VA-Mycorrhizal Spore Populations in Sugar Maple (*Acer saccharum* Marsh. L.) Forest Ecosystems

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ABSTRACT

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Using a modified wet-sieving and centrifugation method, the seasonal changes and effects of cation amendments on the abundance of VAM spores in an acidic hardwood forest soil at St. Hippolyte, Québec were investigated. These data were compared with two more neutral sites in Lacolle, Québec and Waterloo, Ontario. To determine which VAM spores colonized sugar maple, various culturing methods were used and a direct observation method was developed.

Seasonal effects were seen but there was no fertilizer effect on the abundance of spores at the St. Hippolyte site, which had twice as many spores as Lacolle and ten times those at Waterloo. Identified species included: *G. aggregatum*, *G. clarum*, *G. geosporum*, *G. hoi*, *G. macrocarpum*, *G. microaggregatum*, *G. mosseae*, *G. rubiforme*, and *Acaulospora* spp. The major species at St. Hippolyte was *Glomus rubiforme*, a species which, along with other sporocarpic forms, was lacking at the other two sites.

The species occurring on sugar maples could not be determined using traditional culturing methods. Using the direct observation method on roots collected in forest soils it was found that *G. hoi*, *G. macrocarpum*, *G. aggregatum* and *G. rubiforme* grew on maple roots. Of these, *G. hoi* and *G.*

macrocarpum spores were formed throughout the growing season whereas G. aggregatum and G. rubiforme were formed only in the fall.

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TABLE OF CONTENTS

LIST OF FIGURES..... viii

LIST OF TABLES..... ix

1.INTRODUCTION..... 1

2.MATERIALS AND METHODS..... 15

2.1 Spore Populations..... 15

2.1.1 Study sites..... 15

2.1.2 Soil Sampling..... 18

2.1.3 Spore extraction..... 18

2.1.4 Spore quantification..... 19

2.1.5 Statistical analysis..... 20

2.2 Seedling Establishment..... 21

2.3 Symbiont Identification..... 21

2.3.1 Pot culture..... 21

2.3.1.1 Root inoculum..... 22

2.3.1.2 Soil inoculum..... 23

2.3.1.3 Spore inoculum..... 23

2.3.2 Garden experiment..... 26

2.3.3 Aeroponic culture..... 29

2.3.4 Direct observation..... 29

3. RESULTS..... 33

3.1 Spore Populations..... 33

3.1.1 St. Hippolyte..... 33

3.1.2 Comparison between sites..... 45

3.2 Symbiont Identification..... 52

3.2.1 Pot culture..... 52
3.2.1.1 Root inoculum..... 52
3.2.1.2 Soil inoculum..... 57
3.2.1.3 Spore inoculum..... 57
3.2.2 Garden experiment..... 57
3.2.3 Aeroponic culture..... 60
3.2.4 Direct observation..... 60
4. DISCUSSION..... 66
4.1 Spore Populations..... 66
4.2 Symbiont Identification..... 74
4.2.1 Indirect observation..... 74
4.2.2 Direct observation..... 77
5. CONCLUSIONS AND FUTURE DIRECTIONS..... 84
6. REFERENCES..... 88

FIGURES

FIGURE		PAGE
1	Line drawings and photographs of endo and ectomycorrhizal roots.	6
2	Diagram of plot layout-St. Hippolyte.	16
3	Photographs of Spencer Lemaire container system (root inoculum).	24
4	Photograph and line drawing of Spencer Lemaire container system (soil inoculum).	27
5	Photograph and schematic of Aeroponic culture system.	30
6	Photographs of native St. Hippolyte spores.	38
7	Line drawings and photographs of Glomus spore characteristics.	40
8	Line drawings and photographs of G. rubiforme and Acaulospora and their characteristics.	43
9	Graph of St. Hippolyte spore abundances for 1989 and 1990.	46
10	Graph of the seasonal abundances based on total spore numbers.	48
11	Graphs of seasonal spore abundances at the three sampling sites.	53
12	Photographs of spores isolated from root inoculum culture.	55
13	Graph of the mean number of VAM spores recovered in the soil inoculum experiment at various pH's.	58
14	Graph of the mean spore numbers isolated by the Calgon technique.	61
15	VAM spores identified a occurring on the roots of sugar maples at St. Hippolyte.	63
16	SEM photographs of mycorrhizal roots cleaned with Calgon.	79

TABLES

TABLE		PAGE
1	ANOVA table with probability values for the effect of fertilization on spore taxa from St. Hippolyte at various sampling dates.	34
2	ANOVA table with probability values for seasonal effects on spore taxa from St. Hippolyte.	36
3	Mean number of spores per m^2 and the presence or absence of VAM spores at the three sampling sites.	50

1. INTRODUCTION

The soil community consists of fungi, actinomycetes, bacteria, algae, protozoans, and viruses (Alexander, 1977) as well as a range of soil animals. Processes including mineralization, nitrification and denitrification are the results of the metabolism of different combinations of these organisms. A shift in the proportions of bacteria and actinomycetes, which are generally acid-sensitive, to fungi which are much more acid tolerant, could result in a disruption of crucial soil processes. Although fungi are known to be more tolerant of high H+ ion levels than bacteria, they may be adversely affected by the increased metal concentrations associated with acid conditions (Entry et al., 1987) or inhibited by a lack of nutrients produced by cation leaching resulting from proton loading.

This century has seen many declines in sugar maple and other trees. Annual surveys show increasing defoliation and mortality of sugar maple as well as damage to other species (Hendershot and Jones, 1989). This increase in dieback of sugar maple, which has been monitored in Quebec since 1982, threatens a 40 million dollar industry and the livelihood of ten's of thousands of syrup producers (Hendershot and Jones, 1989). In many situations the causal factor or factors of this decline are not known. However, it has been suggested that the greatest mortality corresponds to locations where severe insect attacks, seasonal climatic extremes, low soil

nutrient levels, marginal sites for maple or the highest fallouts of wet acid sulphate and nitrate occur (Hendershot and Jones, 1989). In Quebec, maple stands that show the most decline are located at high elevations and on shallow, droughty soils (Carrier, 1986). One such site is that at St. Hippolyte, where the trees were observed to be in a moderate state of decline at the onset of the study, but later started to slowly recover, just before a fertilizer treatment was applied to boost their health (as part of a larger study (Cooke et al., 1992)). Thus, two predisposing stress factors are the poor soil conditions as well as the marginal climate (Hendershot and Jones, 1989) and if any of the stressors change, the trees will respond accordingly.

Between 1968 and 1985, studies have shown that the relative infertility of soils like those at St. Hippolyte can be explained by an accelerated leaching of soil nutrients, specifically base cations like calcium, magnesium and potassium, due to increased NO_3 and SO_4 loading in precipitation (Wilklander,1980; Ulrich,1987). Furthermore, because the decline covers such a wide geographic region, and variety of soil types, and ecological settings, some ubiquitous environmental impact, like anthropogenic-induced air pollution is likely to be the major factor behind the decline. This could be augmented by natural disturbances which produce subtle stresses that result in "structural or functional alteration of the ecosystem" (McLaughlin, 1985).

There has been little effort put into studying the effects of acidic precipitation on biological processes in the soil until recently, where it can be seen that acid rain and other types of air pollution have significantly altered forest ecosystems. The major problem seems to be the difficulty in studying such a complex system, but it is in these areas where research is required most, specifically the soil ecosystem, where the recycling of nutrients takes place (Déssureault, 1985).

The community of fungi in a northern deciduous forest like that at St. Hippolyte is made up of characteristic species in typical abundances. Seasonal variation in fungal species reflects differences in temperature and moisture levels, but soil properties like pH and the concentrations of some cations have also been shown to be significant factors affecting the fungal community (Christensen, 1969; Bissett and Parkinson, 1979; Widden, 1986a; 1986b). Community composition could be disrupted by a changing soil environment caused by increased acidification. Sulphates introduced into the soil due to acidic precipitation are leached into the soil solution and bound to a cation such as Mg^{2+} or Ca^{2+}, a process which can contribute to the depletion of these nutrients (Binns, 1985). In short term studies, leaching of cations from the soil is a good indicator of acidification rather than a direct measure, due to the high buffering capacity of the organic constituents (McFee and Cronan, 1982). Aluminum, which is present in substantial quantities, is bound to clays and mineral

compounds and is leached into the soil solution more readily in acidic conditions (Havas and Jaworski, 1986; Bache, 1980). This metal has been shown to have adverse effects on mycorrhiza formation (Entry et al., 1987), maple development (Thornton et al., 1986), and the growth of agriculturally important plant species (Havas and Jaworski, 1986).

The mycorrhizal fungi have been shown to be effective in reducing plant stresses caused by nutrient deficiency, drought and soil disturbances (Jasper et al., 1979; 1991; Allen & Boosalis, 1983; Augé et al., 1986; Evans and Miller, 1988; 1990. The roots of 90% of higher plants form a symbiotic relation with fungi, the combination being called a mycorrhiza or fungus-root (Jackson and Mason, 1984). Although mycorrhizae have been known for many years (Dangeard, 1900), it is only in the last 20-30 years that their importance to plant growth and health and their potential use in the improvement of agriculture and the maintenance of forest and grassland ecosystems has been appreciated (Klironomos and Kendrick, in press). It is now widely accepted that mycorrhizae are important components of the root and rhizosphere ecosystem and play a key role in root function and the establishment and maintenance of microbial populations in the soil around them. This zone is now termed the mycorrhizosphere (Linderman, 1988).

There exist two basic types of mycorrhizal associations: ectomycorrhizae (Marx and Krupa, 1978) and endomycorrhizae

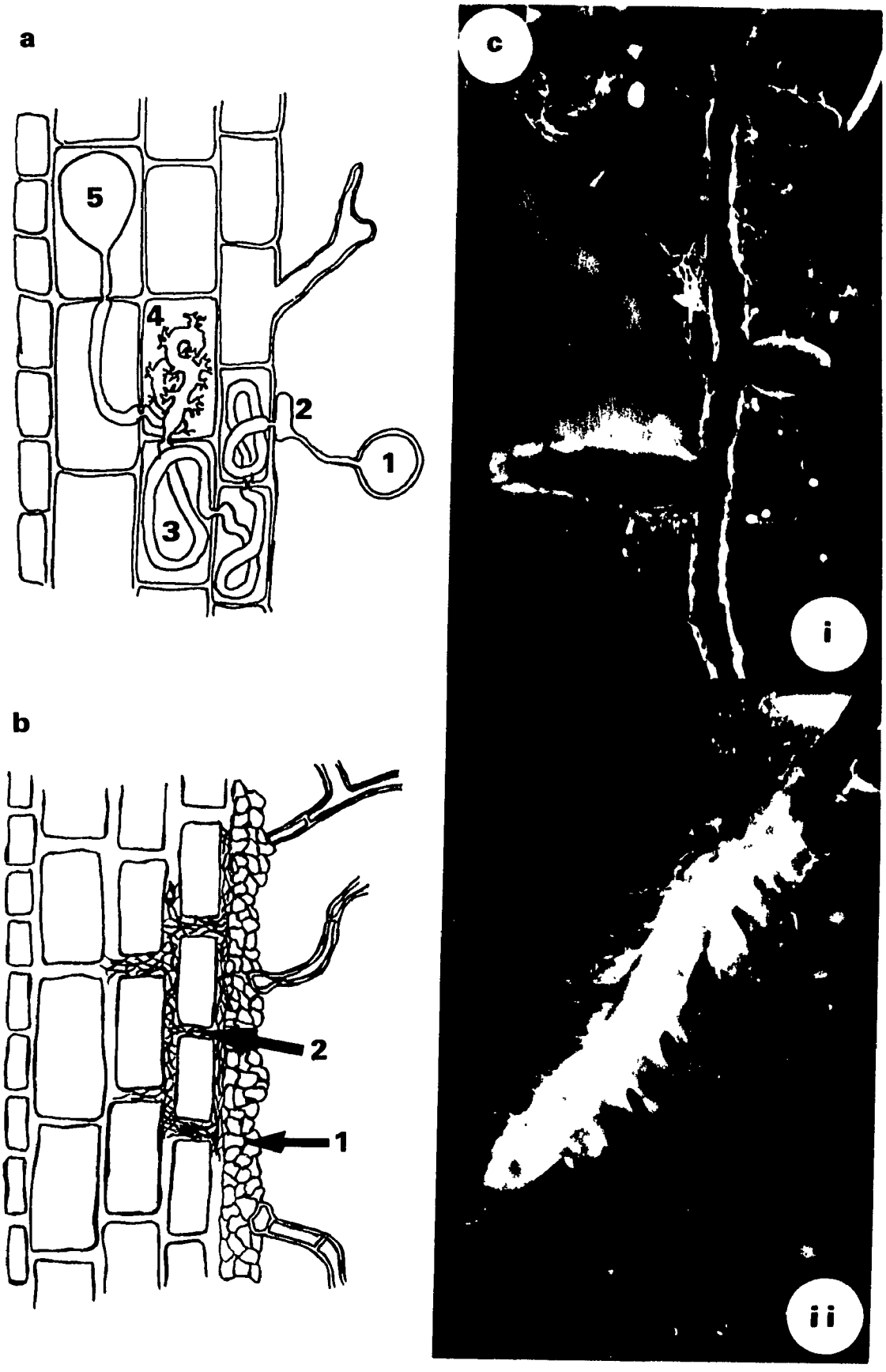
(Hayman, 1978). The fungi in these associations are symbiotic, completing their life cycle in the soil in association with the host plant's roots. In ectomycorrhizae, the fungus forms an external pseudoparenchymous sheath which can be more than 40µm thick and makes up about 40% of the dry weight of the combined fungus-root structure (Hartley, 1965) (Fig. 1b). The fungal hyphae penetrate the intercellular spaces of the epidermis and of the cortical region of the root, forming the characteristic Hartig net, and never invade the living plant cells. The morphology of the root is altered, forming a shorter, dichotomously branched cluster, with a reduced meristematic region.

In contrast to the predominantly exogenous ectomycorrhizae, endomycorrhizae invade the living plant cells in the root, which become filled with mycelial clusters (Hartley, 1965) (Fig. 1a). The most abundant endomycorrhizae are the vesicular-arbuscular (VAM) mycorrhizae, so called because of the presence of vesicles and arbuscules in the cells of the root cortex. VAM fungi colonize roots and discrete areas of infection are evident, each infection unit having a hyphal connection with the extramatricular mycelium in the soil. Within the root, hyphae extend within and between the cells of the cortical layer. Structures that are formed inside the root include: hyphal coils, which may be

Fig.1 (a) Line drawing of an endomycorrhizal (vesicular-arbuscular (VA) mycorrhizal association showing the characteristic structures: (1) chlamydospore, (2) appressorium, (3) hyphal coil, 4) arbusculate coil (maples) and (5) vesicle.

