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The role of vesicular-arbuscular mycorrhizal fungi in the asparagus (Asparagus officinalis L.) agroecosystem

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Tracy Leigh Wacker

has been accepted towards fulfillment
of the requirements for

MS degree in **Botany & Plant Pathology**

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THE ROLE OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN THE ASPARAGUS (ASPARAGUS OFFICINALIS L.) AGROECOSYSTEM

By

Tracy Leigh Wacker

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ABSTRACT

THE ROLE OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI
IN THE ASPARAGUS (ASPARAGUS OFFICINALIS L.) AGROECOSYSTEM

By

Tracy Leigh Wacker

The effect of a mycorrhizal fungus, Glomus fasciculatum, on the severity of Fusarium wilt in asparagus was studied. In the greenhouse, mycorrhizal plants were larger and exhibited less disease severity than non-mycorrhizal plants. There was no apparent effect of mycorrhizal inoculation on disease severity in the field, however dry weights of mycorrhizal plants were greater than non-mycorrhizal plants.

Asparagus fields were surveyed for the species composition of vesicular-arbuscular mycorrhizal fungi. There was a shift in the species composition which was correlated with field age. Younger asparagus fields contained mainly Glomus and Acaulospora spp., while older fields contained mostly Gigaspora spp. Factors contributing to shifts in species composition are discussed.

Ferulic acid, an allelochemical found in asparagus, was shown to inhibit hyphal elongation of G. fasciculatum in vitro. Also, the growth of mycorrhizal asparagus and colonization of asparagus roots by G. fasciculatum were reduced by ferulic acid. Allelochemicals may play an important role in the establishment of mycorrhizal associations in asparagus.

This thesis is dedicated in remembrance of Eric A. Cowling, father, mentor, and friend. He taught me that, upon embarking on the journey into adulthood, there are two things that one must not leave behind: your curiosity and a good sense of humor. I think he'd be proud that I remembered.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I.

EFFECT OF *GLOMUS FASCICULATUM* ON THE INCIDENCE OF FUSARIUM WILT (*FUSARIUM OXYSPORUM* F. SP. *ASPARAGI*) AND GROWTH OF ASPARAGUS (*ASPARAGUS OFFICINALIS* L.)

Vesicular-arbuscular mycorrhizal fungi (VAM) are ubiquitous soil organisms, forming symbiotic associations with a wide array of plant species (25). This symbiosis is a form of mutualism. The VAM (mycobiont) is effective at increasing uptake of mineral nutrients by the host (autobiont), especially phosphorus. The autobiont, in return, provides the fungus with necessary carbohydrates. The symbiosis is essential to the fungus, which requires a host for growth (obligate biotroph). The autobiont can benefit in several ways. Secondary effects of improved plant nutrient status include increased crop yield, improved water relations, and reductions in disease severity (16, 38, 41, 49, 51, 52, 53).

VAM have been shown to increase growth in several field crops, including wheat, maize, oat, rye, barley, potato, and soybean (7, 21, 29, 32, 43). Mycorrhizal associations have also benefited horticultural crops such as chilli peppers, carrot, tomato, pepper, and onion (5, 20, 43, 44, 57). These growth increases are directly related to the improved uptake of phosphorus in mycorrhizal plants. In soils where phosphorus levels were high, the mycorrhizal effect could be overcome and colonization by VAM was significantly reduced (7, 29, 57).

The outcome of interactions between vesicular-arbuscular

mycorrhizal fungi (VAM) and plant pathogens in the rhizosphere could be important when considering biological control methods. For example, the mycorrhizal association could improve the plant's tolerance to soil-borne plant pathogens (63), or interactions between VAM fungi and plant pathogens could involve competition for infection sites in the root. Changes in host nutrition could subsequently affect colonization by the competing organism. Also, there is potential for host tolerance of pathogen infection if VAM associations can compensate for damages caused by the pathogen (25). Zak (65) suggested that mycorrhizal fungi could offer protection to the root by: a) utilizing exuded carbohydrates (thus reducing the chemotactic potential of the plant root toward pathogens), b) providing a physical barrier, c) the production of antibiotics, and d) inducing a rhizosphere environment that is favorable to protective organisms (e.g. antagonistic to plant pathogens). While Zak proposed these mechanisms for ectomycorrhizal fungi, Schenck (52) observed that all of these mechanisms, with the exception of a physical barrier, could be operative in VAM associations. If, however, the VAM association could be formed prior to pathogen invasion, it is possible that the presence of the VAM fungus in the host root would be effective at excluding pathogen colonization, especially those pathogens that cause the most damage by penetrating into the vascular system.

Bagyaraj (4) has proposed several mechanisms that VAM associations could provide to increase resistance (tolerance) to plant pathogens. These can be separated into morphological and physical/biochemical mechanisms. Morphological changes include: a) cell wall thickening (through lignification), which could be effective against penetration, b) greater mechanical strength (due to stronger vascular systems), this

may reduce symptoms of vascular diseases, and c) higher chitinase activity in mycorrhizal tissues, which may diminish or exclude pathogen infection in these areas of the root system. Physical/biochemical mechanisms include: a) higher tissue phosphorus levels in the host (the plant exhibits increased vigor), b) decreased root exudation, c) altered root exudation, which could support a different rhizosphere community (increases in antagonistic spp.), and d) changes in the concentration of certain amino acids within the roots (could lead to reductions in pathogen colonization). However, most studies regarding the interactions between VAM and plant pathogens indicate that the primary effect of VAM associations on disease development is related to changes within the root rather than an alteration of the rhizosphere community (47, 53).

If the effect of VAM associations on disease development is confined to changes in the root, then in order for the symbiosis to effectively inhibit pathogen invasion, the symbionts would require optimum conditions for VAM fungal colonization before exposure to the pathogen (16). Competition for sites between VAM fungi and plant pathogens (simultaneously) would reduce the disease tolerance available through mycorrhizal symbiosis. VAM fungi are capable of colonizing many plant species, whereas many plant pathogens are host specific and aggressive competitors for infection sites. Also, root-rotting fungi and toxin-producing pathogens could cause considerable damage to the root. Dehne (16) suggested that this action could destroy the carbohydrate supply utilized by mycorrhizal fungi within the host root tissue. Highly virulent and aggressive plant pathogens, according to Dehne, will be detrimental to mycorrhizal associations.

Safir (48) was the first to report that VAM inoculation could

decrease disease severity in the host . He found that symptoms of pink rot, caused by Pyrenochaeta terrestris, were reduced in mycorrhizal onion plants. He proposed that this reduction in disease severity was due to a decrease in reducing sugars in the roots of onion. The differences in disease severity and reducing sugars between VAM and non-VAM plants were eliminated when nutrients were added to non-VAM plants. Several researchers have found similar reductions in disease severity of other mycorrhizal crops. Dehne and Schonbeck (17) found that wilt symptoms were diminished in VAM inoculated tomato plants. Caron et al. (9) observed a reduction in both root colonization and sporulation by F. oxysporum f. sp. radicis-lycopersici in tomato plants previously inoculated with the VAM fungus, Glomus intraradices. VAM inoculated tobacco and cotton plants were more resistant to root rot caused by Thielaviopsis basicola (55, 56). The colonization of cotton or tobacco roots by VAM fungi did not affect the infection rate of the pathogen, but the appearance of above-ground symptoms was decreased in mycorrhizal plants. The association of VAM with peanut that had been infected with Sclerotium rolfsii reduced the number of sclerotia produced by the pathogen (35). The pathogen, however, reduced the production of chlamydospores and root colonization by the mycorrhizal fungus.

Biochemical changes in VAM infected plants that may be involved in disease suppression are now thought to be secondary effects of improved phosphorus uptake (22). Graham and Menge (22) studied the effects of Glomus fasciculatum on the severity of take-all disease of wheat, caused by Gaumannomyces graminis. They found that VAM infected wheat was less susceptible to invasion by the pathogen. However, this effect could also be obtained by increasing the soil phosphorus level. Citrus seedlings

inoculated with Glomus fasciculatum grew better in the presence of Phytophthora parasitica than non-VAM plants (15). The number of pathogen propagules was greater when G. fasciculatum was present. The apparent tolerance of VAM - infected citrus to P. parasitica was considered to be phosphorus mediated, as the VAM effect could be duplicated by increasing soil phosphorus. VAM colonization of Poinsettia was higher in plants that were challenged with Pythium ultimum in low phosphorus soils (31). Foliar phosphorus and manganese were higher in VAM plants than non-VAM plants in Pythium infested soils. Also, the populations of Pythium were lower in soil in which VAM - infected Poinsettia were growing.

The presence of a pathogen can cause reductions in VAM colonization. Zambolin and Schenck (66) tested the effect of Glomus mosseae -colonized soybean plants on diseases caused by three fungal pathogens (Macrophomina phaseoli, Rhizoctonia solani, and Fusarium solani). Plants infected with both G. mosseae and any of the three pathogens were intermediate in growth. VAM and control plants were larger, and plants subjected to the pathogen alone were the smallest. There was no significant reduction in disease symptoms due to VAM colonization, and the presence of the pathogen reduced root colonization of soybean by G. mosseae. Schenck and Kinloch (54) also noted a decrease in VAM colonization of soybean in the presence of root-knot nematodes. Baath and Hayman (3) observed that tomato infected with either G. mosseae or G. caledonia did not reduce the severity of Verticillium wilt. The presence of the pathogen reduced plant growth in both VAM and non-VAM plants. The VAM-infected tomato plants were smaller than the non-VAM plants regardless of pathogen treatment.

It has been demonstrated that VAM fungi are capable of reducing disease severity in certain hosts. In others, there is no apparent effect on pathogen colonization or host expression of symptoms. There have also been reports that pathogenicity of VAM fungi can be induced by certain extraneous conditions. Kiernan et al. (33) observed that, at high fertilizer rates, Glomus clarum, G. etunicatum, and G. claroideum could inhibit the growth of sweetgum seedlings. G. fasciculatum was not inhibitory to plant growth. Also, as fertilization levels increased, root colonization and sporulation by these VAM fungi decreased. Modjo and Hendrix (40) found that the amount of sporulation and colonization of roots by Glomus macrocarpum was significantly correlated to the incidence of tobacco stunt disease. The degree of stunting was related to the increased development of arbuscules, hyphae, and vesicles within the host root. G. microcarpum also causes stunt symptoms in tobacco, but is not considered to be as important as G. macrocarpum in the tobacco stunt disease.

Asparagus has been shown to suffer from an overall field decline, resulting in decreases in yield, plant vigor and longevity (1, 24, 39). Several factors contribute to this decline. Environmental and physical stresses play a key role in predisposing the host to pathogen invasion (18, 30, 61). Environmental stresses include nutrient imbalance, low soil pH, and soil moisture. Physical stresses include defoliation by the asparagus beetle and stresses due to cultural practice, such as soil compaction, duration of cutting season, and planting depth (27, 59, 60). However, the major contributors to asparagus decline are the wilt and crown rot organisms. Fusarium oxysporum (Schlect.) Snyd. & Hans. f. sp. asparagi Cohen (Cohen &

Heald) and Fusarium moniliforme (Sheld.) Snyd. & Hans., respectively (13, 23, 24, 30, 61).

Another aspect of the decline problem is the difficulty encountered in attempts to replant asparagus into decline fields. It has been virtually impossible to establish new asparagus stands in fields previously planted to asparagus (24, 36). This problem is partially alleviated by soil fumigation, but asparagus seedlings planted into fumigated soils are often stunted and lacking in vigor (36). While fumigation is effective at removing the fungal pathogens, it is evident that other factors are inhibiting the growth of asparagus seedlings in these soils. It has been hypothesized that there is a build-up of autotoxic chemicals in decline fields that are more inhibitory to asparagus seedlings than to established plants (26, 64, 65). While there is growing evidence to support this hypothesis, another explanation for the stunted growth of asparagus in fumigated fields could be that the fumigation has also destroyed beneficial vesicular-arbuscular mycorrhizal fungi (VAM) and the plant is therefore lacking in essential nutrients.

The deleterious effect of fumigation on resident VAM has been well documented (37). In most cases, plants respond favorably to soil fumigation and this is probably due to the elimination of soilborne plant pathogens. Plants such as citrus, avocado, and cotton often became stunted and chlorotic after fumigation. In these instances, the introduction of VAM into these soils restored plant vigor.

Asparagus has also been shown to respond favorably to VAM inoculation (10, 11, 28, 45, 46). Powell and Bagyaraj (45) side-dressed VAM inoculum into an asparagus nursery that had been fumigated with

methyl bromide ($MeBr_2$) prior to planting. The VAM plants were significantly larger and more vigorous than the non-VAM plants, which exhibited the characteristic stunting mentioned previously. Evans et al. (19) compared the effects of fumigation on asparagus to growth in untreated soils. Asparagus grown in untreated soil had significantly greater fern height and VAM colonization than the fumigated treatment. Asparagus plants from the $MeBr_2$ soils had the shortest ferns and no VAM infection. Also, root phosphorus (P) levels were deficient (0.13 %) in asparagus grown in the fumigated soils.

The deficient levels of phosphorus in asparagus planted into fumigated soils suggests that asparagus may have a high mycorrhizal dependency, at least in the seedling stage. St. John and Coleman (58) pointed out that many perennial plants may be highly mycorrhizal when young, but this requirement would diminish as the storage root system of the autobiont increased. This observation coupled with the apparent dependency of young asparagus on VAM led to questions regarding the importance of VAM in the asparagus decline problem.

The observation that vascular wilt symptoms are almost always lessened in VAM plants (25), coupled with previous findings of VAM-enhanced growth of asparagus seedlings, influenced my decision to study whether VAM-infected asparagus plants were more tolerant of Fusarium wilt than non-VAM plants. Since asparagus is considered a drought tolerant plant (62), the first aspect of this study dealt with the interaction between VAM plants and Fusarium oxysporum f. sp. asparagi grown in the greenhouse at two soil moisture levels. The second portion of this study was concerned with the effect of an introduced VAM fungus on asparagus growth and disease tolerance in the field at two soil

phosphorus levels.

Materials and Methods

Greenhouse study. Inoculum of Glomus fasciculatum (Thaxt. sensu Gerd.) Gerd. & Trappe was obtained by adding chlamydospores of the fungus to pot cultures of sorghum (Sorghum vulgare). The VAM-infested soil and sorghum roots were harvested after 4 months and stored at 4^{o}C for 4 months prior to use.

A 2:1 sand:soil mixture (v:v) was steam sterilized for 24 hours; 500 g of this mix was added to each of 120 4" pots. The soil was either treated with 1 g of Glomus fasciculatum inoculum (50-60 spores/gram soil) or 1 g of sterile inoculum soil, autoclaved for 1 hour on each of 2 consecutive days, plus 1 ml of inoculum wash (-VAM treatments). The inoculum wash was prepared by wet-sieving inoculum through a 38 um sieve into a beaker. This is done so that microbes associated with the inoculum soil can be added to non-mycorrhizal treatments. The inoculum soil in both +/-VAM treatments was placed 1 cm below the roots.

Asparagus plants (UC 157 variety) were transplanted into the prepared soil (one plant per pot) after 8 weeks of growth in a Baccto greenhouse soil. Prior to transplanting, the plants were fertilized weekly with 100 ml of 100 ppm each NPK (20-20-20, Peters' Plant Food) and watered as needed. Each treatment was then split and subjected to soil water potentials of either 0 MPa (+/-VAM) or -1.5 MPa (+/-VAM). The soil water potentials were maintained by weighing the pots daily and bringing them to the required weights for that water potential, which was previously determined by obtaining the soil

moisture curve for the 2:1 sand:soil mix using a soil pressure plate. The plants were fertilized with 100 ml of 100 ppm NPK each week. Under this fertilization regime, the -1.5 MPa treatments were actually subjected to a drying cycle each week but never allowed to dry beyond the -1.5 MPa level.

