidence of arbuscules was also highly correlated with the uptake of Ca, Mg, N and P. The uptake of K seems to stand alone, and essentially be a function of its availability in the fertilizer. Douds and Schenck (1990) also found little relationship between K concentration and VAM in bahiagrass. Plant health and K uptake were negatively correlated, although this was not found to be significant. The fact that high correlations were seen between the incidence of arbuscules and the ratios of Ca, Mg, N and P to K, suggests that arbuscules may be having some kind of mediating effect in controlling the balance of nutrients in the plant. An excess of K could have been involved in the decreased health with increasing fertilizer levels. Seedlings may have been unable to discriminate against K uptake. It is possible that VAM fungi compensate for this by increasing uptake of Ca, Mg, N and P. Azcón et al (1991) have also suggested that VAM fungi may buffer roots against nutrient imbalances.

5. Acknowledgments

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Preface to Chapter 4

Previous experiments examined the incidence and morphology of VAM in a natural sugar maple forest. Feeder roots of mature sugar maples from the field have an abundance of brown pigmentation due to the production of phenolics, making them difficult to clear. The following experiment examines the morphological characteristics of VAM in sugar maple seedlings grown in the greenhouse where the reduced production of phenolics made structures clearer and easier to observe. In this experiment, we were also able to determine the time course of the VAM developments in sugar maple seedlings and study the effects of base cation fertilization over time on the condition (arbuscules or vesicles) of the infection and whether the condition of the infection is related to the health of the seedlings.

CHAPTER 4

THE DEVELOPMENT OF VESICULAR-ARBUSCULAR MYCORRHIZAE IN SUGAR MAPLE (ACER SACCHARUM) AND EFFECTS OF BASE CATION FERTILIZATION ON VESICLE AND ARBUSCULE FORMATION.

1. Introduction

The importance of vesicular-arbuscular mycorrhizae (VAM) to plant nutrition has been well demonstrated in studies involving crop plants and other economically important species (Furlan and Bernier-Cardou, 1989; Manjunath and Habte, 1988; Stribley, 1987), but work with the VAM of temperate forest tree species has been limited. In most studies of VAM, emphasis has been placed upon the quantity or frequency of VAM infection on the one hand (Stribley, 1987; Schultz and Kormanik, 1982), or cytological work on the other hand (Bonfante-Fasolo, 1987; Garriock *et al*, 1989; Gianinazzi, 1991). Little attention has been given to the form or condition of the VAM fungal symbiont, or to its anatomy and development over time (Brundrett *et al*, 1985) and the effects of environmental conditions.

VAM infection begins with the formation of appressoria on the surface of the root. Infection proceeds as the fungus penetrates the epidermis, extending inter- or intracellularly, and sometimes forming coils in the outer cortical layers. Arbuscules then develop in the inner cortical cell layers, where metabolic exchange between host and fungal symbiont occurs (Bonfante-Fasolo, 1986). These arbuscules have been shown to be relatively short-lived structures (lasting only from 4 to 15 days) and, in legumes, function metabolically for only 2.5 days (Alexander _et al_, 1989). The last structures formed by the VAM fungal symbiont are vesicles, which are thought to act as storage organs or resting spores within or between the cells of the root cortex (Bonfante-Fasolo, 1986). In terms of VAM development over time, hyphal penetration of leek roots by _Glomus versiforme_ occurs within two days, arbuscule formation in three to four days, and vesicle formation in four to five days from seedling establishment in pot culture (Brundrett _et al_, 1985).

Under natural forest conditions, VAM associations of sugar maples produce appressoria, inter- and intracellular hyphae, hyphal coils, arbuscules and vesicles (Kessler, 1966). Under experimental conditions, roots of sugar maple seedlings infected with _Glomus etunicatum_ exhibited intracellular hyphae, hyphal coils and arbuscules that appeared as "arbusculate coils", but vesicles were not observed (Yawney and Schultz, 1990). We have examined the morphology of VAM infection on roots of mature trees in a natural sugar maple forest suffering from moderate (25%) decline, and the effects of fertilization with base cations on VAM at this site (Cooke _et al_, 1992a). Infections in these maple feeder roots showed appressoria, inter- and intracellular hyphae, intracellular hyphal coils and vesicles. Arbuscules were very rarely seen in these samples, but those which were observed were similar to the arbusculate coils described by Yawney and Shultz (1990). No effect of base cation fertilization on either VAM infection or tree health was observed (Cooke _et_ _al_, 1992a; Coté, pers. comm.)