(b) Line drawing of an ectomycorrhizal (ECM) association showing the characteristic structures: (1)fungal sheath and (2) hartig net.

(c) Photographs of both types of mycorrhizal root systems: (i) VAM and (ii) ECM (Photograph graciously provided by Suzanne Diamond, University of Manitoba).


a
5
4
2
1
3
b
2
1
c
i
ii

involved in the transfer of nutrients (Cooke et al., 1992), highly dichotomously branched arbuscules, found mainly in the inner cortex and thought to be involved in nutrient transfer between the symbionts (Cox & Tinker, 1976) and vesicles, apical swellings containing large amounts of lipids and probably serving as storage organs (Otto, 1959) (Fig. 1a).

VA mycorrhizae are present in 80% of Angiosperm families. They include groups of great economic importance, such as the Papilionaceae (including those legumes nodulated by the N_2-fixing bacterium, Rhizobium), the Rosaceae and the Graminae (Powel and Bagyarag, 1984). In addition, some Gymnosperms (species of Ginko, Sequoia and Taxus) are examples (Strullu et al., 1981) of those which form VAM associations. Some ferns, lycopods and bryophytes are also known to be colonized by VAM fungi. The taxonomy of VAM fungi remains problematic, with the constant addition of new species and amendation of others. The most recent taxonomic structure, proposed by Morton and Benny (1990), classifies the VAM fungi as follows:

Order: Glomales

Suborder: Glominaceae

Family: Glomaceae	Family: Acaulosporaceae
Genera: Glomus	Genera: Acaulospora
Sclerocystis	Entrophospora

Suborder: Gigasporinae

Family: Gigasporaceae

Genera: Gigaspora

Scutellospora

The major structure used to identify VAM fungi is the external, soil-borne spore. This is a major problem when undertaking ecological studies. The fact that the systematics are in a state of flux due to varying opinions, further complicates such studies. Other problems are the difficulty, inaccuracy, subjectivity and limitations of using morphological criteria in identifying spores to species levels. Although the use of murographs, graphic representations of the spore walls, depicting the number of layers and their type and grouping (Walker, 1983; Morton, 1988), has helped, there is still much room for error. Even with clear descriptions, it is difficult to identify single spores at the species level. This is especially true for field collected spores, which may be at different stages of development; such samples may also lack the necessary components required for identification, such as subtending hyphae, secondary spores, outer walls, suspensors, etc... Colour alone is one of the worst criteria to go by, since the colour of the walls of the spores can change from hyaline at immature stages to different pigmentations according to varying species at maturity (Dalpé, personal communication). An interference-contrast microscope is useful in identifying

the spores. Even then, spores from the field cannot reliably be identified beyond the level of the genus.

The great interest in VAM in recent years has resulted in many surveys enumerating the species (or genera) of VAM fungi in a particular region (Morton, 1986; Sylvia, 1986; Koske, 1987; Warner et al., 1987). The abundance of VAM fungi can be estimated by counting spores in the soil after extraction by wet-sieving (Gerdemann and Nicolson, 1963; modification Pacioni and Rosa, 1985) or separation from organic matter by centrifugation using a sucrose gradient (Daniels and Skipper, 1962). Another commonly employed method is the bioassay of propagules by planting an assay host on dilutions of soil followed by quantitative analysis by the "most probable number" (MPN) procedure (McGraw and Hendrix, 1986; Porter, 1979; Wilson and Trinick, 1983). A number of methods have been designed to increase the efficiency of extracting spores from various soils, where differing levels of organic matter cause them to clump along with soil particles (Ohms, 1957; Ross and Harper, 1970; Furlan and Fortin, 1975; Mertz et al., 1979; Furlan et al., 1980; Tommerup, 1982). There exist other techniques which do not require the use of sieving, these include the concentration of propagules in dry soil (Tommerup, 1982), the Eelworm-counting slide method (described in Daniels and Skipper, 1962) and the plate method (Smith and Skipper, 1979). These techniques may work on certain soil types and not on others, so, as Ianson and Allen (1986) suggested,

several methods should be compared when isolating spores from a soil for the first time in order to determine which method or combination thereof will extract all the species. However, in natural forest soils, most of these methods still leave too much debris for easy counting. Alternative methods, such as the MPN method, which relies on reinfection under lab conditions, assume a total lack of host-specificity in VAM fungi, an assumption that remains problematic. VAM fungi have traditionally been considered to lack host specificity (150 described fungi and approximately 300 000 plant host species), however recent studies suggest that some level of host specificity or preference exists (Dhillon, 1992a; 1992b; Sanders and Fitter, 1992).

Although we are able to determine the quantity and taxa of spores present in the soil, the level of host colonization, and how some environmental factors affect the symbiotic relationship, we still cannot determine exactly which VAM species are infecting any particular host in the field. Many culturing techniques do exist where a variety of hosts can be used to culture several VAM fungi. The classic and still reliable approach, the pot culturing method (Schenck, 1985), involves the use of sterile pots and an inert substrate in which the two symbionts can flourish. Inoculation of sterile plants is carried out in many ways: Spores, VAM hyphae, infected root fragments and possibly some loosened vesicles may serve as the source of inoculum. Any of these infective

units is surface sterilized and mixed in an inert substrate to colonize sterile plant hosts in pot culture. Soil can also be used as inoculum, where a mixture of the aforementioned infective units may colonize plant roots. Aeroponic culturing of VAM fungi (Sylvia, 1986), eliminates the solid substrates which have to be sieved to separate and isolate spores from the pot culturing technique. Although these and other culturing methods have been successful, plants other than trees, usually annuals or crop plants, have been used as host plants. In addition, most of the studies employing these methods were conducted in the greenhouse which, at their best, do not duplicate natural conditions, and therefore cannot be extrapolated to field conditions. Thus, there is a need to improve methods to look directly at plants from the field and determine which species of VAM fungi colonize root systems.

VA mycorrhizal fungi are present in every cultivated or natural ecosystem and play an important role in plant survival and species diversity (Reeves et al., 1979). It is very difficult to discover what factors determine the presence and abundance of VAM fungi. This is because there are differences in edaphic and climatic factors, and differences in host plants and in soil fertility that can stimulate differential sporulation by VAM fungi (Hayman, 1975). Also, spore production is seasonal in many habitats (Hayman, 1970; Giovanetti, 1985; Sylvia, 1986). VAM fungi are now known to play an important role in the mineral nutrition of many

deciduous trees (Kormanik, 1981), but most studies focus predominantly on mycorrhizae in agricultural or sand dune systems and very few on natural forest ecosystems. This is mostly due to the difficulties inherent in studying VAM in forest systems. Because few surveys exist on forest ecosystems, and the ecology of VAM fungi in natural forest systems is not well understood, this study was undertaken, to answer the following questions. (1) Is there a pool of VAM fungal spores present in the soil which differs both qualitatively and quantitatively with respect to geographic locations and seasons? (2) Will fertilization, applied to replace cations lost due to acidification, change the relative abundances of the spores of VAM taxa in a particular soil? (3) Which of the VAM spores present in the soil will form mycorrhizal associations with the roots of sugar maples? (4) Will the types of VAM fungi that colonize the roots of sugar maples differ with respect to geographic location, soil type, climate, etc...?

In order to answer the first question, slight modifications of existing extraction and quantification techniques were made, and sampling was performed on three sugar maple sites (St. Hippolyte and Lacolle, Québec and Waterloo, Ontario) throughout the growing season. In order to answer the 2nd question, the spore populations were continually monitored over 2 growing seasons after the initial surveys were performed, and after fertilizer was added to half

the plots on the St. Hippolyte site. To answer the 3rd question, a variety of culturing methods, inocula and substrates were used, and a direct method of observation of the sugar maple roots, collected from the sites, was also developed to determine the identity of the VAM symbionts on sugar maples. To answer the 4th question, the fungi on the roots of maples from the two study sites were compared, using the direct observation method.

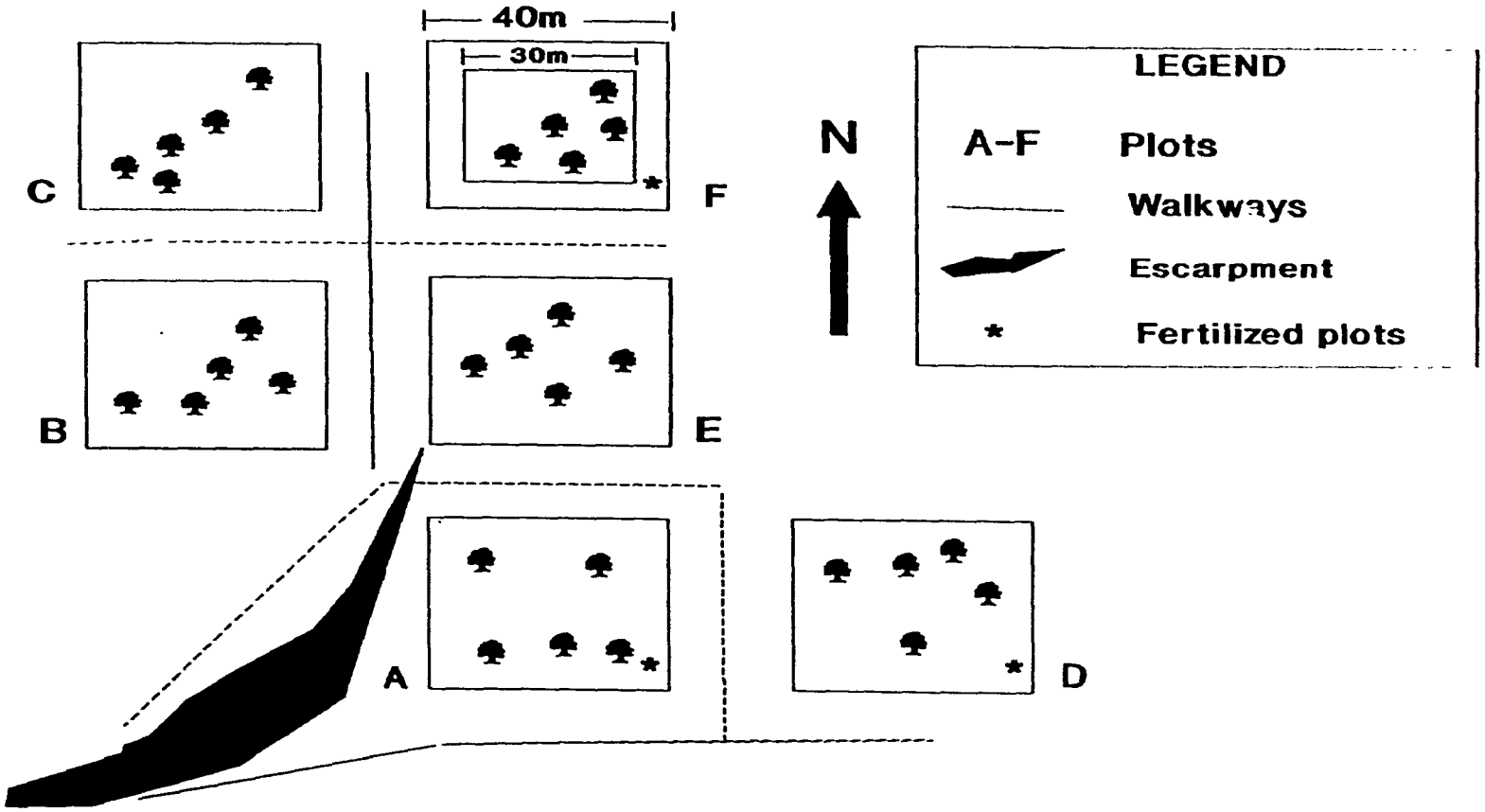
2. MATERIALS AND METHODS

2.1 Spore Populations

2.1.1 Study sites

The study sites were deciduous forests with sugar maples (Acer saccharum Marsh.) as the dominant trees, with varying soil and climatic conditions. The first site was located in the Laurentian mountains, northwest of Montréal, at St. Hippolyte, Québec, Canada (University of Montréal, Biological Field Station), where the soil was acidic (pH=4.2-4.3). The site consisted of six plots (30m x 30m) with a ten meter buffer zone (Fig. 2), located in a 60-80 year-old stand which was predominantly sugar maple mixed with white birch (Betula papyrifera Marsh.) and striped maple (Acer pensylvanicum L.). The soil is an Orthic Ferro-Humic Podzol receiving an annual rainfall with a pH of about 4.3. Fertilization was carried out on June 9, 1989 on three of these plots with a base cation mixture composed of 500 kg/ha K_2SO_4, 250 kg/ha $CaCO_3$ and 250 kg/ha $CaMg(CO_3)_2$, which were the deficient alkaline metals detected in the larger study (Cooke et al., 1992). The second site was located in the Lower St. Lawrence Floodplain, 8km south of Lacolle, Québec, Canada, where the soil was less acidic (pH=5.0-5.8) (described in Widden, 1979). The third study site was located 15km west of Waterloo, Ontario, where the soil was similar to the second site (pH=5.0-5.8) (described in Brundrett & Kendrick, 1988).

Fig. 2 Diagram of the plot layout at the St. Hippolyte sampling site showing the dimensions of the plots and the relative position of the trees.


40m
30m
C
F
B
E
A
D
N
LEGEND
A-F Plots
Walkways
Escarpment
Fertilized plots

2.1.2 Soil Sampling

In each of the six St. Hippolyte plots, five trees of even age were chosen based on trunk diameter, and served as reference points where three samples were collected at random compass points 5m from the base of each tree, yielding a total of 90 samples per sampling period. Each sample consisted of a 2.0 x 15 cm (deep) core, since preliminary study showed this region to be the rooting zone. Samples were bagged and stored in a cold room at 5°C until they were processed. Sampling was done prior to fertilization on May 9, 1989 and after fertilization on July 15 and Aug. 15, 1989, and May 22, July 10, and Sept. 25, 1990.

The other two sites, described above were used as comparative sites. However, having previously sampled the primary site, it was determined, using the "Power Approach" for planning of sample sizes (Neter, et al., 1985), that the sampling needn't be as intensive as it was for St. Hippolyte. Five samples were taken at random compass points around three trees (selected just as for the St. Hippolyte site), bagged and stored in the cold until they were processed. Sampling was done in May, July, October, and February of 1991.

