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The effects of the plant parasitic nematodes, Xiphinema americanum and Meloidogyne hapla on the endomycorrhizae of sugar maple, Acer saccharum.

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The Effects of the Plant Parasitic Nematodes, *Xiphinema americanum* and *Meloidogyne hapla* on the Endomycorrhizae of Sugar Maple, *Acer saccharum*

A Thesis Presented by

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ABSTRACT

Sugar maple seedlings (Acer saccharum Marsh) were inoculated with two species of plant parasitic nematodes, Meloidogyne hapla and Xiphinema americanum to investigate possible effects on the seedlings' endomycorrhizae. X. americanum failed to reproduce in the greenhouse in all tests conducted. Inoculation with M. hapla resulted in galled roots and the production of giant cells and erratic xylem in greenhouse experiments. Also, the fungal symbiont failed to invade tissues galled by the nematodes. After overwintering it was noticed that mycorrhizal infection in trees that had been inoculated with nematodes the previous year appeared more senescent, i.e. more vesicles were present and arbuscules were in more advanced stages of digestion than control seedlings.

A study was made of declining maples on the campus of the University of Massachusetts at Amherst to look for possible correlation between decline symptoms nematodes and endomycorrhizae. No definite correlation was found, however, degree of decline exhibited by the crown of the tree and the degree of mycorrhizal infection in the fine feeder roots were found to be related.

CHAPTER I

INTRODUCTION

Nematodes have been implicated in many cases of decline and stunting of forest and nursery trees. It is also known that the endomycorrhizal state is natural for the great majority of plants. Plants normally endomycorrhizal must be so to be able to compete with other plants where nutrition is the limiting factor; indeed, some plants are dependent on the fungal symbiont for nutrient absorption even when the supply of nutrients is not limiting and show symptoms of stunting and decline when not infected.

Since nematodes are soil organisms which feed on roots and fungal mycelium, it seems logical that they might affect the endomycorrhizae although little is known of these effects. Symptoms of stunting and decline on woody plants may be due to poor root development because of the presence of certain plant parasitic nematodes, lack of mycorrhizae or a number of other causes.

As we become increasingly aware of the complexity of the rhizosphere, more questions are being raised about the multitude of organisms inhabiting it and their interrelationships. Some of the modifications in plant growth due to the feeding of these nematodes on the tissues of the root may be actually due to the effects of the nematodes on the endomycorrhizal fungus. Perhaps some of the decline symptoms exhibited by sugar maple may be due to the destruction or improper development of its endomycorrhizae by the feeding of nematodes. This

study is meant to investigate this possibility. The two species of nematodes were chosen because of their availability and their being implicated as pathogens of woody plants. Xiphinema americanum Cobb is a migratory ectoparasite, Meloidogyne hapla Chitwood is a sedentary endoparasite.

The main objectives of this study are:

1. To see if Meloidogyne hapla is a pathogen of sugar maple.
2. To observe maple seedlings inoculated with M. hapla and Xiphinema americanum to see if they exhibit symptoms of maple decline.
3. To make histological examinations of roots of maples that are exhibiting symptoms to look for possible effects of the nematodes on the endomycorrhizae.
4. To examine roots of declining maples in the field by doing histological examination of their endomycorrhizal condition and to take soil samples to determine nutrient status and what species of nematodes are present for possible correlation of these factors and the state of the endomycorrhizal fungus.

CHAPTER II

REVIEW OF LITERATURE

Sugar maple (Acer saccharum Marsh) is a tree of great economic and aesthetic importance in the Northeast. It has been estimated that there are twelve million sugar maple trees in the state of Massachusetts

alone and maple products contribute several million dollars to the gross income of the state (DiSanzo, 1967). Since 1957, dieback of both roadside and woodland sugar maple trees has been reported in the Northeast (Hibben, 1964) and was first noticed by most growers in Massachusetts in 1961 (DiSanzo, 1967). This problem has come to be called maple decline. The main symptoms as described by Kessler (1962) are premature leaf coloration and leaf fall, usually preceded by chlorotic and sparse foliage. Affected trees may also show marginal leaf scorch that sometimes extends to cover the entire leaf and wrinkled leaves can be observed starting in June. Chlorosis and yellowing of the leaves of affected trees appear between June and August followed by defoliation of terminal twigs and branches in August or September, while normal leaf coloration and fall occurs in October. Severity of symptoms varies from trees showing a few dead twigs and branches to trees exhibiting dieback of more than 50% of the crown.

In the twenty odd years that scientists have been working on maple decline (Riffle, 1962; Houston and Kuntz, 1962; Kessler, 1962; LaCasse and Rich, 1964; Hibben, 1964; Mader et al., 1969; DiSanzo and Rohde, 1969; others), no single pathogen has been implicated. It is presently believed that a combination of factors are involved: environmental stress predisposes the trees to attack by a particular pathogen or attack by a pathogen weakens the tree so it is more susceptible to environmental stress.

Woody plants and their associated rhizosphere microorgani[illegible] in

general are poorly understood. Plants normally endomycorrhizal must be so to be able to compete with other plants where nutrients are limiting, even to survive where nutrients are plentiful and easily available in species exhibiting a high degree of mycorrhizal dependency (Aldon, 1975; Crush, 1974; Gerdemann, 1968, 1974; Kleinschmidt and Gerdemann, 1972; Strzemska, 1973). Mycorrhizal dependency is the degree to which a plant is dependent on the mycorrhizal condition to produce maximum growth and yield at a given level of soil fertility (Gerdemann, 1974). Dependency varies from 0 for a nonmycorrhizal species such as cabbage to a high degree of dependency for plants such as citrus that remain stunted even in soils of high nutritional status. Most plants fall between these two extremes. One can "assume that at low nutrient levels all plants that normally produce VA mycorrhizae are benefited by the infection" (Gerdemann, 1974). VA mycorrhizae is the most common form of endomycorrhizae, forming arbuscules and vesicles within the root. In this paper the two terms are synonymous.

Little is known about the mycorrhizal dependency of sugar maple, indeed little work has been done on the endomycorrhizae of sugar maple at all (Clark, 1969; Kessler, 1966; Kessler and Blank, 1972). Clark showed interspecificity of a fungus forming VA mycorrhizae with sugar maple in an experiment in which he obtained successful infection of sugar maple (Acer saccharum), and other tree species with Endogone gigantea.

Kessler (1966) showed that the beaded appearance of sugar maple rootlets was due to intermittent growth of the rootlets following fluctuation in moisture levels in the upper part of the soil. Long non-beaded rootlets as well as the short beaded ones were found to be infected. Uninfected rootlets were rarely found in soil layers containing humus (Morrow, 1950). In another study, endomycorrhizae were found on at least 95% of the sugar maple roots examined in all plots. "These mycorrhizae were found in all seasons of the year when the roots were growing..... It is significant that the mycorrhizae were widespread, although individual development was not great on relatively fertile soils of relatively high pH" (Morrow, 1950).

Similar observations have been confirmed many times by studies with different plant species; mycorrhizal development is best under conditions of moderately low fertility. Since most healthy sugar maple roots were found to be heavily infected by the fungus and in a normal growing situation sugar maple must compete with many other plants and microorganisms for nutrients in the soil, one might hypothesize that the fungus is very beneficial to the tree even though the degree of mycorrhizal dependency in unknown. Kessler and Blank (1972) estimated 2,600,000 sporocarps per acre in an undisturbed forest of mature sugar maple. One might wonder what the effects would be on a tree so seemingly adapted to the mycorrhizal state as sugar maple of any factor causing a reduction in the amount of infection.