It has been shown previously that fertilization with N,P, and K can change the relative frequency of vesicles and arbuscules in maple trees (Schultz _et_ _al_, 1981), as can the state of health of the tree (Spitko _et_ _al_, 1978). We therefore undertook this study, using maple seedlings grown under greenhouse conditions to determine the following:-

1. What is the normal time of development for VAM in maple seedlings, and what are the morphological characteristics of the infection?

2. Does fertilization affect the rates of infection or the state of the infection (arbuscules vs vesicles)?

3. Is the health of the seedlings related to the state of the VAM infection?

Materials and Methods

2.1.Seedling establishment

Sugar maple seeds, which had been produced by a single maple tree on the Niagara Peninsula Conservation Authority Property (Comfort Maple Location), were obtained from the Petawawa National Forestry Institute, Chalk River, Ontario (seed lot # 8830273). Sterile seedlings were germinated and established in the greenhouse as previously described (Chapter 3).

2.2. Substrate

Soil was taken from the top 10cm of forest floor of a sugar maple forest described previously (Chapter 2) at St. Hippolyte, Québec, Canada (Univeristy of Montréal, Biological Research Station), and sterilized by gamma irradiation for 24 hours. Natural inoculum was provided to all treatments by mixing sterile soil with an equal weight of the non-sterile soil.

2.3. Experimental design

Experimental treatments consisted of 4 fertilizer levels and 4 harvest periods. Each treatment was replicated 6 times. Fertilizer levels included a control (0 fertilizer), level 1, 0.45g per pot (equivalent to 1000 kg/ha), level 2, 0.90g per pot (equivalent to 2000 kg/ha), and level 3, 1.35g per pot (equivalent to 3000 kg/ha) of a base cation mixture composed of 2 parts (by weight) of fertilizer grade K_2SO_4, 1 part $CaCO_3$, and 1 part $Ca,Mg(CO_3)_2$. This mixture contained a final analysis of 10% Ca, 3% Mg and 22.7% K. Seedlings were harvested after 30, 60, 90 or 120 days of growth.

2.4. Plant analyses

At the end of the specified growth period, the plants were removed from the pots and the shoots and roots were cut apart using scissors. The roots were carefully separated from the soil by washing in tap-water and then seperately placed in formalin-acetic acid-alcohol (FAA) for subsequent analysis of VAM structures. Whole roots were analyzed for VAM in the 30, 60 and 90 day-old seedlings, while a sub-sample of root tissue was analyzed in the 120 day-old seedlings. The shoots were oven-dried at 65^{0}C for 48h to determine dry weight. The root samples were kept in FAA for at least 24h, after which they were cleared and stained using the chlorazol black E method (Brundrett et al, 1984) as modified by Cooke et al (1992a).

The percent of VAM infection for each seedling was estimated using the grid-line intersect method (Giovannetti and Mosse, 1980), by evaluating 100 intersects for each root system using a Wild M3 dissecting microscope (mag. 32x). The thickness of the grid-lines was 0.5mm. The measures of infection recorded were the number of intersects at which 1) only internal mycelium, 2) vesicles, or 3) arbuscules were seen. Mycorrhizal structures were further examined and photographed using a Nikon Optiphot differential interference contrast microscope, at a magnification of either 200x or 400x.

2.5. Data Analysis

All statistical analyses were performed using the SPSS-X (4.0) computer program (Norusis, 1985). ANOVA was performed to assess effects of treatment. Where main effects were significant, post-hoc Tukey tests (P=0.05) were performed to determine group differences. Correlation analyses were performed on dependent variables to examine trends in the data not revealed through mean-variance analysis (ANOVA).

3. Results

VAM infection proceeded via an initial penetration point (appressorium)(Plate 1.1), from which a hyphal coil formed in the underlying cortical cell (Plate 1.2-3). Hyphal coils proliferated through the adjacent cortical cells and arbuscules appeared to develop from them (Plate 1.4-6), forming arbusculate coils similar to those illustrated by Yawney and Schultz (1990). Linear hyphae running from one cell to the next were not normally observed, and intercellular hyphae were rarely observed. Fungal hyphae were seen to constrict (Plate 1.4-5) upon passage through cell walls as infection advanced longitudinally and laterally from cell to cell within the root cortex (Plate 1.4-6).

