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INFLUENCE OF BIOLOGICAL AGENTS ON ASPARAGUS DECLINE SYNDROME

By

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ABSTRACT

INFLUENCE OF BIOLOGICAL AGENTS ON ASPARAGUS DECLINE SYNDROME

By

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Asparagus decline syndrome is caused by *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM). Effective methods for controlling *Fusarium spp.* in asparagus are not available currently. Resistant varieties have not been found in asparagus. Chemical treatments have failed to increase crown survival or yield of asparagus.

Brassica spp. residues have been shown to control *Fusarium* diseases in other crops. Seven brassicas were incorporated in the field and greenhouse. The brassicas included 'Premier' kale (*B. oleracea* L. *virdis*), 'Rondo' turnip (*B. rapa* L.), 'Global' canola (*B. napus* L. *annua*), 'Glacier' canola (*B. napus* L. *biennis*), yellow mustard (*B. nigra* L.), 'Dwarf Essex' oilseed rape (*B. napus* L. *biennis*), 'Humus' oilseed rape (*B. napus* L. *biennis*) and sorghum-sudangrass (*Sorghum bicolor sudanense*) as the control. Brassica residues had no effect on FOA or FM, but were phytotoxic to several indicator crops. Chemical extraction of the brassica residues did not yield any *Fusarium*-inhibitory compounds.

Several soil-borne microorganisms have been found inhibitory to Fusarium diseases in other crops. A novel *Streptomyces spp.* (ME2-27-19A) was isolated from

asparagus field soil and found inhibitory to FOA and FM in vitro. Chemical extraction of ME2-27-19A yielded a fraction that was inhibitory. The inhibitory fraction was purified by vacuum-liquid chromatography and C18 medium-pressure chromatography and found inhibitory at 50 $\mu g \cdot ml^{-1}$. ME2-27-19A extract produced variable control FOA or FM in vitro, and was phytotoxic at 1000 $\mu g \cdot ml^{-1}$. In soil, ME2-27-19A extract reduced the *Fusarium* population at 100 $\mu g \cdot ml^{-1}$, but also reduced the asparagus shoot length.

ME2-27-19A cells, applied as a root-dip in vitro, reduced the disease incidence in asparagus. In soil, ME2-27-19A decreased the number of root lesions caused by *Fusarium spp.*, but also reduced the root length of the asparagus. ME2-27-19A cells colonize the entire root system in sterilized soil, but not at sufficient concentration to inhibit FOA and FM. Maltodextrin was a satisfactory carrier for ME2-27-19A and other biological control agents. ME2-27-19A may compete with FOA and FM, but it is not capable of controlling disease.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2:

Chapter 3:

Chapter 4:

LIST OF APPENDICES

CHAPTER I

LITERATURE REVIEW

Introduction

Asparagus (*Asparagus officinalis* L.) is an important vegetable crop in the United States. The total acreage in the United States increased from 86,675 in 1978 to 97,335 in 1987. Michigan acreage increased from 19,719 to 23,426 (Bureau of Census, 1978, 1987). The average price received at the wholesale level increased from $24.30/cwt in 1975 to a high of $59.00/cwt in 1988 (Michigan Agriculture Statistics Service, 1980, 1992). In 1991, Michigan produced 12,950 tons of "snapped" asparagus with a value of $14.1 million (Michigan Agriculture Statistics Service, 1992). Snapped asparagus is hand picked by breaking the spears near the ground, giving less waste during packaging.

Michigan asparagus production averaged 1200 pounds per acre over the past decade (Michigan Agriculture Statistics Service, 1992). However, yields of 3000 pounds per acre are achieved in some fields (Hodupp, 1983), indicating that a majority of the acreage is not producing at its full potential. Replanting these declining field is difficult because 50% of young asparagus plants die within five years of planting and replanted fields often yield only half of the expected production (Hodupp 1983).

Asparagus stands generally have a profitable life of 10 to 20 years (Tiedgens, 1924, 1926; Grogan and Kimble 1959). They reach peak production in the eighth year,

then spear size gradually decreases until the product is unmarketable (Grogan and Kimble, 1959). In recent years, fields have been removed after six to eight years because of declining yields (Takatori and Souther, 1978). This phenomenon of early decline, known as Asparagus Decline Syndrome (ADS), threatens the profitability of the affected fields (Grogan and Kimble, 1959). Additional symptoms of ADS are stunted, yellow, wilted plants and blank spaces in the rows. It is caused by a complex of organisms and allelopathic chemicals (Hartung, 1987).

Decline of asparagus was first reported as "Fusariosis" by Stone and Chapman in 1908 (Cohen and Heald, 1941). Cook (1923) described a "Dwarf Asparagus" disease resembling ADS. The plants were stunted and yellow, having vascular discoloration and occurring in circular areas. Cohen and Heald (1941) were the first to attribute the disorder to *Fusarium oxysporum* (Schlecht) emend Snyder and Hansen f.sp. *asparagi* Cohen (FOA). *Fusarium moniliforme* (Seld.) emend Snyder and Hansen (FM) also is recognized as a primary disease-causing organism (Johnson et al, 1979). Both are isolated readily from asparagus seed and most cultivated soils (Correll et al., 1986; Elmer, 1989). Inglis (1980) reported that up to 9.3% of the asparagus seed in Washington state was infected with FOA and FM. Soil which had no history of asparagus production had low populations of FOA and FM (Hartung et al., 1990).