The pathogen, Fusarium oxysporum (Schlect.) Snyd. & Hans. f. sp. asparagi Cohen (Cohen & Heald), which will be referred to as "FOA", was maintained on Komada's medium (34). The pathogen, FOA, was added to the asparagus as a conidial suspension 4 weeks after the VAM inoculation. Sterile glass-distilled water was added to petri plates containing 4 week old cultures of the pathogen (FOA). The conidia were dislodged, and the collected suspension diluted with sterile glass-distilled water to an approximate concentration of 2×10^4 conidia/ml, with a ratio of 7 macroconidia to 1 microconidium. The plants were inoculated with either 1 ml of sterile glass distilled water (-FOA) or 1 ml of the conidial suspension (+FOA) using a hypodermic needle placed just beneath the crown of the plant. The FOA treatments (+/-) were applied to half of each of the previously stated treatments and the plants were placed in a complete randomized block design consisting of 15 replications (pots) for each of 8 treatments.

Plants were harvested 8 weeks after the FOA inoculation (4 1/2 mo. old). Plants were rated for FOA severity using % root affected (lesions, reddening) as the criterion. A rating system was devised such that each integer represented the percent of the root system affected by lesions or reddening: 1 = 0 to 10 %, 2 = 11 to 20%, 3 = 21 to 30%, 4 = 31 to 40%, and 5 = over 40% of the roots affected.

Roots were then removed (0.5 g per plant) at random and stained with acid fuchsin in lactophenol (42). Root segments (1 cm in length) were placed on slides for observation using light microscopy. There were 20 segments per treatment. Segments were rated for VAM infection by the following scale: 0 = no infection, 1 = 1 to 25% of segment infected, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = over 75% infection. All internal fungal structures were included in the rating (arbuscules, vesicles, and intercellular hyphae). Fresh and dry weights were obtained and tissue phosphorus assays (6) performed to determine the P concentration within the plant. The data were subjected to analysis of variance and Student-Newman-Keuls mean separation test. This experiment was repeated once.

Field study. Inoculum of Glomus fasciculatum was prepared as mentioned previously. Fusarium oxysporum f. sp. asparagi was grown on PDA at room temperature (20^{o}C) for 7 days. Millet seed (200 g) was placed in 1-liter Erlenmyer flasks with 100 ml of distilled water. The millet seed was autoclaved for 1 hour at 121^{o}C on two consecutive days. When cool, the flasks were shaken vigorously to eliminate large clumps of millet seed. One 4-mm-diameter plug of Fusarium-infested PDA was added to each flask. Flasks were shaken every day to facilitate distribution of the fungus. After 14 days, the millet seed was allowed to dry at room temperature (20^{o}C) for 6 days.

Asparagus seeds (UC 157 variety) were surface-sterilized with a 10% Chlorox solution plus Tween 20 (0.1 ml per 100 ml Chlorox solution) for 30 min., then placed in a 1:10 Benlate(50W):acetone mixture and shaken for 24 hours (14). The seeds were rinsed with acetone to remove the

Benlate residue, then rinsed with 100 ml of distilled water six times. The seeds were place on sterile moistened filter paper in petri dishes and allowed to germinate for 7 days. Germinated seeds were placed in flats containing Baccto greenhouse mix. Prior to seed placement, either 5 g of G. fasciculatum inoculum (50-70 spores/gram soil) or 5 g of autoclaved G. fasciculatum soil plus 1 ml of inoculum wash (to replace the microbes that are commonly associated with these soils) was added to the soil mix in the flats. After one month, the asparagus was transplanted into 4" clay pots maintaining the Baccto mix. VAM-treated plants were selected at random and infection levels were assessed. At this stage root colonization appeared to be less than 10%. The plants were fertilized every 2 weeks with a 100 ml solution containing 100 ppm each of NPK (20-20-20) and watered as needed. Asparagus plants were transplanted to the field at 4 months of age.

A 20 ft. x 60 ft. area (located at the Asparagus Research Farm in Hart, MI (sandy loam soil, pH = 7.0, soil P_2O_5 = 185 ppm) was deep-tilled (12 inch depth) and fumigated with methyl bromide ($MeBr_2$, rate: 400 lb/acre) in September, 1985. The soil was randomly sampled and tested for the presence of Fusarium species and vesicular-arbuscular mycorrhizal fungi. The fumigation was apparently adequate, since no viable inoculum of either fungal type was found within the plow layer (8" depth).

Rye (Secale spp.) seeds, surface sterilized with a 10% Chlorox solution for 30 minutes, were broadcast over the entire field plot to form a dense cover crop. It was treated with RoundUp in May, 1986 and tilled under to increase the soil organic matter. On 17 June, 1986, the entire field area was divided into three replications with four

blocks each. Two blocks within each replication received phosphorus, added as P_2O_5 at 231 lb/acre (116 ppm), to attain a final soil P level of 350 ppm. The entire field plot was fertilized with nitrogen (46-0-0) and potassium (0-0-60, K_2O) to levels of 75 lb/acre (38 ppm) and 146 lb/acre (73 ppm), respectively.

In preparation for transplanting, rows were placed 3 ft. apart and holes dug every 1 ft. within each row to accomodate 24 plants per block (3 rows of 8 plants in each block). The pathogen, Fusarium oxysporum f.sp. asparagi, was added as 15 g of infested millet seed per plant. Since infection levels of VAM on the asparagus were lower than anticipated, 5 g of G. fasciculatum inoculum (50-70 spores/g) or 5 g of autoclaved inoculum was added. The VAM treatment was split so that each soil P level had both VAM and non-VAM treatments. Asparagus was transplanted into the field on 14 June, 1986. Plants were placed directly over the millet seed and +/- VAM soil at a depth of 4 inches. The experiment was set up as a 2 x 2 factorial in a randomized complete block design with 3 replications. The four treatments were labeled -M-P (control); -M+P (high P); +M-P (VAM treatment); and +M+P (VAM and high P). FOA was included in all treatments. Fern heights and diameters were measured each month and above-ground volumes were calculated from these data. Eight plants per treatment in each replication were harvested in September, 1986 and rated for disease severity and VAM colonization (staining procedure and rating system are outlined above). Fresh weights of ferns and roots were obtained and dry tissue was assayed for P concentration. The same kinds of data were collected for plants in the 1987 growing season, except plants were harvested in August. Data were subjected to analysis of variance and an LSD mean

separation test.

Results

Greenhouse study. There was little or no difference in fresh weights of plants grown at the two soil matric potentials (Table 1.1). At 0 MPa, fresh weights of plants inoculated with *Glomus* or FOA or both tended to be greater than those of non-inoculated plants. However, at -1.5 MPa, fresh weights were not significantly different. Dry weights tended to follow the same trend (Table 1.1).

Mean root ratings for disease severity and estimated populations of FOA are shown in Table 1.2. Plants inoculated with FOA developed root lesions. These symptoms were reduced in mycorrhizal plants at 0 MPa, but not at -1.5 MPa. Rhizosphere populations of FOA reflected the disease severity, in that the greatest propagule numbers were found in plants without *Glomus* at 0 MPa.

Mycorrhizal colonization was reduced in plants inoculated with FOA at 0 MPa, but not at -1.5 MPa (Table 1.3). Tissue phosphorus concentration among the treatments was not significantly different at either 0 MPa or -1.5 MPa.

Field study. In 1986, mean shoot volumes of mycorrhizal asparagus plants were greater from August on (Figure 1.1). There was no apparent effect of phosphorus on shoot volumes until the second (1987) growing season, when mycorrhizal plants receiving supplementary phosphorus had smaller shoot volumes than mycorrhizal plants grown without additional phosphorus. Shoot volumes of mycorrhizal plants without additional P decreased during the period from 16 July, 1987 to 17 August, 1987 due to invasion of plots by *Fusarium moniliforme* and to cultivation injury (see

discussion). At harvest, mycorrhizal plants exhibited slightly greater shoot volumes than non-inoculated plants. However, differences were not separable by the LSD mean separation test ($P = 0.05$).

Fresh weights of mycorrhizal plants were greater than those of non-inoculated plants in 1986 but not in 1987 (Table 1.4). However, dry weights of mycorrhizal plants tended to be greater than non-mycorrhizal plants in 1987.

In 1986 and 1987, the numbers of shoots/plant were similar among the treatments; only mycorrhizal asparagus without supplementary P had significantly more shoots than those without P (Table 1.5). Root:shoot ratios calculated from 1987 dry weight data showed that plants subjected to both *G. fasciculatum* and phosphorus addition had significantly lower root:shoot ratios than control plants ($P < 0.05$).

The percentage of the roots affected by FOA lesions in 1986 did not differ significantly (Table 1.6). In 1987, plants subjected to both *G. fasciculatum* and phosphorus addition had lower ratings than the control plants. Numbers of FOA propagules/g rhizosphere soil did not differ significantly among the treatments in 1986 (Table 1.6). However, in 1987, mycorrhizal plants at low soil P exhibited the highest number of propagules/g soil and soils supplemented with phosphorus yielded the lowest populations.

All plants were uniformly mycorrhizal by the end of the first growing season (Table 1.7). However, in 1987, root colonization tended to be greatest in low phosphorus soils ($P < 0.05$). Tissue phosphorus levels were not significantly different in 1986-harvested plants (Table 1.7). In 1987, tissue phosphorus levels were greatest ($P = 0.05$) in control plants.

Table 1.1. Effect of Glomus fasciculatum and Fusarium oxysporum f. sp. asparagi infections on growth of asparagus in a greenhouse experiment at two soil matric potentials.

Soil Moisture	Fresh Wt. (g)		Dry Wt. (g)	
0 MPa:				
-M-F[a]	32.1	c[b]	7.9	bc
-M+F	38.6	abc	8.8	abc
+M-F	44.3	a	10.3	a
+M+F	40.9	ab	9.6	ab
-1.5 MPa:				
-M-F	36.1	bc	8.0	bc
-M+F	37.9	abc	8.0	bc
+M-F	39.5	abc	7.9	bc
+M+F	35.4	bc	6.9	c

[a] M: G. fasciculatum; F: Fusarium oxysporum f. sp. asparagi.

[b] Values within columns followed by the same letter are not significantly different at the 0.05 level (Student-Newman-Keuls mean separation test).

Table 1.2. Effect of Glomus fasciculatum inoculation of asparagus on Fusarium wilt severity and on soil populations of Fusarium oxysporum f. sp. asparagi in asparagus rhizospheres. Plants were grown in the greenhouse at two soil matric potentials.

Soil Moisture	FOA Rating[a]	FOA Populations[b]
0 MPa:		
-M-F[c]	1.0 c[d]	---
-M+F	2.7 a	39.8 a
+M-F	1.0 c	---
+M+F	2.1 b	8.3 b
-1.5 MPa:		
-M-F	1.0 c	---
-M+F	1.6 bc	18.5 b
+M-F	1.0 c	---
+M+F	1.6 bc	17.5 b

[a] Rating system ranges from 1 to 5 and reflects the percentage of the root system affected by lesions or reddening.

[b] Square-root transformations of (number of propagules/g soil + 0.05).

[c] M: Glomus fasciculatum; F: Fusarium oxysporum f. sp. asparagi.

[d] Values within columns followed by the same letter are not significantly different at the 0.05 level (Student-Newman-Keuls mean separation test).

Table 1.3. Effect of Glomus fasciculatum and Fusarium oxysporum f. sp. asparagi infections on mycorrhizal colonization and tissue phosphorus levels of asparagus grown in the greenhouse at two soil matric potentials.

Soil Moisture	VAM Rating [a]	Tissue P (% dry weight)
0 MPa:		
-M-F [b]	0 c [c]	0.2 b
-M+F	0 c	0.2 b
+M-F	1.6 a	0.2 ab
+M+F	0.8 b	0.2 ab
-1.5 MPa:		
-M-F	0 c	0.2 ab
-M+F	0 c	0.2 ab
+M-F	1.6 a	0.3 a
+M+F	1.6 a	0.3 a

[a] Ratings are based on the percent of root segments colonized by G. fasciculatum and range from 0 (no infection) to 4 (over 75% of the root segment infected).

[b] M: G. fasciculatum; F: Fusarium oxysporum f. sp. asparagi.

[c] Values within columns followed by the same letter are not significantly different at the 0.05 level (Student-Newman-Keuls mean separation test).

Discussion

The results of this study indicate that asparagus seedlings respond favorably to mycorrhizal inoculation. The increased growth in the field for the first year and in the greenhouse under adequate soil moisture supports the findings of previous researchers (10, 11, 28, 45, 46). However, FOA infection reduced the growth of mycorrhizal asparagus plants at 0 MPa (Table 1.1). Also, the plants that were infected with the pathogen alone grew as well as those inoculated with G. fasciculatum, or both G. fasciculatum and FOA at 0 MPa. At -1.5 MPa, there were no significant differences between fresh and dry weights of any treatments. Wilcox (62) studied the effect of soil matric potential on growth of asparagus. Her findings indicate that asparagus is a drought tolerant plant, as asparagus is capable of maintaining a high relative water content despite prolonged exposure to low soil matric potentials (-1.5 MPa). Previous studies regarding the increased drought tolerance in mycorrhizal plants involved plants that do not exhibit these characteristics of drought tolerance (2, 8, 41, 50, 51). It is possible that asparagus, because it already possesses xerophytic characteristics, does not respond to mycorrhizal colonization under drought stress. If this is the case, the lack of a growth response in mycorrhizal asparagus at low soil matric potentials is probably not due to an inhibition of VAM infection. The VAM infection ratings at -1.5 MPa were not significantly different than the VAM rating for the 0 MPa mycorrhizal plants (without FOA, Table 1.3).

In the greenhouse, disease severity was the greatest in plants inoculated with FOA at 0 MPa (Table 1.2). However, the percent of the

Figure 1.1. The effect of Glomus fasciculatum and supplementary soil phosphorus on shoot volumes of asparagus plants grown in the field in the presence of Fusarium oxysporum f. sp. asparagi. "-M": without G. fasciculatum; "+M": G. fasciculatum added; "-P": no phosphorus added to the soil (185 ppm); "+P": additional soil phosphorus (350 ppm). From August, 1986 to 14 July, 1987 the mean shoot volumes/plant in mycorrhizal plants were significantly greater than non-mycorrhizal plants ($P = 0.05$). From 7 May, 1987 to 14 July, 1987, mycorrhizal plants without added soil phosphorus had significantly greater shoot volumes than all other treatments ($P = 0.01$).

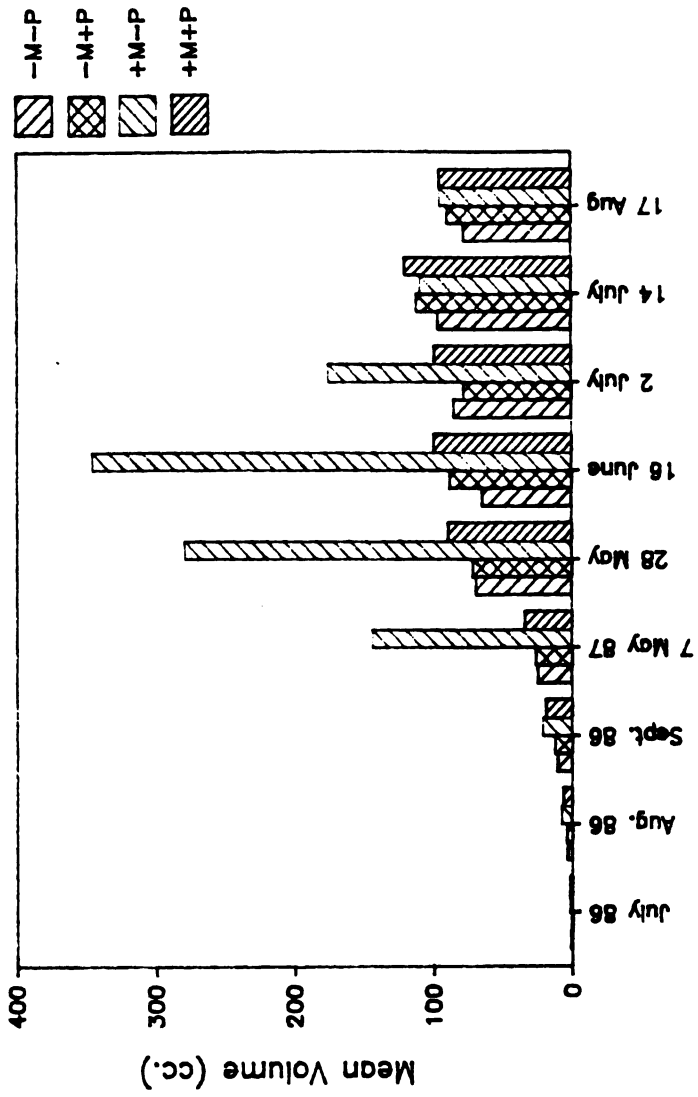

Figure 1. Mean shoot volumes/plant for field experiment.