Coarse aseptate hyphal coils were always formed in the outermost root cortex stemming directly from the appressoria (Plate 1.1-2), and were also observed in the inner cortical layers (Plate 1.3). Two major types of arbuscule were observed. Arbusculate coils, as mentioned above, formed from hyphal coils which had budded and then branched (Plate 1.5, 2.7). These were formed from coarse, seemingly single coils, in an almost haphazard way (Plate 2.7), and also from apparently tiered coils, in a more deliberate fashion (Plate 2.9). The second type of arbuscule arose from extensions of linear hyphae within the cell as they extended intracellularly from cell to cell (Plate 2.9-12). Arbuscules were never isolated, but occupied clusters of cells in a section of root, were usually associated with hyphal coils, and were not restricted to the inner cortical layers. Degenerating arbusculate structures were never observed in these sugar maple seedlings.

Elongate (Plate 3.13) and spore-like (Plate 3.14) vesicles formed singly and terminally on inter- and intracellular hyphae, and sometimes occupied the entire cortical cell (Plate 3.16). Vesicles often formed in groups (Plate 3.15) and were occasionally observed to stem from arbuscules occupying the adjacent cell (Plate 3.17).

Arbuscules were formed in sugar maple seedlings within 30 days in all treatments. Vesicles appeared later, forming between 30 and 60 days in the untreated and level 1 fertilizer treatment, and between 60 and 90 days in the level 2 and 3 treatments (Fig. 1).

More arbuscules than vesicles were observed in all treatments. The incidence of arbuscules was highest in the control and level one fertilization treatment, whereas vesicles were highest in the level one fertilization treatment. At the higher levels of fertilizer there was a large, significant reduction in infection levels for both vesicles and arbuscules (Fig. 1), and plant growth was reduced (Fig. 2).

Plate 1

Figs. 1-6. Progression of VAM development from appressorium to arbuscule. **Fig. 1.** Appressorium (arrow) on surface of rootlet (Mag. 400x). **Fig. 2.** Hyphal coil formed in first layer of cortical cells beneath initial penetration point (appressorium) (Mag. 200x). **Fig. 3.** Coarse aseptate hyphal coils in cortical cells of feeder root (Mag. 400x). **Fig. 4.** Presumed stages in formation of arbusculate coils (b) from branched hyphal coil (a). Longitudinal development (arrow) is seen as hypha passes from cell to cell. (Mag. 200x). **Fig. 5.** Arbusculate coil shows original coiled hypha and fine dichotomous branching. Arrow shows constriction as hypha passes from one cell to the next. (Mag. 400x). **Fig. 6.** Presumed stages of arbuscule development form coil (a) to arbuscule (b). The cell between a and b shows early development of arbuscular structure. (Mag. 200x).

1

2

3

4

5

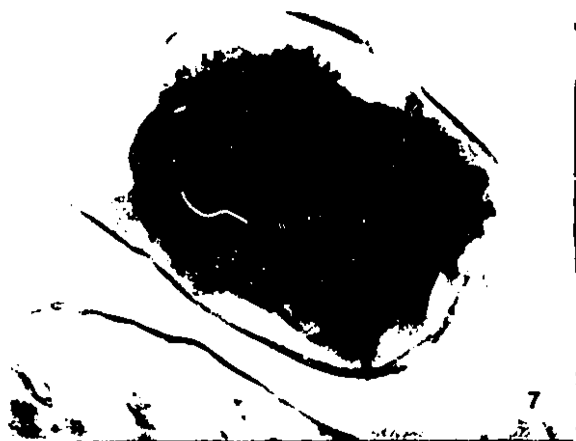
a

b

6

Plate 2

Figs. 7-12. Different types of arbuscules. **Fig. 7.** Arbuscule formed from coarse hyphal coil (Mag. 400x). **Fig. 8.** Arbuscule formed from finer, tiered hyphal coil (Mag. 400x). **Fig. 9.** Arbuscule forming from linear hypha as it passes through the cell (Mag. 400x). **Fig. 10.** More fully developed arbuscule formed in the same way as that in Fig. 9 (Mag. 400x). **Fig. 11.** Arbuscule formed from linear hypha as it extends intracellularly (arrow) from cell to cell (Mag. 200x). **Fig. 12.** Detail of arbuscule seen in Fig. 11 (Mag. 400x).