Fusarium wilts are among the most common diseases of horticultural crops. They affect asparagus, banana (*Musa X paradisiana* L.), cabbage (*Brassica oleracea* var. *capitata* L.), celery (*Apium graveolens* L. var. *dulce*), cotton (*Gossypium hirsutum* L.), cucumber (*Cucumis sativus* L.), flax (*Linum usitatissum* L.), muskmelon (*Cucumis melo*),

onion (*Allium cepa* L.), pea (*Pisum sativum* L.), sweet potato (*Ipomea batatus* Lam.), tomato (*Lycopersicum esculentum* L.), watermelon (*Citrullus lanatus* (Thumb.) Matsum. & Nakai), aster (*Aster dumosus* L.), carnation (*Dianthus caryophyllus* L.), chrysanthemum (*Chrysanthemum X morifloium* Ramant.), gladiolus (*Gladiolus X hortulanus* Bailey), and tulip (*Tulipa gesnerana* L.)(Louvet et al., 1981). *Fusarium spp.* account for 80-90% of the total fungal populations in the rhizosphere of these crops (Toussoun, 1975).

FOA causes vascular discoloration and reddish-brown, elliptical lesions on the roots and crowns of asparagus (Grogan and Kimble, 1959). The lesions are associated with the points of attachment of the lateral roots (Graham 1955). Fibrous roots may be shrivelled or absent. *Fusarium oxysporum* strains are indistinguishable except by pathogenicity tests (Correll et al. 1986). The strains that are pathogenic on asparagus also infect onion, gladiolus and celery (Elmer and Stephens, 1989) and cause stunting in sorghum (*Sorghum bicolor* (L.) Moench) and sweet pepper (*Capsicum annuum* L.) (Tu and Cheng, 1990). However, strains that are pathogenic on cotton, cucumber, muskmelon, radish and sweet potato are not pathogenic on asparagus (Elmer and Stephens, 1989). In addition, FOA can survive on other crops and weeds (Damicone et al., 1988). Alfalfa (*Medicago sativa* L.), oats (*Avena sativa* L.), red clover (*Trifolium pratense* L.) and soybeans (*Glycine max* (L.) Merrill) tended to increase disease incidence, whereas potatoes (*Solanum tuberosum* L.) maintained a low level of inoculum (Graham 1955).

In asparagus, FM causes stem and crown rot which leads to yellowing, stunting and wilting of the fern (Johnson et al., 1979). FM is cross-pathogenic between corn (*Zea mays* L.) and asparagus (Damicone et al., 1988), and spreads rapidly through airborne conidia (Johnson et al., 1979). Damicone and Manning (1985) concluded, "It is impossible to grow *Fusarium*-free asparagus," since *Fusarium* was isolated from every crown after just one growing season.

FOA and FM may invade at an early stage of plant growth and live as saprophytes until a stress alters the host physiology, allowing the fungi to become pathogenic (Nigh 1990). Injuries make asparagus more susceptible to *Fusarium* (Cohen and Heald 1941; Cook 1923). Lacy (1979) found more Fusarium infection in hand-weeded than in herbicide-treated asparagus.

Nigh (1990) suggested that FOA and FM might not be a problem if asparagus could be maintained in an unstressed condition. Asparagus seedlings emerged best at 35% of the water holding capacity (WHC) of the soil and emergence was lower at 10 and 80% WHC (Nigh, 1990). However, FOA showed no change in activity from 20 to 80% WHC. Therefore, moisture stress predisposes the seedling to preemergence infection. Cohen and Heald (1941) observed that Fusarium wilt was more abundant in low areas and on steep, sandy slopes, where plants are likely to suffer from water stress.

Another factor of ADS is that asparagus produces several autotoxic compounds including asparagusic, caffeic, ferulic and methylenedioxycinnamic acids (Hartung, 1987; Hartung et al., 1990). These autotoxins are heat-stable and water-soluble, and should leach out of the soil (Hartung, 1987; Yang, 1982). However, asparagus debris still

contained some autotoxin eight months after the death of the plants (Yang, 1982). These inhibitors have detrimental effects on certain microflora organisms including *Pythium* (Hartung, 1983; Peirce and Colby, 1987). Caffeic and ferulic acid inhibit *F. oxysporum* f.sp. *radicis-lycopersici* (Kasenberg and Traquair, 1988). However, FOA, FM and some soil bacteria are insensitive to the allelopathic substances (Hartung, 1987; Hazebroek et al., 1989). Peirce and Colby (1987) determined that the filtrate from asparagus roots predisposed asparagus seedlings to FOA infection and stimulated growth of FOA chlamydospores. Caffeic and ferulic acids inhibited growth of asparagus seedlings and appeared to damage root cells by erosion of the cell wall (Peirce and Miller, 1993).

Attempted Controls

Resistance. Numerous methods have been investigated in attempts to control Fusarium wilt diseases in vegetables. Resistant varieties have been the most effective control of Fusarium diseases in other vegetable crops (Mace et al., 1981). Although numerous varieties of asparagus have been developed, resistance to FOA and FM has not been found in *A. officinalis* (Hartung, 1987). Stephens et al. (1989) found 'Lucullus 234' and 'Lucullus 328' were the most tolerant cultivars. Sadowski and Knaflewski (1990) found 'Huchel's L', ST-3, and 'Danische Riesen' were the most tolerant cultivars. However, progeny of parents selected for *Fusarium*-resistance were among the most susceptible when exposed to *Fusarium* (Bussell and Ellison, 1987). *Asparagus densiflorus* (Kuath) Jessop 'Sprengeri' and 'Myersii' were resistant to FOA and FM, and offer potential for resistance through recombinant breeding techniques (Stephens et al., 1989).