Figure 1.1. The effect of Glomus fasciculatum and supplementary soil phosphorus on shoot volumes of asparagus plants grown in the field in the presence of Fusarium oxysporum f. sp. asparagi. "-M": without G. fasciculatum; "+M": G. fasciculatum added; "-P": no phosphorus added to the soil (185 ppm); "+P": additional soil phosphorus (350 ppm). From August, 1986 to 14 July, 1987 the mean shoot volumes/plant in mycorrhizal plants were significantly greater than non-mycorrhizal plants ($P = 0.05$). From 7 May, 1987 to 14 July, 1987, mycorrhizal plants without added soil phosphorus had significantly greater shoot volumes than all other treatments ($P = 0.01$).

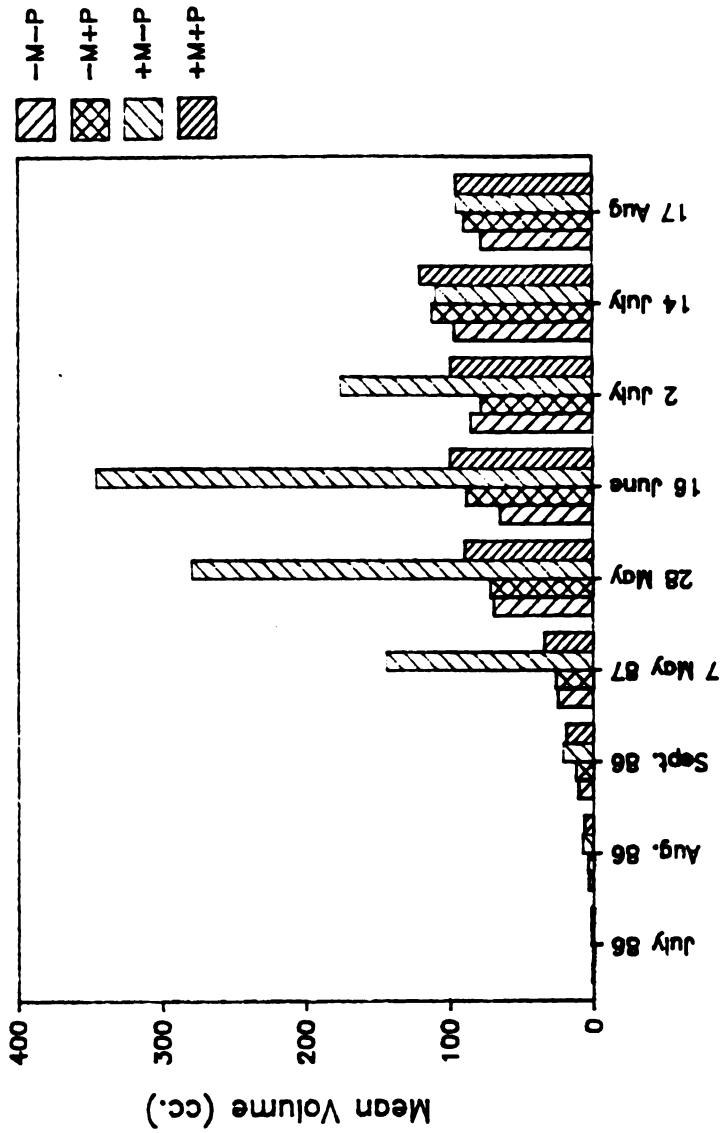

Figure 1. Mean shoot volumes/plant for field experiment.

Table 1.4. Effect of Glomus fasciculatum infection and supplementary soil phosphorus on growth of asparagus grown in the field in the presence of Fusarium oxysporum f. sp. asparagi.

	Fresh Wt. (g)		Dry Wt. (g)	
1986:				
-M-P[a]	443.6	c[b]	---	
-M+P	613.5	bc	---	
+M-P	922.7	a	---	
+M+P	785.2	ab	---	
1987:				
-M-P	833.5	a	302.6	b
-M+P	817.4	a	297.1	b
+M-P	906.7	a	321.5	ab
+M+P	1001.7	a	397.8	a

[a] M: G. fasciculatum; P: supplementary phosphorus.

[b] Values within columns and within the same year that are followed by the same letter are not significantly different at the 0.05 level (LSD mean separation test).

Table 1.5. Effect of Glomus fasciculatum infection and supplementary soil phosphorus on the number of shoots/plant and root:shoot ratios of asparagus grown in the field in the presence of Fusarium oxysporum f. sp. asparagi.

	# shoots/plant		Root:shoot ratio	
1986:				
-M-P[a]	8.7	b[b]	----	
-M+P	10.5	ab	----	
+M-P	12.9	a	----	
+M+P	11.7	ab	----	
1987:				
-M-P	11.1	a	1.23	a
-M+P	11.0	a	1.19	ab
+M-P	11.2	a	0.99	ab
+M+P	8.1	a	0.98	b

[a] M: G. fasciculatum; P: supplementary phosphorus.

[b] Values within columns and within the same year that are followed by the same letter are not significantly different at the 0.05 level (LSD mean separation test).

Table 1.6. Effect of supplementary soil phosphorus and Glomus fasciculatum inoculation of asparagus on Fusarium wilt severity and soil populations of Fusarium oxysporum f. sp. asparagi in asparagus rhizospheres. Plants were grown in the field and FOA was present in all treatment soils.

	FOA Rating[a]		FOA Populations[b]	
1986:				
-M-P[c]	1.9	a[d]	17.5	a
-M+P	2.2	a	18.3	a
+M-P	1.9	a	13.9	a
+M+P	2.1	a	17.2	a
1987:				
-M-P	2.2	a	49.9	ab
-M+P	1.6	ab	39.1	c
+M-P	1.8	ab	52.7	a
+M+P	1.5	b	39.6	bc

[a] Rating system ranges from 1 to 5 and reflects the percentage of the root system affected by lesions or reddening.

[b] (Number of propagules/g soil + 0.5)$^{1/2}$.

[c] M: G. fasciculatum; P: supplementary phosphorus.

[d] Values within columns and within the same year that are followed by the same letter are not significantly different at the 0.05 level (LSD mean separation test).

Table 1.7. Effect of supplementary soil phosphorus on mycorrhizal colonization and tissue phosphorus concentrations of asparagus grown in the field in the presence of *Fusarium oxysporum* f. sp. *asparagi*.

	VAM Rating [a]		Tissue P (% dry weight)	
1986:				
-M-P [b]	1.7	a [c]	0.4	a
-M+P	1.8	a	0.3	a
+M-P	1.6	a	0.4	a
+M+P	1.7	a	0.2	a
1987:				
-M-P	1.0	ab	0.5	a
-M+P	0.8	b	0.3	b
+M-P	1.1	a	0.4	b
+M+P	0.9	b	0.3	b

[a] VAM (mycorrhizal colonization) rating of asparagus root segments. Values range from 0 (no infection) to 4 (over 75% of root segment infected).

[b] M: *G. fasciculatum*; P: supplementary phosphorus.

[c] Values within columns and within the same year that are followed by the same letter are not significantly different at the 0.05 level (LSD mean separation test).

root system affected by FOA infection was significantly lower in plants that were also infected with Glomus fasciculatum at 0 MPa. Disease severity was lower in plants grown at -1.5 MPa, but these were not significantly different than mycorrhizal plants at 0 MPa. These results are not surprising since high soil moisture can facilitate the initial infection of plant roots by soilborne pathogens (12). Once infection is established, then low soil moisture can increase pathogen movement inside the host by causing an increase in transpiration. The number of FOA propagules was affected by mycorrhizal colonization of asparagus at 0 MPa. Reducing the soil matric potential also led to a decrease in the FOA propagules/g soil (Table 1.2). The number of FOA propagules was lower in the rhizospheres of mycorrhizal asparagus at 0 MPa than plants grown at -1.5 MPa, although they were not significantly different. Generally, if the mycorrhizal association reduces disease severity or sporulation by the pathogen, the effect is regarded as positive in terms of the benefit to the host (47). By this definition, the pre-infection of asparagus with G. fasciculatum at high soil moisture leads to a reduction in the severity of Fusarium wilt on both counts (in the greenhouse). However, a reduction in the number of propagules in the soil does not necessarily correlate with a reduction in inoculum potential (there are several other factors involved). Unfortunately, information regarding the relationship between propagule number and inoculum potential of FOA is not currently available.

Colonization of asparagus roots by G. fasciculatum was reduced when FOA was also present at 0 MPa (Table 1.3). The presence of FOA apparently affected the ability of G. fasciculatum to extend into uninfected root tissue (plants were mycorrhizal prior to FOA addition).

This may explain the slightly depressed plant growth exhibited by these plants. The reduction in mycorrhizal colonization of asparagus infected with both fungi may be due to the pathogen causing damage to the root that subsequently cuts off the carbohydrate supply to the mycorrhizal fungus (16). At -1.5 MPa, mycorrhizal colonization of asparagus was not affected by the presence of FOA, but these plants also exhibited less disease severity than the plants grown at 0 MPa and infected with both fungi.

Tissue phosphorus levels of non-mycorrhizal plants at 0 MPa approached a phosphorus deficiency level (0.13 %, Table 1.3) and the levels for mycorrhizal plants at 0 MPa were slightly, but not significantly, higher than the non- VAM plants. At -1.5 MPa, there was no significant difference between VAM and non-VAM asparagus tissue P concentrations. All -1.5 MPa treatment tissue P levels were above deficiency levels. Mycorrhizal plants grown at -1.5 MPa had greater tissue P concentrations than non-mycorrhizal plants. The mycorrhizal plants at -1.5 MPa had significantly greater tissue P concentrations than the non-mycorrhizal plants at 0 MPa. This suggests that the mycorrhizal association may still benefit the host by improved phosphorus uptake under drought conditions.

Inoculation of asparagus transplants with G. fasciculatum significantly increased the growth of these plants compared to non-inoculated asparagus in the field (first season data). This effect was demonstrated in both the fresh weights and mean shoot volume (Table 1.4, Figure 1.1). In 1987, the growth stimulating effect of mycorrhizal inoculation was diminished. This was due, in part, to the colonization of non-inoculated plants by indigenous VAM fungi. These fungi may have

re-entered the research area via wind dispersal or the fumigation may not have eliminated these fungi below the 8 inch sampling depth. The most prominent indigenous VAM fungus identified was G. aggregatum.

From July to harvest (August), there were significant reductions in the shoot volumes of mycorrhizal plants grown without supplementary phosphorus (Figure 1.1). However, prior to this decrease, these plants exhibited significantly greater shoot volumes than all other treatments ($P = 0.01$). This indicates that the growth effect was still operative prior to this decrease. This reduction in shoot volume was compounded by the increased size (specifically height) of these plants. Some of this reduction can be attributed to injury due to cultivation practices, but the decrease in shoot volume is partially due to invasion of the test plots by Fusarium moniliforme. Crown symptoms did not differ between treatments, but asparagus colonized by G. fasciculatum produced larger shoots in 1987, and exhibited more shoot collapse symptoms than plants with smaller shoots.

The average number of shoots/plant in 1986 was significantly greater if soil phosphorus was increased or G. fasciculatum was added (Table 1.5). In 1987, there was no difference between treatments in the number of shoots/plant. However, when considering these data in conjunction with the shoot volume data (Figure 1.1) and the root:shoot ratios (Table 1.5), it appears that G. fasciculatum-inoculated asparagus plants are producing larger shoots (root:shoot ratios < 1.0).

In 1987, disease severity was greater in non-inoculated asparagus planted in low phosphorus soils than all other treatments (Table 1.6). Only those asparagus plants inoculated with G. fasciculatum and subjected to higher soil phosphorus were significantly different than

the control plants (Table 1.6). Plants subjected to high soil phosphorus or G. fasciculatum were intermediate in disease severity. The number of FOA propagules/g soil was also lowest in the high phosphorus soils, and greatest in the G. fasciculatum treatment (Table 1.6). This is contrary to the greenhouse results, where VAM inoculation reduced disease severity and FOA propagule number at 0 MPa and had no effect on FOA infection or sporulation at -1.5 MPa.

Pre-inoculation of asparagus seedlings with G. fasciculatum led to a reduction in disease severity in the greenhouse. The asparagus transplants for the field experiment had also been pre-inoculated, but the infection levels were very low, so inoculum was added to the soil prior to planting. The VAM infection levels may have been low because a greenhouse potting soil was used. It is possible that high phosphorus levels in the potting mix inhibited VAM colonization. If VAM colonization could have been well-developed in the field plants before introducing the pathogen, the same response may have been exhibited as in the greenhouse. In most field situations, pre-inoculation of the host with VAM fungi would be impractical. However, pre-inoculation of crops with VAM fungi could be beneficial if seedlings are raised in a nursery, then transplanted in the field (4). The potential benefit of VAM pre-inoculation is two-fold: a) only small amounts of inoculum would be necessary to produce healthier seedlings (field inoculation would require extremely large amounts of inoculum), and b) mycorrhizal seedlings would probably withstand transplant shock better and may increase host tolerance of pathogens.

Currently, asparagus is either direct-seeded into field nurseries or transplanted from greenhouse-grown seedlings (46). With the

increased emphasis on biotechnology, the use of tissue-culture asparagus as transplants may become a reality in the near future. Mycorrhizal inoculation of these transplants could help to alleviate field stresses on transplanted asparagus.

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CHAPTER II.

ANALYSIS OF SPECIES COMPOSITION OF VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGI IN VARIOUS AGED ASPARAGUS (*ASPARAGUS OFFICINALIS* L.) FIELDS

"Population biology is the study of numbers of organisms, and how differences in time and place can affect the number of organisms present" (19). It also takes into account the effects of the physical environment, including environmental and biological stresses on population levels of organisms. Environmental factors include temperature, light, and edaphic conditions. Biological factors would include interspecific and intraspecific competition, and predation. These factors are directly associated with the selective processes in nature that lead to change within a community.

Field surveys designed to study the populations of vesicular-arbuscular mycorrhizal fungi (VAM) have been conducted with limited success in agricultural systems. There are several factors that hinder studies of this nature. In early studies, researchers concentrated their efforts on host-fungus interactions and the growth benefit afforded to the host by this association. The focus of most VAM population studies was on the amount of host root colonized rather than the number of propagules overwintering in the soil. In some cases, it was presumed that greater colonization was correlated with greater reproductive output by the fungus, but this is frequently not the case (25). In perennial systems where the climate is temperate,

spore production can be extremely low despite high root colonization. Baylis (2) suggested that there is no evolutionary stimulus for spore production in systems where root growth is essentially continuous. Conversely, if spore numbers are low, plants may become infected relatively slowly even if they exhibit a high mycorrhizal dependency (49). Vesicular-arbuscular mycorrhizal fungal inoculum can include mycelium and vesicles, as well as spores. However, the infectivity of hyphae that have overwintered in the soil would be extremely difficult to assess. The potential of vesicles as propagules has been suggested but not confirmed for all species of VAM fungi (18). Therefore, when studying the fitness and succession of individuals within a particular environment, the number of spores produced and their survival into the next growing season are the most reliable measures of population size available at this time.

Number of spores in a site can vary due to location, host plant, and seasonal changes. Mosse et al. (41) found extreme variability between samples of VAM fungal spores in a single site, which was attributed to an uneven distribution of spores within the site. Spores can become aggregated in soil due to favorable microclimates, and the suitability of symbionts (addressed later). Seasonal changes also affect spore populations. Sutton and Barron (53) observed that spore populations increased into late summer, and declined into the following spring. Population decline could be attributed to spontaneous germination and subsequent death, ingestion by soil fauna, destruction by parasites, or by stimulation of germination in the presence of a living host. Senescence of the host in late season would lead to a loss of substrate for the fungus. Since fungal spore germination had been

stimulated by the host, which is no longer an effective symbiont, the fungus would have exhausted its endogenous nutrient reservoir and death would be eminent. Mosse (39) suggested that spore numbers reflect the nutritional status of the host and/or the soil, the onset of adverse conditions. In general, factors which result in maximum plant growth result in maximum sporulation by the fungus (27). However, Koske (33) pointed out that conditions that are favorable for spore production by one VAM spp. may not be favorable for another species. In agricultural soils VAM populations can vary considerably due to cultivation practices (24).