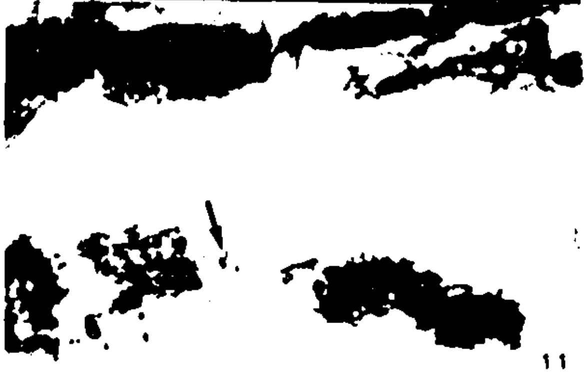


7

8

⑨

10



11

12

Plate 3

Figs. 13-17. Types and associations of vesicles. **Fig. 13.** Elongate vesicle (Mag. 400x). **Fig. 14.** Spore-like vesicle (Mag. 400x). **Fig. 15.** Vesicles formed in groups (Mag. 200x). **Fig. 16.** Vesicles occupying, and assuming the shape of the entire cortical cell (Mag. 200x). **Fig. 17.** Vesicle in association with an arbuscule in the adjacent cell (Mag. 400x).

13

14

15

16

17

Fig. 1. Means for the percent incidence of a) vesicles and b) arbuscules at different levels of cation fertilization and seedling age. Error bars represent 95% confidence limits. N=6.