2.1.3 Spore Extraction

Each sample was sieved according to the sieving and decanting procedure of Gerdemann and Nicolson (1963), using a high pressure water hose to wash the spores onto the final

sieve, where they were ultimately collected. To further clean the spores, centrifugation using sucrose (Ohms, 1957; Daniels and Skipper, 1962; Ross and Harper, 1970) was used, though modified as follows in order to remove the high organic contents present in the soil: The spore suspension was equally divided into two 50ml centrifuge tubes, and layered on top of a 60% sucrose cushion. This was then centrifuged at 400g for 20 minutes using an IEC universal model, swinging bucket, table top centrifuge. The water portion was collected with a pasteur pipette, and passed through a millipore filter apparatus. All the spores were filtered onto a 37mm (1.2µm pore size) gridded nitrocellulose filter, and washed with distilled water to remove excess sucrose, and to distribute the spores evenly over the entire grid. The filters were then stored in a small plastic petri dish on a moist Whatman #1 filter paper for subsequent quantification.

2.1.4 Spore Quantification

A total spore estimate was made by counting all the spores present on ten squares in a predetermined pattern covering the entire surface of the gridded filters. A subsample of all the spores from a square which was completely covered was chosen for species identification and relative frequency determination. Using fine needles, spores were mounted on a glass slide into a polyvinyl-alcohol mounting medium (Omar et al., 1979). Identification was done using

differential interference-contrast microscopy and the key and descriptions to the VAM fungi by Berch (1988). From this, the proportion of spores belonging to each taxon was estimated.

It was determined that the recovered spores covered an area of 9.621cm^2 on the original gridded filter paper. Each square covers an area of 0.111cm^2, so that the total area counted was 1.11cm^2. Multiplying by a factor of 8.569 gave an estimate of the total number of spores in the core. As the cross sectional area of the corer was determined to be 3.1 x 10^{-4}m^2, the total number of spores per m^2 could be calculated from each core sample by dividing by the area of the corer. From the identification of each genus, the total number of spores, or the proportion for that particular genus per meter squared (to a depth of 15cm) could also be estimated.

2.1.5 Statistical Analysis

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

2.2 Seedling establishment

Sugar maple seeds from a single maple tree in the Niagara Peninsula Conservation 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 140 seeds. Prior to sterilization, they were immersed for 24 hours in distilled water. The following day the seeds were surface sterilized in 35% hydrogen peroxide for 1/2 hour, rinsed 3 times in sterile distilled water, transferred singly to 16mm test tubes containing 1% water agar slants, and kept at 5°C in the dark until germination (approximately three months). The seedlings were removed from the test tubes at the primary leafing stage and used in the following culturing experiments: root, soil and spore inoculum.

2.3 Symbiont Identification

To determine the species of VAM fungi that infect maples in the field, a number of methods of culturing the fungus with its plant host or hosts were attempted. It must be kept in mind that none of the following experiments were designed to produce quantitative data, but just to identify the fungal symbiont(s):

2.3.1 Pot culture

The first approach taken was pot culturing using various

forms of inoculum (root, soil, and spore). The substrates used in the potting of the plants were inert materials and included: sand, perlite, vermiculite, and combinations of these that were autoclaved for 20 minutes prior to use. In addition to this, space saving "Spencer Lemaire" [small=(2.5cm x 2.5cm); large=(6cm x 4cm)] containers and 3 inch plastic pots were tried, to optimize conditions for the symbionts to flourish. All plants were kept in the greenhouse under a 16hr. photoperiod, maintained on half-strength Hoagland's nutrient solution (Morton, 1992) once a week and watered as required.

2.3.1.1 Root inoculum

Sugar maple roots obtained from the field were surface sterilized as follows: Roots were submerged in a 6% sodium hypochlorite (Javex) solution for 2 minutes and then rinsed three times in sterile distilled water. Following this, the roots were cut up into small fragments with a sterile scalpel and used as inoculum to infect sterile maple seedlings. Two small "Spencer Lemaire" container systems (Fig. 3) were used, and the potting substrate consisted of a mixture of fine and coarse vermiculite which was mixed with the inoculum, aseptically transferred to the containers and then subsequently planted with the sterile maple seedlings and maintained in the greenhouse. A total of 30 plants were planted in each of two container systems, one was given

Hoagland's solution set at pH=4.3 while the other was given Hoagland's set at pH=6.5. Plants were harvested after 5 months to determine if there was any spore production as well as to observe any effect of colonization on plant health.

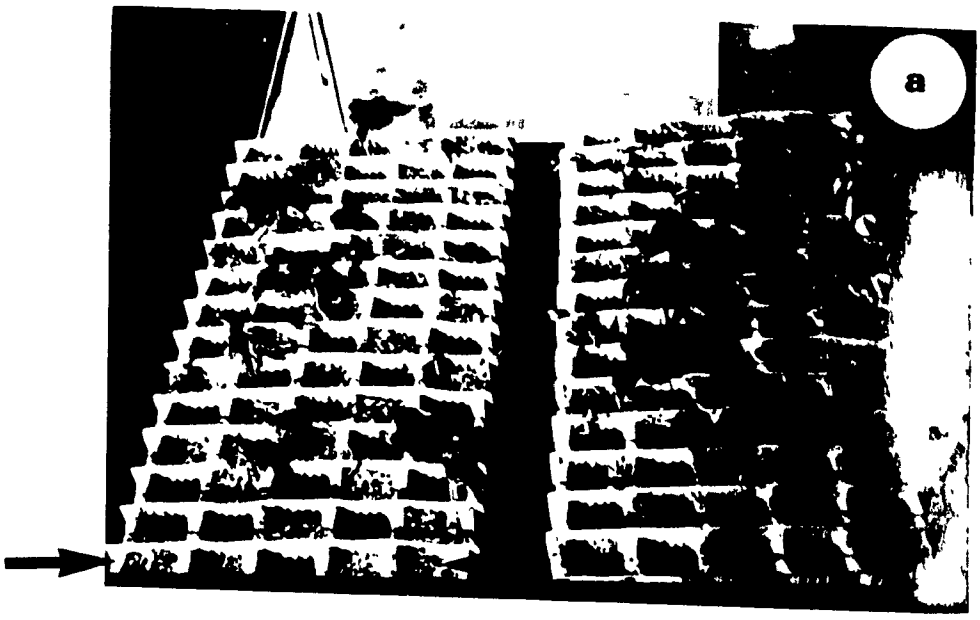
2.3.1.2 Soil inoculum

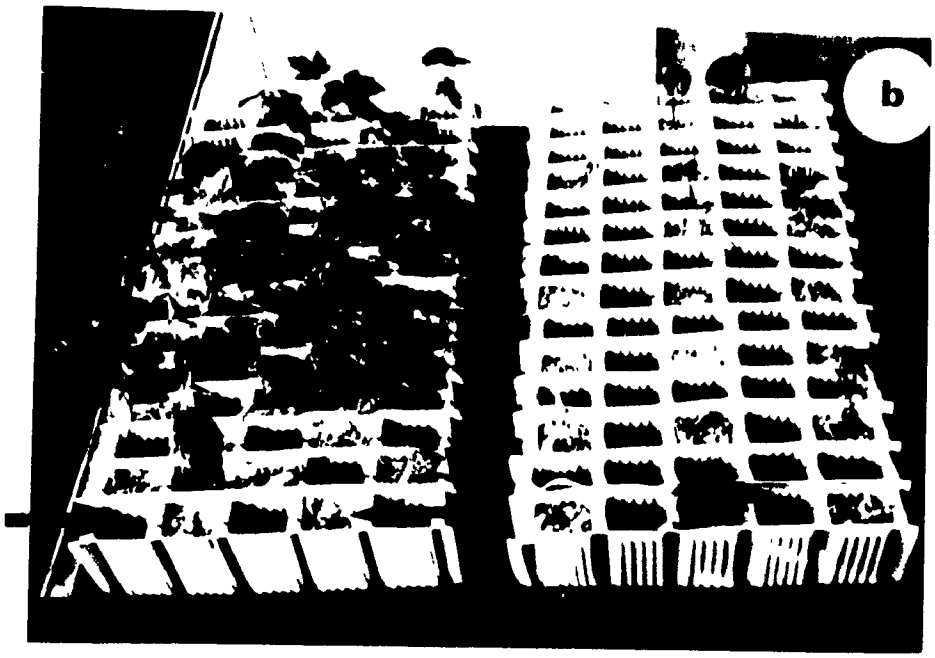
In this experiment, soil inoculum from St. Hippolyte and Waterloo was introduced as a thin layer towards the bottom of the container and covered by a larger layer of sterile vermiculite where the sterile sugar maple seedlings were transplanted. The roots were allowed to grow into the inoculum, become colonized and produce a new crop of spores in the vermiculite layer (Fig. 4b). Thirty plants were transplanted to each of two container systems and the conditions were maintained as in 2.3.1.1. Because of the poor growth of the plants in the smaller container system, the experiment was also repeated using the large Spencer Lemaire container systems and with varying pH (pH=3.5; 4.3; 5.5; 6.5) to see if this would have any effect on the types of spores produced (Fig. 4 a and b). The plants were maintained as above and harvested after 5 months.

2.3.1.3 Spore inoculum

Spores extracted from the soil from the three study sites were surface sterilized for 10 minutes in 0.5% sodium

Fig.3 Photograph of the Spencer Lemaire container system using root inoculum and maintained at (a) pH=4.3 and (b) pH=6.5. Horizontal rows (arrows) show the position of the control plants.


a


b

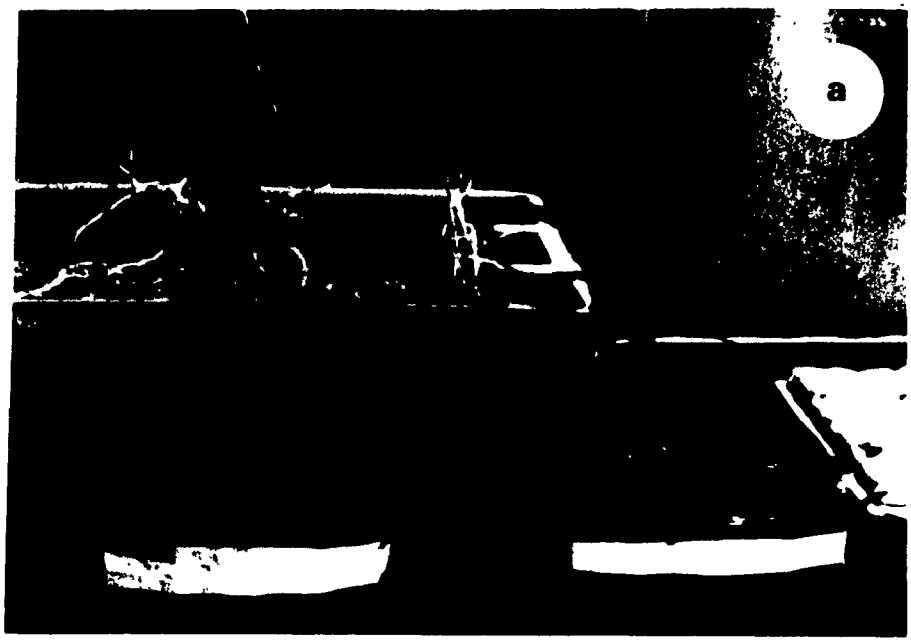
hypochlorite (Javex) and rinsed seven times with sterile distilled water. A solution containing 1ml of each of two antibiotics (2.00mg/ml Streptomycin, 1.00mg/ml Gentamycin) and two drops of Tween20 (0.05% v/v) (surfactant) was used to further sterilize the spores for 1 minute, followed by several washings with sterile distilled water (Mertz et al., 1979). After this, the spores were pregerminated on nutrient agar, 1% agar, or filter paper and then inoculated onto sterile sugar maples either in pot culture or in test tubes where the maple seeds were germinated.

2.3.2 Garden experiment

A derivative of the pot culturing method, referred to as the "garden experiment", was used. Thirty maple seedlings (2-3 year olds) were collected from the field each month, surface sterilized with a 6% sodium hypochlorite solution (Javex), rinsed 3 times with sterile distilled water and potted. Half the plants were maintained in compartmentalized plexiglass growing boxes, while the other half were potted in the small Spencer Lemaire container system. A total of 320 plants (80 for each) were potted in various sterile inert potting substrates (sand, sand:perlite, perlite:vermiculite, vermiculite). The pH of the nutrient solution was also varied (half were kept at pH=4.3 and half at pH=6.5). Plants were maintained in the greenhouse as previously described. After 5 months in the greenhouse, plants were harvested to determine

Fig.4 (a) Photograph of the Spencer Lemaire container system (SLCS) using soil inoculum and maintained at various pH`s.

(b) Line drawing of SLCS depicting how the inoculum was introduced and the pH settings.



b

STERILE SUBSTRATE

CONTROLS ROW

INOCULUM

3.5 4.3 5.5 6.5

STERILE SUBSTRATE

pH TREATMENTS

COTTON

whether any spore production had occurred.

2.3.3 Aeroponic culture

The Aeroponic culturing of VA mycorrhizal plants (Sylvia, 1990) was also attempted. Two aeroponic boxes (Fig. 5a,b) were constructed according to Zobel et al. (1976) with some modifications to the dimensions. Thirty-two sugar maple seedlings from St. Hippolyte were surface sterilized just as for the root inoculum, and planted in each box. The nutrient solution and water provided to one box was set at pH=4.3, while the other was set at pH=6.5. The plants were allowed to grow over one year and every two weeks, as the nutrient solution was changed, pH was measured and the solution was sieved to determine if any spores were produced.

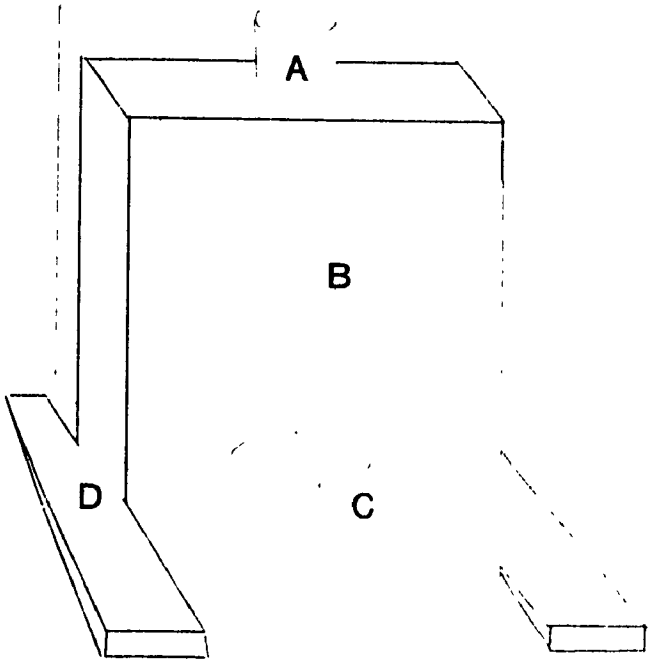
2.3.4 Direct observation

All the above techniques use greenhouse culture as a means for determining which VAM species colonize sugar maple. Because these methods were time-consuming and because the early results were not promising, a method for the direct observation of the maple root system was developed, using the soil dispersant, sodium hexametaphosphate ("Calgon"). Throughout the 1992 growing season, 20 maples (2-3 year olds) were collected arbitrarily, using a 6.0cm root corer, from the St. Hippolyte and Waterloo sites every 2 weeks. These were subjected to a gentle series of washings performed in 1000ml

Fig.5 (a) Photograph of Aeroponic culture system. Arrows indicate the position of the plants.