Resistance does not require elimination of *Fusarium spp.* from the plant roots. In celery, the roots of the resistant cultivar were colonized with a population similar to that of the susceptible cultivar, except that the *Fusarium* did not penetrate into the roots (Elmer and Lacy, 1987). Resistance also can be overcome by pathogens. In cabbage, monogenic type A resistance to Fusarium Yellows was overcome by *Fusarium oxysporum* f.sp. *conglutinans* Race 5, demonstrating the potential for the organism to mutate and the need for additional controls (Ramirez-Villapudua et al. 1985).

Chemicals. Chemical treatments have not proven effective for control of *Fusarium spp.* in asparagus. Fumigation with Vorlex (1,3-dichloropropene, 1,2-dichloropropane, methyl isothiocyanate) gave variable results (Lacy 1979). Captafol drench after steaming was effective but not practical for field situations (Komada, 1975). Crown-dip treatments with benomyl [methyl 1-(butylcarbamoyl)-1H-benzimidazol-2-ylcarbamate] (300 ppm) and captan [N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide] (1800 ppm) produced no positive effect on survival or yield of asparagus (Lacy, 1979). Falloon and Fraser (1991) observed some control with an acid formulation of thiabendazole, but it was too expensive for field applications.

Salt. Liberal applications of rock salt (NaCl) once were recommended for production of asparagus (Rudolph, 1921). Elmer (1992) found that asparagus seedlings grow best when fertilized with NH_4NO_3 and NaCl. Treatments with 560-1120 $kg \cdot ha^{-1}$ of NaCl suppressed FOA and increased asparagus dry weight, but were not herbicidal (Elmer

1992). The colony-forming units of FOA per centimeter of root was correlated with the ratio of K^+ to Cl^- ions. Therefore, the traditional practice of applying rock salt ($2qt/yd^2$) may have benefitted the asparagus plants as well as controlling weeds (Elmer, 1989).

Chloride ions were shown to inhibit FOA (Elmer 1992). Salt (NaCl) may suppress *Fusarium spp.* through a fungistatic effect or by stimulating host resistance in the asparagus (Elmer 1992). Chloride ions are associated with nitrate uptake and malate synthesis, which may affect root exudation (Elmer 1992).

Suppressive Soils

Naturally occurring *Fusarium*-suppressive soils have been identified for over 100 years (Campbell, 1989). Disease-suppressive soils impede the development of specific soil-borne diseases when susceptible plants are cultivated.

The classic example of naturally occurring suppression of *F. oxysporum* f.sp. *cubense* in banana was studied by N.J. Volk (United Fruit Company, La Lima, Honduras) and summarized by Stover (1962). Volk found that pH affected the productive life of banana plantings (Wardlaw, 1972). Reinking and Manns (1933) conducted extensive physical and chemical analyses of the suppressive soils, but did not identify the *Fusarium*-suppressive element(s). Since montmorillonite clay was found in all of the suppressive soils, Reinking and Manns (1933) concluded that soil type affected the survival of *Fusarium spp.* rather than pH. However, sandy loam soils also have been found to be suppressive (Scher and Baker, 1980).

Other *Fusarium*-suppressive soils also have been found that have a high clay content (Walker and Snyder, 1933) or a high WHC (Cook and Schroth, 1965; Burke 1965). Stover (1962) suggested that the clay mineralogy maintained the soil moisture content, stimulating antagonistic bacteria. Stover (1953) studied the use of flooding for control of *Fusarium spp.* in banana in Central America. All of the *Fusarium* species tested were reduced by 50-85% soil saturation. Six months of flood fallowing were sufficient to eliminate *F. oxysporum* f.sp. *cubense* from the soil (Wardlaw, 1972).

Other researchers have found that soil pH influences germination and growth of *Fusarium spp.* The optimum pH for germination of *Fusarium oxysporum* is 5-6 (Griffin, 1981). Jones et al. (1989) report that liming soils to pH 6.5-7.5 decreased Fusarium wilt of tomato (*F. oxysporum* f.sp. *lycopersici*). Raising the soil pH to 7.0 was as effective as pasteurizing soil with a pH of 5.5 (Jones and Overman, 1971). Searseth (1945) reported complete control of Panama disease of banana (*F. oxysporum* f.sp, *cubense*) with heavy lime applications (12-15 tons/acre) and deep plowing (2 ft). Liming could be effective in asparagus because asparagus tolerates pH up to 7.5. Alabouvette (1990) claims that high pH does not directly affect *Fusarium spp.*, rather it influences the microbial interactions. Actinomycetes and bacteria are favored by high pH (Waksman, 1927), making them more competitive with *Fusarium spp.*

Introduced Suppression

Couteaudier et al. (1985) found that transferring 1 ppm of the Fusarium-suppressive Chateaurenard soil established suppression in a conducive soil. Loss of the

suppression by autoclaving and selective growth of saprophytic *Fusarium spp.* in the suppressive soil support the case for microbial competition (Smith and Snyder, 1972). However, transferring suppressive soil to conducive soil is not a practical method of introducing suppression. Therefore, a basic concept of introduced suppression is to isolate antagonistic microorganisms from suppressive soils and inoculate them into conducive soil. The information on introduction of organisms is complicated and fragmented (Mutitu et al., 1991).