Injury to plants by nematodes has been grouped into three categories

by Rohde (1960): (1) formation of galls, (2) necrosis of cells, (3) blinding of root tips (feeding of the nematode stops further growth and cell division of the root tips but they are not killed). Nematodes seldom kill their host plant outright. Plants parasitized by nematodes generally decline slowly, common symptoms of decline exhibited being dieback, yellowing, wilting and premature shedding of foliage (Sasser, 1954). Parasitized plants are often more susceptible to other pathogens as well as winter injury and drought (Riffle, 1962; Griffin and Epstein, 1964). Nematodes have been implicated in many cases of decline and stunting of forest trees (Nickle, 1960; Haasis et al., 1961; Riffle and Kuntz, 1967; Ruehle, 1968, 1969, 1971; Churchill et al., 1971).

Xiphinema americanum, the dagger nematode, is a migratory ectoparasite. "Although it is found occasionally around the roots of corn, oats, and some of the grasses, this dagger nematode probably attains greatest importance as a parasite of trees and shrubs" (Christie, 1953). Xiphinema sp. feeds on cells of the cortex and phloem and feeds along the sides of roots as well as the root tips with only its stylet entering the root (Mountain, 1954). Several different species of Xiphinema have been studied as pathogens of woody plants showing general stunting and decline symptoms. Ruehle and Sasser (1962) found X. americanum along with other nematodes to be "the primary inciting agents of stunting of pines on sand sites in southeastern North Carolina." Baker (1955) found that by feeding along the tips and sides of roots X. americanum devitalized the root tips and caused extensive injury to laurel oak.

White (1959) and Thorne (1961), in separate studies, found X. americanum in orchards showing symptoms of decline. White (1959) carried his studies further to show X. americanum was able to reproduce on apple seedlings in the greenhouse.

There have been several reports on the association of X. americanum with sugar maple but little real evidence of its pathogenicity. Zuckerman and Coughlin (1960) found X. americanum when they sampled soil from sugar, red (A. rubrum), silver (A. saccharinum), Norway (A. platanoides) and boxelder (A. negundo) maples. Stessel (1961) found X. americanum on sugar maple. Riffle (1962) did an exhaustive study on nematodes associated with declining maple stands in Wisconsin and found X. americanum there, however no species of plant parasitic nematodes were found to be associated exclusively with healthy or diseased maple trees. Hibben (1964) found mostly Xiphinema sp., Helicotylenchus sp. and Tylenchus sp. associated with sugar maple in New York State but he failed to find a correlation between dieback and the nematodes.

Probably the most conclusive study on the subject was done by DiSanzo (1967). He showed that sugar maple seedlings in the greenhouse that had been inoculated with X. americanum developed symptoms of decline similar to those observed on the foliage and root systems of naturally declining maple trees. He also set up glass observation boxes and observed and photographed the actual feeding of X. americanum on the roots. However, at the end of the experiments he failed to recover X. americanum from the soil of the trees that were exhibiting symptoms.

The root knot nematode, Meloidogyne spp. is one of the most important genera of nematodes economically, including twenty-six species of plant parasites (Rohde, informal communication). It is a sedentary endoparasite. The preparasitic second stage larvae attack the region of primary meristematic tissue just behind the root cap. The larvae move intercellularly through the cortex until they reach the pleurome where they begin to feed, stimulating the formation of giant cells or synctia in the vascular tissue as well as hypertrophy and hyperplasia in the root. The efficiency of roots in supplying water and minerals to the rest of the plant is seriously impaired by the disruption of the vascular tissue. Even if the nematodes fail to reproduce in the host, just by their feeding they can stimulate cortical hypertrophy and hyperplasia, and normal functioning of the root is disrupted.

There have been several reports of Meloidogyne spp. being pathogenic on maple. Barss (1929) found Meloidogyne spp. was pathogenic on bigleaf maple (Acer macrophyllum). M. incognita produced galls on roots of Japanese maple. Riffle (1963) found sugar maple trees in Wisconsin exhibiting slow growth, sparse and chlorotic foliage. On examining their roots he found many rootlets swollen into galls. He described a new species of nematode, Meloidogyne ovalis, that was parasitic on sugar maple. He also found it to attack American elm, white ash, paper and yellow birch, boxelder, Norway and red maple. Ruehle (1971) tested the pathogenicity of Meloidogyne hapla and M.

incognita on Acer rubrum as well as other southern hardwoods but failed to obtain infection. There are no reports of the northern root knot nematode, M. hapla, being pathogenic on maple.

Plant-parasitic nematodes are soilborne organisms and feed on the roots of plants and may directly or indirectly have an effect on the root inhabiting fungi. It is only recently that scientists have become aware that the fungus within the root tissue is only part of the organism, that there is actually an extensive hyphal network in the soil (Rhodes and Gerdemann, 1975) that is critical to the increased absorption of nutrients by the fungus. These hyphae in the soil as well as the coarse distributive hyphae on the surface of the roots are of an ephemeral nature and might be very subject to disturbance by nematodes. Yet studies on interaction of VA mycorrhizae and nematodes are few. Mycorrhizae formed by Endogone mosseae on tobacco increased resistance to Meloidogyne incognita (Baltruschat et al., 1973). Fox and Spasoff (1972) found that Heterodera solanacearum and Endogone gigantea each adversely affected the reproduction of the other and attributed it to competition for the same living space. Bird et al (1973) found an increase in VA infection of cotton in soils that had been treated with nematicides and a decrease in infection in cotton roots infested with endoparasitic nematodes. In a related study Rich et al. (1974) showed that VA infection early in the season kept down populations of plant parasitic nematodes. Marx (in Bird et al., 1973) found that endomycorrhizae would colonize roots infected by Meloidogyne

spp. but the fungus would not invade tissues galled by the nematodes.

Recent studies by Schenck and Kinloch (1974) and Schenck et al. (1975) on mycorrhizal soybean in Florida support these findings. They found high populations of Meloidogyne incognita and Heterodera glycines were consistently associated with low degrees of infection by the mycorrhizal fungus, and that high nematode populations caused a decrease in the number of spores produced by the fungus.

There have been no studies done to date on the interaction of nematodes and the endomycorrhizae of woody plants.

CHAPTER III

MATERIALS AND METHODS

i. Inoculations of Potted Strawberries

Attempts to culture X. americanum in pure culture have so far proved unsuccessful. In order to have large amounts of nematodes to use as inoculum an attempt was made to raise the nematodes on strawberry since strawberry is a good host (Perry, 1958; White, 1959; Schindler and Braun, 1957). In September 1975, twenty-four bare-rooted strawberry plants from a strawberry nursery specializing in producing virus free stock were planted in steam sterilized soil made up of 50% sand and 50% loam in six inch plastic pots. When the plants became established and were exhibiting active growth, twenty-one of them were inoculated with 200 hand-picked juvenile and adult X. americanum each. The X. americanum inoculum was obtained by using Jenkins (1964) sugar flotation to extract the nematodes from soil samples

taken from a yard overgrown with sugar maple seedlings in Amherst. Part of the surface soil of each plant was scraped away and the water-nematode suspension was then poured onto the exposed roots. The control plants were treated with distilled water only. The plants were kept evenly moist and maintained in the greenhouse for three months.

ii. First Year Inoculations with X. americanum

In March 1976, 100 six to eight inch sugar maple seedlings were planted as bare rootstock in a 1:1 mixture of steam sterilized sand and loam in six inch plastic pots, two seedlings per pot. A small amount of the nursery soil the trees came packed in was sprinkled on the surface of each pot to insure the trees' mycorrhizal infection. The first week of June, after the trees had broken dormancy, thirty of the pots were inoculated with 100 X. americanum each, in the same manner as described previously except that the nematodes were extracted from the soil using Cobb's sifting and gravity method (1918) rather than sugar flotation. Eight trees served as controls. The trees were maintained in the greenhouse for three months during which they were carefully observed for symptom development.

iii. First Year Inoculations with M. hapla

In February 1976 tomato seeds, Lycopersicum esculentum cv. "Rutgers," were planted in root knot infested soil containing approximately forty larvae per 500 g of soil. By June 1976 the roots were heavily galled with hypertrophy and hyperplasia affecting more than 90% of the roots. With the plants watered so the soil moisture was at field capacity the soil

containing the infected roots was chopped up and 20 g of the infected mixture was mixed with the top one inch of soil of twelve maple trees. These trees were kept evenly moist and observed carefully for three months in the greenhouse.