(b) Schematic of Aeroponic culture box (61cm x 122cm x 46.7cm). [A] Motor: 1.4 kg ball bearing/1.4 horse power/350 rpm/0.6 amp/115 volts. [B] Shaft: stainless steel, fitted on motor with 0.32cm set screws. [C] Spinner: plastic, home humidifier spinner. [D] Motor support: steel, 63cm x 47cm.

b



beakers, in the Calgon solution (0.5%) followed by 1L rinsings in tap water, until the root system was free of all or most debris except for attached extramatrical VAM hyphae and spores. The number of spores on the entire plant root system was counted, and samples from this and other experiments were transferred to FAA (formalin, alcohol, acetic acid) for later quantification and identification using a DIC microscope. Spore samples were taken to Dr. Yolande Dalpé at the Biosystematics Institute at Agriculture Canada in Ottawa for confirmation to species level. In addition to this, root samples with attached spores were prepared for scanning-electron microscopy (SEM) by the following procedure: The roots were placed in 50% ethanol, which was then substituted for 40% and finally 30% ethanol. They were then transferred to a phosphate buffer (pH=7.1) for 24 hours. Dehydration in an acetone series was followed by critical point drying, mounting and sputter coating with gold. Observations were made using a Hitachi S520 electron microscope.

3. RESULTS

3.1 Spore Populations

3.1.1 St. Hippolyte

Preliminary analysis showed that fertilization had no effect on the number of spores present at the St. Hippolyte site (Table 1). Since no treatment effects were observed, the data were pooled to examine the relative abundance and seasonal trends of VAM genera at St. Hippolyte. The major taxa present in the soil as spores were found to be species of Glomus (Nicolson & Gerdemann), which included, G. (Sclerocystis) rubiforme (Gerdemann & Trappe), G. clarum (Nicolson & Schenck), G. aggregatum (Schenck & Smith emend. Koske), G. geosporum (Nicolson & Gerdemann), G. hoi (Berch & Trappe), G. macrocarpum (Tulasne & Tulasne), G. microaggregatum (Koske, Gemma & Olexia), G. mosseae (Nicolson & Gerdemann) and Acaulospora spp. (Fig. 6). It must be noted that these species were only identified as being present but the only species that could be readilly quantified was G rubiforme.

Glomus species were the most abundant spores, forming globose, ellipsoid, or irregular spores, 20-400 µm in diameter with walls up to 30 µm thick. The spores are hyaline, yellow, red-brown, brown, or black and form at a hyphal tip, usually one per tip, but occasionally several are formed. By maturity, the spore contents are separated from the attached

Table 1: ANOVA table with probability values for the effect of fertilization on spore taxa from St. Hippolyte at the various sampling dates.

TAXON	DATE	SSx10^6	DF	MSx10^6	F	SIG. of F
Glomus	May.89	93.8	24	3.05	1.28	.217
	Aug.89	67.5	24	2.81	.53	.955
	Oct.89	52.3	24	2.18	.93	.563
	May.90	66.9	24	2.79	.88	.626
	Aug.90	21.9	24	.91	.45	.982
	Oct.90	509.7	24	21.20	1.06	.414
Glomus (Sclerocystis) rubiforme	May.89	34.0	24	1.42	1.53	.093
	Aug.89	163.7	24	6.82	.95	.540
	Oct.89	63.9	24	2.66	.50	.578
	May.90	45.1	24	1.88	.63	.894
	Aug.90	570.4	24	23.80	.97	.510
	Oct.90	438.9	24	18.30	1.03	.450
Acaulospora	May.89	9.7	24	.41	.88	.630
	Aug.89	7.2	24	.30	.84	.669
	Oct.89	.3	24	.01	.50	.965
	May.90	63.1	24	2.63	1.07	.405
	Aug.90	.4	24	.02	.57	.933
	Oct.90	2.3	24	.10	.66	.869

TABLE 2: ANOVA table with probability values for seasonal effects on spore taxa from St. Hippolyte.

FUNGAL TAXON	SOURCE OF VARIATION	DF	SSQx10^6	MSQx10^6	F RATIO	F PROB.
Glomus 1989	btwn. seas.	2	3.0	1.51	.43	.650
	wthn. seas.	258	904.1	3.50		
	total	260	907.2			
Glomus rubiforme 1989	btwn. seas.	2	54.0	27.00	7.14	.001
	wthn. seas.	258	976.1	3.80		
	total	260	1030.2			
Acaulospora 1989	btwn. seas.	2	29.3	14.70	52.47	.000
	wthn. seas.	258	72.1	.28		
	total	260	101.5			
Glomus 1990	btwn. seas.	2	88.6	44.30	5.09	.007
	wthn. seas.	259	2251.3	8.69		
	total	261	2239.9			
Glomus rubiforme 1990	btwn. seas.	2	106.8	53.40	3.52	.031
	wthn. seas.	259	3928.4	15.20		
	total	261	4035.3			
Acaulospora 1990	btwn. seas.	2	2.6	1.28	1.51	.223
	wthn. seas.	259	220.1	.85		
	total	261	222.6			

Fig.6 Plate of photographs with some representative species present in the soil at the St. Hippolyte site: (a) G. rubiforme, (b) an aggregating Glomus sp. (c) G.geosporum, (d) G. mosseae (e) G. clarum, (f) Acaulospora spp., (g) and (h) other Glomus spp.

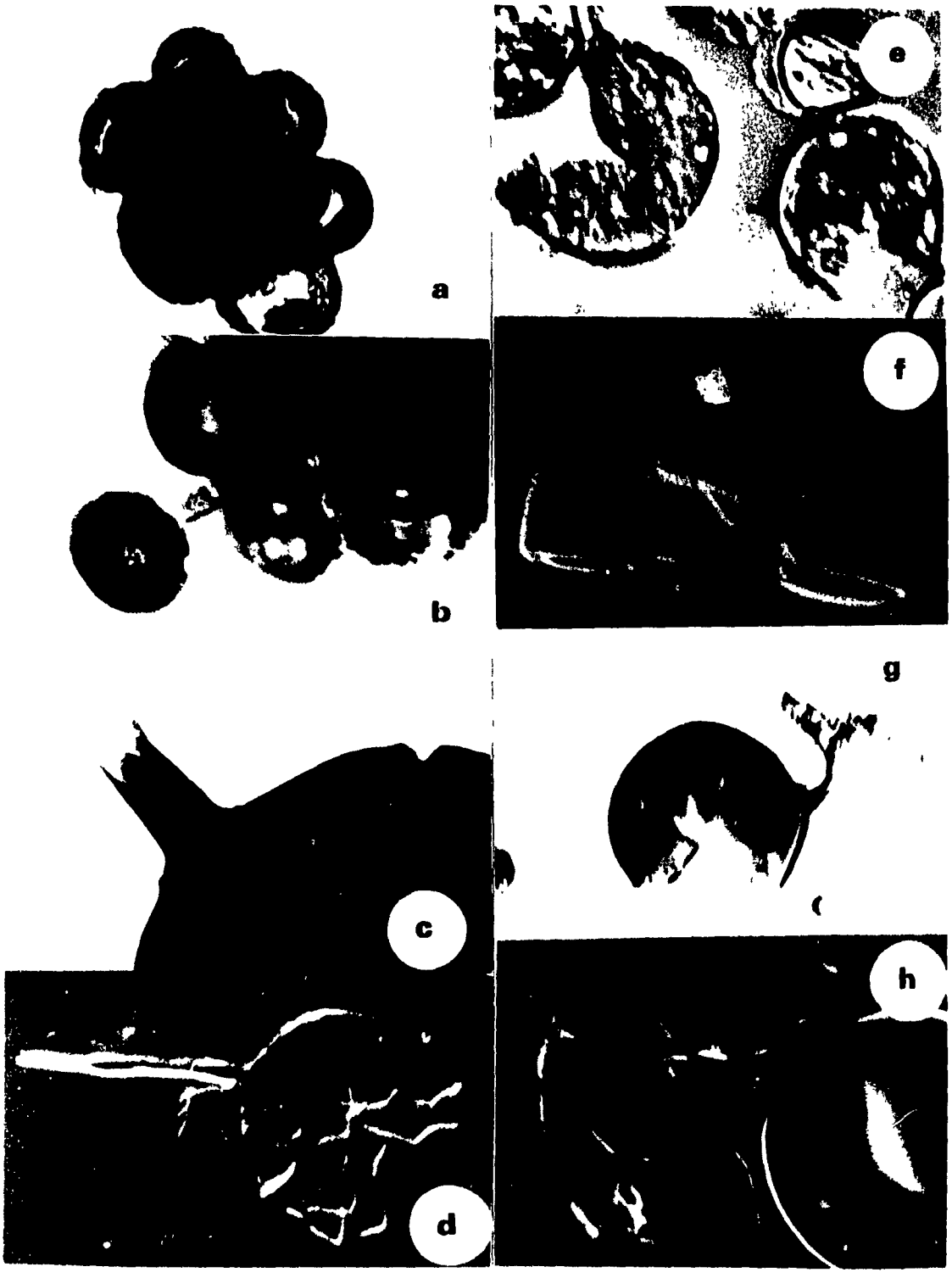

a
b
c
d
e
f
g
h

Fig.7 (a) Line drawing of Glomus spore showing a typical septum (arrow).

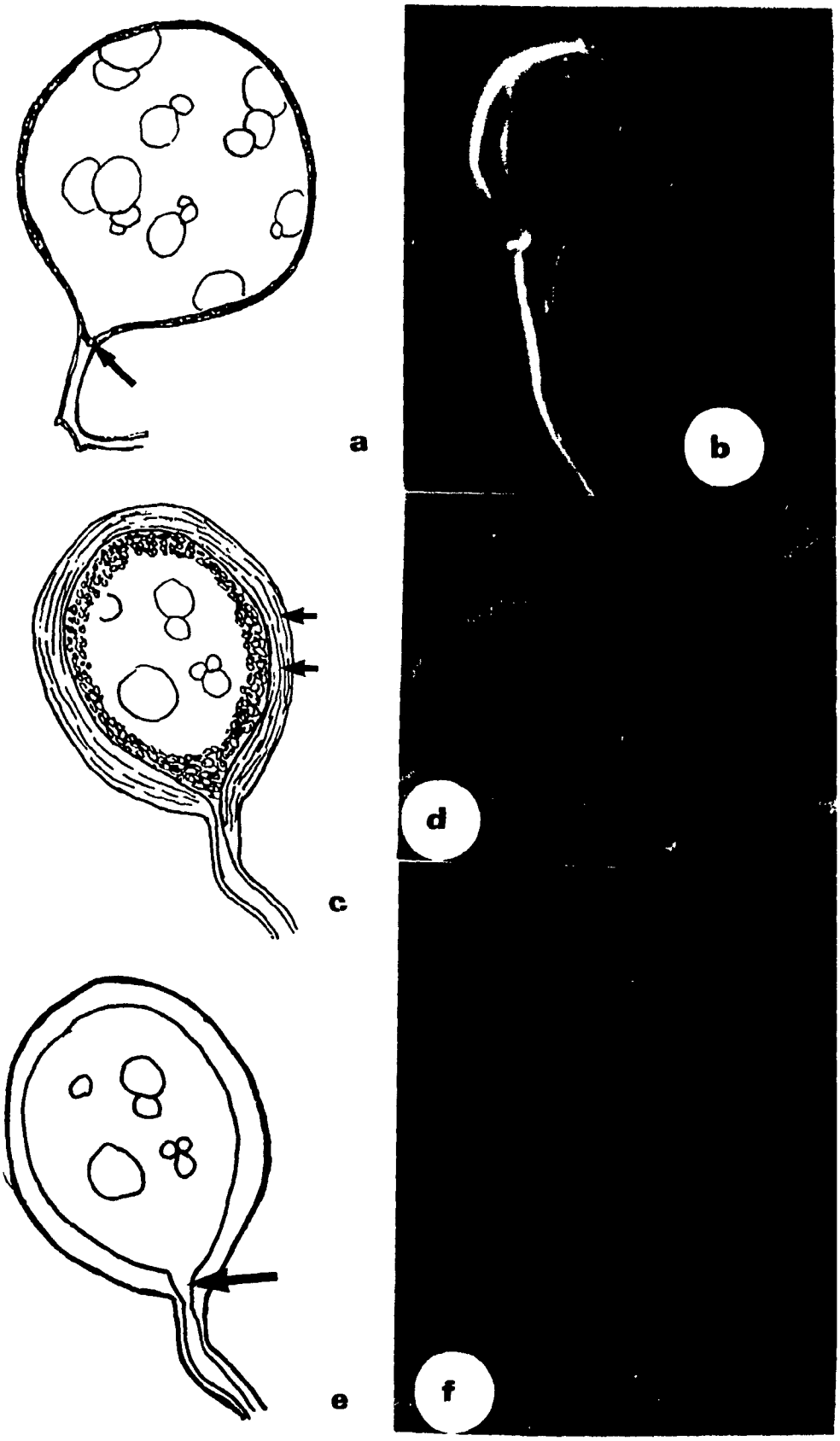
(b) Photograph of G. mosseae from St. Hippolyte site showing the presence of a septum (arrow).

(c) Line diagram of a mature Glomus spore with a laminar wall (arrows).

(d) Photograph of G. clarum from the St. Hippolyte site showing the presence of a laminar wall (arrow).

(e) Line drawing of Glomus spore with the characteristic funnel-shaped subtending hypha (arrow).

(f) Photograph of Glomus sp. from St. Hippolyte showing the presence of the funnel-shaped subtending hypha (arrow).


a
b
c
d
e
f

hypha by a septum (Fig. 7a,b) or by an occlusion with deposits of wall material. Spores of most Glomus species are borne singly in the soil, but some may also form in sporocarps (Gerdemann and Trappe, 1974; Schenck and Smith, 1982). One main characteristic that distinguishes Glomus from other genera, is the presence of a funnel-shaped subtending hypha (Fig. 7e,f) and a smooth or slightly roughened single wall which may contain various layers and or laminations (Fig. 7c,d).