Most obligate biotrophic fungi are host specific. Mycorrhizal fungi are the exception, and are capable of colonizing a wide range of host plants. There is no known case of host specificity in any vesicular-arbuscular mycorrhizal (VAM) association (18), although in nature, there is an apparent selectivity between certain hosts and fungal symbionts (24). Mosse (40) observed variations in mycorrhizal colonization of apple, leek, oats, and strawberry. The extent of colonization by VAM species was dependent on the host. Kruckelmann (35) monitored the effect of six crops grown in monoculture for 16 years on the frequency of VAM fungi in arable soils. His results suggest that the autobiont can affect sporulation and possibly the survival of VAM fungi over time. Schenck and Kinloch (50) monitored a newly cleared woodland site, after planting with six agronomic crops, for 7 years. They observed that Gigaspora spp. were more numerous in soybean monocultures, whereas Glomus and Acaulospora spp. predominated in the rhizospheres of monocotyledonous crops. Sanders et al. (49) noted differences in VAM spp. growth-promoting properties on the same host.

Several factors could account for the variation in VAM effectiveness in different hosts. For instance, the extent and distribution of active mycelium in the soil would affect the rate of colonization by the mycobiont and subsequent uptake of nutrients (40). There could be physiological differences between VAM species that would affect the rate of nutrient uptake, as well as translocation and release of these nutrients. Differences in the ability of VAM fungi to utilize plant metabolites may be directly related to the longevity of hyphae and arbuscules within the host root. All of these factors could be important in the succession of VAM fungi in agricultural systems, especially perennial systems and crops grown in monoculture for several years.

The interaction of VAM fungal species with the soil is also important when considering the effectiveness of the fungus (25, 40). Hayman (25) stated that the suitability of the soil is more important to the fungal colonization of roots than specificity between symbionts. She found that Acaulospora laevis and Glomus fasciculatum grew well in acid soils, while G. mosseae was better suited to more alkaline soil conditions. She also noted that if soil pH was very high, all VAM activity could be inhibited. Green et al. (15) observed that G. mosseae germinated better at neutral pH (7.0), while Gigaspora heterogama and G. coralloidea germinated best under acid conditions in vitro. They suggested that temperature and pH are major factors controlling the distribution of VAM spp. in natural soils. However, due to the ubiquity of VAM fungi in soils, generalizations regarding the effect of soil factors on various species should be approached cautiously. Individuals within the same species may respond differently to temperature, pH, etc.

depending on the type of soil the organisms are native to.

Other than the studies by Schenck and Kinloch (50) and Kruckelmann (35), most research on VAM populations has been concerned with mixtures of species at a fixed point in time (or within one growing season). As population biology is concerned not only with species interactions, but with the responses of organisms to these interactions over time (19), the sequence of these events can reveal how interference by neighbors (and hosts, in the case of VAM fungi) can affect species survival in a particular environment.

Other variables can also influence the population dynamics of VAM fungi. The host plant can affect root colonization, sporulation and survivability of the fungus. Soil type can alter the species composition of VAM fungi, and there may be an interactive effect between soil type and host species. Microbial antagonism may play an important role in VAM succession within the rhizosphere of the autobiont. Changes in host root exudation can alter the microbial composition of the rhizosphere and subsequently affect the species composition of VAM fungi also (4).

Exudation from roots can be affected by a variety of factors such as soil moisture stress, temperature, light intensity, and mineral nutrition (17). Compounds exuded are involved in the ecological succession of microorganisms and in interactions with other plants. The spacial distribution of individuals within a community has traditionally been attributed to competition between organisms, but there is growing evidence that chemical inhibition or stimulation (allelopathy) may play a major role in the spatial distribution and succession of organisms, especially in the rhizosphere community (46, 47).

Dighton *et al.* (12) conducted a survey of the species of ectomycorrhizal fungi associated with *Betula* spp. and found a predictable succession of these fungi over time. They suggested that early colonizers have a low energy requirement, a greater capacity for extracting soil nutrients, and a high competitive ability. Late colonizers would require greater amounts of energy from the host and exhibit a slower growth rate (e.g. less competitive) than early colonizers. However, the late colonizers should benefit the host by their ability to take up nutrients from a diminished nutrient pool. St. John and Coleman (51) stated that in perennial systems, seedlings probably exhibit a greater mycorrhizal dependency than mature plants. This is because seedlings have a relatively smaller proportion of storage roots (compared to fibrous roots) than mature perennials, and may have less extensive root systems in relation to total living biomass. If mature perennials do not require mycorrhizal associations to the extent that seedlings do, then are the late colonizing species of mycorrhizal fungi still maintaining a mutualistic association with the host plant? Does the cost of a potentially higher energy drain on the host outweigh the benefit of the fungus' ability to improve uptake from a diminished nutrient pool? These questions may be important when considering perennial crops that suffer from decline problems due to root diseases. If late colonizing species of mycorrhizal fungi are eliciting an increased carbohydrate drain from the host, then this could provide sufficient stress to predispose the plant to pathogen invasion.

Asparagus is a perennial crop that suffers from decline problems (1, 16, 20, 30, 38). This decline has been attributed to invasion by *Fusarium moniliforme* and *Fusarium oxysporum* f. sp. *asparagi*. Several

factors have been implicated as stress-causing agents that predispose the crop to these pathogens. These include soil moisture stress, soil compaction, pesticides and cultivation practices, and the exudation of allelochemicals that are autotoxic (20, 21, 22, 30, 36, 38, 54, 55, 56, 59, 60, 61). Vesicular-arbuscular mycorrhizal fungi have been shown to increase growth and improve phosphorus uptake in young asparagus plants (7, 8, 13, 31, 44, 45, 52). VAM fungi isolated from established asparagus stands in Ontario, Canada, however, were shown to depress growth in young asparagus plants (31). Unfortunately, the VAM fungal species were not identified and it is unclear whether the inoculum was added as soil or the spores were isolated prior to addition to the medium used for this study. If the inoculum was added as soil obtained directly from the field, it could contain pathogens that would debilitate the growth of asparagus seedlings, and it would be difficult to attribute the depressed growth solely to the colonization of young asparagus roots by the VAM fungi found in these fields.

The onset of asparagus decline has affected the productivity and longevity of asparagus in these fields. Asparagus has the potential to remain productive for 20 years or more (1). It is highly probable in a perennial system such as this, that the exudation from roots of the crop has a pronounced effect on the composition of rhizosphere microflora. To my knowledge, there has been only one attempt to assess the possible succession of VAM fungi in perennial crops (Schenck, unpublished data). He monitored the composition of VAM fungi in coffee plantations for four years and found that the composition shifted from a mixed community to Acaulospora spp. The elucidation of successional changes of these fungi is essential before the importance of the previously mentioned factors

(selectivity, allelopathy, antagonism, etc.) can be assessed. With this in mind, I surveyed various asparagus fields of different ages (as well as non-asparagus soils) for VAM species composition. Ideally, to study succession of species, it would be desirable to monitor the same fields over time. However, as a preliminary study to determine whether succession may be occurring, this would obviously be impractical. My survey was done at the end of the growing season (1987). The soil type was the same for all fields tested (sandy loam), and several of the fields belonged to the same farmer (3, 5, 10, 15, and 18 years), indicating that cultivation practices would be consistent. Since all samples were taken at the same point in time, then the VAM fungi would have been exposed to the same climatic changes during the season. By minimizing the variability of these factors and by analyzing all data by non-parametric statistics, any significant differences should reflect true successional stages of VAM fungi in the asparagus agroecosystem.

Materials and Methods

Samples of rhizosphere soil were obtained from asparagus fields ranging in age from 1 to 18 years (5 core samples per age category, 6-12 inch sampling depth). Single core samples were also taken from non-cultivated areas bordering asparagus fields (1, 8, and 15 year fields, 0-8 inch sampling depth) and two orchard (cherry and peach) sites in Oceana county, Michigan. The herbaceous vegetation in the orchard sites was representative of species occurring in late old field succession in western Michigan. This included *Plantago* spp., *Trifolium repens* L., *Trifolium pratense* L., *Hieracium pratense* Tausch., *Vicia cracca* L., and *Cerastium arvense* L., among others. The cherry orchard was an

established orchard in production and the peach orchard had been abandoned for several years. The orchard and "border" sites served as the control ("0 year"). These results were averaged and were analyzed as one treatment to account for possible variation due to location and vegetation.

Five soil samples (5 gram each) per treatment were wet sieved and spores of vesicular-arbuscular mycorrhizal fungi (VAM) were separated from the soil by a modified centrifugation-flotation technique (41), which involved forming a Ficoll (Sigma Chemical Co., St. Louis, MO.) gradient (60, 50, 45, 30, and 15% densities were used). The gradient is useful because organic debris can be separated from the VAM spores. Prior to centrifugation, spores migrate to the upper portion of the tube (lower density), and organic matter remains in the higher density Ficoll solution. Centrifugation forces most organic matter into the pellet. After centrifugation, the VAM spores were in the supernatant. Spores were isolated from this solution using a 38 um cup sieve. Fungal spores were subjected to several washings with distilled water to remove any Ficoll residue and rinsed into a petri dish for initial spore counts.

Organic debris was removed by hand using a Pasteur pipette under a dissecting microscope. Spores were removed from the petri dish with a hemacytometer, which minimized the amount of water that was transferred. Spores were then mounted on a glass slide coated with Andre and Hoyer's medium (10). Spores were arranged on the medium with a needle under a dissecting microscope so that hyphal attachments were visible and then permanently mounted. VAM fungal species were identified under light microscopy using a computerized key developed by R. Hetrick (29) which is based on Trappe's "Synoptic Key to the Endogonaceae" (58).

Root samples were also obtained from the various aged asparagus fields. Roots were removed at random and included both storage and feeder roots. The roots were stained with 0.1% acid fuchsin in lactophenol (42) and 1 cm segments were placed on slides for observation under the light microscope. There were 20 feeder root and 5 storage root samples per field. The stained root segments were rated for VAM infection using the following scheme (per 1 cm segment): 0 = no infection, 1 = 1 to 25%, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = over 75% infection. The infection ratings were analyzed by the Kruskal-Wallis one-way ANOVA and means were separated by the Bonferroni pairwise comparison procedure.

After identifying VAM species from all samples, data pertaining to the possible succession of VAM fungi in asparagus were analyzed. Relative densities and frequencies were calculated for each species. Relative density is a measure of species density at one successional stage compared to the density of that species over all successional stages (5). Conversely, frequency is a comparison of one species to all other species represented at the same successional stage. Frequencies of species in relation to the age of the field and the presence of other species were analyzed by Spearman's rank-order correlation.

The diversity and dominance values of each field site were also compared. After frequencies were calculated, they were used to determine the Shannon-Weaver Index for each site. The index, H', is a measure of diversity which ranges from 0 to 1; values close to 1 represent greater diversity than values nearer 0. 1 - J' dominance was expressed by calculating H_{max}', an estimate of maximum species diversity at one stage; 1 - J' is equal to 1 - H'/H_{max}'. Again, values range

from 0 to 1, low values correspond to low dominance and high values to high dominance. Shannon-Weaver diversity indices and dominance values were compared using the Kruskal-Wallis one-way ANOVA.

Since diversity indices are based on species frequencies and don't take differences in species make-up into account, the similarity in species composition between the various aged fields was analyzed. Sørensen's coefficients were calculated using the 0 year treatment data and data from each asparagus field. Coefficients of similarity were also computed for comparison of each stage with the successive stage (i.e. 0 year and 1 year fields, 1 year and 3 year fields, etc.). Differences in Sørensen's coefficients of similarity were analyzed by Spearman's rank-order correlation.

Soil samples from the various sites were sent to the Soil Testing Laboratory, Michigan State University, for analysis of macro- and micronutrients and soil pH. This was done to determine if differences in soil nutrient levels or soil acidity could aid in interpretation of changes in species composition over time. The data were compared using Spearman's rank-order correlation.

Results

Eight different species of VAM fungi were isolated. These have been tentatively identified as Acaulospora trappei Ames & Lind., Glomus aggregatum Schenck & Smith, Glomus fasciculatum (Thaxt. sensu Gerd.) Gerd. & Trappe, Glomus intraradices Schenck & Smith, Glomus mosseae (Nic. & Gerd.) Gerd. & Trappe, Scuttelospora calospora (Nicol. & Gerd.) Gerd. & Trappe, Gigaspora gigantea (Nicol. & Gerd.) Gerd. & Trappe, and an unidentified Gigaspora species, designated "G2".

Root colonization in the 3 year field differed significantly (P = 0.07) from that in the 8 or 10 year fields (Figure 2.1). There was an apparent increase in root colonization until the 8^{th} year; after the 10^{th} year, there was a steady decline in VAM root infection (Spearman's rank-order correlation, r_{sp} = 0.2174, r_{sp} = - 0.2418, respectively; critical $value_{0.05}$ = +/- 0.1853). Total numbers of spores were greatest in the 8 year field, but this was not significantly different from the other values (Figure 2.1). There was no correlation between field age and total number of spores from 0 to 8 years, but there was a significant negative correlation from the 8 to the 18 year field (r_{sp} = -0.9383, critical $value_{0.05}$ = +/- 0.8221).

Shannon-Weaver indices for diversity and dominance values were not significantly different (Table 2.1, P = 0.45 for both expressions). However, the Sørensen's coefficients of similarity for the comparison of the 0 year to all other stages suggest that similarity in VAM species composition decreases with increasing field age (Table 2.2, r_{sp} = -0.9077, critical $value_{0.05}$ = +/- 0.6266). Comparisons of each field with the preceding stage for similarity were not significant (r_{sp} = 0.3410, $P > 0.05$).

Frequencies of the eight species in each age class are shown in Figure 2.2. Several of these frequencies were correlated with each other (Table 2.3): G2, *G. gigantea*, and *G. fasciculatum* were positively correlated with field age (as well as with each other). All other species were inversely correlated with increasing field age, but only *G. mosseae* showed significant correlations. *G. aggregatum* and *G. mosseae* showed significant inverse correlations with G2. Similar correlations were observed between *G. mosseae* and *G. gigantea*, *G. aggregatum* and *A.*

Figure 2.1. VAM root ratings and number of spores per gram of soil versus asparagus field age.

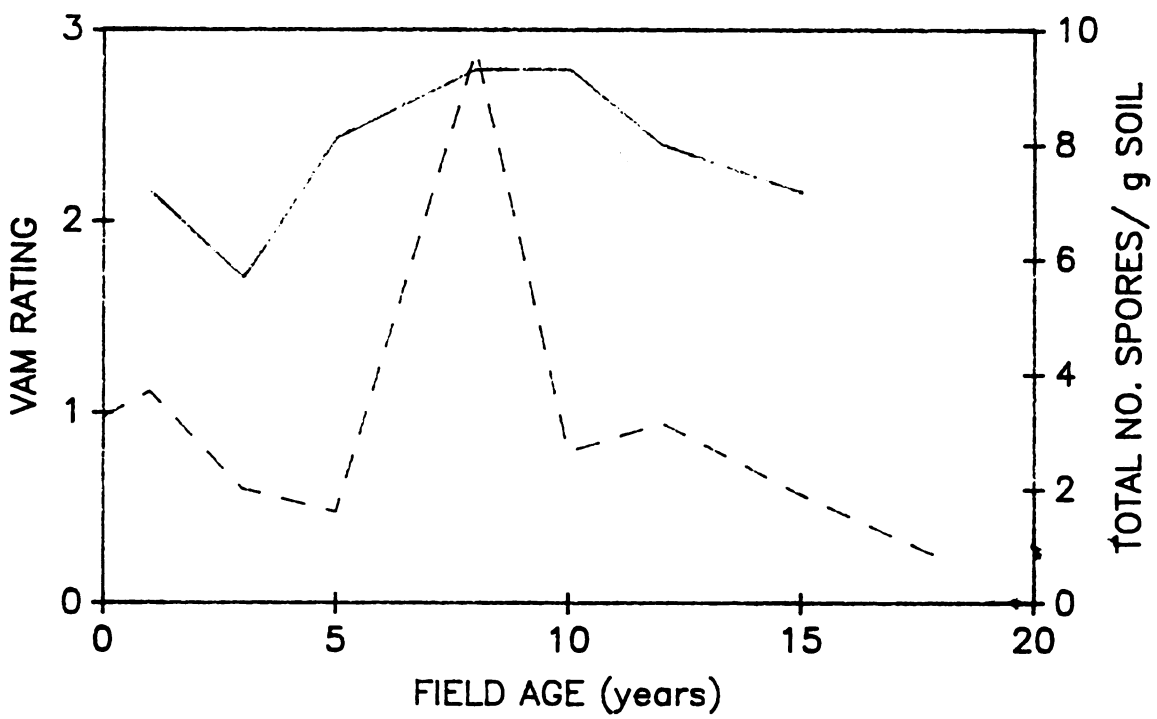

Table 2.1. Shannon-Weaver indices and dominance values for various aged asparagus fields. H' values close to 1 represent high diversity, 1 - J' values close to 1 represent high dominance.