Fusarium. Several microorganisms have been isolated from *Fusarium*-suppressive soils. It was observed that the population of non-pathogenic *Fusarium spp.* in the suppressive Chateaurenard soil was ten times greater than in a similar conducive soil (Alabouvette et al., 1983). Several non-pathogenic fusaria were isolated and tested for suppression of *F. oxysporum* f.sp. *melonis*. Non-pathogenic *Fusarium* could compete directly with the pathogen for nutrients or infection sites (Alabouvette, 1990). The ratio of pathogenic:non-pathogenic *Fusarium* affects the amount of suppression observed (Alabouvette et al., 1983). Carbon and iron were the main nutrients for which microorganisms competed; carbon seemed to be more important (Alabouvette, 1990). Non-pathogenic isolates, which inhibited germination of *F. oxysporum* f.sp. *lini*, had a high affinity for glucose (Couteaudier, 1992). The isolates with the best ability to inhibit germination of pathogenic chlamydospores also reduced wilt symptoms (Alabouvette and Couteaudier, 1992). Damicone and Manning (1982) found a non-pathogenic *Fusarium* isolate that protected asparagus seedlings for 8 weeks.

Other researchers have suggested that inoculation with non-pathogenic *Fusarium* stimulates the defenses of the host, known as induced resistance (Kuc, 1990). Ogawa and Komada (1983) found that dipping tomato seedlings in *F. oxysporum* f.sp. *dianthi* protected the plants from *F. oxysporum* f.sp. *lycopersici* for several weeks.

Pseudomonas. Suppressiveness in soil generally is attributed to *Pseudomonas spp.* (Alabouvette, 1990). *Pseudomonas* are gram-negative bacteria that produce siderophores (Miller 1990). They comprise 0.5% of the soil bacteria, and colonize the rhizosphere more than the rhizoplane (Miller 1990). Cassini et al. (1985) found a *Pseudomonas* that reduced *Fusarium* infection in asparagus. Tari and Anderson (1988) found that *Pseudomonas putida* suppressed *Fusarium oxysporum* f.sp. *cucumerinum* in soil. It was suspected that the organisms were competing for iron (Tari and Anderson 1988).

Siderophores are iron-binding organic molecules that inhibit fungi. The siderophore produced by *P. putida* is pyoverdine (Paulitz and Loper, 1991). Sneh et al. (1984) found a correlation between siderophore production and *Fusarium* chlamydospore inhibition. *Fusarium* also was inhibited by the synthetic siderophore, ethylenediamine-di[(o-hydroxyphenyl)acetic acid] (EDDHA) (Sneh et al., 1984). The affinity for iron was highest in EDDHA, slightly less for the plant and lowest for *F. oxysporum* (Campbell, 1989), giving the *Pseudomonas* and the plant a slight advantage. *Pythium* was inhibited by both EDDHA and a *Pseudomonas* isolate (Becker and Cook, 1988; Paulitz and Loper, 1991). However, the inhibition was overcome by the addition of $FeCl_3$, supporting the concept of competition for iron (Becker and Cook, 1988).

Siderophore production may not be necessary for biocontrol, since eight siderophore-negative mutants did not inhibit *Pythium*, but did protect cucumber plants from infection (Paulitz and Loper, 1991). Levy et al. (1989) isolated 1-hydroxy-phenazine as an antibiotic produced by *Pseudomonas* which inhibited *F. oxysporum* f.sp. *vasinfectum*. A *Pseudomonas spp.* was isolated from the surface *F. oxysporum* f.sp. *lini* hyphae in a *Fusarium*-suppressive soil (Scher and Baker, 1980). It was not clear whether *Pseudomonas* could parasitize *Fusarium*.

Actinomycetes. Merriman et al. (1974) found a *Streptomyces griseus* isolate that was antagonistic to *Rhizoctonia solani* in barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), wheat (*Triticum aestivum* L.), and carrot (*Daucus carota* var. *sativa* L.). It did not control the root disease, but did give higher yields, suggesting that it produced a growth regulator (Merriman et al. 1974). Sabaou and Bounaga (1987) found *Nocardiopsis dassonvillei* and *Streptomyces alni* reduced populations of *F. oxysporum* f.sp. *albedinis* in date-palm soil. Thus, actinomycetes offer some potential for control of *Fusarium spp.*

Actinomycetes are gram-positive, coccus and rod-coccus bacteria. Most are saprophytes with resistant spores (Goodfellow and Williams, 1983) comprising 20% of the soil bacteria, but only 4% of the rhizosphere bacteria (Nemec et al. 1989). Mayfield et al. (1972) found *Streptomyces spp.* in discreet, spherical sites, associated with dead root fragments.

Streptomyces spp. produce some of the known antibiotics, such as cycloheximide (Leach et al., 1947), tetracycline (Conover et al., 1953), nystatin (Hazen and Brown,

1951), and streptomycin (Schatz et al., 1944). However, there has been much debate as to whether antibiotics are produced in soil (Gottlieb, 1976). Inoculation of natural soil with *Streptomyces* did not result in detectable amounts of antibiotics, but addition of glucose and *Streptomyces* did yield some antibiotic compounds (Williams, 1982). Williams (1982) suggested that the soil is a nutrient-limiting environment and antibiotics are produced when nutrients are available. Nitrogen content of the organic material also may limit antibiotic production in the soil (Isizawa and Araragi, 1976). Antibiotics still have not been isolated from natural, unamended soil (Maplestone et al., 1992).