In October 1976, fifteen three inch sugar maple seedlings from the forest were potted in three inch plastic pots of steam sterilized forest soil and kept in a protected outdoor area to go through a hardening off period and winter dormancy. All fifty pots of seedlings from the previous year's experiments were also moved to a protected outdoor area and allowed to overwinter. In the first week of January all seedlings were brought inside the greenhouse, watered and maintained until they broke dormancy. The first signs of the trees breaking dormancy were shown by the forest seedlings in the three inch pots (see Tables 1,2,3). The seedlings from the previous year's experiments in the six inch pots broke dormancy somewhat later, although most of the control trees leafed out before the trees that had been inoculated. Two pots of each treatment were examined to determine root and nematode development. Xiphinema americanum in the soil were recovered by sugar flotation. Meloidogyne hapla in the roots were examined by staining with acid fuchsin (Goodey, 1951).

By simmering the roots for an hour in 10% KOH and then staining with Trypan Blue, which stains mycorrhizal mycelium within the cortical cells and on the surface of the root, the degree of mycorrhizal infection can be assessed (Phillips and Hayman, 1970).

For histological analysis , the roots were fixed in FAA and dehydrated via Johansen's tertiary butly alcohol series (1940), embedded in paraffin and sectioned on a rotary microtome into 12 u sections, and stained in Safranin and Fast Green.

iv. Second Year Inoculations of Three Inch Forest Seedlings

Ten forest seedlings in three inch pots were inoculated with 100 Xiphinema americanum nematodes, as described previously, in March 1977 when they had completely broken dormancy and were exhibiting an active flush of new growth. Five were maintained as controls. Two of the control seedlings were sacrificed as soon as soon as they had broken dormancy and their root system subjected to the three analytical procedures previously mentioned. The soil was analyzed for nematode content by sugar flotation. The thirteen remaining trees were maintained in the greenhouse at even moisture and temperature for the next three months.

v. On Campus Maple Survey

The last part of this study involved an investigation of declining maples on the campus of the University of Massachusetts at Amherst. Twelve trees were divided into four groups from healthy to severely declined: 1 = healthy, 2 = slightly declined with some bare twigs showing in the crown, 3 = 1/3 of the crown died back, 4 = 1/2 or more of the crown died back. Soil samples were taken for pH and nutritional analysis (done by the West Experiment Station), and analyzed for nematodes by sugar flotation. Root samples from four sites around each tree were taken and sub-

Table 1. Dormancy broken by trees inoculated with X. americanum in 6/76

X_1	3/2/77	X_{16}	3/2/77
X_2	3/2/77	X_{17}	3/2/77
X_3	3/2/77	X_{18}	3/2/77
X_4	3/2/77	X_{19}	3/2/77
X_5	2/18/77	X_{20}	3/2/77
X_6	2/28/77	X_{21}	3/2/77
X_7	2/24/77	X_{22}	3/2/77
X_8	2/28/77	X_{23}	2/24/77
X_9	3/2/77	X_{24}	2/28/77
X_{10}	3/2/77	X_{25}	2/28/77
X_{11}	3/2/77	X_{26}	3/2/77
X_{12}	3/2/77	X_{27}	2/18/77
X_{13}	2/28/77	X_{28}	2/18/77
X_{14}	2/22/77	X_{29}	3/2/77
X_{15}	2/12/77	X_{30}	3/2/77

Table 2. Dormancy broken by trees inoculated with *M. hapla* in 6/76 (M) and control trees (C).

M_1	2/22/77	C_1	2/18/77
M_2	3/10/77	C_2	2/22/77
M_3	2/28/77	C_3	2/28/77
M_4	2/22/77	C_4	2/18/77
M_5	3/2/77	C_5	2/22/77
M_6	2/22/77	C_6	2/28/77
M_7	3/10/77	C_7	2/22/77
M_8	2/22/77	C_8	2/28/77
M_9	2/24/77		
M_{10}	2/18/77		
M_{11}	2/24/77		
M_{12}	3/2/77		

Table 3. Dormancy broken by forest seedlings in 3" pots

F_1	1/27/77
F_2	"
F_3	"
F_4	"
F_5	"
F_6	"
F_7	"
F_8	"
F_9	"
F_{10}	"
F_{11}	"
F_{12}	"
F_{13}	"
F_{14}	"
F_{15}	"

jected to the same analyses described previously.

CHAPTER IV

RESULTS

i. Inoculations of Potted Strawberries

When working with nematodes one of the problems frequently encountered is getting enough nematodes of a particular species to work with. "Xiphinema is probably an obligate parasite of higher plants" (Cotten, 1973). Attempts to grow Xiphinema americanum in axenic culture have so far been unsuccessful so inoculum must be obtained from soil around the roots. Strawberry is known to be a host for several different species of Xiphinema (Schindler and Braun, 1957; Perry, 1958; White, 1959); therefore, it was hoped that by inoculation with the nematodes, sufficient populations would be built up for later use as maple tree inoculum. After three months the plants were examined for root damage and the soil analyzed via sugar flotation.

The roots of the strawberry plants, upon examination under a dissecting scope, showed normal healthy growth as compared with the controls and no nematodes were recovered from the soil.

These results are not wholly unexpected as X. americanum is notoriously difficult to raise in the greenhouse (Griffin and Darling, 1964; DiSanzo, 1967; Cohn and Mordechai, 1970). Attempts were made to insure conditions of constant moisture and temperature. Plants were fed no nutrient solution after they were inoculated since Cohn and Mordechai (1970) found soil drenches with a normal concentration of

nutrient solution had disastrous effects on the population levels of several species of Xiphinema they were working with. Using sugar flotation as the method of extracting the original inoculum may have been the reason no nematodes survived since the extraction procedure involves a brief period in a sugar solution of high concentration that is quite stressful to the nematodes. Also copper screens were used in the extraction procedures; there have been reports (D. Kaplan, personal communication) of copper ion toxicity to nematodes.

ii. First Year Inoculations with X. americanum

In the first set of inoculations on the potted maples the nematodes used were hand picked from soil samples that had been processed by Cobb's sieving and Baermann funnel technique thus avoiding the physiological shock of concentrated sugar solution. The thirty pots inoculated with Xiphinema americanum in June 1976 were carefully observed for symptoms of decline, such as chlorosis, premature coloration, wrinkles or scorch. Throughout this period (June, July, August) the trees did not exhibit any variations in growth as compared with the controls. At the end of the three month period it was discovered that no nematodes that had been applied as inoculum had survived. The root tissues showed the outer layers of cortical cells filled with coils of hyphae and the inner layers of cortical cells heavily infected with well developed arbuscules (Figs. 1 and 2) similar to those described by Kessler (1966). Only a few vesicles were in evidence; this was a young and thriving infection as judged from the arbuscular condition. It

Fig. 1. Outer layers of cortical cells of sugar maple root showing coils of intracellular mycelium (430X).