Table 2.1. Shannon-Weaver indices and dominance values for various aged asparagus fields.

Field Age	H'	1 - J'
0	0.62	0.11
1	0.46	0.34
3	0.55	0.29
5	0.54	0.10
8	0.54	0.23
10	0.46	0.34
12	0.51	0.28
15	0.56	0.20
18	0.62	0.11
	N.S.	N.S.

(Kruskal-Wallis rank test)

Table 2.2. Sørensen's coefficient of similarity (CC_S) for various aged asparagus fields. Each asparagus field was compared to the control (0 year) or the different aged fields were compared to the stage previous to them. The data were analyzed by Spearman's rank-order correlation.

Table 2.2. Sørensen's coefficient of similarity (CC_S) for various aged asparagus fields.

Comparison years	CC_S	Comparison years	CC_S
0/1	0.80	0/1	0.80
0/3	0.80	1/3	1.00
0/5	0.67	3/5	0.67
0/8	0.80	5/8	0.67
0/10	0.60	8/10	0.80
0/12	0.60	10/12	1.00
0/15	0.40	12/15	0.80
0/18	0.40	15/18	1.00
r_{sp}= -0.9077, P < 0.05		r_{sp}= 0.3410, P > 0.05	

Figure 2.2. Species frequencies of six VAM fungi in various aged asparagus fields. S. calospora and G. fasciculatum are not included because of extremely low frequencies.

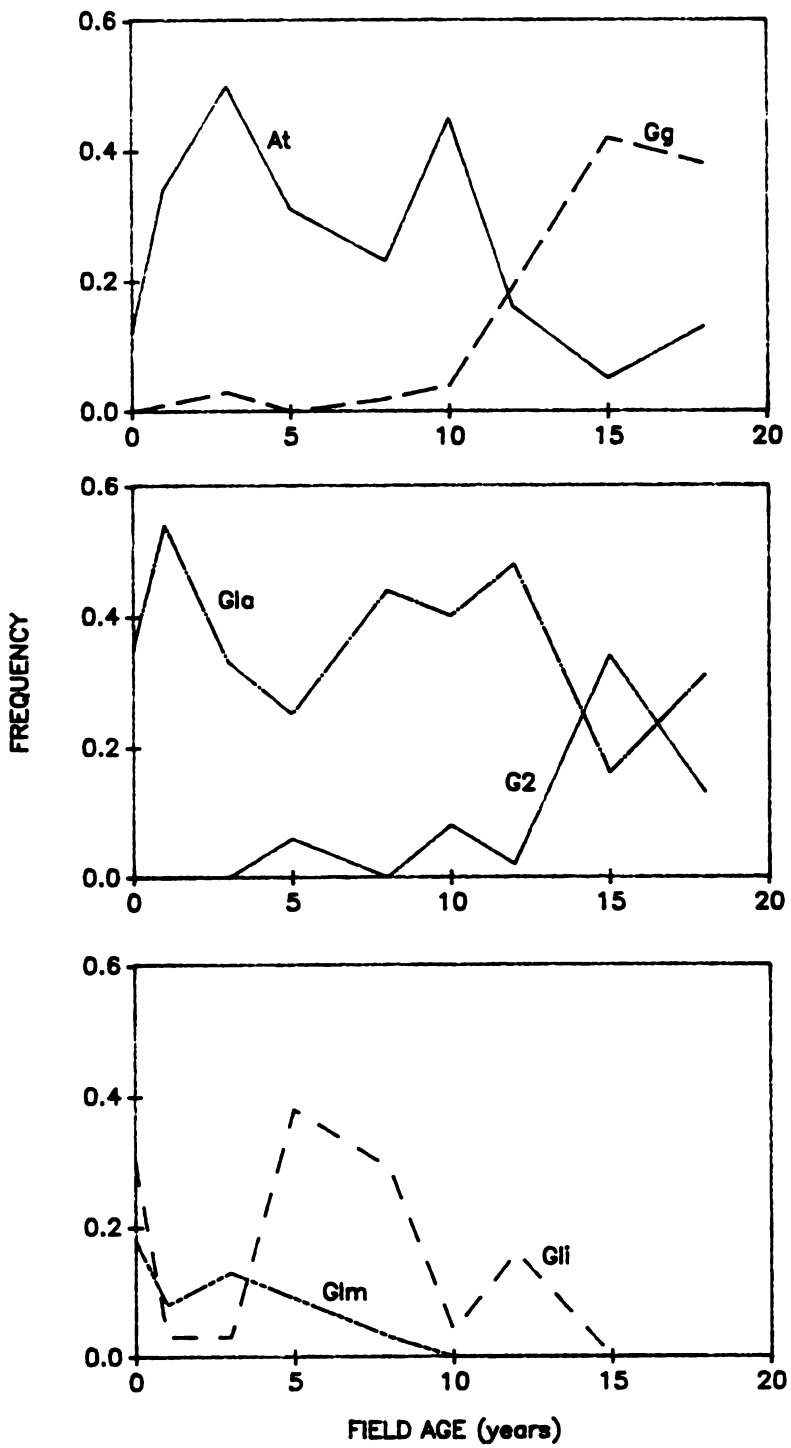

Table 2.3. Spearman's rank-order correlation matrix for field age and VAM species in asparagus fields.

	Age	G2[a]	At	Gg	Gla	Glf	Gli	Glm	Sc
Age	1.0000[b]								
G2	.8269	1.0000							
At	-.3667	-.3917	1.0000						
Gg	.8787	.6949	-.3013	1.0000					
Gla	-.3333	-.6180	.3500	-.2427	1.0000				
Glf	.7303	.7389	-.5934	.7104	-.5934	1.0000			
Gli	-.5210	-.4476	.1008	-.7764	.1933	-.7250	1.0000		
Glm	-.8398	-.8581	.1826	-.5867	.3104	-.4500	.1933	1.0000	
Sc	-.5477	-.3576	-.4108	-.4813	.0000	-.1875	.4143	.6000	1.0000

[a] G2: Gigaspora spp. (red-brown spore); At: Acaulospora trappei; Gg: Gigaspora gigantea; Gla: Glomus aggregatum; Glf: Glomus fasciculatum; Gli: Glomus intraradices; Glm: Glomus mosseae; Sc: Scutellospora calospora.

[b] Critical value for $r_{sp(0.05)}$ = + or - 0.5861 (one-tail).

Figure 2.3. Relative densities of six VAM fungi in various aged asparagus fields. S. calospora and G. fasciculatum are not included due to low relative densities. Relative density measures an individual species' density at one stage (site) in relation to that species' density at all stages.

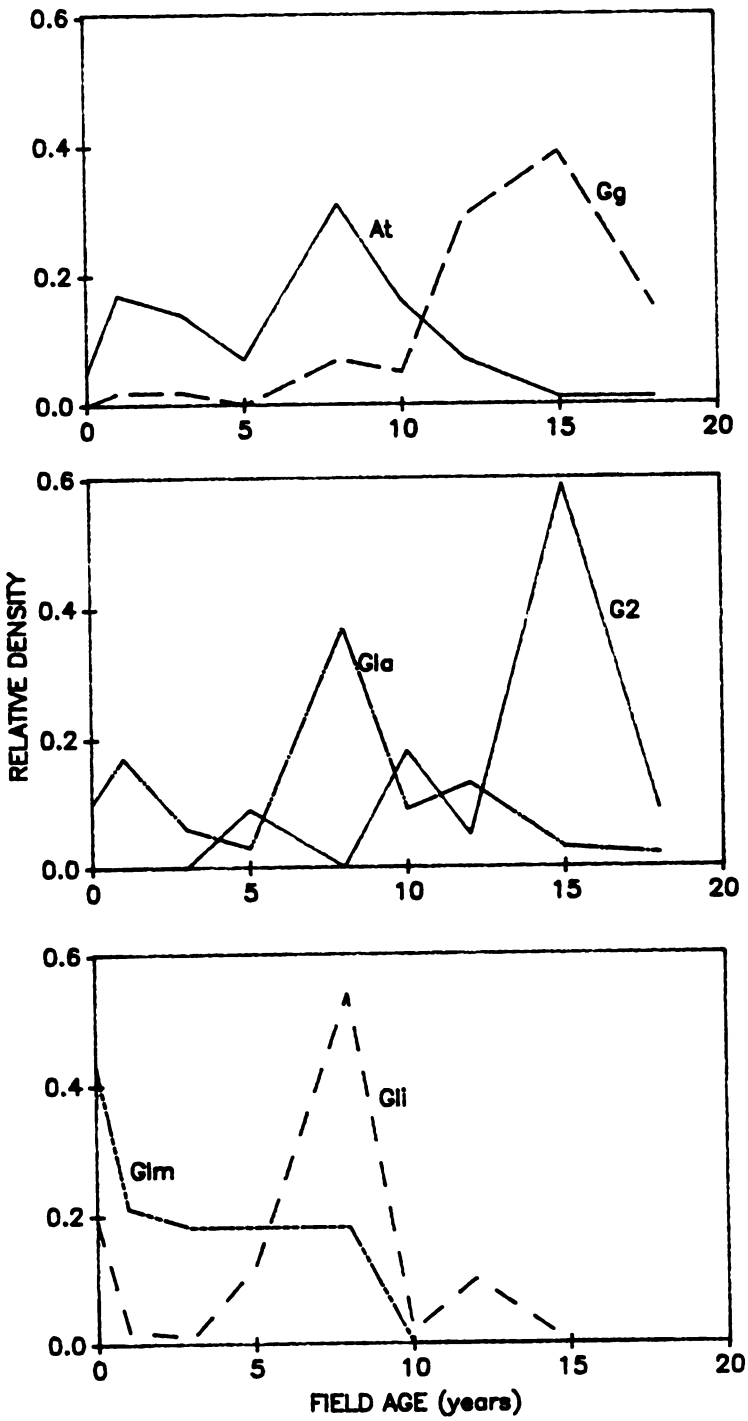

Table 2.4. Soil nutrient analysis for various aged asparagus fields. Values represent parts per million.

Field Age	P [a]	K	Ca	Mg	Zn	Mn	pH [b]
Control soils	100	36	283	153	0.5	2	5.0
1 year	186	378	517	110	2	16	5.0
3 years	87	226	470	99	2	5	5.5
5 years	62	174	235	32	1	2	5.0
8 years	75	188	329	58	1	7	5.2
10 years	60	216	900	80	1	1	6.8
12 years	51	221	423	84	1	10	5.3
15 years	75	142	329	110	2	6	5.9
18 years	40	266	376	106	1	2	6.0

[a] $r_{sp} = -0.8285$, $P < 0.05$, Spearman's rank-order correlation.

[b] $r_{sp} = 0.7459$, $P < 0.05$, Spearman's rank-order correlation.

trappei with G. fasciculatum, and G. intraradices with both G. gigantea and G. fasciculatum.

Changes in relative species density are useful in examining successional changes within a community (5). The relative densities of A. trappei, G. gigantea, G. aggregatum, G2, G. mosseae, and G. intraradices are shown at each successional stage in Figure 2.3. G. mosseae was highest in the non-asparagus soils, but was not evident after 8 years of asparagus cultivation. A. trappei, G. aggregatum, and G. intraradices increased in abundance and attained peak productivity at 8 years. G. gigantea and G2 slowly increase through the 10^{th} year, reaching highest abundance at 15 years. However both species were greatly reduced by the 18^{th} year.

There were no correlations of field age with potassium, calcium, magnesium, manganese, or zinc concentration in soil (Table 2.4). Extractable phosphorus declined significantly with increasing field age from 100 ppm P_2O_5 to 40 ppm (r_{sp} = -0.8285, critical $value_{0.05}$(2-tail) = +/-0.6642), whereas other elements remained constant or were variable. The soil pH of the various fields increased significantly with age from approximately 5.0 to 6.0 (r_{sp} = 0.7459, critical value same as above).

Discussion

The results of this survey suggest that succession of VAM fungi may be occurring in asparagus fields. The frequency of G. mosseae was highest in the control sample and spore numbers decreased until there were none evident after 8 years (Figure 2.2). Spores of Acaulospora trappei, G. aggregatum, and G. intraradices were comparatively high in number and did not begin to decline until 15 years. These VAM species,

due to their ubiquity in all soils surveyed, should be tested for their importance in asparagus mycorrhizae. Populations of G. gigantea, G2, and G. fasciculatum increased with time, although G. fasciculatum populations remained low. Future studies regarding the effect of these late colonizers on asparagus decline should concentrate on G. gigantea and G2.

The diversity and dominance values for the fields surveyed were not significantly different (Table 2.1). These data, when viewed with the the relative densities (Figure 2.3), frequency correlations (Figure 2.2, Table 2.3), and coefficients of similarity (Table 2.2), provide further support to the idea that shifts in species composition occur with increasing field age. Since the diversity and dominance values did not differ significantly with age, the number of individuals per species at any given stage are fairly constant. However, comparisons of relative densities, species frequencies and coefficients of similarity show that, even though site (age) diversities are not different, there is a tendency toward change in the species that are colonizing those sites over time. Sørensen's coefficient of similarity values suggest that these changes are gradual: when various aged fields are compared to the control, there is evidence of decreasing similarity between sites, but when sites were compared with fields that precede them in age, there was no apparent difference in species composition ($P < 0.05$, $P > 0.05$, respectively).

Changes in species composition of VAM fungi in asparagus could be due to several factors, including soil factors and host selectivity. Hetrick and Bloom (28) compared the VAM fungal species composition of tall grass prairie soil to that of adjacent wheat fields. They found

that all species in the wheat fields were present in the prairie soils, but the prairie soils contained species that were absent from the wheat fields. This would suggest that wheat could affect the survivability of certain VAM fungi that are indigenous to the prairie soils. They also noted that the total spore numbers in the prairie soils were much greater than those in the wheat monoculture. They attributed this decrease to late season colonization of wheat which may not provide enough time for spore production to occur. Liberta and Anderson (37) found no significant differences in spore abundance in corn monoculture and adjacent prairie soils in Illinois. This suggests that corn does not exhibit selective pressures on VAM colonization (at least on those species of VAM fungi that are native to Illinois prairie soils). However, phosphorus levels of the agricultural soils versus the prairie soils may play a very important role in VAM species composition in both wheat and corn. Both crops are subject to heavy fertilization, and any differences between the prairie soils and the crop soils may be due to changes in soil factors, such as phosphorus addition. Also, the comparison of prairie versus wheat hosts is a comparison of dominance by C_4 versus C_3 plants (VAM composition differed), whereas the prairie/corn comparison is of two systems dominated by closely related C_4 taxa (no significant difference in VAM composition).

Koske (33) found differences in the VAM species composition of sand dune sites. He concluded that the presence of any species of VAM fungi does not reduce or increase the frequency of occurrence of other VAM fungi in the rhizosphere of dune plants. He suggested that host species and soil conditions were major determinants in VAM species composition. However, his study did not rule out the possibility that

stimulation/inhibition of one VAM species by another was occurring, but stressed that the environment was of greater importance to species occurrence.