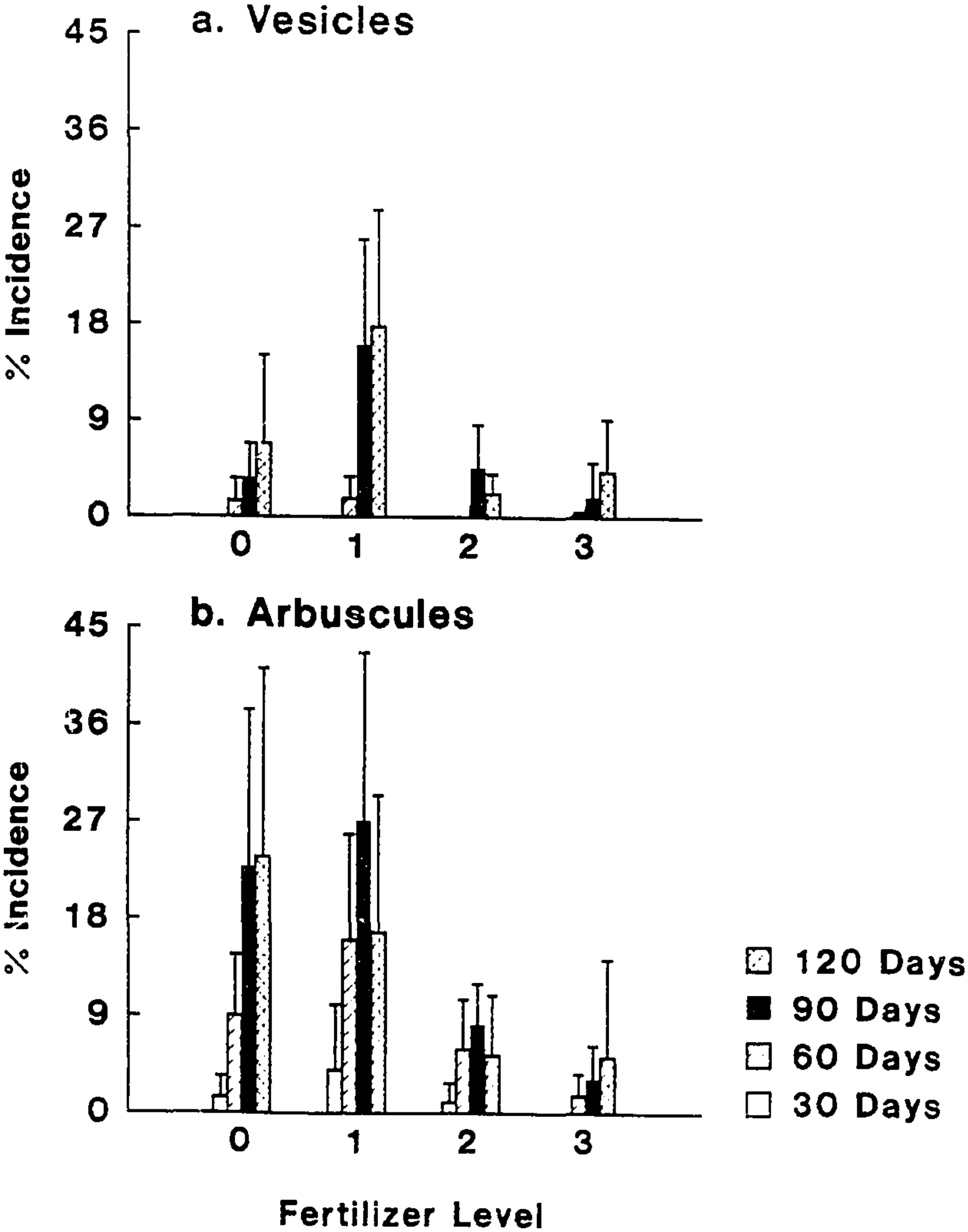
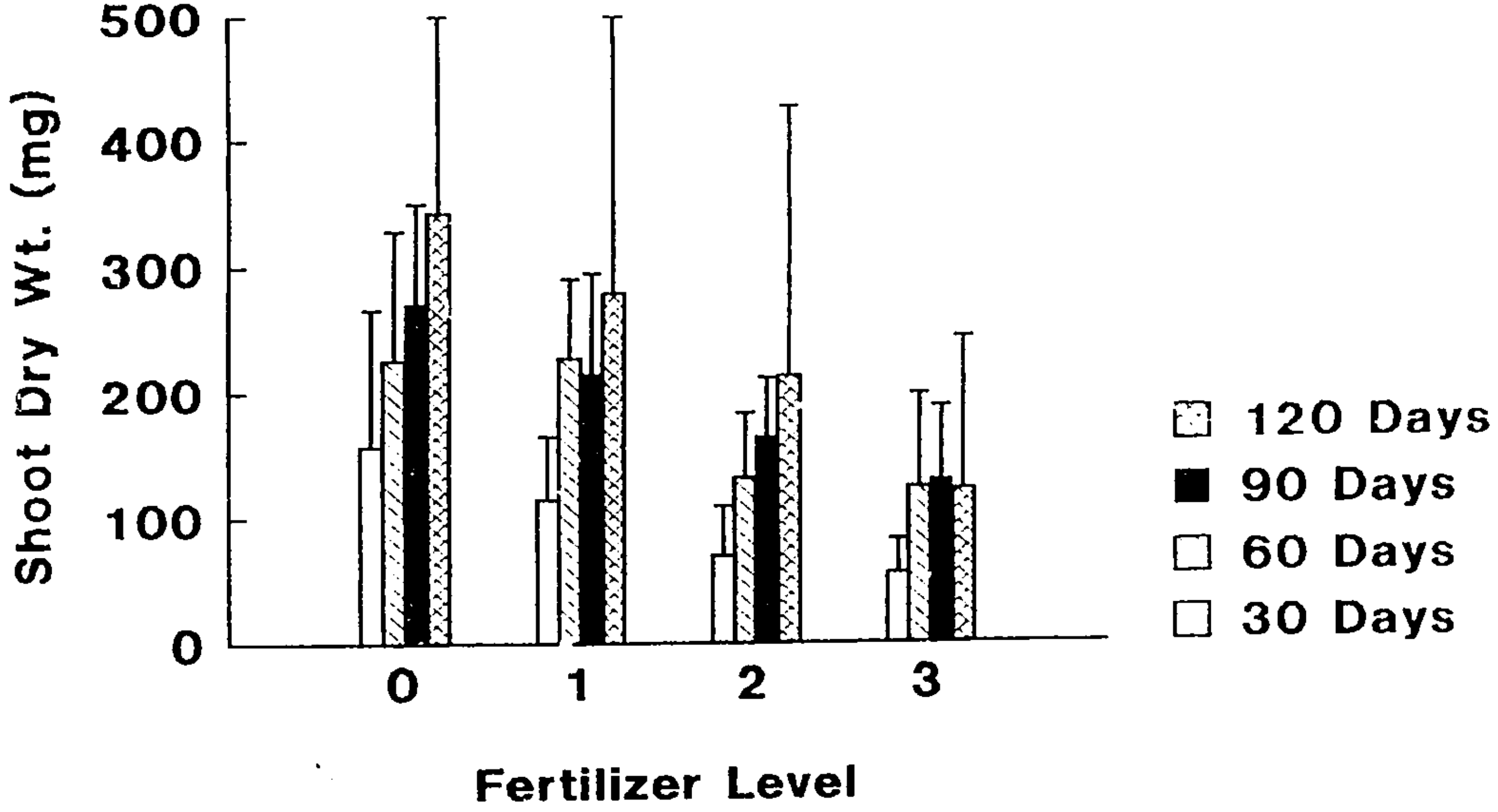

a. Vesicles
b. Arbuscules
% Incidence
0
9
18
27
36
45
0
1
2
3
Fertilizer Level
120 Days
90 Days
60 Days
30 Days

Fig. 2. Means for sugar maple shoot dry weight (mg) at different levels of cation fertilization and seedling age. Error bars represent 95% confidence limits. N=6.