Glomus (Sclerocystis) rubiforme was distinguished from other Glomus species during the study for two reasons, the first being that it was easy to identify due to its characteristic sporocarpic form (Fig. 8a,b), and the second being that it was classified in Sclerocystis until recently when it was incorporated into the genus Glomus (Almeida and Schenck, 1990). To determine the number of spores in each sporocarp, they were broken apart and single spore numbers were recorded. Single spores of G. rubiforme were also present and may have been quantified as Glomus spp., for it is difficult to recognize the species when it appears as single spore.

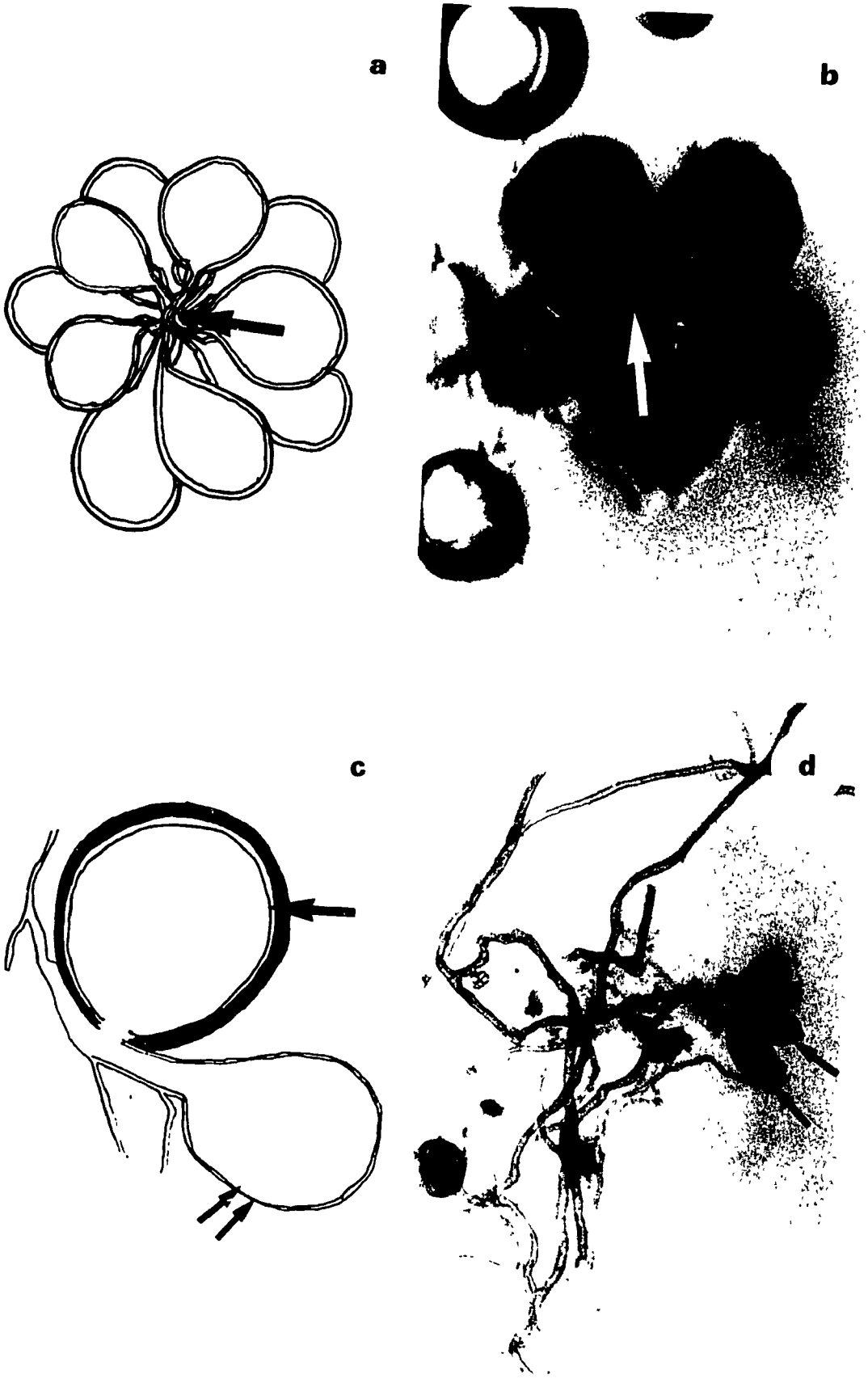
Acaulospora differs from Glomus and G. rubiforme, in that it is produced as a bud off a primary spore or swollen hyphal terminus. This primary spore collapses at maturity and may cling to the secondary spore, but most often it falls off, leaving behind a small pore with an inconspicuous rim. The

Fig.8 (a) Line diagram of G. (Sclerocystis) rubiforme sporocarp showing the central plexus of hyphae (arrow) where the spores are attached.

(b) Photograph of G. (sclerocystis) rubiforme sporocarp from the St. Hippolyte site showing this central mass of mycelium where all the spores originate from (arrow).

(c) Line diagram of Acaulospora sp. showing the formation of a secondary, ornamented spore (arrow) and the remains of the primary spore (arrows) that may or may not remain attached.

(d) Photograph of Acaulospora sp. from Waterloo site stained with cotton blue to show differential darker staining of multiple walls (arrows).


a
b
c
d

spores are borne singly in the soil and are globose or ellipsoid, 100-400 µm in diameter. They are distinguishable by their multiple walls and the ornamented nature of the external wall (Fig. 8c,d).

In 1989 (Fig. 9a), there was no significant difference among seasons with regards to the abundance of Glomus spp. (Table 2). This is not surprising since this taxon included many species that were lumped together because of the difficulty of classifying spores to species level. However, G. rubiforme, showed significantly higher numbers of spores in the fall compared to the spring (Table 2). Acaulospora spp. showed a significant decreasing trend throughout the 1989 growing season (Table 2). In 1990 however (Fig. 9b), Glomus spp. differed in abundance ($p<0.05$) between the summer and fall sampling seasons (Table 2). No significant seasonal effects were observed during 1990 for G. rubiforme and Acaulospora spp. (Table 2). However, it is quite clear that the most abundant spores on the site were those of G. rubiforme, representing 20-40% of the spores in 1989 and 30-50% in 1990. This number is probably an underestimate as single spores of this species may have been identified under Glomus spp..

3.1.2 Comparison between sites

The Québec site differs from the other two sites in that the latter have a significantly lower abundance of total

Fig.9 Bar graph of the abundance of VAM spores at St. Hippolyte during the (a) 1989 and (b) 1990 sampling season. Error bars represent the 95% confidence limits for the mean.

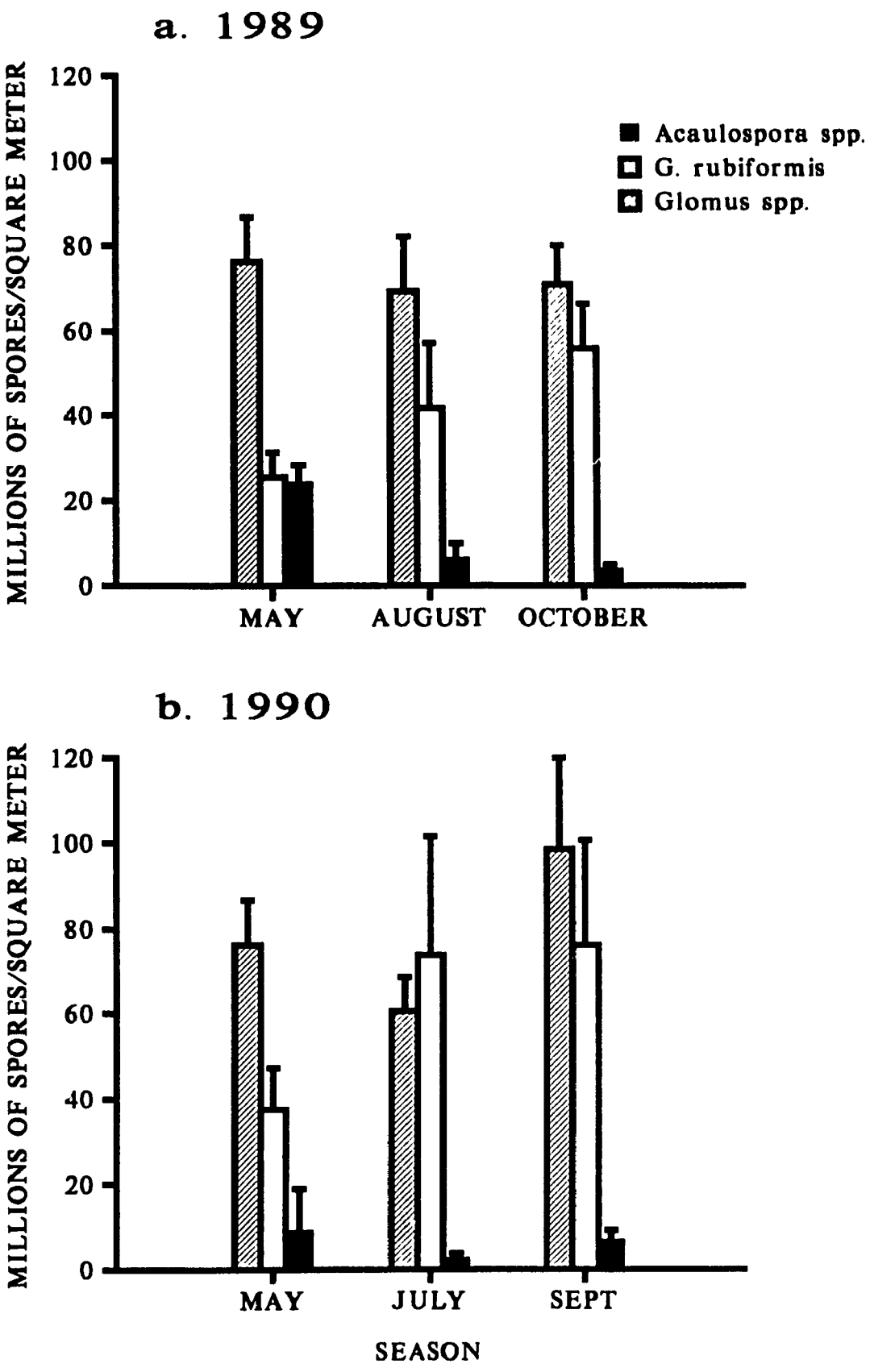

a. 1989
MILLIONS OF SPORES/SQUARE METER
120
100
80
60
40
20
0
Acaulospora spp.
G. rubiformis
Glomus spp.
MAY
AUGUST
OCTOBER
b. 1990
MILLIONS OF SPORES/SQUARE METER
120
100
80
60
40
20
0
MAY
JULY
SEPT
SEASON

Fig.10 Bar graph of the seasonal abundances for total spore numbers for the three sampling sites. Error bars represent the 95% confidence limits for the mean.

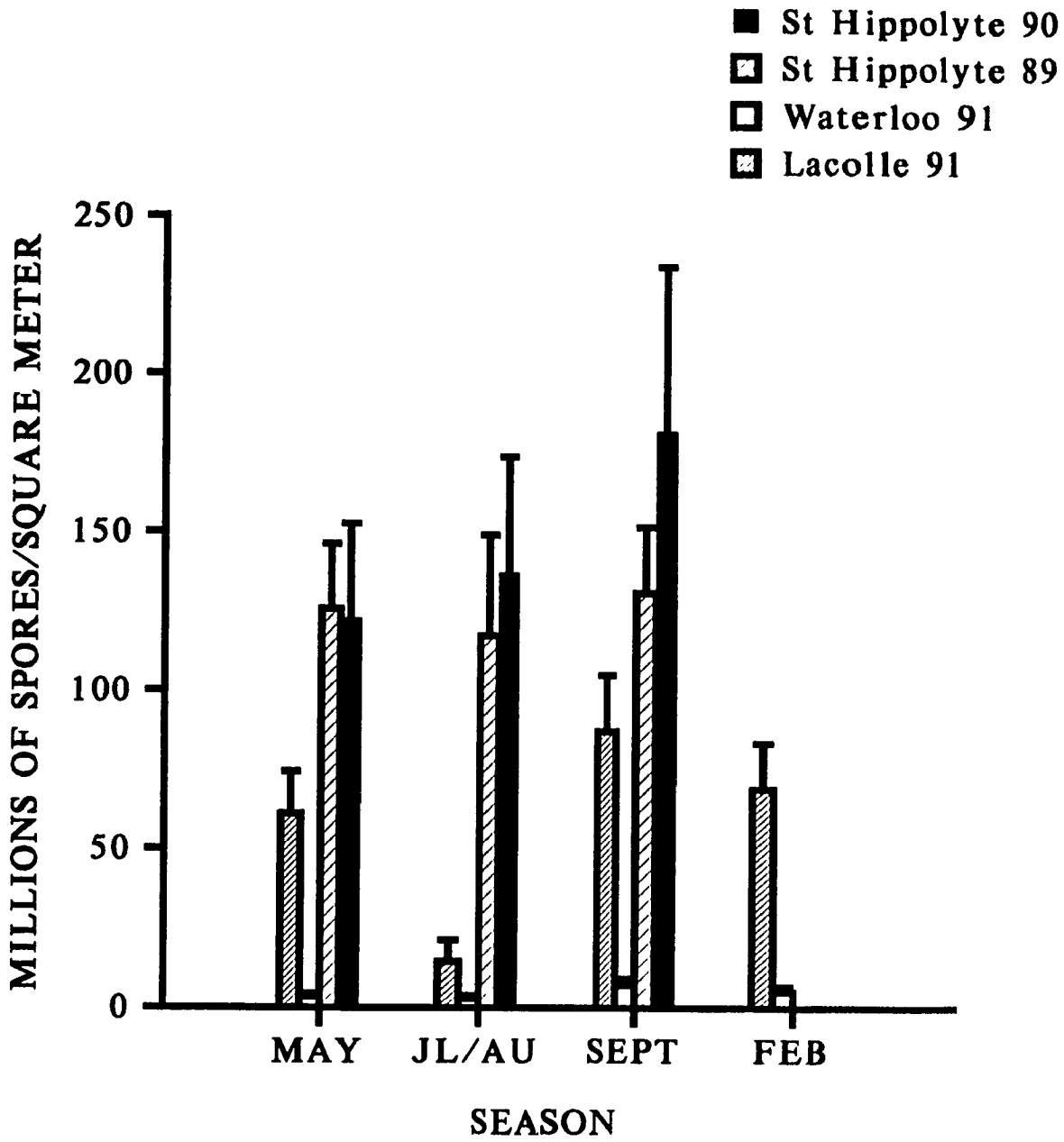

St Hippolyte 90
St Hippolyte 89
Waterloo 91
Lacolle 91
MILLIONS OF SPORES/SQUARE METER
250
200
150
100
50
0
MAY
JL/AU
SEPT
FEB
SEASON

Table 3: Mean number of spores per m^2 of counted taxa and the presence (+) or absence (-) of other species of VAM spores at the three sampling sites.