The soil nutrient analyses from the various aged asparagus fields showed that there was a significant decrease in phosphorus levels with increasing field age (Table 2.4). Also, the soil pH was positively correlated with increasing field age. If soil factors are affecting the sporulation and/or survivability of VAM fungi in asparagus, then the potential of phosphorus and soil pH as determinants in VAM species composition should be addressed. It should be noted, however, that high soil phosphorus has been shown to inhibit VAM fungi in several crops (18). This inhibition has also been shown to occur in asparagus in the field (Chapter I). Since phosphorus levels were inversely correlated with increasing field age, then the expected outcome would be higher colonization and increased spore production as soil phosphorus diminished. This tendency is visible from the 1 to 8 year time frame. Total root colonization significantly increased until 8 years, whereas spore numbers were not significantly different (Figure 2.1). However, from 8 to 18 years, there was a significant reduction in both root colonization and total spore numbers. The period from 8 to 18 years was also when Gigaspora gigantea, G2, and Glomus fasciculatum were most evident (Figures 2.2, 2.3). This suggests that the early colonizing species may be better suited to high soil phosphorus or that some other factor is instrumental in the decline of early colonizers (Acaulospora and Glomus spp.) and the appearance of Gigaspora spp. and G. fasciculatum.

It is difficult to implicate host selectivity as a major factor in

the change of VAM species composition in asparagus fields. If host selectivity were operative in this situation, then the initial colonizers would be expected to decrease in number more rapidly than my results suggest. However, asparagus is a perennial crop and the needs of mature host plants may be quite different from those of seedlings (51). The increase in spore numbers of late colonizing species may be due initially to seasonal variation in VAM fungal activity. Mature asparagus has well developed storage roots, and relies on these reserves for early season growth. There is evidence that mature asparagus does not begin to take up phosphorus until mid-season (6). The timing of VAM colonization with nutrient uptake could be critical to the overall well-being of the host asparagus plant. VAM fungal species that are capable of colonizing asparagus roots early would provide little increased phosphorus uptake by the host and may, in fact, disrupt host metabolism by imposing a considerable carbohydrate drain on the host. Fungi that colonize later in the season would be more beneficial if colonization coincided with (or followed) the exhaustion of reserves by the host plant. Also, the nutritional requirements of the VAM fungi found in asparagus fields may be quite different. Colonization by early versus late species may be due to a superior ability of late colonizers to metabolize compounds exuded by mature asparagus plants.

Asparagus suffers from replant as well as decline problems (20, 21, 36). Soil fumigation partially alleviates the replant problem by the elimination of Fusarium pathogens. However, seedlings planted in fumigated soils are stunted and lacking in vigor. It has been postulated that allelopathic compounds that persist in the soil may be involved and there have been several reports of autotoxicity in

asparagus (20, 21, 22, 59, 60, 61). The stunting of asparagus seedlings planted into fumigated soils has also been attributed to the destruction of VAM inoculum by fumigation (13, 44, 52). Young asparagus plants have been shown to respond favorably to VAM inoculation when planted into fumigated soils (44, Chapter I). However, the mycorrhizal dependency of seedlings may be different than that of mature plants (51), and late successional species of mycorrhizal fungi may have a higher energy requirement than early colonizers (12). If this is the case, then replanting asparagus seedlings into older fields containing the late colonizing species of VAM fungi could result in a situation where the VAM fungal drain on host carbohydrate or disruption of host metabolism outweighs the benefits to the young plant. This imbalance between benefit and carbohydrate drain has been shown to occur in other mycorrhizal crops (23).

Several studies indicate that allelochemicals can inhibit root colonization and the survivability of both VAM and ectomycorrhizal fungi (3, 9, 11, 14, 32, 34, 43, 46, 57). If asparagus seedlings benefit from VAM associations with fungi that are not useful (and possibly deleterious) to established asparagus plants, could the exudation of allelochemicals by mature asparagus plants be an effective deterent to root colonization by these fungi? Inhibition of early VAM species could be direct or mediated by an enhancement of rhizosphere microflora that are antagonistic to these VAM fungal species.

There is some evidence that Gigaspora species may be more resistant to inhibitory compounds than other VAM fungal species. In a study to determine the lack of herbaceous understory plants in Ponderosa pine plantations, Kovacic et al. (34) found that allelochemicals produced by

Ponderosa pine were inhibitory to several VAM fungal species. The only VAM fungus found to coexist with the pines was an unidentified Gigaspora species. St. John and Coleman (51) observed that several soil organisms can suppress VAM fungi in field soils. Suppression may affect both root colonization and spore production, and may also affect the extramatrical phase of the fungus. They suspected actinomycetes as the primary antagonists responsible for this suppression. Koske (33) tested the effects of antibiotics produced by actinomycetes on VAM fungi and found no inhibition of spore germination of a Gigaspora species.

Gigaspora species may also be less susceptible to attack by soil microflora. Ross and Ruttencutter (48) observed the effects of mixtures of Glomus macrocarpum and Gigaspora gigantea in association with soybean and peanut monocultures. G. macrocarpum spore production was reduced in plots that also contained G. gigantea, but root colonization was greater when both species were present. Spore production by G. macrocarpum decreased in the second year of monoculture, regardless of the presence of G. gigantea. They attributed the reduction in spore production by G. macrocarpum to hyperparasites. Hyperparasites were found only in association with G. macrocarpum in the field, but infection of G. gigantea could occur in culture. They suggest that hyperparasites may affect both the population and function of some VAM fungi in the field.

In conclusion, there are several hypotheses relating to the succession of VAM fungi in asparagus that need to be tested before their role, if any, in asparagus decline can be elucidated. Also, the potential superiority of Gigaspora species in their ability to function in the presence of toxic compounds and harmful microflora warrants

further investigation. However, prior to investigations of this nature, there is a need to expand upon the analysis of successional species of VAM fungi in asparagus. This would involve increasing the number of fields sampled in each successional stage, to diminish the likelihood that the changes observed in this study were due to aggregations of spores in microsites. The use of transect sampling would be preferable to the random sampling method used in this study. It would be of interest to sample both the rhizosphere and non-rhizosphere soils in asparagus fields and determine the species and their frequencies in these sites. Single-spore inoculum culture of the various species found in the soils tested should be attempted and maintained for positive identification of VAM fungal species and for further testing of their effectiveness in asparagus mycorrhizae.

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CHAPTER III.

EFFECT OF FERULIC ACID ON GLOMUS FASCICULATUM AND ASSOCIATED EFFECTS ON GROWTH OF ASPARAGUS

The effect of plant root exudates on soil microorganisms has been well documented (19, 45, 46, 47, 48). In most cases, the researchers have been concerned with changes in exudation of amino acids and sugars, and how these changes can influence the composition and succession of rhizosphere microflora. Another aspect concerns the effects of allelochemicals on rhizosphere microflora. In most studies regarding allelopathic effects, the main topic of interest is with plant-plant interactions (41, 43, 44). However, in recent years, there has been a growing interest in the potential of allelochemicals to stimulate or inhibit soil microorganisms and the subsequent effect this could have on the plant's well-being (43, 44).

Vesicular-arbuscular mycorrhizal fungi (VAM) are common in most plant rhizospheres (20, 30, 34). VAM colonization of plants is inhibited by increases in phosphorus concentration in the roots (1, 18, 25, 26, 31, 42). Increased P concentration in plant roots leads to a decrease in exudation, which is probably due to a reduction in membrane permeability (42).

There have been few studies regarding the effect of allelochemicals on VAM fungi. Initial investigations of possible allelopathic effects on VAM dealt with the suppression of VAM infection and spore production in

host crops when they were grown in rotation with a non-host cruciferous crop (5, 23). Reductions in root colonization by VAM fungi were observed when the host crop was either onion or barley. However, other studies showed no effect on VAM colonization when crucifers were rotated with lavender or lettuce (36). These researchers attributed the failure of VAM fungi to infect non-host species to an intrinsic resistance of the root epidermis or cortex. Morley and Mosse (33) studied the effect of lupin (non-host) root exudates on clover mycorrhizae. The presence of lupins caused the formation of abnormal appressoria and intercellular hyphae in VAM on clover growing in the same pots. The repression of VAM infection on clover was attributed to either root exudates or to seed coat exudates.

More recently, it has been suggested that allelochemicals produced by the donor plant may interfere with the growth of the target plant by repressing the formation of VAM or ectomycorrhizal associations. For example, the inability of forest trees to invade certain prairies and grasslands can be attributed, in part, to allelopathic inhibition of mycorrhizal fungi commonly associated with tree species (37). Eucalyptus baxteri produces a zone of suppression beneath its canopy which is a direct consequence of the production of allelopathic compounds. This zone is effective in inhibiting the encroachment of heathland plant species and is maintained by the direct inhibition of mycorrhizal formation in these species (13). A similar effect was demonstrated in the interference by Festuca silvatica with reforestation attempts using Abies alba (3). There was a direct allelopathic effect of F. silvatica on A. alba, but there were also signs of a selective detrimental effect on the mycorrhizal fungi of A.

alba. Other interaction effects associated with a repression of mycorrhizal fungi by allelopathic exudates include: a) Pinus resinosa and mortality of hardwood seedlings (50); b) Ponderosa pine and changes in herbaceous understory composition (27); c) litter from broomsedge, fescue, and blackberry and the reduced growth of black walnut seedlings (39); and d) mature Pinus radiata and the growth retardation of P. radiata seedlings (9).

Asparagus has also been shown to produce allelopathic chemicals which are autotoxic (21, 22, 51, 52, 53). Hartung (22) isolated and characterized several of allelochemicals from asparagus root tissue, and identified, among others, ferulic, iso-ferulic and caffeic acids. She suggested that allelochemicals might be active on the root surface and could affect the plant via uptake of these allelochemicals. This effect could be mediated by the alteration of the plant's biochemical processes or by directly inhibiting beneficial rhizosphere microorganisms.

These three compounds are cinnamic acid derivatives (44). Einhellig et al. (15) found that four cinnamic acid derivatives, each at a concentration below the minimum required to reduce growth of sorghum, were capable of inhibiting both germination and growth of sorghum in various combinations of two, three, and all four compounds. Newman and Miller (35) provided evidence that allelopathic root exudates are capable of inhibiting phosphorus uptake in Trifolium grown in combination with Anthoxanthum sp. Glass (17) found that cinnamic acid derivatives at concentrations ranging from 5×10^{-5} M to 1×10^{-3} M could also reduce uptake of P_i by barley seedlings.

Ferulic acid is derived from several crop residues and is

persistent enough in soil to be implicated as a potential toxicant (22). It was also capable of inhibiting phosphate uptake by excised roots of three varieties of soybean (29). There was, however, no apparent effect on the translocation of absorbed P in intact seedlings. In similar studies using cell cultures of rose (`Paul's Scarlet'), ferulic acid inhibited uptake of macronutrients and ^{86}Rb, but the inhibition varied with the age of the culture. The uptake of macronutrients was greatly inhibited in young cells, while the uptake of ^{86}Rb was less so. The reverse was true for older cell cultures (11). Ferulic acid was also found to be inhibitory to the uptake of ^{14}C by rose cell cultures (10). The exposure of cells to ferulic acid resulted in the diversion of ^{14}C to lipid synthesis, rather than protein and amino acid synthesis. Ferulic acid has also been shown to inhibit protein synthesis in lettuce seedlings (6) and chlorophyll synthesis in soybean (14). These are possibly secondary effects; the inhibition of mineral uptake, such as phosphorus, may be the main deleterious effect of ferulic acid.

The possible inhibition of phosphorus uptake by ferulic acid is of interest when considering the role of VAM fungi in asparagus growth. VAM have been shown to increase growth of asparagus (7, 8, 24, 40). However, this response was in young asparagus plants and the production of allelochemicals by asparagus plants may not be at toxic concentrations at such an early age. Of specific interest is the effect ferulic acid might have on VAM colonization of asparagus. One might suppose that VAM colonization could increase if ferulic acid were limiting P uptake at or near the root surface. Under these conditions, any mechanism (such as VAM colonization) capable of extending the volume

of soil for P uptake beyond this inhibition zone would be of great benefit to the host. However, ferulic acid is a cinnamic acid derivative, and could be classified with phenolic inhibitors which have been implicated in the altered functioning and/or death of several soil microorganisms (44, 45, 54). My studies were designed to test the hypothesis that ferulic acid is capable of direct inhibition of VAM growth and colonization of asparagus.

Materials and Methods

Germination and hyphal elongation. A modified version of a medium containing root exudates that had previously been shown to stimulate hyphal elongation (16) was prepared. This medium contained (per 100 ml of glass distilled water): 2 g Difco agar, 5 ml of Hoagland's solution (10X strength, without phosphorus), and 5 ml of rehydrated clover root exudate (taken from one-week-old plants grown axenically in Hoagland's solution, then lyophilized and stored frozen until ready for use). The pH was adjusted to 6.8 and the medium autoclaved for 20 min. at 121^{o}C. Ferulic acid was added to this medium at concentrations of 0, 50, 200, and 400 ppm. The ferulic acid was first dissolved in 10 ml glass distilled water (this required steaming for one hour), and the pH was adjusted to 6.8. The ferulic acid solutions were then passed through a Millipore filter (0.45 um pore size) and added to the sterile medium with a small amount of streptomycin sulfate to reduce bacterial contaminants. Ten ml of the ferulic acid/clover root exudate agar was added to each petri plate.

The VAM fungal species, Glomus fasciculatum (Thaxt. sensu Gerd.) Gerd. and Trappe was grown in sorghum (Sorghum vulgare) pot cultures in

the greenhouse for 4 months and stored at 4°C for 4 months prior to use. Chlamydospores were isolated from the soil surrounding the sorghum roots by wet sieving, followed by a modified centrifugation-flotation technique (38). This modification involved the formation of a density gradient using 15, 30, 45, 50, and 60% Ficoll (Sigma Chemical Co., St. Louis, MO.) to separate the chlamydospores from the inoculum soil. After centrifugation, the chlamydospores were suspended in the supernatant. The chlamydospores were collected in a 38 um cup sieve and washed exhaustively with distilled water. Organic debris was removed by hand from the final spore suspension using a Pasteur pipette under a dissecting microscope. The spores were then surface sterilized in 30 ml of a solution containing 2% (w/v) chloramine-T, 0.02% (w/v) streptomycin sulfate and a small amount of sodium laurel sulfate. The chlamydospores were incubated under vacuum in this solution for 30 min. and washed with sterile distilled water (28). The chlamydospores were stored at 4°C in a solution containing 0.25% (w/v) streptomycin sulfate and 0.17% (w/v) gentomycin sulfate for use within 5 days.

Five spores taken from the above solution were added per petri plate at each concentration of ferulic acid using a sterile Pasteur pipette. There were 10 plates per ferulic acid concentration (50 spores per treatment). After the spores were added, the petri plates were sealed with Parafilm and stored at 30°C. Hyphal growth was measured every 7 days. Spores were considered germinated if the hyphal length equalled the diameter of the spore. The experiment was terminated after six weeks. Final growth data were analyzed by one-way ANOVA and Student-Newman-Keuls mean separation test. This study was repeated once.

Growth of asparagus with and without VAM. Asparagus seeds (Asparagus officinalis L., var. UC 157) were surface sterilized for 30 min. in a 10% Chlorox solution and rinsed several times with sterile glass distilled water. The seeds were subjected to further sterilization with a 1:10 solution of benlate(50W):acetone (w/v), which was rotary shaken for 24 hr. The seeds were washed exhaustively with acetone to remove all benlate residue, then rinsed with 100 ml sterile glass distilled water six times (10). The seeds were allowed to germinate for 7 days on sterile moist filter paper. Germinated seeds were planted into 4 inch diameter pots containing Baccto greenhouse mix. Beginning 2 weeks after planting, the asparagus plants were fertilized each week with 100 ml containing 100 ppm each of NPK (20-20-20, Peters' Plant Food). After four months, plants were transplanted into a steam sterilized 2:1 (v:v) sand:soil mix in 5 inch diameter pots. One half of the plants received 1 g of G. fasciculatum inoculum (50-60 spores/g soil), which was placed 1 cm below the roots at time of transplanting. The control plants were inoculated with autoclaved inoculum soil plus 1 ml of inoculum wash, both of which were placed 1 cm below the roots at time of transplanting. The inoculum wash was prepared by wet-sieving inoculum soil through a 38 um sieve and collecting everything that passed through the sieve in a beaker. This is done so that microbes that are associated with the inoculum soil can be added to non-mycorrhizal treatments.