Shoot Dry Wt. (mg)
500
400
300
200
100
0
0
1
2
3
Fertilizer Level
120 Days
90 Days
60 Days
30 Days

4. Discussion

A major advantage of examining VAM structures formed on greenhouse-grown seedlings is the limited amount of brown pigmentation due to phenolics. These phenolics are abundant on roots from the field, making them difficult to clear. VAM structures are therefore much harder to observe in roots from the field than in greeenhouse grown seedlings. Thus, our results are similar to those from field-collected roots in a previous study 'Cooke et al, 1992a), but we have been able to observe more detail in the seedling roots.

In general, the VAM infections in the seedlings were similar to those observed by Kessler (1966) and Yawney and Schultz (1990) in that the infections began with the formation of appressoria on the surface of the feeder root, the formation of coils in the underlying cells and the subsequent formation of arbuscules. Our results also are in agreement with the above authors in showing that hyphal growth was primarily intracellular and that intercellular hyphae were rare. However, while Yawney and Schultz (1990) observed large intracellular hyphae passing through cortical cells unchanged, this was never observed in the present study, unless the hypha terminated in a vesicle. This difference may be a function of the VAM fungal species involved, since Yawney and Schultz (1990) were using a single species inoculum. The hyphae constricted upon passage through the cell walls (Plate 1.5) in a similar manner to that observed by Yawney and Schultz (1990), but Kessler (1966) stated that these constrictions were rare. Kessler also observed that the orientation of hyphal coils was parallel to the longitudinal axis of the root, and this was always apparent in the present study. He also noted that hyphal coils often exhibited irregular protuberances. This phenomenon was observed in the present study as hyphal coils gradually developed into arbuscules (Plate 1.4, 1.6, 2.8-9).

Very few infected cells contained what appeared to be terminate fungal structures (Yawney and Schultz, 1990), and apart from vesicles (which are by definition terminate structures) no terminate fungal structures were seen in the present study. There were however, arbuscules that appeared to be advancing, but had not yet moved into adjacent cells, apparently because the plant had been fixed before penetration of the next cell had occurred.

Kessler (1966) has suggested that the morphology of the fungal symbiont is highly organized, with the hyphal layer forming in the outer cortex and the arbuscular and vesicular layers forming in the inner cortex. Although some level of organization was observed in the present study, arbuscules and vesicles were not restricted to the inner cortical cell layer, and hyphal coils were not restricted to the outer cortex, but tended to be closely associated with the formation of arbuscules. Vesicles were observed to form in isolation from other structures (Plate 3.13-16) or, occasionally, in association with arbuscules (Plate 3.17). The normal development of the VAM association in sugar maple was as follows: appressoria, hyphal coils, followed by the proliferation of arbusculate coils. Vesicles did not appear to be a part of this progression, but were formed in a manner independent of the other processes.

The different forms of arbuscules observed in this study are similar to those shown by Yawney and Schultz (1990) although these authors made no comment of their development from coils, nor speculated on their differences. Since a single type of arbuscule occupied an individual infection focus, it may be that specific foci were formed by infection by different VAM fungal species. It is also possible that the form of arbuscular development depends upon the physiological state of either the host cell or the fungus. The form of the arbuscule could therefore be a response to environmental conditions, at the cellular level or beyond. However, arbuscule types did not change with the health of the sugar maple seedlings, the same types being seen in unhealthy plants at the highest fertilizer levels as were seen in healthy plants.

Similar arbuscular forms have been observed in other studies of sugar maples (Kessler, 1966; Brundrett and Kendrick, 1988; Yawney and Schultz, 1990), as well as in <u>Erythronium</u> <u>americanum</u>

(dogtooth violet), gathered from a sugar maple forest (Brundrett and Kendrick, 1988). In annual crop plants, arbuscules have been shown to be of the classical type, which arise as dichotomously branching structures from a single, unbranched hypha (Bonfante-Fasolo, 1986). Our data leads us to question whether VAM associations of perennial, or woody species are in fact similar to those of annual species used in agriculture. This is difficult to determine from the literature, since most research with crop plants has been concerned with the benefits of VAM in increased production rather than VAM morphology. Yawney and Schultz (1990), for example, have studied the VAM fungus Glomus etunicatum on maples, and while this species has been used with annuals, no morphological data were provided.