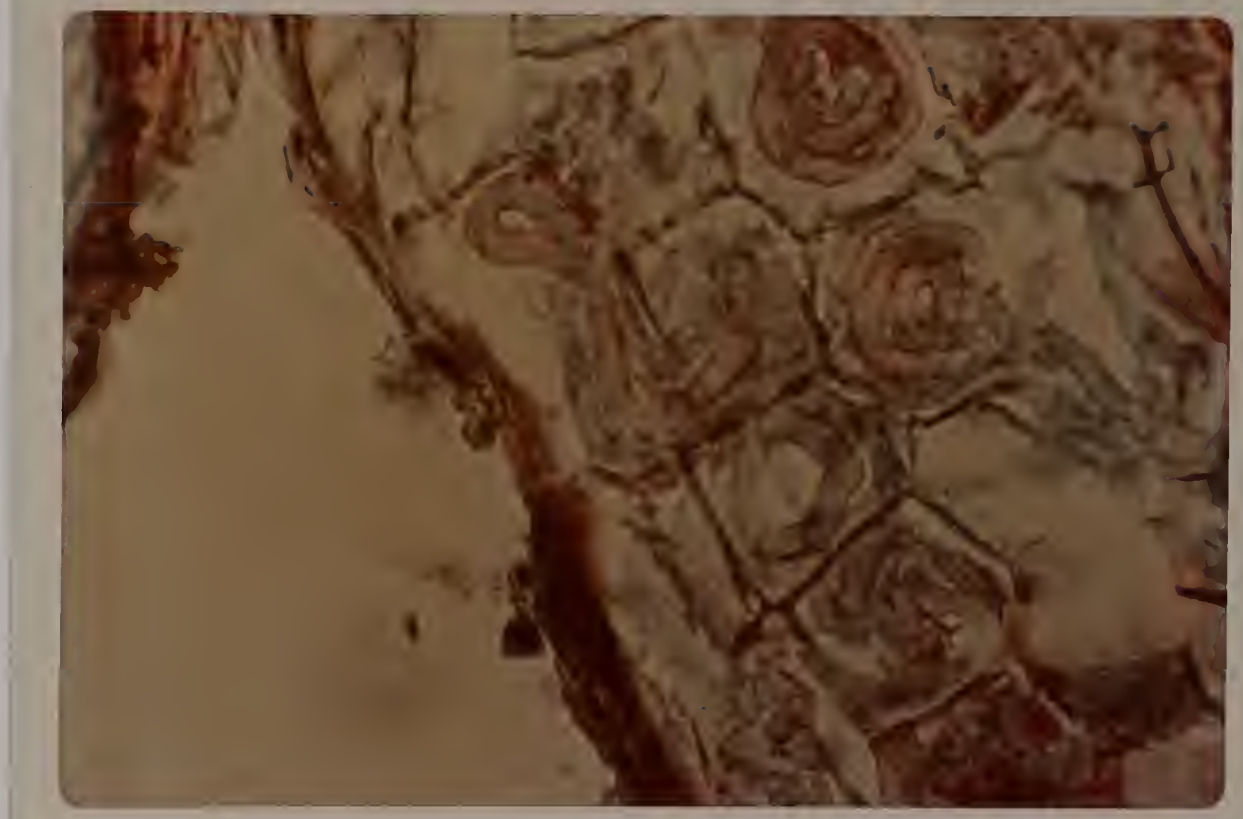
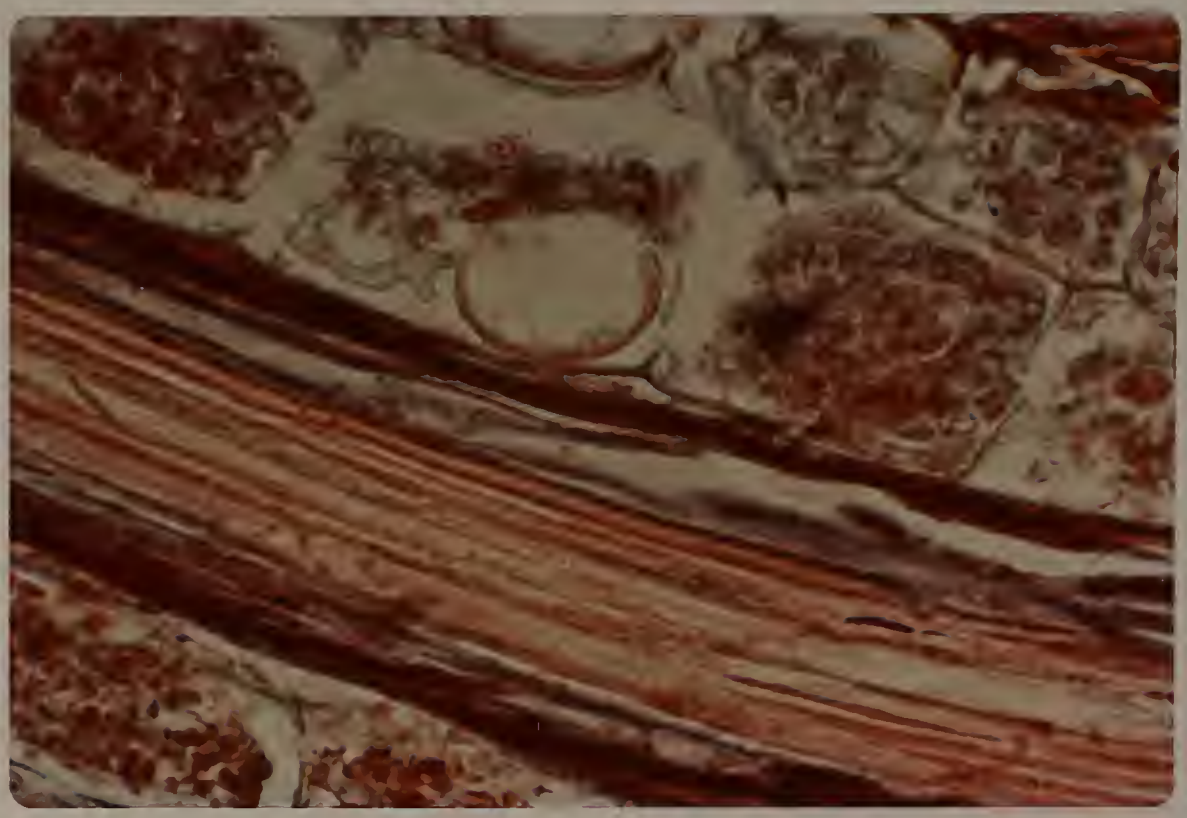


Fig. 2. Inner layers of cortical cells packed with arbuscules. Two vesicles can also be seen in upper center of picture. (430X)



was impossible to tell from this experiment if the nematodes had any effect on the endomycorrhizae of sugar maple.

iii. First Year Inoculations with M. hapla

In late July it was noted that four of the twelve trees inoculated with Meloidogyne hapla were exhibiting leaf scorch, chlorosis, and a reddish cast to all of the leaves (Fig. 3). Roots exhibited several gall-like swellings as well as some lesions. Swollen females were evident protruding from the smaller galls on the fine feeder roots stained with acid fuchsin and larvae in various stages of development were teased from galls (Fig. 4).

The histological examination of the galls showed typical giant cell formation (Fig. 5) along with the production of anomalous xylem (Fig. 6). Several sections also showed the nematodes within the root itself or protruding from it (Fig. 6 and 7). This is the first report of M. hapla attacking sugar maple, at least under the artificial conditons of the greenhouse. Although only four of these trees showed symptoms, root knot larvae were recovered from the soil of trees that did not show symptoms. The eight trees that had been inoculated the previous year, but had not shown any symptoms, were reinoculated. At the end of three months only one tree was exhibiting any symptoms. Upon examining the roots of all eight trees, only the one that was exhibiting symptoms was found to have galls on its roots.

iv. Examination of Trees from Previous Year's Experiments After Overwintering

Fig. 3. Chlorotic foliage shown by sugar maple seedling inoculated with root knot nematode.