Ferulic acid solutions were prepared in distilled water at concentrations of 0, 50, 100, 200, and 400 ppm. The solutions were placed in a boiling water bath for 1 hour to aid in dissolving the ferulic acid and the pH was adjusted to 6.8. Ferulic acid solutions

were added to the appropriate pots (+/- VAM) at a rate of 100 ml per week. Fertilization was continued at 100 ppm NPK per week and plants were watered as needed. There were five plants per treatment, arranged in a complete randomized block design. Plants were harvested at 8 months of age.

Roots (0.5 g/plant) were removed at random and stained with a 0.1% solution of acid fuchsin in lactophenol (38). Root segments (1 cm length) were mounted on slides for observation under the light microscope. There were 40 observations per treatment. Segments were analyzed for VAM infection using the following rating system: 0 = no infection, 1 = 1 to 25% root segment infected, 2 = 25 to 50%, 3 = 50 to 75%, and 4 = over 75% infection. Infection ratings were analyzed by Spearman's rank-order correlation. Fresh and dry weights were recorded and root:shoot ratios were calculated. Phosphorus was extracted from dry tissue (0.004 g per Kjeldahl flask, four samples per treatment) using a modification of Bartlett's technique (2, Appendix A). Absorbance values at 620 nm were read from a Perkin-Elmer spectrophotometer. The percent dry weight of tissue phosphorus was calculated after obtaining the approximate concentration (ug/g) in each sample by comparison to a standard curve. Growth data and tissue P data were subjected to analysis of variance. This study was repeated once.

Results

Germination and hyphal elongation. The germination of spores was not simultaneous as spores continued to germinate throughout the experiment (Figure 3.1). The final germination was 68% for 0 ppm, 64% for 50 ppm, 72% for 200 ppm, and 60% for 400 ppm. These percentages did not differ

significantly. However, hyphal elongation after the first week was inhibited in the 200 and 400 ppm treatments (Figure 3.2). Hyphal length in the 50 ppm treatment continued to increase until the third week, after which it remained fairly constant. Hyphal length in the 0 ppm treatment (control) increased slightly through the third week, and at four weeks had increased dramatically (3 week mean: 6.18 mm, 4 week mean: 21.39 mm). The hyphal lengths for all ferulic acid treatments were significantly less than the control at the sixth week (Figure 3.3, $P = 0.012$). The reduction in hyphal length (sixth week data) with increasing ferulic acid concentration is not a linear response.

Growth of asparagus with and without VAM. There were significant differences in fresh and dry weights due to VAM inoculation ($P < 0.02$ for both data sets, Figures 3.4, 3.5). The differences between fresh and dry weights for VAM and non-VAM plants due to ferulic acid concentration were not significant at $P = 0.23$ and $P = 0.33$ respectively. The trend toward growth reduction within the VAM treatment with increasing ferulic acid concentration was not statistically significant ($P = 0.19$ for both data sets, Figures 3.4, 3.5).

There was also a significant difference in the root:shoot ratio of asparagus plants due to VAM inoculation ($P = 0.04$, Figure 3.6). There was a slight (but not significant) reduction in root:shoot ratios of VAM plants with increasing ferulic acid concentration ($P = 0.21$, Figure 3.6). Tissue P concentrations were not significantly different regardless of VAM treatment or ferulic acid concentration ($P > 0.50$, Table 3.1). The colonization of asparagus roots by G. fasciculatum

Figure 3.1. Germination of Glomus fasciculatum spores subjected to increasing concentrations of ferulic acid in clover root exudate/Hoagland's agar over a six-week period. Ferulic acid concentration did not affect spore germination ($P > 0.05$). a) 0 ppm; b) 50 ppm; c) 200 ppm; and d) 400 ppm ferulic acid added.

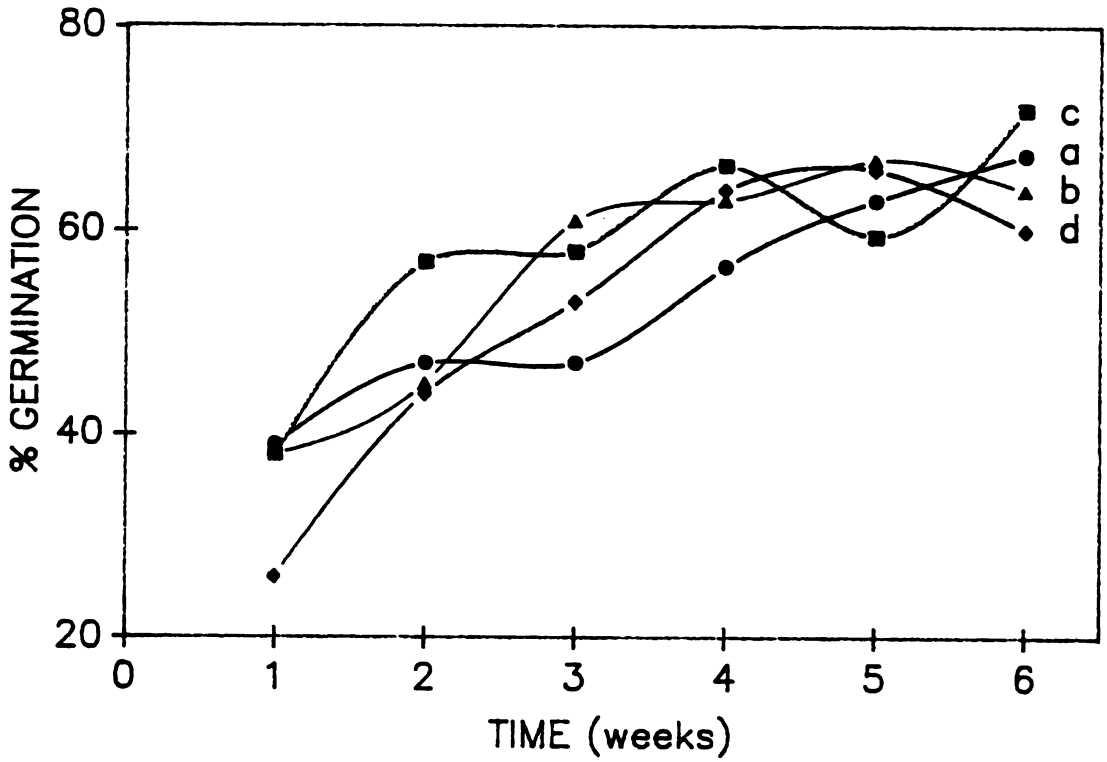
80
60
40
20
% GERMINATION
0
1
2
3
4
5
6
TIME (weeks)
c
a
b
d

Figure 3.2. Hyphal elongation of Glomus fasciculatum spores subjected to increasing concentrations of ferulic acid in clover root exudate/Hoagland's agar over a six-week period. a) 0 ppm; b) 50 ppm; c) 200 ppm; and d) 400 ppm ferulic acid.

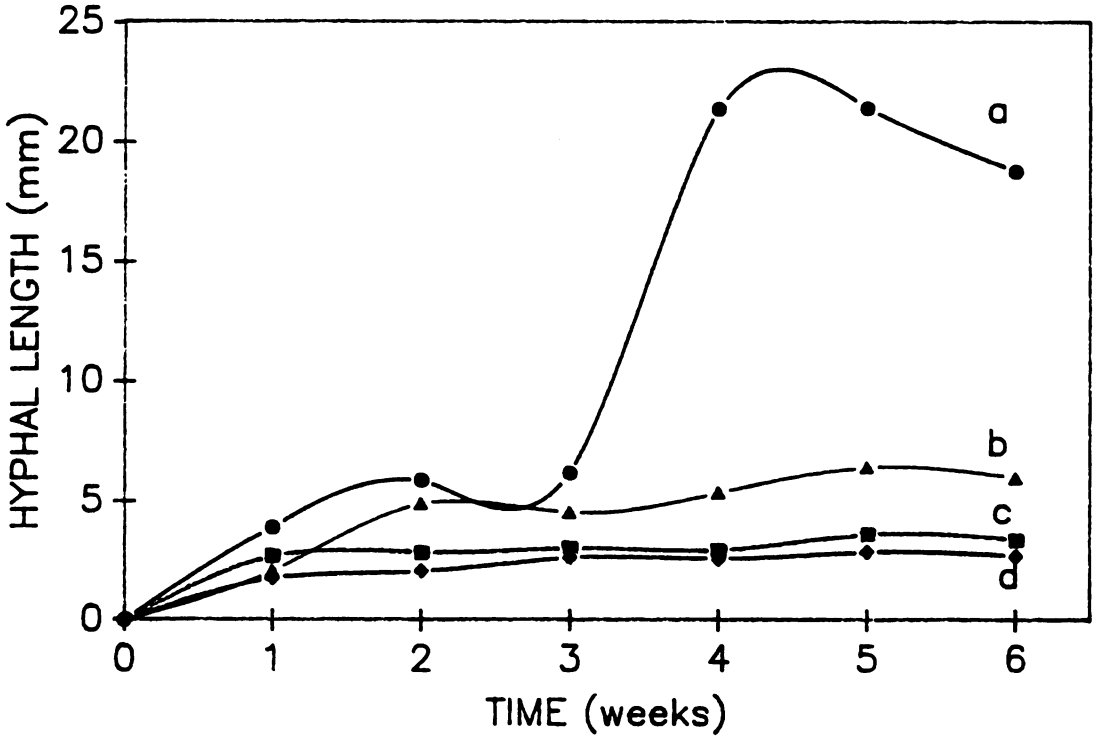
HYPHAL LENGTH (mm)
25
20
15
10
5
0
0
1
2
3
4
5
6
TIME (weeks)
a
b
c
d

Figure 3.3. Hyphal lengths of Glomus fasciculatum spores subjected to increasing concentrations of ferulic acid after six weeks. Spores from the control group exhibited significantly greater hyphal elongation than any other treatment ($P = 0.012$).

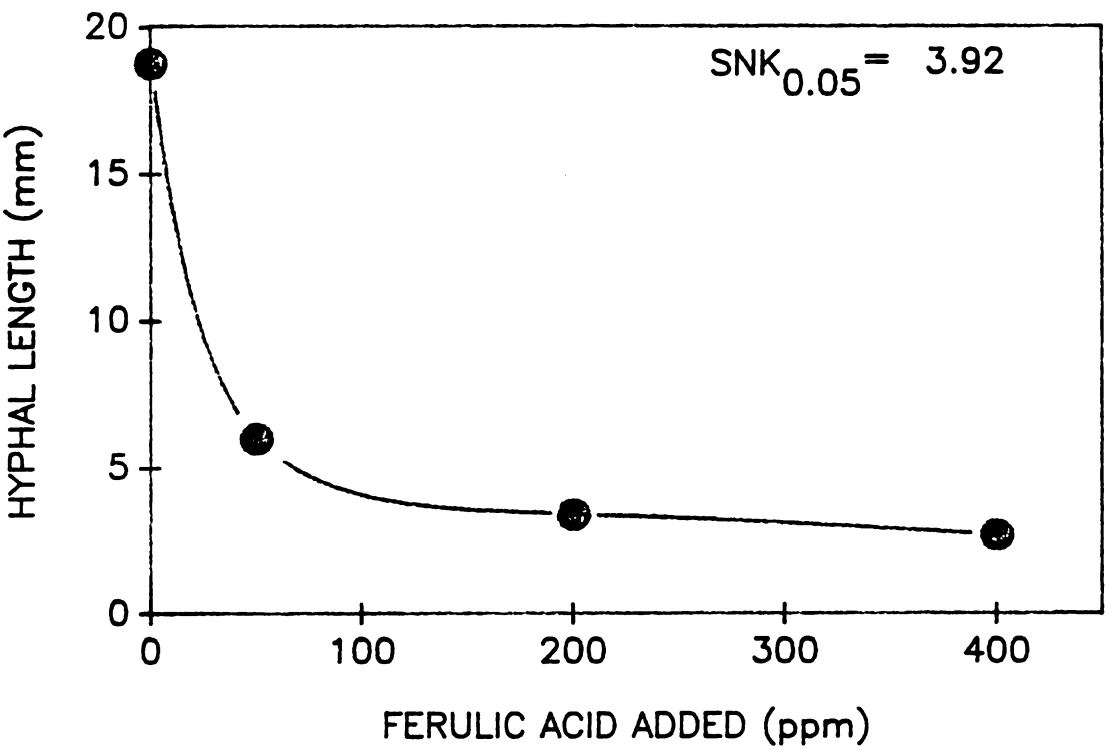
20
15
10
5
0
HYPHAL LENGTH (mm)
SNK0.05= 3.92
0
100
200
300
400
FERULIC ACID ADDED (ppm)

Figure 3.4. Fresh weights of VAM and non-VAM asparagus plants growing in a greenhouse and subjected to increasing concentrations of ferulic acid. VAM-colonized asparagus plants were significantly smaller than non-VAM plants ($P < 0.02$). The effect of ferulic acid on fresh weight was not significant ($P = 0.23$). Non-VAM: ———; VAM: ------.

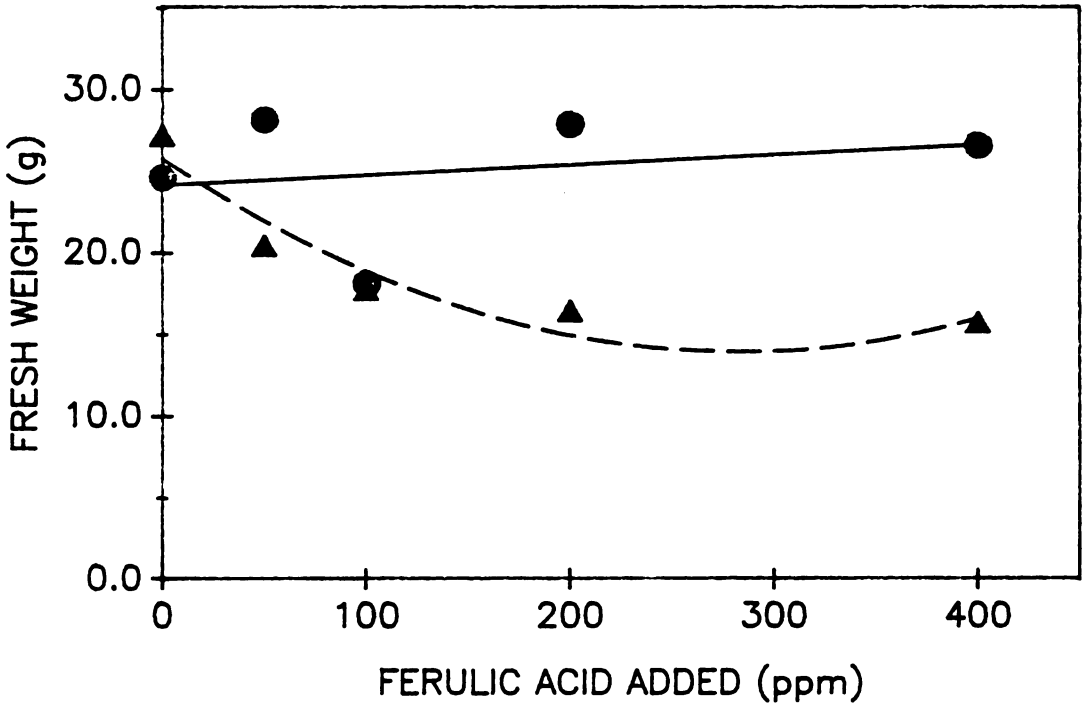
30.0
20.0
10.0
0.0
FRESH WEIGHT (g)
0
100
200
300
400
FERULIC ACID ADDED (ppm)

Figure 3.5. Dry weights of VAM and non-VAM asparagus plants growing in a greenhouse and subjected to increasing concentrations of ferulic acid. VAM-colonized plants were significantly smaller than non-VAM plants ($P < 0.02$). Ferulic acid concentration did not affect plant dry weight ($P = 0.33$). Non-VAM: ———; VAM: ------.