TAXON	MEAN NUMBER OF SPORES PER M^2			
	St. Hippolyte (1989)	St. Hippolyte (1990)	Lacolle (1991)	Waterloo (1991)
Glomus spp.	7.22×10^7	7.84×10^7	5.78×10^7	4.32×10^6
G. geosporum	+	+	6.08×10^5	1.17×10^5
G. clarum	+	+	-	-
G. hoi	+	+	-	+
G. macrocarpum	+	+	+	+
G. aggregatum	+	+	-	-
G. microaggregatum	+	+	-	-
G. mosseae	+	+	+	+
G. rubiforme	4.10×10^7	6.25×10^7	-	-
Acaulospora spp.	1.16×10^7	6.43×10^6	3.33×10^5	3.63×10^5

\+ Species present at the site but not quantified.

\- Species not detected at the site.

spores by at least a factor of two (Fig. 10). No spores of G. rubiforme were found at Lacolle or Waterloo, in fact, no sporocarpic forms were observed at either of these two sites (Table 3) in this study and in a more intensive sampling carried out by Klironomos (PhD Thesis, in preparation). The number of Acaulospora spores was higher at St. Hippolyte than at Lacolle or Waterloo by about ten fold (Fig. 11). The Québec sites, on the other hand, are similar in that ignoring G. rubiforme, the relative number of Glomus spores are not that different. However, these sites differ from the Ontario site in that the latter has a ten fold lower number of Glomus spores.

3.2 Symbiont Identification

3.2.1 Pot culture

3.2.1.1 Root inoculum

No useful data as to which VAM spores were actively growing on the sugar maple roots was obtained. This was because very few spores were produced and those that were produced did not mature during the period of experimental observation so that identification could not be made beyond the genus level. The spores could be best described as young immature Glomus (Fig. 12). The experiment did show that the health of the infected plants was much better than the control plants, which all died.

Fig.11 Log scale bar graphs of the seasonal spore abundances at the three sampling sites (a) St. Hippolyte (1989), (b) St. Hippolyte (1990), (c) Lacolle (1991) and (d) Waterloo (1991). Error bars represent the 95% confidence limits for the mean.

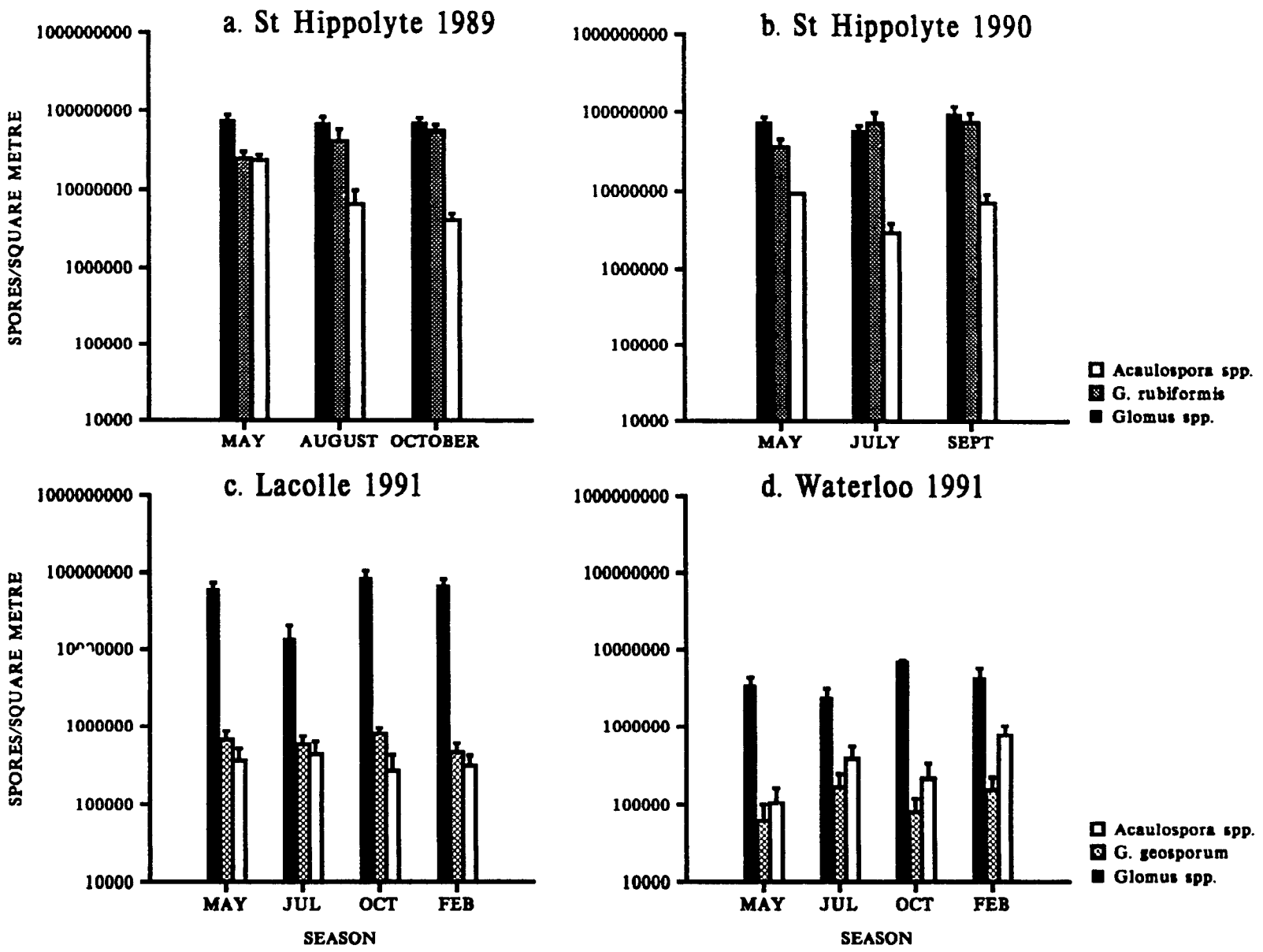

a. St Hippolyte 1989
b. St Hippolyte 1990
c. Lacolle 1991
d. Waterloo 1991
SPORES/SQUARE METRE
1000000000
100000000
10000000
1000000
100000
10000
MAY
AUGUST
OCTOBER
JULY
SEPT
JUL
OCT
FEB
SEASON
Acaulospora spp.
G. rubiformis
G. geosporum
Glomus spp.

Fig.12 (a) Photograph of young, immature spores of Glomus spp. isolated from the root inoculum experiment.

(b) Photograph of these immature spores stained with Chlorazol Black E. and attached to the root (arrow) along with vesicles (arrows).


a


b

3.2.1.2 Soil inoculum

The soil inoculum procedure produced spores which were mature and identified as Glomus hoi. The control plants remained uninfected throughout the experiment. From Fig. 13, one can see that the plants produced more spores at the two extreme pH levels (pH=3.5 and pH=6.5), but this difference was not significant at $p<0.05$.

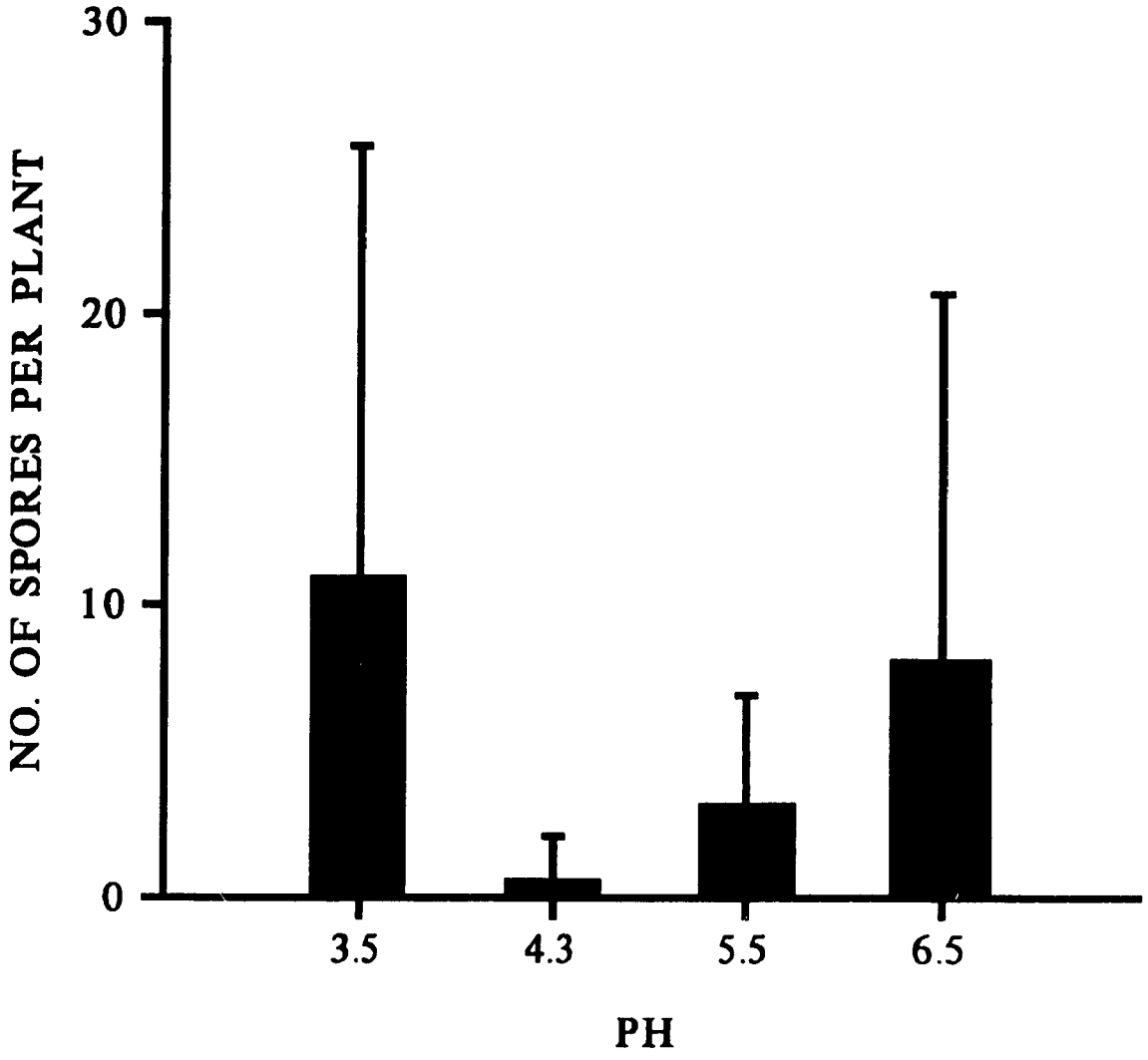
3.2.1.3 Spore inoculum

Infection of sterile sugar maples with pregerminated spores proved to be ineffective because of the abundance of contaminants that were inside the spores. Endoparasites as well as other endogenous fungi, other than VAM, infect the spores in the field (Linderman, 1988) and thus, no spores were successfully pregerminated without contamination.

3.2.2 Garden Experiment

In the greenhouse, the "garden experiment" yielded no production of spores and several problems including dormancy served as major setbacks. Although plants initially seemed to grow well and become acclimatized to the greenhouse conditions, they would suddenly lose their leaves and go into dormancy. Attempts were made to revive the dormant plants by subjecting them to cold shock in a 5°C cold room, but this was only successful on a few plants.

Fig.13 Bar graph of the mean number of VAM spores present on the root systems of maples in the soil inoculum experiment at various pH's. Error bars are 95% confidence limits for the mean.


30
20
10
0
NO. OF SPORES PER PLANT
3.5
4.3
5.5
6.5
PH

3.2.3 Aeroponic culture

The maple saplings in the aeroponic "garden experiment" also showed problems with dormancy. Although chilling the maples for two months did break the dormancy, additional technical difficulties with the system resulted in no data. However, some relevant physiological information came out of this experiment in that the plants from the field, in the box set at pH=4.3 grew much better and produced a more extensive root system than those set at the "optimum" pH=6.5 described in the literature for best growth (Jarstfer & Sylvia, 1991). This was due to the fact that these seedlings were taken from St. Hippolyte, where the trees grow in a more acidic environment.

3.2.4 Direct observation

Direct observation using Calgon treated roots provided data over an entire sampling season for the St. Hippolyte site, but no spores were ever observed attached to roots of sugar maples from Waterloo (Fig. 14). Species that were identified were: Glomus macrocarpum (Fig. 15d) and Glomus hoi (Fig. 15a,b), which were observed throughout the entire sampling season, and Glomus aggregatum (Fig. 15c) and Glomus rubiforme (Fig. 15e,f) whose spores appeared only in the fall. Overall, 50.56% of the plants did not show any spores attached to their root system, only extramatrical mycelium. An ANOVA showed no significant seasonal effect on total spores

Fig.14 Bar graph of the mean number of VAM spores over a sampling season isolated by the "Calgon" technique at St. Hippolyte. Error bars are the 95% confidence limits for the mean.