Fig. 4. Swollen M. hapla larva teased from a gall on a fine feeder root of sugar maple. (100X)

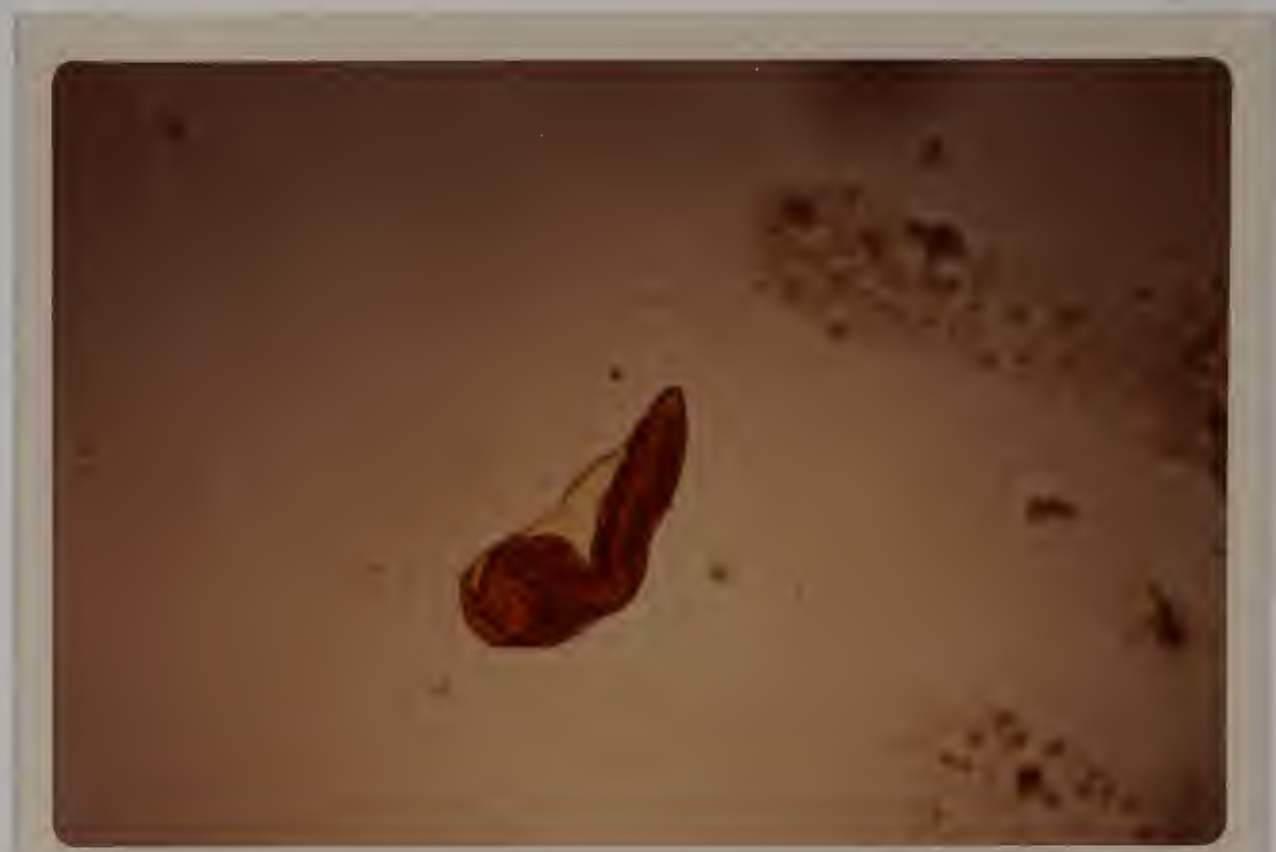


Fig. 5. Cross section through sugar maple root and root knot nematode (left of root), showing thick walled giant cell formation and beginning production of erratic xylem. (100X)

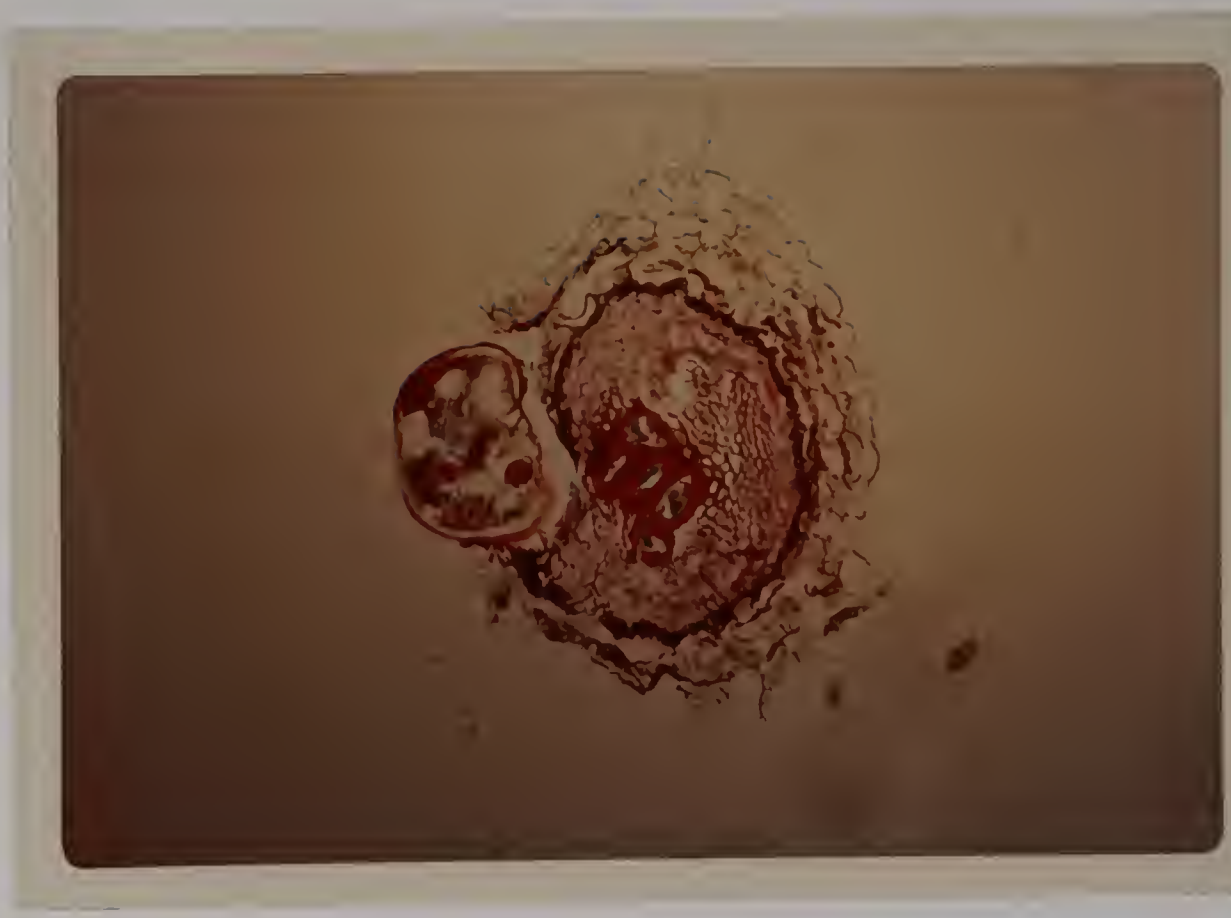


Fig. 6. Cross section of gall with embedded nematode (upper right) and erratic xylem. (430X)