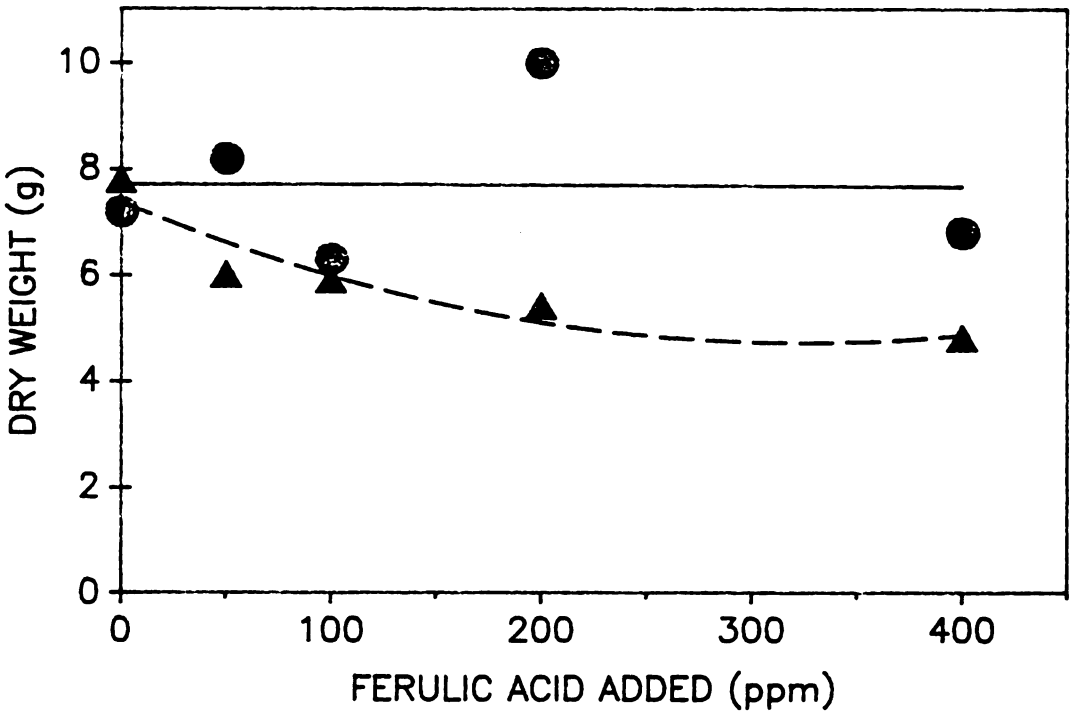
10
8
6
4
2
0
DRY WEIGHT (g)
0
100
200
300
400
FERULIC ACID ADDED (ppm)

Figure 3.6. Root:shoot ratios for VAM and non-VAM asparagus plants growing in a greenhouse and subjected to increasing ferulic acid concentrations. VAM-colonized asparagus plants had significantly lower root:shoot ratios ($P = 0.04$). There was a slight, but not significant, reduction in the root:shoot ratios of VAM plants with increasing ferulic acid concentration. Non-VAM: ———; VAM: ------.

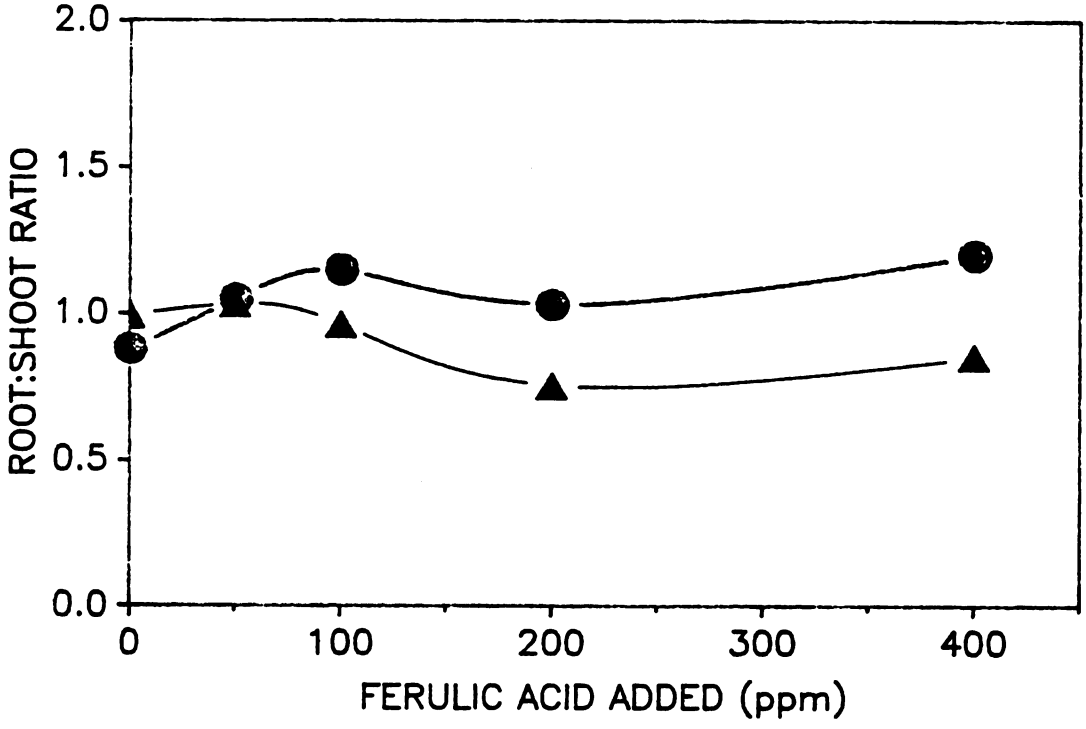
2.0
1.5
1.0
0.5
0.0
ROOT:SHOOT RATIO
0
100
200
300
400
FERULIC ACID ADDED (ppm)

Figure 3.7. Infection ratings for VAM asparagus plants grown in the greenhouse and subjected to increasing ferulic acid concentrations.

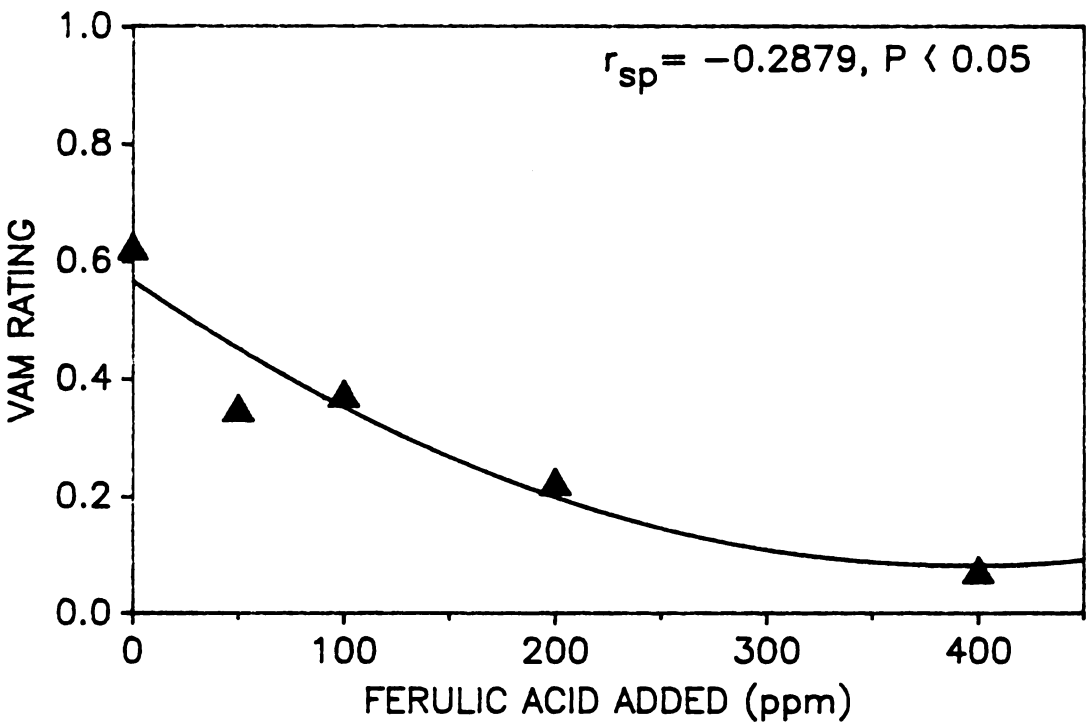

Table 3.1. Tissue phosphorus (P) levels in VAM and non-VAM asparagus at increasing concentrations of ferulic acid in soil in the greenhouse.

Ferulic Acid (ppm)[a]	Tissue P (% dry weight) non-VAM	VAM
0	0.33	0.38
50	0.32	0.39
100	0.42	0.33
200	0.33	0.35
400	0.37	0.34
ANOVA	N.S.	

[a] Ferulic acid was applied each week at a rate of 100 ml/pot.

diminished with increasing ferulic acid concentration (r_{sp} = -0.2879, critical value at 5% level for a one -tail comparison is -0.1167, Figure 3.7).

Discussion

The inhibition of growth of Glomus fasciculatum by ferulic acid was not due to a reduction in germination of chlamydospores (Figure 3.1), but instead to a decrease in hyphal elongation (Figures 3.2, 3.3). This inhibition was not correlated to concentration, as all concentrations of ferulic acid were similarly inhibitory. However, it cannot be assumed that this effect would also occur in the field. Schroth and Hildebrand (48) pointed out that a compound may inhibit an organism in an artificial medium, but may have little effect when added to soil because of factors such as pH, adsorption to clay particles, and microbial degradation. It is therefore important to design an experimental environment that corresponds to field conditions.

The greenhouse experiment was designed to mimic field conditions as closely as possible. The soil mix was comparable to the sandy loam soils of commercial asparagus fields. The pH ranged from 6.7 to 7.0, which is within the optimal range for asparagus growth, and the fertilization regime, though added weekly, was meant to maintain soil nutrient levels comparable to field levels. Soil moisture, however, was higher than soil moisture levels found in most Michigan asparagus fields.

Increasing concentrations of ferulic acid led to a decrease in colonization of asparagus by G. fasciculatum (Figure 3.7). It is possible that the toxin may affect the formation of arbuscules and vesicles within the host root. There was a conspicuous decline in the

amount of these structures present in the roots. Unfortunately, no attempt was made to quantify this observation, and further studies should address this phenomenon. The reduction in VAM colonization of asparagus by ferulic acid was not as dramatic as the reduction in hyphal elongation (Figures 3.3, 3.7). However, if ferulic acid is affecting the hyphal elongation of G. fasciculatum in the soil, this could account for the reduced host colonization.

The lesser effect of ferulic acid on colonization of asparagus roots by G. fasciculatum than on hyphal growth in vitro could also be due to soil variables. High moisture could have facilitated leaching of the toxin from the soil, but since the compound was added at the stated concentrations every week, the level of ferulic acid in the soil should have been fairly constant. That microbial alteration of the toxin occurred in the soil environment is suggested by a previous study in which asparagus residues were less toxic when subjected to an aerobic environment (22). Phenolic acids added to soils growing wheat were rapidly degraded by soil microbes (49) so that at harvest their amounts were not different than those in control soils. Soil biomass increased proportionally with increased addition of phenolic acids, suggesting that the soil microbes were able to metabolize the phenolic compounds.

There was no apparent effect of ferulic acid on the uptake of P or tissue P levels (Table 3.1). It is possible that the fertilization schedule coupled with the relatively high soil moisture levels enabled soil P to be readily available to the plant and in high enough concentrations that ferulic acid did not affect uptake. High soil P levels can cause reductions in VAM colonization (1, 4, 18). Even if soil P was at concentrations that inhibit VAM, this would not account

for the gradual reduction in infection with increasing ferulic acid concentration, because all treatments received the same amount of added phosphorus.

The response of asparagus to ferulic acid was not expected. Plants without VAM did not appear to be affected by increased concentrations, and exhibited no apparent pattern in growth response (Figures 3.4, 3.5). The VAM-infected asparagus plants tended to decrease in biomass with increasing concentrations of ferulic acid. The root:shoot ratios also tended to be reduced in VAM treatments (Figure 3.6). The decrease in biomass and root:shoot ratios of asparagus plants is probably not due to a direct effect of ferulic acid on the plant or the non-VAM plants would have shown a similar response. This indicates that the impaired growth of VAM-infected asparagus plants at higher ferulic acid concentrations may be mediated by a reduction in the symbiotic effectiveness of G. fasciculatum. Interestingly, the level of infection by the fungus also diminished with increasing concentrations of the toxin. Kiernan et al. observed a similar pattern in mycorrhizal sweetgum seedlings at high fertilizer concentrations (26). In previous studies, G. fasciculatum was capable of eliciting a positive growth response in asparagus (see Chapter I), so the reduced growth in the present study cannot be explained by an incompatibility between symbionts. Since fungal colonization is decreased and arbuscule formation is apparently diminished, the amount of nutrients provided to the host by the mycobiont would also be decreased. Thus, the fungus could be acting parasitically or pathogenically in these situations, depleting the host of carbohydrates. This would lead to the reduced growth that was evident at high ferulic acid concentrations.

The results of this study pose several questions regarding the role of VAM in the growth of established asparagus plants. If the allelochemicals can inhibit root colonization by VAM fungi, and if those fungi that infect the roots can act as mild parasites, then is the asparagus plant's growth also debilitated through the loss of VAM-improved nutrient uptake by an essential mycobiont (as well as through the loss of carbohydrates to the fungus)? Also, is the production of allelopathic compounds instrumental in the succession of VAM fungi in asparagus fields, and if so, what mechanism(s) is involved that allows the late colonizers to overcome the effect of these toxins? Schenck (unpublished data) found that there was a shift in VAM species infecting coffee to Acaulospora spp. after four years. He suggested that shifts in species composition of VAM fungi may contribute to decline problems in perennial crops. Coffee also produces allelochemicals and suffers from decline problems (44). The possibility of allelochemical mediation of VAM succession in decline situations should not be ignored. Are the late successional species of VAM in asparagus fields providing the benefit to the host that is commonly associated with mycorrhizal symbiosis, or are these fungi extremely aggressive colonizers eliciting greater stresses on the host plant? Further studies on the role of VAM fungi in the asparagus decline complex should address these questions. It is also imperative that the effect of other allelochemicals on VAM fungi be addressed. These allelochemicals could be acting either additively or synergistically in the inhibition of VAM colonization of asparagus roots, or other compounds produced by asparagus could mask the effect of ferulic acid on mycorrhizal infection in asparagus.

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APPENDIX

Appendix A. Procedure for Tissue Phosphorus Assay

1. Weigh < 1 to 50 mg dry tissue into a 10 ml Kjeldahl flask.

2. Add 0.5 ml 10 N sulfuric acid to each flask. The amount of sulfuric acid is critical. To make 10 N sulfuric acid, combine 270 ml concentrated H_2SO_4 and 730 ml distilled water.

3. Heat at # 3 on a microkjeldahl apparatus.

4. When white fuming starts, reduce the heat to # 2 and reflux for 30 minutes.

5. Cool the tubes and add 0.5 ml 30% hydrogen peroxide (H_2O_2).

6. Shake gently, repeat steps 3 and 4.

7. Cool tubes again and add 5.0 ml glass-distilled water.

8. Heat tubes in a boiling water bath (100°C) for 10 minutes.

9. Cool tubes and add 0.2 ml of 5% ammonium molybdate.

10. Add 0.2 ml Fiske-Sabbarow* reagent and vortex.

11. Heat in boiling water bath (100°C) for 7.5 minutes.

12. Read absorbance at 620 nm.

* Fiske-Sabbarow reagent:

1.0 g 1-amino-2-napthol sulfonic acid
1.0 g sodium sulfite (Na_2SO_3)
58.4 g sodium meta-bisulfite ($Na_2S_2O_5$)

Mix and grind to a dry powder. Dissolve 0.77 g in 5.0 ml warm glass-distilled water 15 minutes before use.