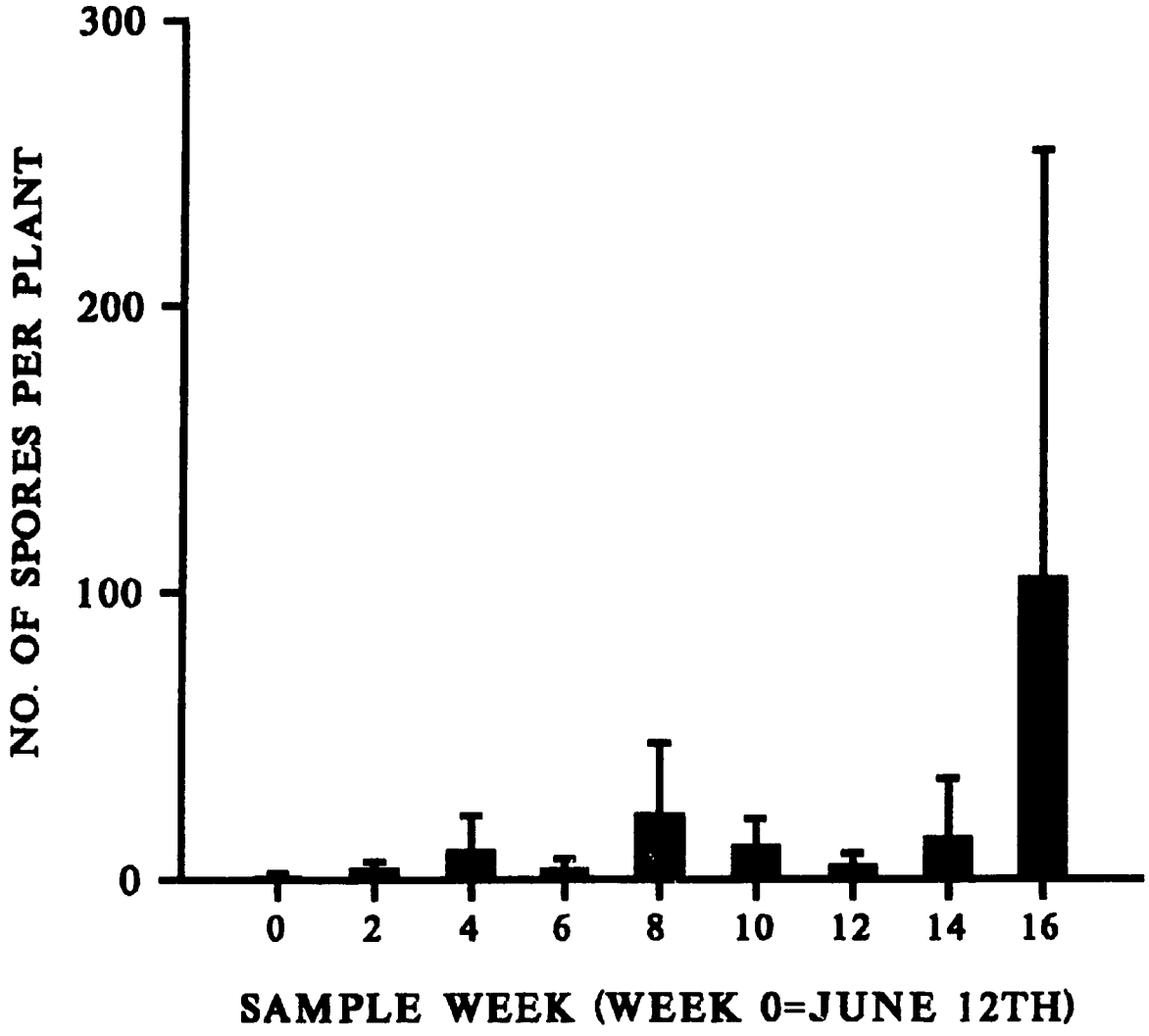
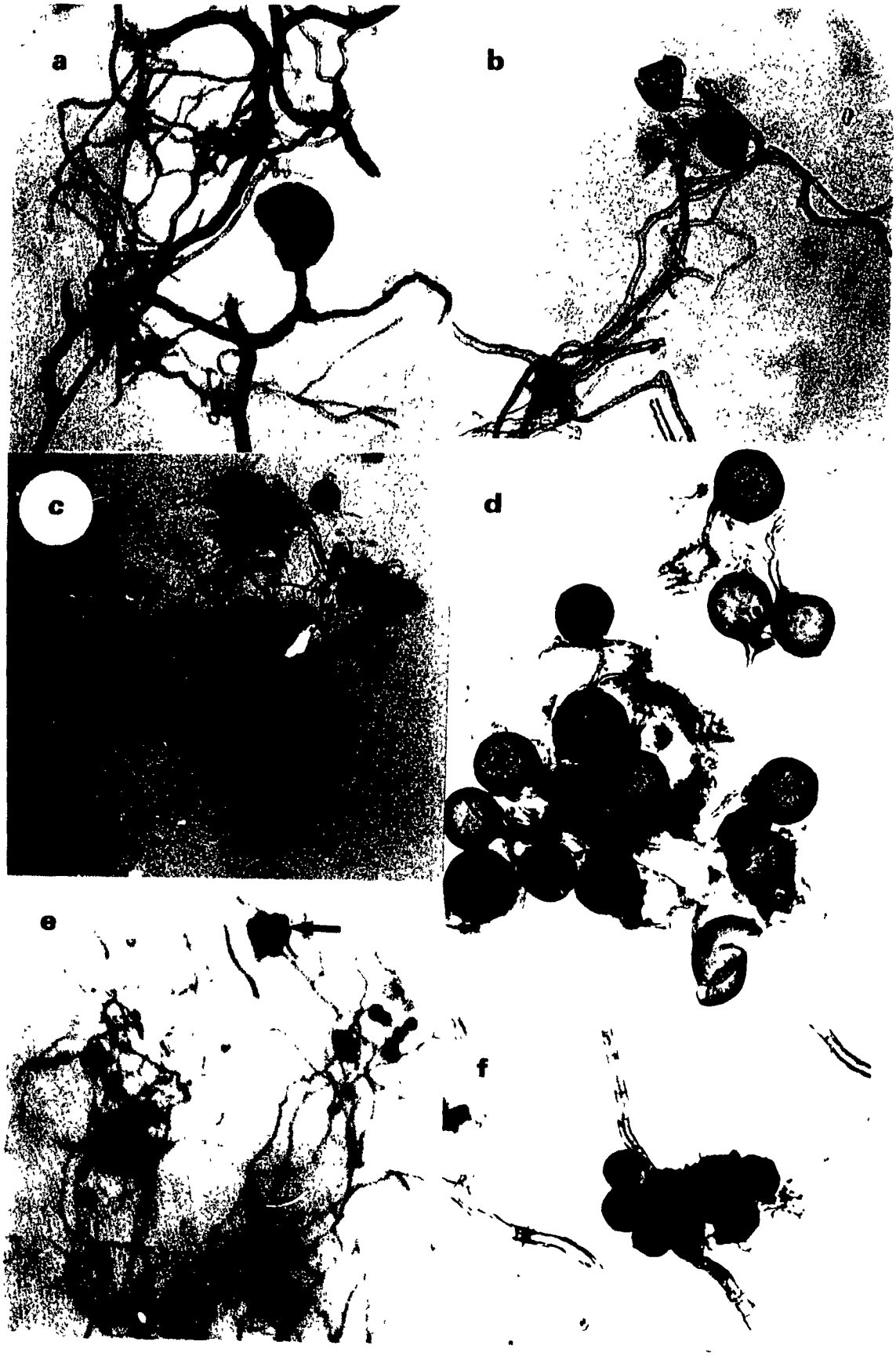

300
200
100
0
NO. OF SPORES PER PLANT
0
2
4
6
8
10
12
14
16
SAMPLE WEEK (WEEK 0=JUNE 12TH)

Fig.15 VA mycorrhizal spores that were identified on the roots of sugar maples at St. Hippolyte after calgonization: (a) a mature spore of G. hoi stained with lactophenol cotton blue; (b) young, immature spores of G. hoi in a polyvinyl-alcohol mounting medium (PVLG); (c) a cluster of G. aggregatum in PVLG; (d) an aggregate of G. macrocarpum in PVLG; (e) G. rubiforme (arrow) and (f) a close-up of a sporocarp of G. rubiforme attached to the hyphal system that was teased from a maple root.


a
b
c
d
e
f

occurring on the roots of sugar maples at $p<0.05$.

4. DISCUSSION

4.1 Spore Populations

VAM fungi have several periods of maximum sporulation (Gemma et al., 1989; Gemma and Koske, 1988b). Differences in the seasonality of sporulation of the VAM species colonizing sugar maple may be correlated with the incidence of VAM fungi present in the roots, although this has yet to be demonstrated because we still cannot identify the fungus by the internal mycelium within the plant root. Furthermore, the activity of the VAM fungi over a growing season may be synchronous with the intermittent growth of the maple feeder roots which occurs in response to the moisture changes in the soil, giving the roots their characteristic beaded appearance (Brundrett and Kendrick, 1988). VAM fungi have also been shown to be affected directly by moisture availability (Hetrick, 1986), so it is possible that VAM hyphal activity (expansion) and subsequent sporulation are correlated with new root growth. Therefore in order to have a better understanding of the seasonal dynamics of VAM fungi, biotic and abiotic variables have to be measured simultaneously with sporulation rates and colonization rates of the mycorrhizal fungi involved.

The population survey, at St. Hippolyte, has shown that VAM fungal spore numbers in this natural maple stand vary over a growing season, and can differ from year to year, probably due to local environmental variations. The addition of the

cation fertilizer to the soil didn't have any effect on the VAM spore populations even one year after the initial application. However, because the identifications were limited to the genus level, it is possible that effects occurred at the species level. On the other hand, one could also argue that due to the extremely efficient buffering capacity of the soil, no differences would be expected.

If one just considers the families, the Glomaceae were the most abundant spore type present in the soil, representing 80% of the total spores at the site, while the Acaulosporaceae made up the remaining 20% of the spores present. This was consistent for both 1989 and 1990. The only species at St. Hippolyte that could be distinguished and readily quantified was Glomus rubiforme which comprised 20-40% of the total spores at the site, and was the most abundant spore type at St. Hippolyte. The other members of the Glomaceae found included: G. aggregatum, G. clarum, G. geosporum, G. hoi, G. macrocarpum, G. microaggregatum and other unidentifiable species but none were as abundant as G. rubiforme.

From Table 3 we can see that for Glomus spp., there were $7.2x10^7$ - $7.8x10^7$ spores per m^2. Similarly, for G. rubiforme approximately $4.1x10^7$ to $6.3x10^7$ spores were found, and for Acaulospora spp., there were $6.0x10^7$ - $1.2x10^7$ spores per m^2 of soil, in and around the rooting zone of the maples. Comparing the spore abundance with other surveys is rather difficult because experimental designs differ between surveys, along

with the fact that very few surveys have been done in this type of ecosystem.

For agricultural soils, it has been determined that varying numbers of VAM spores are present and Sutton and Barron (1972) found 70 ± 17 spores/gram dry soil under maize, and up to 86 ± 11 spores under strawberry, all in the top 24cm of soil. Recalculation of my data shows that throughout any particular growing season, about 1000-1600 spores per gram of soil were found in the first 15cm of soil at St. Hippolyte. It is therefore quite evident that there are 10-20 times more spores present in the St. Hippolyte soil than in the agricultural soils studied by Sutton and Barron. Kessler and Blank (1972) estimated that the upper 10cm of soil in a Michigan hardwood forest that was dominated by maple contained nearly 7 million VAM sporocarps per hectare. One hectare is 10 000 m^2, so that would result in roughly 700 sporocarps per m^2 in the first 10cm of soil. For the sake of comparison, this is the equivalent of 1050 sporocarps per m^2 in 15cm of soil. Counts of G. rubiforme sporocarps from St. Hippolyte, showed approximately 800 000 sporocarps/m^2, in the first 15cm of soil. Therefore it is quite evident that a significantly larger number of sporocarps (800 times more) are present at the St. Hipployte site, than were found at the Michigan site.

The surveys showed that total spore numbers were significantly lower in the neutral sites, than those observed at St. Hippolyte. When the taxa were taken into consideration

(Table 3), it was found that the St. Hippolyte site had a significantly higher number of spores of Acaulospora compared to the other two sites. Excluding G. rubiforme, the two Québec sites were not that different with respect to the numbers of spores of Glomus, but had significantly higher populations of these spores compared to the Ontario site. A total of 133-782 spores per gram of soil were found at the Lacolle site, and only 27-65 spores/g soil at the Waterloo site were found. It is quite evident that the numbers of spores at the Waterloo site are much more similar to those found by Sutton and Barron (1972). One cannot compare the spore numbers with the study by Kessler and Blank (1972) because they reported sporocarps only and there were none present at either Lacolle or Waterloo.

The use of spore counts as opposed to the most-probable number (MPN) method has an advantage in that it doesn't assume a lack of host specificity by VAM fungi. The data illustrate this well, in that G. rubiforme was the most abundant spore type in the St. Hippolyte soil and was totally lacking in the soils of the other two sites. This fungus has not been successfully cultured, and has never been detected on maples planted in these soils. It is thus unlikely that it would be recovered using other trap plants. Only by means of the spore counts or the direct observation method could it be detected, even though it is clearly an important member of the VAM community at the St. Hippolyte site.

It is possible that G. rubiforme is adapted to acid soils as it was only found at the acid site. Other collections made from acid sites have also found this species to be present and behaving in a similar seasonal fashion (Dalpé, pers. comm.). Thus, this species and others similar to it may prove to be useful and important, even essential where agricultural crops or nursery trees are being transplanted to acid soils.

Environmental factors and soil conditions influence the occurrence of mycorrhizal associations in ecosystems, but it is difficult to study the direct impacts of these factors on the fungi because, in nature, they do not occur without a host plant, and in the lab, they have never been grown in pure culture (Harley and Smith, 1983). Nevertheless, some proof of the physiological diversity of these fungi has been provided by comparing experimental responses to soil pH, soil nutrient levels, soil moisture, salinity, temperature and other factors (Abbott and Robson, 1990; Daniels Hetrick, 1984; Morton, 1988; Slankis, 1974; Trappe and Molina, 1986). The majority of these data have been obtained by the use of simple experimental systems which allow the effect of a single factor on one mycorrhizal fungus to be studied, but some field data can also be found and used for comparisons.

Specific VAM fungi have been previously shown to prefer specific pH conditions (Powell, 1975). For example, certain species of Glomus, such as G. mosseae prefer more neutral conditions, while others like G. rubiforme and other

aggregated forms prefer acidic conditions, much like species of Acaulospora (Kruckelmann, 1975). In an analysis of the two secondary sites (Klironomos et al., in press) the pH was found to be highly correlated with the abundance of certain spores. However, pH is only one of the many factors that determine the presence and absence of spores.

As this was one of the first attempts to examine the occurrence and abundance of these spores in a temperate deciduous forest, available techniques for the extraction and quantification of the spores had to be modified. There have not been many studies on the validity of sampling methods (Tews and Koske, 1986), so in trying to determine the most suitable experimental design for the primary study site, the most important factor seemed to be to minimize the variation. The same was also true for the other two sites involved in the study, but since the St. Hippolyte study was done first, this served as a model for subsequent surveys. Anderson et al., (1983) showed that the variance of spore counts was positively correlated with the size of the sampling area. In one survey, 9 samples were required (each of 10ml of soil) in order to observe all 9 species present in a particular site (Tews and Koske, 1986), whereas 26 samples of the same size were needed to find all 18 species present at another site. In addition to this, it is also known that spores and mycorrhizal hyphae formed by VAM are not found randomly in the soil (St. John and Hunt, 1983; Porter et al., 1987), but they occur in close

proximity to the roots of their host plants. For these reasons, trees were used as reference points and equidistant sampling was performed so that comparable parts of the rooting system were being sampled.