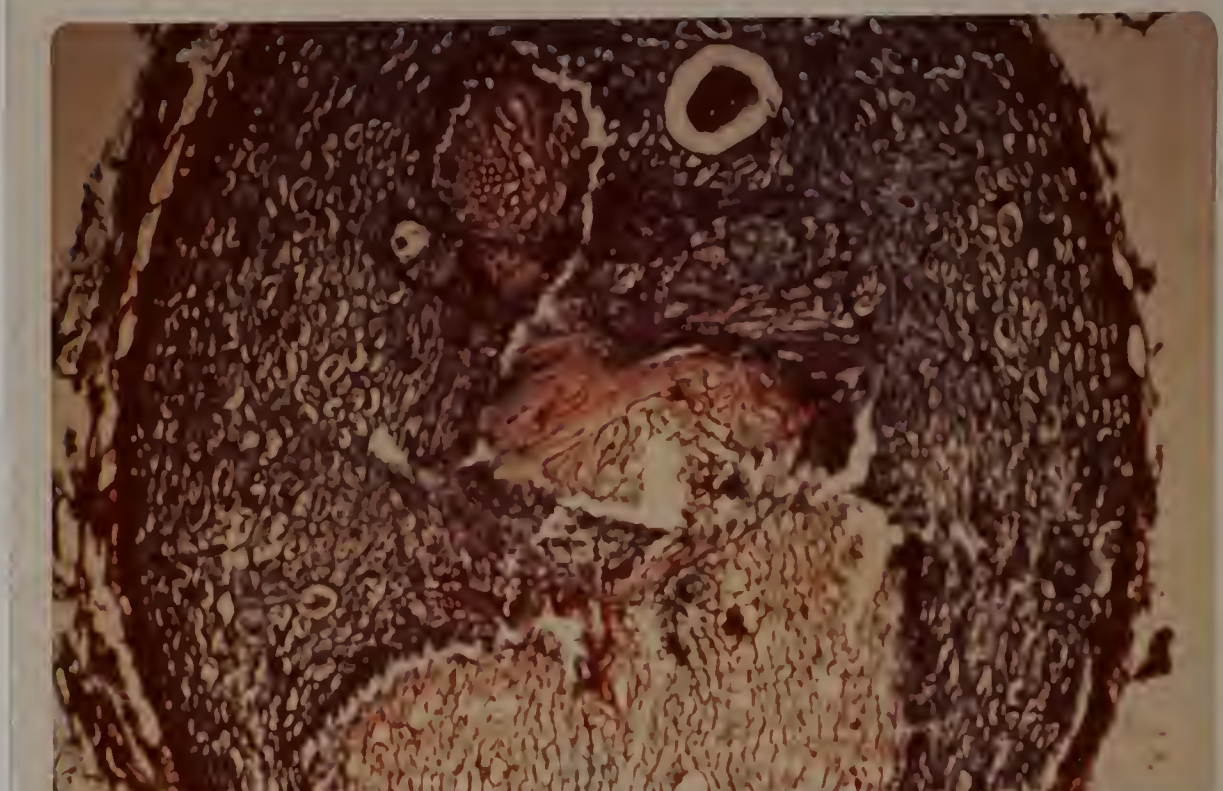
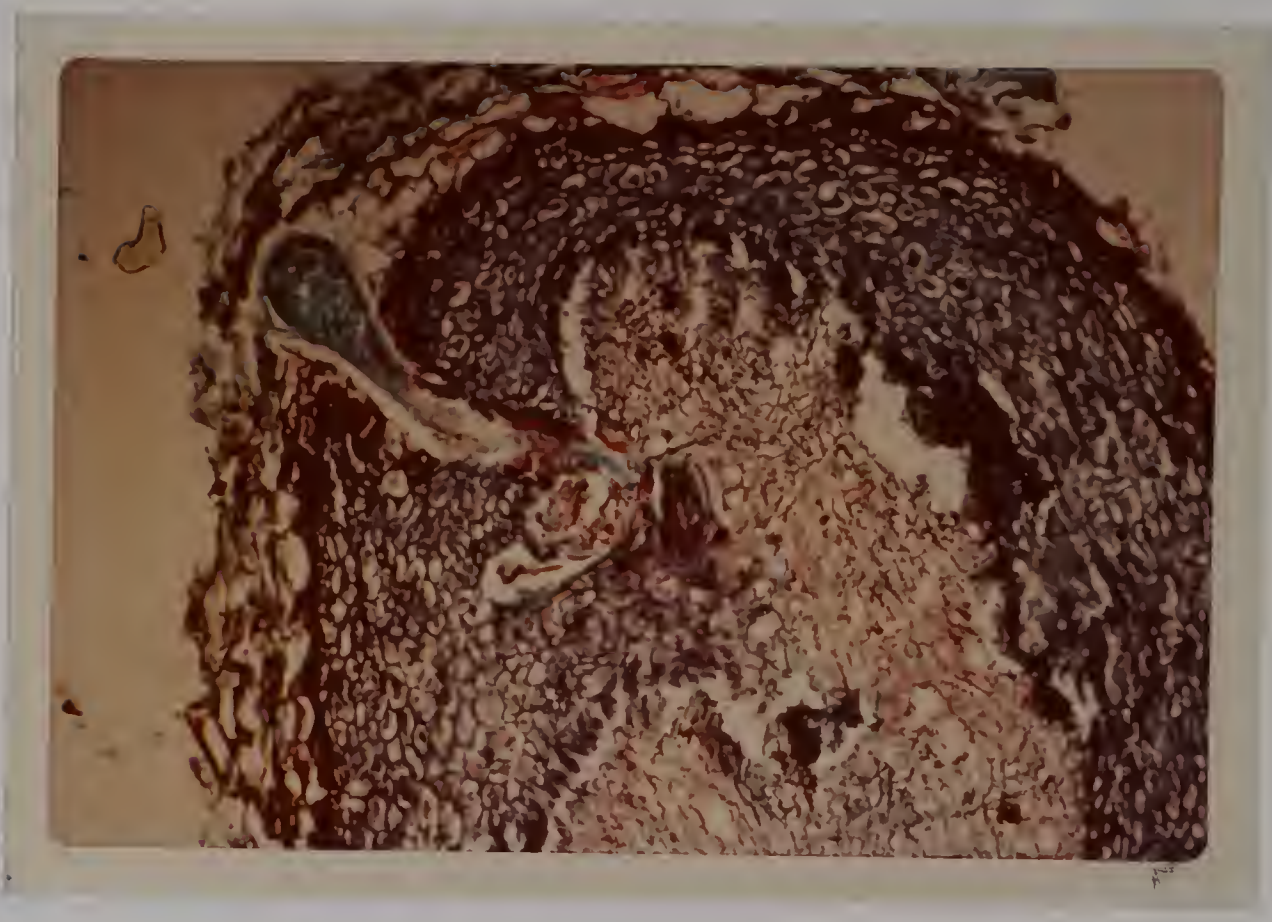


Fig. 7. Cross section of root showing erratic xylem and root knot nematode protruding laterally from root (left upper corner). (430X)



The six seedlings from the previous year's experiments, two that had been inoculated with X. americanum, two that had been inoculated with M. hapla and two control seedlings, were found, using sugar flotation, to contain no living nematodes after overwintering. When stained with acid fuchsin (Goodey, 1951), none of the inoculated or control trees showed any sign of endoparasites being present.

Using the KOH procedure (Phillips and Sanders, 1970) it was noted that the degree of mycorrhizal infection in all three sets of roots was very similar. However, there was no mycorrhizal infection in the tissues galled by M. hapla. The cortical cells of the galls looked normal as far as could be determined from examination under a light microscope, but they were not invaded by the fungus.