Mayfield et al. (1972) found a *Streptomyces spp.* that colonized fungal hyphae, but it was not clear if the actinomycete penetrated the cell walls. Sutherland and Lockwood (1984) found that actinomycetes parasitized the oospores of *Phytophthora megasperma* f.sp. *glycine*. Some actinomycetes use chitin as a carbon source, so it is possible that they may parasitize fungi for the chitin in the cell walls.

In 1987, Tahvonen (1988) isolated a *Streptomyces spp.* from a light-colored Sphagnum peat. It was found to control *F. oxysporum* f.sp. *dianthi* in carnations (Lahdenpera, 1987). It also reportedly inhibited *Alternaria*, *Botryis*, *Pythium*, *Rhizoctonia*, and *Verticillum* (Lahdenpera, 1987, 1990). The organism was formulated by Kemira Oy into a powdered preparation called Mycostop (Lahdenpera, 1987). Tahvonen (1988) claimed it was effective in peat, sand or clay. Although the yield of treated plants increased by 20-35% when using Mycostop, they rarely reached the yield of healthy control plants (Lahdenpera, 1987).

Smith et al. (1990) found that *S. griseus* controlled FOA in asparagus. It increased root dry weight when applied as a root dip. The antibiotic compound was extracted and named Faerifungin (Nair et al. 1987). Faerifungin inhibited FOA at 25 ppm (Smith et al., 1990).

Other organisms capable of inhibiting *Fusarium spp.* were *Arthrobacter* (Barrow-Broaddus and Kerr, 1981), *Aspergillus, Penicillum, Trichoderma* (Marois and Mitchell, 1981); *Bacillus* (Mutitu et al., 1991). *Trichoderma harzianum* colonized the rhizosphere of tomato and tobacco, reducing the *Fusarium* population from 8.8×10^4 to 6.3×10^2 cfu/g of soil (Sivan and Chet, 1989). However, the effect was nullified by the presence of excessive root exudates. *Trichoderma* also increased the rate of seedling emergence, suggesting that it produces plant growth substances (Windham et al., 1986).

Colonization. The ability of any microorganism to protect plant roots depends on colonization of the root. Colonization has been studied more thoroughly for *Pseudomonas*. Although *Pseudomonas* have flagella, and Weller (1983) found colonization along the entire root system of wheat, other researchers have not found evidence for active movement (Dijkstra et al., 1987; Howie et al., 1987). Percolating water enhanced movement of the organism along the root (Chao et al., 1986; Liddell and Parke, 1989). Passive movement on the root tip was suggested by Dijkstra et al. (1987) and Howie et al. (1987) but Liddell and Parke (1989) found little evidence for such movement. Tan et al. (1991) suggested that adsorption on the soil particles was the key factor affecting movement of *Pseudomonas* in soil.

Colonization and consistency of the biological control are dependent on several environmental conditions (Lemanceau and Alabouvette, 1991). Soils with a matric potential of less than -.05kPa were too wet and greater than -4.0 kPa was too dry for *P. fluorescens* (Howie et al.,1987). Suppression was more effective at neutral to alkaline pH (Park et al., 1988).

Colonization was also temperature-sensitive. *P. aeruginosa* and *P. fluorescens* both survived temperatures from 4 to 42C. However, *P. aeruginosa* had a maximum cell yield at 28C and a maximum siderophore yield at 19C. *P. fluorescens* had the maximum cell yield at 19C and maximum siderophore yield at 12C (Seong et al., 1991). Weller and Cook (1983) found that the *Pseudomonas* population declined to 2.8×10^3 cfu/g over winter, but increased 10-fold in spring and remained at that level. Miller et al. (1990) interpreted the frequently observed decline in *Pseudomonas* as the inability of the plant to support the organism in later development.

Rhizobacteria can be very specific in their interaction with the host plant and their antibiosis (El-Meleigi, 1989; Huber et al., 1989). Weller and Cook (1983) found that a combination of isolates was more effective than single isolates for control of *Pythium* in wheat. Lemanceau and Alabouvette (1991) found that a particular strain may not have the same effect on two different Fusarium diseases. Stotzky (1964) found that *Achromobacter* inhibited *F. oxysporum* if inoculated three days before exposure to the *Fusarium*, but not if the microorganisms were co-inoculated.

Weller (1983) found that *Pseudomonas* populations were higher on roots infected with *F. oxysporum*. Nemec et al. (1989) also reported the *Pseudomonas* population was

higher with *F. solani* in citrus. Park et al. (1988) found that *Pseudomonas* and non-pathogenic Fusarium controlled *F. oxysporum* in cucumber in combination, but were ineffective alone. Lemanceau and Alabouvette (1991) found that a *Pseudomonas* isolate plus non-pathogenic Fusarium controlled *F. oxysporum* f.sp. *radicis-lycopersici* in tomato under greenhouse conditions. Park et al. (1988) suggest that *Fusarium* infection induces increased root exudation, which leads to competition for iron by the microorganisms.

Organisms that are introduced into soil must compete for nutrients with the organisms that are already established (Schroth and Hancock, 1982; Campbell 1989). Therefore, biological controls often fail unless large quantities of inoculum are applied, or the soil is partially sterilized. It has been economically unfeasible to apply concentrated inoculum on a regular basis. Direct application to seed or roots was more feasible (Schroth and Hancock, 1982). However, Zablotowicz et al. (1989) used an in-furrow delivery system which required only one liter of *Pseudomonas* cell suspension per hectare.