After sampling the sites, the routine sieving and decanting procedure employed by Gerdemann and Nicolson (1963) proved to be the best initial step in separating larger particles but this had to be further complimented by the use of centrifugation to remove fine organic debris that is quite abundant in these forest soils. In soils with high organic matter, small spored species may not be loosened from the substrate and will therefore be missed (St. John et al., 1983). This was one of the primary concerns when the extraction procedure was being refined. To confirm that no spores were missed, all the discardable material was checked under the microscope for the presence of any VAM propagules, before being discarded.

In our study, it was determined that a swinging bucket rotor, as well as an increase in the centrifugation speed and duration, used in the method described by Furlan et al., 1980, was required for a horizontal separation of the sediment. In addition to this, the method for the extraction of VAM spores usually calls for a sucrose gradient (Furlan et al., 1980), but it was found that with soil samples from this type of natural forest ecosystem, a 60% sucrose layering alone worked remarkably well, yielding a clearer sample and a greater

number of spores that could easily be isolated, transferred and quantified. It was found that the best condition was a 60% sucrose cushion spun at 400g for 20 minutes using a swinging bucket rotor. The use of gridded filters allowed for estimates of spore numbers in the entire soil core. Furthermore, knowing the area of the corer, these numbers could be calculated per m^2 of forest floor, to the depth chosen.

Detailed surveys of VAM fungal spores have provided information as to the number of mycorrhizal species present in particular plant communities. From surveys like these, it may be remarked that the soil in one place contains more than one VAM fungus, in fact it may support a wide diversity of these fungi. It is difficult to compare surveys because of the differences in sampling methodologies (sampling intensity and the area surveyed vary considerably). Spore occurrence data may be misleading because spore abundance is usually poorly correlated with mycorrhizal colonization (inside the root). Fungi that do not produce recognizable spores may be present (Johnson, 1977; McGee, 1989; Morton, 1988) and roots of different plants often intermingle so spores could occur under plants that they are not necessarily associated with. The spore characteristics used to identify to species level are quite conservative so that genetic variations may exist within a species of VAM fungus (Morton 1990) and this intraspecific variation may also have to be considered.

The presence of many VAM fungi in soils or within roots suggests that both interspecific and intraspecific competition between them is possible. Koske (1981) was unable to prove that any of the VAM species present in a coastal ecosystem were better competitors than others, but rather, he suggested that environmental factors and host plants were more important factors influencing their distribution. Contrary to this, Gemma et al. (1989), found seasonal differences in spore production between co-existing species and suggested that the fact that the abundant spore production by one VAM fungus was usually correlated with lower levels of spore production by others may have been due to antagonism between species.

4.2 Symbiont identification

4.2.1 Indirect observation

Propagules of VAM fungi consist of spores, root fragments containing vesicles and intradical mycelium, and soil hyphae (Manjunath and Bagyaraj, 1981; Tommerup and Abbott, 1981; Biermann and Lindermann, 1983). The results of the St. Hippolyte maple root inoculum experiment were promising, yielding spores in some containers. All the control, non-inoculated, plants died during the experiment, which told us that the conditions, most likely the containers, were not adequate to support the growth of the sugar maples. Unfortunately, the spores that were produced were immature (Fig. 12) and could only be classified to the family

Glomaceae. It is interesting that these maples did much better at pH=6.5 than at pH=4.3. One explanation for this could be that the seeds used for this experiment were obtained from sugar maples growing in neutral soils as opposed to the seedlings that were collected from St. Hippolyte, used in the "garden" culturing experiments. This is supported by Stahl et al., 1988, who remarked that several VAM fungi seem to posses a limited tolerance range to environmental conditions and may only be present in some types of soils (Lambert et al., 1980). This may also occur with their symbiotic plant partners.

Many mycorrhizologists now consider the large soil-borne spores to be the most important type of inoculum even though the numbers are poorly correlated with mycorrhizal formation in soils (Abbott and Robson, 1984; 1990; Schmidt and Reeves, 1984; Mukerji and Kapoor, 1986; Ebbers et al., 1987; McGee, 1989). Pregermination of these spores was necessary, prior to inoculation, to ensure viability and colonization. Viable VAM spores in the soil may not function as propagules if they are inactive because soil conditions are not good or because they posses an inherent period of dormancy, a mechanism allowing them to survive long unstable soil conditions (Tommerup, 1987). The experiment failed because almost 100% of the field collected spores were non-viable either because they were contaminated by endophytes other than VAM, or they were parasitized.

The final type of inoculum used was soil, in a method similar to that used by INVAM (Klironomos, pers. comm.). The result of this was quite successful, because the larger Spencer Lemaire containers resulted in improved sugar maple growth especially since the control (uninoculated) plants survived and showed no signs of colonization or sporulation. The inoculated plants were colonized and the spores produced were determined to be mature and identified as G. hoi. Nevertheless, a high rate of mortality was still observed even in this experiment, with over 75% loss of sugar maples.

It is very difficult to work with sugar maples. They are very sensitive to stresses and shocks, and thus very difficult to establish in lab culture because they immediately go into a dormant state (Webb, 1977). Although we have been able to germinate sterile seeds in test tube culture, it is a long process, taking 3 months of cold storage to break seed dormancy and produce a radicle, and then about two weeks to one month to obtain a seedling with primary cotyledons and lateral root development. This, along with the fact that successful colonization by VAM requires about 2-4 weeks and subsequent sporulation requires several months, makes these experiments very time-consuming. Furthermore, the mortality rate observed in greenhouse experiments with sugar maple (greater than 55% mortality, Diamond, unpublished data) is so high that one has to increase the number of replicates to ensure the possibility of obtaining results. It is clear,

therefore, that there is a need for alternate techniques for determining which VAM species form associations with sugar maple.

From both the "garden" experiments (Spencer Lemaire container and Aeroponic box system), it was observed that the sugar maples initially did well, but approximately 2-3 weeks later, they would suddenly go dormant. Dormancy can occur with the slightest physical or environmental stress (Webb, pers. comm.). Cold chilling is the only way in which to break dormancy (Webb, 1967, 1977). This was not very successful, for the container-grown maples showed very sparse recovery, and it was determined that another factor that could be limiting their growth was the pot size. The aeroponic plants, on the other hand, did show a general recovery when they were reintroduced into the greenhouse. In addition to this, the plants from the field preferred the box which had been set at pH=4.3, similar to that in their natural habitat from which they were collected. This was not surprising since it has been previously observed that mycorrhizal plants and spores will do better in their native soils (Henkel et al., 1989). Regardless of all this, no VAM sporulation was ever observed throughout this experiment, thus demonstrating the need for a different approach.

4.2.2 Direct observation

The use of sodium hexametaphosphate or Calgon, a soil

dispersant, had previously been used by Bardgett (1991) to extract hyphae from the soil and estimate hyphal lengths. In an unpublished study by Miller et al., Calgon was used as the soil dispersant to extract extraradical mycorrhizal hyphae from silt loam to silty clay loam tallgrass prairie soils. In the present study, this compound was used to disperse the soil clumped around the root systems of maple saplings collected from the field and resulted in a variety of species being detected on the roots which could also be quantified. It can be clearly seen from SEM photographs (Fig. 16), that the treatment cleans the sample and yields a virtually perfect specimen for identification. It was found that spores of G. macrocarpum and G. hoi were present regularly throughout the season on the root system whereas G. aggregatum and G. rubiforme appeared only at the end of the season at the St. Hippolyte site.

It has never before been shown which VAM species infect sugar maples in nature, even though Yawney and Schultz (1990) did successfully infect sugar maple seedlings with Glomus etunicatum under artificial conditions. Schultz and Kormanik (1982) successfully infected sugar maple in pot culture with G. fasiculatum, and a mixed culture of G. mosseae, and Gigaspora species, but the inoculum was made up of root fragments from pot cultures of sorghum, and the purity of the isolates had not been proven in this study. In the Compilation of the Endogonaceae, Berch (1988) quoted Clark

Fig.16 Scanning electron microscope (SEM) photographs of mycorrhizal roots from the field cleaned with Calgon: (a) spores attached to maple root; (b) close-up of single spore from (a) (arrows) with uncharacteristic spine (appendage); (c) (d) and (e) photographs of spores and hyphae attached to maple roots; (d) emerging directly from the root; (e) the presence of a vesicle (arrow) and (f) arbusculate coils (arrow), in fractured cortical cells from "Calgonized" maple roots.


a
b
c
d
e
f

(1969) as saying that sugar maples are infected by Gigaspora gigantea, but Clark does not make this claim. It is also of interest that this is the first report of G. hoi occurring in Eastern Canada, prior records coming from British Columbia, Oregon and Washington. This is also the first report of a natural plant host which supports G. rubiforme. However, we don't know whether this species is also occurring on the roots of the other VAM plants present at the site.

One can see from the graph in Fig. 14 that the frequency of spores per plant starts off low in the spring and increases at the end of the season. The plants collected from the Waterloo site however, never showed any evidence of spores attached to the extramatrical mycelium, which was abundant. Surprisingly, when the plants were left in beakers submerged in water, after the Calgon treatment, they eventually sporulated, producing spores typical of the Glomaceae. No spores were detected close to the roots, possibly because the mechanism or strategy of infection may be different than that at St. Hippolyte. Further support for this hypothesis can be seen from the population survey, which showed few spores present at the Waterloo site as opposed to the Lacolle and the St. Hippolyte site. Hyphal abundance was much higher at Waterloo than at Lacolle suggesting that colonization of new sugar maple roots may be taking place via hyphae rather than spores (Klironomos et al., in press). As VAM hyphae in the soil cannot be reliably differentiated from the hyphae of

free-living fungi, we cannot be sure that this alternate strategy of infection is occurring, but it remains a possibility (Warner, 1984).

The question of host-specificity is of considerable interest in natural ecosystems, where it is not safe to assume that any VAM species present can infect all potential host species. No doubt some species are better able to compete for one host than another, even when they may be able to infect either host under ideal conditions. The use of a direct observation technique has proved that sugar maples are colonized by G. aggregatum, G. hoi, G. macrocarpum and G. rubiforme in a forest system. Recently, the development of specific monoclonal antibodies to specific species of VAM fungi has proved useful in identifying fungi in plant tissues (Wright et al., 1987). It has also been shown that isozymes extracted from VAM spores have different electrophoretic mobilities on polyacrylamide gels (Sen and Hepper, 1986) for different species. The method is very sensitive, reproducible, and quantitative. By far the most promising work has been from Simon et al. (1992) who reported the amplification of the nuclear genes coding for the small ribosomal RNA subunit for Glomus intraradices, and Gigaspora margarita, and were the first to sequence the rDNA genes from these VAM fungi. This also allowed them to design a VAM fungal-specific primer (VANS1) that can be used to amplify rDNA genes from other VAM fungal species using the polymerase

chain reaction. This could allow for the production of DNA probes designed to detect and identify other VAM fungi even when spores are not available. However, since this technology was not available, during this study, we had to rely on the classical approaches to determine which VAM fungi are occurring on particular host plants. With the "Calgonization Method" for the direct observation of spores occurring on natural field root system, valuable information is provided without the use of sophisticated and expensive equipment.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Although no fertilization effect was found at St. Hippolyte, the surveys suggested that sporulation occurs mainly in the fall, although differences in spore abundances are apparent throughout the season. The data shows that the sampling procedure used, in conjunction with the use of gridded filters, made it possible to obtain estimates of the spore populations at all 3 sites with error terms (95% confidence intervals) that are small enough to detect seasonal and site differences.

Environmental pressures elicit significant physiological stresses that affect the growth and development of plants (Powell and Bagyaraj, 1984). The most common stress is nutrient deficiency due to low concentrations in the soil solution or decreased availability because of the high P-fixation capacity of soil. It has been shown in several greenhouse experiments that plants colonized with VAM fungi are much healthier under stressful conditions than uncolonized plants.

The "Calgonization" technique has proven to be an inexpensive and effective method for detecting spores on plant roots and opens possibilities for future surveys of spore populations that will be more informative because not only can one obtain an idea of the relative abundance of VAM spores in a particular soil, but also which spores are occurring on

which plants and at what times of the year they are sporulating. Furthermore, treatment effects can be monitored more closely and it is possible that had this method been available at the onset of the study, the effect of the nutrient deficiencies and cation fertilization could have also been investigated in the sugar maple seedlings. In addition to this, the VAM spores present on the sugar maple root systems may also have shown differences in sporulation in response to the treatment, which could have also been detected using this technique.

The use of spore counts and the direct observation technique have the advantage that they do not assume a lack of host specificity by VAM fungi, and made it possible to clearly show that sugar maples are infected by G. aggregatum, G. hoi, G. macrocarpum and G.rubiforme at St. Hippolyte. Moreover, the data from the St. Hippolyte study, showed that G. rubiforme sporulation increased towards the end of the season, and this was confirmed by the Calgon experiment, in that spores of this species occurred on plant roots only in the fall.

In natural ecosystems, host specificity must not be taken for granted. There is no doubt that some VAM species have a wider range of potential hosts, especially when grown under ideal conditions in a lab, but different strategies of survival are important in all organisms. Again the most striking example being that of G. rubiforme, which has never

been truly cultured, even in the present study, using a variety of classical and modified culturing practices, and would have remained undetected using other trap plants. Only by using the spore counts and the direct observation technique could this species, which is clearly an important member of the VAM community at St. Hippolyte, have been detected. Until techniques are developed that can identify the symbionts occurring within the root systems of plants, one cannot be certain how many species are colonizing a particular host plant, nor which are abundant under certain conditions. Thus methods like the most probable number method still leave too many gaps in the data, whereas the method described here gives a more accurate representation of the association and can be used to determine effects from a variety of factors that can affect VAM fungi.

Identification of the clonal isolates of mycorrhizal fungi that would result in the greatest benefit to host plants and how they interact with environmental factors or soil conditions is just beginning. In the future, careful identification of mycorrhizal isolates, and progress in VA mycorrhizal taxonomy is required. Implementation of molecular tools in the field, like the work of Simon et al. (1992), will probably demonstrate higher degrees of specialization, and could allow for concrete host-symbiont relationships to be established. In depth taxonomic studies and investigations of mycorrhizas in undisturbed ecosystems, should be considered to

be essential foundations which ultimately benefit all mycorrhizal research. These types of studies will one day be very beneficial to forestry, revegetation, and other research, as well as aiding mycorrhizologists in comprehending the functioning of this symbiosis in agricultural situations.

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