After fixing, dehydrating and sectioning the root tissues from the two sets of trees that had been inoculated with X. americanum the previous year, it was noted that the mycorrhizal infection looked more senescent that in the controls. Many arbuscules were in their sporangiolar state; what used to be considered reproductive propagules by Kessler (1966) and others are now known to be arbuscules in a moderate to advanced state of senescence (Fig. 8). The arbuscules in this state are undergoing active digestion of the fine branches which makes them appear globular (Kinden and Brown, 1975). Also, vesicles were more abundant in the tissues inoculated the previous year, a condition indicative of the infection being in an advanced or stressful state. Vesicles are thick walled, resistant structures that

store accumulated nutrients for the fungus when the infection is no longer actively growing. This can be due to natural senescence of the fungus or a decrease in carbohydrates and other nutrients available to the fungus due to an unhealthy host.

v. Second Year Inoculations of Three Inch Forest Seedlings

The roots of two of the control three inch seedlings growing in steam sterilized forest soil were analyzed using Phillips and Sanders' (1970) KOH procedure. They were found to be heavily infected by the fungus; almost all cortical cells on the feeder roots were filled with arbuscules with almost no vesicle formation (Figs. 9 and 10). These trees were seen to be much more heavily infected by the fungus than the control trees from the previous year's experiments that had been planted as bare rootstock in steam sterilized soil. Many more cortical cells were filled with the mycelium of the fungus.

The ten trees inoculated with 100 X. americanum each (three were maintained as controls) were observed carefully for three months. At the end of the time period only one of the trees was showing any aberrant growth. Its leaves remained green but the top three sets of opposite leaves remained very small and did not expand. Nematodes were recovered from only one tree, the tree that had been appearing stunted. Five X. americanum were recovered from the soil of this tree.

The roots of these trees were analyzed by the KOH procedure (Phillips and Sanders, 1970). The tree from which the nematodes had been recovered was less highly infected than the controls or the other

Fig. 8. Advanced stage of mycorrhizal infection: arbuscules are deteriorating to their "sporangiole" state, vesicles are forming in many cells. (430X)

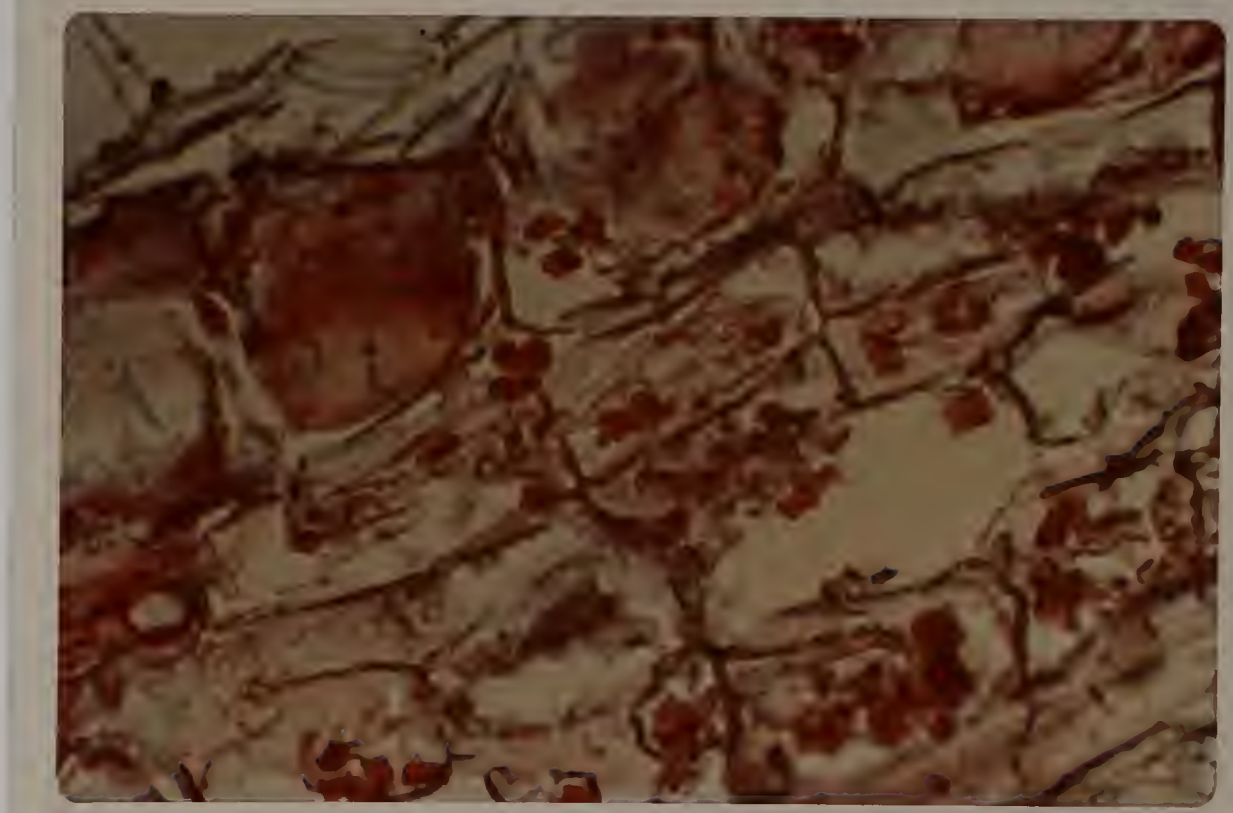


Fig. 9. Portion of root cleared and stained with Trypan Blue.

Note cortical cells packed with arbuscules. (100X)

Fig. 10. Cortical cells cleared and stained with Trypan Blue containing arbuscules and coils of mycelium. (430X)

nine trees from which no nematodes were recovered. The roots also had some definite areas of necrosis.

vi. On-campus Maple Survey

Xiphinema americanum was found in soil samples from every tree, ranging from 5 to 25 individuals per 500 grams of soil. In the course of two years many soil samples from many different locations and plants have been analyzed and it is only when soil from around sugar maple roots was sampled that X. americanum was found consistently. With a sample of only twelve trees it is impossible to draw any statistically significant conclusions, yet it is possible to form some hypotheses that might be supported later by larger sampling. The numbers of X. americanum in these samples was highly variable, yet the nematodes were found around all trees sampled. Also, the mean number of X. americanum found around the class 1 and class 4 trees was seven and nine, respectively; for the class 2 and 3 trees, twelve and fourteen, respectively, which is consistent with the findings of DiSanzo (1967) who found higher numbers of X. americanum around moderately declining maples than healthy or severely declined ones. No Meloidogyne hapla were found at all in any soil samples processed. Root samples from all trees were stained with acid fuchsin and no endoparasitic nematodes were seen (Table 4).

The degree of mycorrhizal infection was estimated as per cent infected cortical cells per 1 cm root using Phillips and Hayman's (1970) method (see Table 5). The degree of mycorrhizal infection of the roots

was found to be roughly correlated to the amount of decline shown by the crown. Healthy trees had extensive mycorrhizal development characterized by abundant arbuscule formation and much attached extramatrical mycelium (Fig. 11). The amount of mycorrhizal infection decreased steadily and became much more localized in trees from classes 2 and 3. Many holes had to be dug for trees of crown class 4 before adequate root samples could be found. Root samples that were found were black and any mycorrhizal development would have been obscured.

Whether decreasing mycorrhizal infection with increasing decline is a cause or a result of decline could not be determined from this study, but it is without doubt that declining trees exhibit poor mycorrhizal infection.

CHAPTER V

DISCUSSION

The results of this study, as in many other studies with nematodes, have led to their being implicated as pathogens but no positive proof of their pathogenicity was demonstrated. The more positive results came from the greenhouse inoculations with the northern root knot nematode Meloidogyne hapla. In the first year's inoculations, only four out of twelve inoculated trees became infected and showed symptoms of decline; in the second year's experiments only one out of eight became infected. In the campus study of declining maples using the acid fuchsin stain for endoparasites (Goodey, 1951) no endoparasites were found.