Induced Suppression

Suppression may be induced by germination and lysis of the pathogen propagules or by promotion of native antagonists. Cook and Schroth (1965) found that *F. solani* f.sp. *phaseoli* chlamydospores require exogenous sources of carbon and nitrogen for germination. They hypothesized that reduction of the fungal propagules should be feasible by adding the appropriate nutrients in the absence of a susceptible host. Asparagine and ammonium-nitrogen were identified as the preferred sources (Cook and

Schroth, 1965). Sivan and Chet (1989) found maximum germination of *F. oxysporum* chlamydospores with 0.4 mg glucose and 0.08 mg of asparagine per gram of soil. The success of this type of control depends on the inability of the organism to form daughter chlamydospores. If daughter chlamydospores form, the inoculum density will increase (Papavizas and Lumsden, 1980).

A natural germination-lysis system was found in the coniferous forests of California. These forest soils lacked *Fusarium spp.* propagules (Toussoun et al., 1969). *F. oxysporum* and *F. solani* could be isolated from seedlings grown in a nursery, but the *Fusarium* populations rapidly declined in the forest. Aqueous extracts of conifer needles (*Pinus ponderosa* Laws., *Abies concolor* (Gord. & Glend.) Hoeps., *Sequoia sempervirens* (Lamb) Endl.) caused 96-98% germination of the chlamydospores, but the germ tubes lysed before daughter chlamydospores could form. The germ tubes formed abnormally small tips before lysis (Toussoun et al., 1969). Additional studies revealed that water extracts of conifer needles contained shikimic, malic, citric, quinic and phosphoric acids (Muir et al., 1964). The germination of *F. oxysporum* f.sp. *lini* chlamydospores was directly proportional to the concentration of the shikimic and quinic acids (Hammerschlag and Linderman, 1975). Hammerschlag and Linderman (1975) concluded that if the germination-lysis reaction occurred repeatedly, *Fusarium* eventually would be eliminated from the soil. The *Fusarium* isolated from the seedlings was associated with the grass between the rows in the nursery, since Graminae species selectively encourage *Fusarium spp.* (Toussoun, 1975).

Crop rotations have been effective for reducing disease in some crops. *Fusarium oxysporum* f.sp. *apii* was reduced by rotating celery with onions (Elmer and Lacy, 1987). Soluble residue extracts from the onions may have stimulated chlamydospore germination in the absence of a suitable host (Elmer and Lacy, 1987).

Organic Amendments. Many organic amendments have been tested to induce suppression of *Fusarium spp.* in soil. Mitchell and Alexander (1961) found that application of 200 lbs/acre of chitin reduced the severity of *F. solani* f.sp. *phaseoli* and *F. oxysporum* f.sp. *conglutinans* in radish. They suggested that chitin encouraged the development of actinomycetes which degrade the cell walls of fungi. However, the application rate was not economically feasible and was replaced by other materials in later experiments. Adachi et al. (1987) revived the work on *F. solani* f.sp. *phaseoli*, but found that the effects of chitin decreased after three cultivations.

Zakaria and Lockwood (1980) added barley, wheat, corn, soybean, sugarbeet pulp, alfalfa meal, linseed meal, cottonseed meal, soybean meal and ground crab shells to *Fusarium*-infested soil. They found that 2% linseed, cottonseed or soybean meal reduced the populations of *F. moniliforme* and *F. solani* from 10^5 to 10^1-10^2 cfu/g of soil after 4 weeks in containers. Soybean meal was the most effective treatment, reducing the *Fusarium* population at a concentration of 0.25% (w/w) over a range of WHC. However, field experiments did not support these greenhouse results. In addition, linseed and cottonseed meals were phytotoxic. They concluded that toxic, volatile substances

were produced during the decomposition of the oilseed meals. Ammonia was thought to be the active component (Zakaria and Lockwood, 1980).

Tu and Cheng (1990) applied a complex mixture of bagasse (4.4%), rice husk (8.4%), oyster shell powder (4.2%), urea (8.2%), $CaHPO_4$ (13.1%), potassium nitrate (1.0%) and slag (60.5%) and found that asparagus growth improved and the population of saprophytic *Fusarium* increased. Inoculating soil with saprophytic *Fusarium* at 4.5×10^4 cfu/g reduced *Fusarium* infection from 24.45 to 12.16% (Tu and Cheng, 1990).

Mutitu et al. (1991) found that farmyard manure and coffee hulls reduced *Fusarium* in beans. They isolated a *Bacillus spp.* as the active organism, which suggests that organic amendments control *Fusarium spp.* by modifying the soil microflora.

Brassica Residues. Ramirez-Villapudua and Munnecke (1988) added residues of cabbage (*B. oleracea* var. *capitata* L.), kale (*B. oleracea* var. *virdis* L.), mustard (*B. nigra* L.), cauliflower (*B. oleracea* var. *botrytis* L.), broccoli (*B. oleracea* var. *italica* L.), collard (*B. oleracea* var. *virdis* L.), Brussels sprouts (*B. oleracea* var. *gemmifera* L.), turnip (*B. rapa* L.), radish (*Raphanus sativus* L.), alfalfa hay (*Medicago sativa* L.), wheat straw (*Triticum aestivum* L.), chicken manure, and steer manure to *Fusarium*-infested soil. They found that 2% dry cabbage, kale, or mustard reduced the population of *Fusarium oxysporum* f.sp. *conglutinans* (FOC), whereas wheat residue, chicken manure and steer manure increased the populations. A combination of solar heating and cruciferous residues reduced the population and disease incidence to

near zero. Solarization also reduced populations of *F. oxysporum* f.sp. *lycopersici* in tomato (Greenberger et al. 1987).