Infection foci could be seen where hyphae had moved through a number of adjacent cells forming arbuscules, with no signs of degeneration in the older arbuscules (Plate 1.4, 1.6). Both Kessler (1966) and Yawney and Schultz (1990) reported the presence of degenerating arbuscular clumps. This phenomenon was not seen in the present study, nor was it observed in a previous study, under natural conditions (Chapter 2). Our data therefore suggest that arbuscules in sugar maple may be more durable than the arbuscules formed in annual plants, such as some legumes, where they live from 4-15 days (Alexander et al, 1989).

In the present study, arbuscules were observed within 30 days in all treatments, while Yawney and Schultz (1990) first detected infection only at day 35, and did not observe vesicle formation even after 75 days. Treatment or plant health had no effect on the morphology of VAM in sugar maple seedlings. The only effect of fertilizer treatment in the present study was a decrease in the incidence of VAM structures, as well as a decrease in plant health. More vesicles were observed at level 1 fertilizer treatment, while arbuscules were greatest in the control and level 1 fertilizer treatments. It is possible that the preponderance of vesicles in the level 1 treatment was a response by the fungus to stress in the plant. This thesis is supported by Spitko et al (1978) who observed more vesicle formation in sugar maples as tree health declined. Thus, an abundance of vesicles and scarcity of arbuscules may be an indicator of plant stress.

The present study supports the results of a previous field study (Chapter 2). The only difference between results from the field and the greenhouse is that few arbuscules and an abundance of vesicles were observed in feeder roots of mature sugar maples, while the situation was reversed in greenhouse grown sugar maple seedlings. This discrepancy may be a result of plant age. However, we have observed the development of VAM in sugar maple seedlings gathered from the study site during their first growing season. These seedlings exhibit mycorrhizal infections consisting mainly of vesicles (unpublished data). It is therefore possible that the abundance of arbuscules on greenhouse-grown seedlings was an artifact produced by the conditions of growth, even though the soil was the same. No difference in the morphology of the VAM association was observed between seedlings in the field and those in the greenhouse.

We have determined that VAM infection in sugar maples occurs within one month of exposure to inoculum. This system then, is clearly different from the development of VAM in legumes, where infection occurs at a faster rate (Brundrett _et_ _al_, 1985). While in the past the arbuscule has been viewed as a separate structure (Bonfante-Fasolo, 1986), we have determined that arbuscules in sugar maples are formed through a progression of hyphal coil, to arbusculate coil, to arbuscule. The data from the present study is also in disagreement with previous work that indicates that the arbuscule is a very short-lived structure. We did not observe degenerating arbuscules either in field samples or in greenhouse seedlings. This suggests that arbuscules in sugar maples are a more 'lasting' structure compared to those of crop plants. Our data suggest that an abundance of vesicles in sugar maple feeder roots may be an indicator of plant stress. While plant health had no effect upon the morphology of the VAM association, the addition of basic cations, which led to a cation imbalance in the plants (Chapter 3) increased vesicle formation. A direct relationship between nutrient availability, plant health and condition of VAM fungal association is therefore indicated.

5. Acknowledgments

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CHAPTER 5

CONCLUSION

1. VAM in Sugar Maple

This thesis represents the only thorough examination of VAM associations of sugar maple using natural inoculum. That such a study has been avoided in the past is understandable given the complexity of the system and techniques required.

Because root samples from the forest were so heavily pigmented by phenolics, techniques normally used in the clearing and staining of mycorrhizal roots (Brundrett et al, 1984) required substantial modification. Although sugar maples grown under greenhouse conditions produced fewer phenolics, and therefore presented less problems, these modified methods were still superior to published methods for both field and greenhouse grown material.

The complexity of the VAM system requires that the mycorrhizal system be cultured as a unit, with the fungal component grown in association with a living host. Unlike ectomycorrhizal systems, where the fungal component may be grown separately on agar under laboratory conditions, VAM fungi had not been successfully cultured in such a system until 1988, when Bécard and Fortin established an Ri T-DNA transformed carrot-root system which could be grown on agar in association with Gigaspora margarita. Such systems however, preclude the examination of naturally occurring VAM associations.