Table 4. Numbers of *M. hapla* and *X. americanum* found in soil samples from 12 declining maples on UMass campus, spring 1977.

crown class	*M. hapla*	*X. americanum*	tree #
1	0	7	8
1	0	7	12
2	0	9	7
2	0	22	9
2	0	6	11
3	0	5	1
3	0	25	2
3	0	15	5
3	0	12	10
4	0	4	6
4	0	11	3
4	0	12	4

Table 5. Degree of mycorrhizal infection of 12 sugar maple trees in various stages of decline, spring 1977.

crown class	sample number	% mycorrhizal infection
1	8	90+
1	12	90+
2	7	90+
2	9	30-40
2	11	50-60
3	1	30-40
3	2	10-20
3	5	30-40
3	10	30-40
4	6	0-10
4	3	0-10
4	4	0-5

Fig. 11. Extramatrical mycelium of fungal symbiont with attached vesicles. (100X)

When the potted maples that had been inoculated the first year were brought inside to break their winter dormancy, it was found that no nematodes had survived overwintering.

These factors lead one to hypothesize that M. hapla is an incidental pathogen of sugar maple where winters are less severe its seriousness as a pathogen might increase considerably. There is little doubt that M. hapla can infect sugar maple under greenhouse conditions. Giant cell formation as well as erratic xylem can be clearly seen in the sections made of infected roots. Riffle (1973) found similar erratic xylem in Pinus ponderosa infected with a Meloidogyne species. Whether M. hapla can actually complete its development on sugar maple is debatable since swollen females but no egg masses were found. Perhaps if the experiments were allowed to continue for a longer time period this could have been observed. Nematode feeding and partial development might disrupt the normal physiological functioning of the roots so they are no longer inhabitable by the fungus. Why the endoparasites invaded some of the trees in the greenhouse and not others could not be determined from this study since all trees were treated in the same way; perhaps genetic variability makes some trees more susceptible to nematodes than others.

Working with Xiphinema americanum presented many problems one of which was merely obtaining enough nematodes to work with. Since X. americanum cannot be raised in axenic culture, "pot cultures" on strawberry plants were hoped to support enough nematodes to inoculate

the sugar maple seedlings with high numbers of nematodes. It was originally thought that using sugar flotation (Jenkins, 1964) might be injurious to the nematodes and that was why the cultures did not reproduce; throughout the rest of the experiments the nematodes were extracted using a modified Cobb's (1918) sieving and gravity method to avoid this. Six inch plastic pots were used originally for the strawberry plants and the first year's maple seedlings to minimize drying out and a well aerated soil mixture was used to insure adequate supply of oxygen to the nematodes. No fertilizers were applied to any of the pots since these are known to be detrimental to nematode populations (Cohn and Mordechai, 1970). Unfortunately, even under these conditions the nematodes failed to reproduce. In the following year's experiments the three inch forest maples were potted in steam sterilized forest soil in place of the 1:1 sand and loam greenhouse mix used previously to try to duplicate natural conditions as much as possible. Also, distilled water and stainless steel screens were used in extracting the nematodes to avoid excess copper ions, which have been considered toxic to nematodes (D. Kaplan, personal communication). Three inch plastic pots were used to hopefully keep the nematodes confined to the root area more closely, but in spite of all the precautions taken the nematodes still failed to survive on all but one maple seedling. Only small amounts of inoculum, 100 individuals per tree, could be used since the nematodes had to be picked one by one from the processed soil samples; accumulating enough inoculum to do many trees is a tedious and time-consuming task.

On the one seedling that did support a nematode population there was less mycorrhizal infection when the roots were analyzed using Phillips and Hayman's technique (1970). Perhaps the probing of the cortical cells with their stylets when the Xiphinemas were searching or feeding led to modification of the cells so they were no longer able to be infected by the fungus. The reverse situation may also be true; perhaps because this one maple seedling was less highly infected by the fungal symbiont it was a more suitable host for the nematodes. Since nothing is known about the effects of VA infection of sugar maple on nematodes believed to pathogenic on it, it is impossible to determine which really happened. The senescent appearance of the two sets of nematode infected trees in the six inch pots, i.e. the arbuscules being in their "sporangiolar" state and the presence of vesicles, led to analysis of the rest of the trees that had been inoculated with Xiphinema the previous year. Observations of these trees further supported the idea that the nematodes had had some sort of stressful effect, causing the infection to senesce.

In attempting to do an integrated study such as this many problems of defining unforeseeen variables arise. As well as little being known about the cultural conditions of X. americanum, little is also known about the mycorrhizal dependency of sugar maple. Differences were noted in the degree of infection of control seedlings when they were planted as bare rootstock and those inoculated with the fungal symbiont by sprinkling forest soil on the top of the soil in the pots and when they were dug up from forest soil with their root systems naturally infected.

In the study of the on-campus maples, nutritional and pH factors did not seem to be related to the decline of the crown and root systems (Table 6). There did seem to be a correlation between the degree of decline shown by the crown and the health of the fungal symbiont in the roots. From trees of classes 2 to 3 to 4 there was an increasing amount of blackening of the roots and a corresponding decrease in the amount of fungal infection. In trees of class 4 what root system could be found was completely blackened, with most of the feeder roots rotted off, so no fungal infection could be found at all. Trees of class 1, exhibiting healthy growth, had young infections showing characteristic arbuscule development and much extramatrical mycelium with attached chlamydospores. In classes 2 and 3, as well as there being a lessening of the amount of arbuscular infection, there was an increase in the amount of vesicle formation, at times to the point where the cortical cells were obviously disrupted by their presence.

Xiphinema americanum was found associated with the roots of every tree sampled, from healthy to severely declined. It is relatively safe to assume, from this work and studies previously mentioned, that X. americanum is indeed a pathogen of sugar maple. Whether the feeding of this nematode effects the endomycorrhizae indirectly by disturbing cortical cells by its feeding behavior cannot be conclusively determined from this study; however, some interaction between the two is indicated. Perhaps the case here is similar to that postulated by Fox and Spasoff

Table 6. pH and nutritional analysis of 12 sugar maple trees, UMass campus, spring 1977

Tree #	crown class	pH	Ca	K	P	Mg	NO_3	NH_4
1	3	6.3	M	L	MH	H	L	H
2	2	5.9	M	M	L	H	M	L
3	4	6.4	MH	MH	MH	H	M	L
4	4	6.5	MH	M	H	H	M	-
5	3	5.7	L	M	L	H	M	-
6	4	6.2	M	M	MH	H	L	-
7	2	6.3	M	H	MH	H	M	L
8	1	6.2	M	M	M	H	L	L
9	2	6.3	M	M	L	H	L	L
10	3	6.3	M	M	L	H	L	L
11	2	6.4	MH	M	M	H	M	L
12	1	6.2	MH	M	L	H	M	L

Key to rating by letter: PPM in soil

	NO_3	NH_3	P	K	Ca	Mg
EH	75	200	200	350	3500	-
VH	50	175	150	300	2500	-
H	30	150	100	250	1600	125
MH	20	80	50	180	1200	50
M	10	35	25	120	900	25
L	5	12	12	60	500	12

(1972) in that competition between the fungal endophyte and the nematodes for the same living space results in their having a detrimental effect on each other.

This study raised many questions and answered few. Much needs to be learned about the mycorrhizal dependency of sugar maple. What caused the roots of sugar maple to turn black and decay remains to be determined. Why X. americanum is so difficult to raise in the greenhouse is not known. In a study involving organisms inhabiting an environment as complex as the rhizosphere it become difficult to tell which is the cause and which is the effect. The two species of nematodes, in their feeding activities, could be detrimental to the fungal symbiont causing a condition of stress. This could lead to decline itself, or weaken the tree so it is more susceptible to other conditions causing decline.

CHAPTER VI

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