Additional research with dry cabbage residue indicated that survival of the FOC propagules was directly proportional to the concentration of the amendment and the time of exposure (Ramirez-Villapudua and Munnecke, 1988). In sealed jars, FOC survival was reduced to zero after 15 days. However, in open jars, the population increased sharply, then returned to near control levels, indicating the loss of volatile compounds. Radial growth of the fungus was inhibited by the volatiles of cabbage after 24 h, but resumed its original growth rate when transferred to fresh air. While the total culturable fungi decreased with cruciferous residues, the total culturable bacteria increased 16-fold. Ramirez-Villapudua and Munnecke (1988) suggested that volatiles stimulate germination of FOC propagules but continued exposure makes them susceptible to attack by other microflora.

The effects of cruciferous residues on fungi have been studied more thoroughly in *Aphanomyces euteiches* root rot of pea (*Pisum sativum*). Papavizas and Lewis (1971) incorporated 0.5% corn, Brussels sprouts, collards, soybeans, cress, turnip, mustard, kohlrabi, kale, or cabbage into dry soil. They found that cruciferous and soybean residues were effective against *A. euteiches* and did not reduce pea stand if added 3 weeks before planting. The beneficial effects may be due to the fumigation action of volatile sulfur-containing compounds arising from microbial decomposition of the residue (Papavizas and Lewis, 1971). Suppression of the *Aphanomyces* root rot lasted for 15 weeks (Papavizas 1966).

Lewis and Papavizas (1971) found that isothiocyanate (ITC) was extremely toxic to *Aphanomyces*. Growth was prevented by 0.7 ppm, and the fungus did not recover when transferred to fresh air (Lewis and Papavizas, 1971). Allyl-isothiocyanate (AITC) and methyl-isothiocyanate (MITC) were the most effective forms. MITC is used in the commercial fumigant Vorlex (1,3-dichloropropene + MITC). It also results from the decomposition of dazomet [3,5-dimethyl-tetrahydro-1,3,5,2H-thiadiazine-2-thione] and metham [sodium N-methyl-dithiocarbamate (anhydrous)]. Dazomet was effective against *Aphanomyces* at 50-200 ppm, although lower concentrations were required in closed containers (Papavizas and Lewis, 1971). ITC also is derived by enzymatic degradation of glucosinolates found in the *Brassica spp.*

Brassica Compounds. Glucosinolates are a group of secondary compounds produced by the *Brassica spp.* The glucosinolate-thioglucosidase system is a defining character of the Capparales family (Chew, 1988; Larsen 1981). The thioglucosidase enzymes (myrosinases) are produced by the plants, but are isolated from the glucosinolates in idioblast cells (Davis 1988). When the plant is injured, the myrosinases interact with glucosinolates and release isothiocyanate. Certain bacteria, fungi, mammals, and the cabbage aphid have thioglucosidase enzymes (Davis 1988; Larsen 1981). Glucosinolates are hydrophilic, non-volatile compounds that are stable at neutral pH (Larsen, 1981).

Isothiocyanates are volatile, highly reactive compounds with a strong smell and taste (Larsen 1981). Isothiocyanates have been shown to reduce fungal growth. Allyl

isothiocyanate is a known inhibitor of protein synthesis, but is believed to dissipate too quickly to affect seed germination and viability (Mason-Sedun 1986).

The morphology of the sexual structures and mycelium of *A. euteiches* were affected adversely by ITC. Zoospore motility and formation stopped after 2-6 h of exposure to ITC (Lewis and Papavizas, 1971). Hyphae exhibited abnormal protrusions, and the oogonia were deformed (Papavizas and Lewis, 1971). Chan and Close (1987) conclude that soil with a disease severity index of 50 could be reduced to a level of no risk by one or two plantings of rape.

Brassicas are known to contain volatile compounds that are phytotoxic (Ramirez-Villapudua, 1985). High concentrations of cabbage caused browning and stunting of pea roots (Papavizas and Lewis, 1971). Ramirez-Villapudua and Munnecke (1988) found that tomatoes quickly wilted and died in cabbage-amended soil, indicating a residual allelopathic effect. The effects may last at least six months in the field (Papavizas and Lewis, 1971).

The concentration of glucosinolates in the various Brassica species has been investigated in relation to phytotoxicity to subsequent crops. *B. campestris* residue inhibited wheat growth by 43-57% (Mason-Sedun 1986). Many of the *Brassica spp.* have different quantities of glucosinolates depending on cultivar, seeding date, and harvest date (Davis 1988). The toxicity range from lowest to highest is: *B. oleracea* < *B. campestris* < *B. napus* < *B. nigra* < *B. kaber* (Mason-Sedun 1986). The conditions that increase the phytotoxicity of brassica residues are high temperatures (>30C), long days, low nutrition, water stress, and autoclaving (Mason-Sedun 1986). The

glucosinolate concentration also depends on location within the plant. Glucosinolates are 10-fold higher in the seed meal than in leaves and stems of brassica plants (Davis 1988).

Most soils suppress disease or pathogens to some degree (Hornby, 1983). Addition of organic amendments may improve the antagonistic qualities of these soils or may cause germination and lysis of the pathogen propagules. The purpose of this dissertation was to examine two methods for enhancing the natural suppressiveness and reducing Asparagus Decline Syndrome.