This thesis has provided information regarding the ecology of VAM in a natural sugar maple forest growing in unfavorable conditions. The rates and seasonality of infection, as well as VAM morphology under natural field and greenhouse conditions have been described. The effects of ameliorative base cation additions on VAM were evaluated under both field and greenhouse conditions. Additionally, the effects of natural VAM associations on tree health and nutrient uptake were determined.

The present research began with an examination of the incidence and morphological characteristics of VAM over several growing seasons in a natural sugar maple forest suffering from moderate decline symptoms, and the effects of ameliorative base cation additions on these associations (Chapter 2). Because inconclusive results were obtained in terms of the effects of base cation fertilization, research was continued under controlled greenhouse conditions, using soil from the forest site as substrate and source of VAM fungal inoculum. Here, the effect of base cation fertilization on VAM colonization rates of maple seedling roots, seedling growth and nutrient uptake was determined, and the effect of VAM infection on either nutrient uptake or seedling health was examined (Chapter 3). The study was completed under greenhouse conditions as morphology and development of VAM over time were determined (Chapter 4). The questions of whether fertilization affects the development or the state (i.e. arbuscules, vesicles) of VAM infection, and whether the state of the infection is related to seedling health were also addressed (Chapter 4).

The data show that the incidence of VAM fungi in sugar maple feeder roots of moderately declining trees fluctuates throughout the growing season, with infection rates being lowest in mid-summer. This is in agreement with Brundrett and Kendrick (1988), and may be related to seasonality of sporulation of the fungal species involved or to variations in moisture availability during the growing season. Infection rates were also seen to vary from year to year, possibly a reflection of local climatic variations.

The addition of basic cations to the soil in the maple stand did not affect VAM infection rates, even one year after fertilization, but under greenhouse conditions, VAM colonization rates were found to decrease as base cation fertilization increased. In the greenhouse, seedling growth, and the uptake of Ca, Mg and N decreased with fertilization, while K uptake increased. It is suggested that VAM may have a mediating effect in controlling the balance of nutrients in sugar maple seedlings, since the incidence of VAM was highly correlated with the uptake ratios of Ca, Mg, N and P, to K.

Fertilized plots in the field also exhibited elevated foliar K levels, even 3 years after fertilization, while Ca levels fell 2 years after, and Mg levels remained elevated 1 year after fertilization (Coté, pers. comm.). While it is not feasible to extrapolate results from the greenhouse experiment to field application, it is apparent that excess K application may have resulted in limiting the uptake of other elements, especially Ca.

The morphology of the VAM associations in sugar maple were similar in the feeder roots gathered from mature trees in the field and those of seedlings grown in the greenhouse, with the exception that vesicles predominated under natural conditions, while arbuscules predominated under greenhouse conditions. It is suggested that this may be an artifact produced by differing growth conditions, even though the soil was the same. It is also possible that a preponderance of vesicles is a response by the fungus to stress in the plant.

A major finding in this study is the discovery that arbuscules in sugar maples form directly from hyphal coils, and not as separate structures, and that arbuscules in sugar maple are longer-lived structures than was previously thought.

2. Contributions to Original Knowledge

The elements of this thesis that are considered contributions to original knowledge include:

1. The development of modified techniques for the clearing and staining of sugar maple roots.

2. An ecological survey of VAM associations in sugar maple growing in a natural forest setting.

3. The morphological examination of natural VAM associations of sugar maple.

4. The effects of added base cations (Mg, Ca, K) on the morphology, incidence and condition of the VAM associations of sugar maple.

5. The relationship between the incidence and condition of the VAM fungal symbiont and sugar maple health and nutrient uptake.

2. Directions for Future Research

Though this research has answered many of the questions regarding the role of VAM in sugar maple forests, the following indicates research required to further understand this system:

1. Which VAM species infects sugar maples in nature? How many species of VAM fungus exist in a natural sugar maple forest, what are their relative frequencies, and is this affected by forest site, nutrient availability or degree of tree decline? These questions are presently being answered through continuing work in our laboratory.

2. What is the effect of acid precipitation on the natural VAM associations in sugar maple? Under greenhouse conditions, this question could be answered through manipulation of substrate pH under constant nutrient availability.

3. Is the condition of the VAM fungal symbiont related to the degree of stress of the plant? Varying stresses, such as drought, substrate pH, decreased photosynthesis (defoliation) and subsequent evaluation of the state of the VAM association (arbuscule, vesicle) may help to answer this question.

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