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CHAPTER 2

INFLUEUNCE OF *BRASSICA SPP.* RESIDUES ON *FUSARIUM SPP.* IN ASPARAGUS

Abstract

Brassica spp. residues have been shown to control *Fusarium* diseases in other crops. Seven brassicas were incorporated in the field and greenhouse. The brassicas included 'Premier' kale (*B. oleracea* L. *virdis*), 'Rondo' turnip (*B. rapa* L.), 'Global' canola (*B. napus* L. *annua*), 'Glacier' canola (*B. napus* L. *biennis*), yellow mustard (*B. nigra* L.), 'Dwarf Essex' oilseed rape (*B. napus* L. *biennis*), 'Humus' oilseed rape (*B. napus* L. *biennis*) and sorghum-sudangrass (*Sorghum sudanense* (Piper) Stapf.) as the control. Brassica residues had no effect on disease incidence in asparagus or *Fusarium* populations. Addition of plastic covers, which produced solarization, did not affect the disease incidence. Brassica residues were phytotoxic to several indicator crops, including asparagus. Chemical extraction of the brassica residues did not yield any *Fusarium*-inhibitory compounds.

Introduction

Asparagus (*Asparagus officinalis* L.) is an important vegetable crop in Michigan. In 1991, Michigan produced 12,950 tons of asparagus with a value of $14.1 million on 23,500 acres of land (Michigan Agricultural Statistics Service, 1992). Michigan asparagus production has averaged 1200 pounds per acre over the past decade (Michigan Agricultural Statistics Service, 1992). However, yields of 3000 pounds per acre are achieved in some fields (Hodupp, 1983), indicating that a majority of the acreage is not producing its full potential. Replanting these fields has limited benefit, because 50% of young asparagus plants die within five years of planting and replanted fields often yield only half of the expected production (Hodupp, 1983).

The phenomenon of declining yields has been termed Asparagus Decline Syndrome (Grogan and Kimble, 1959). It is characterized by stunted, yellow, wilted plants and blank spaces in the rows. The major causes are thought to be soil microorganisms and allelopathic chemicals which adversely affect the growth of asparagus plants (Hartung, 1987). The primary crown and root rot organisms are *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM).

Methods for controlling *Fusarium spp.* in asparagus currently are not available. Resistant varieties, which have proven effective for control of *Fusarium spp.* in other vegetable crops (Mace et al., 1981) have not been found in asparagus (Stephens et al., 1989). Chemical treatments with benomyl [methyl 1-(butylcarbamoyl)-1H-benzimidazol-2-ylcarbamate] and captan [N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide] produced no positive effects on crown survival or yields (Lacy, 1979).

Ramirez-Villapudua and Munnecke (1987) found that cabbage (*Brassica oleracea* var. *capitata*), kale (*B. oleracea* var. *virdis*) or mustard (*B. nigra*)residue at 2% of the soil dry weight reduced the population of *F. oxysporum* f.sp. *conglutinans* in cabbage. The addition of a plastic cover, which produced solar heating, reduced the *Fusarium* populations to near zero.

Brassica spp., such as cabbage, kale or mustard, contain glucosinolates which degrade to isothiocyanate. The beneficial effects of brassica residues may be caused by the fumigation action of these volatile, sulfur-containing compounds arising from microbial decomposition of the residue (Papavizas and Lewis, 1971). Isothiocyanates are extremely toxic to some fungi and are used in the commercial fumigant Vorlex (1,3 dichloropropene + methylisothiocyanate). Many *Brassica spp.* contain higher concentrations of glucosinolates than cabbage (Davis, 1989), and may be more effective for controlling pathogens.

Brassicas also are phytotoxic. Ramirez-Villapudua and Munnecke (1987) found that tomatoes quickly wilted and died when transplanted into cabbage-amended soil. Mason-Sedun (1986) found that wheat yields were reduced 43-57% when following a canola crop. The objectives of this study were to determine the effect of brassica residues on FOA and FM; and to determine the duration of *Brassica* phytotoxicity.

Materials and Methods

Plants, inocula and media. The asparagus selection 'Syn 4-56' (Nourse Farms Inc., South Deerfield, MA) was used in all experiments. Seeds were surface-disinfected with

0.5% NaOCl (20% bleach) for 20 min, followed by infusion with benomyl (2.5g) in acetone (100ml) for 18-20 h (Damicone et al., 1981). Seeds were rinsed with acetone, followed by sterile deionized water and then dried on a sterilized clay tile (Stephens and Elmer, 1988). Seedlings were germinated on 0.6% water agar and grown in 96-cell (5 x 28 x 53 cm) plastic trays. Seedlings were fertilized once a week with 1000 ml/tray of 200 ppm Peters 20N-8.7P-16.6K fertilizer. Transplants used in the experiments were washed to remove the soil and inspected for absence of Fusarium symptoms.

Single-spore cultures of FOA (isolate FOA50) and FM (isolate F12) were obtained from cultures stored in muck soil and grown on potato dextrose agar (PDA). For in vitro studies, spores were washed from 14-day-old culture plates and diluted to 10^6 colony forming units per milliliter (cfu/ml). For soil studies, a 5-mm agar plug of FOA or FM was inoculated into autoclaved fescue seed and grown for two weeks, with mixing each day. The fescue seed then was dried and ground to pass a 2-mm sieve in a Wiley mill.

The soil used in all experiments consisted of a Spinks loamy sand (Psammetitic Hapludalf, sandy, mixed, mesic, pH 6.4, P 195 $kg \cdot ha^{-1}$, K 110 $kg \cdot ha^{-1}$, Ca 538 $kg \cdot ha^{-1}$, Mg 179 $kg \cdot ha^{-1}$, CEC 2 $meq \cdot 100g^{-1}$, bulk density 1.4-1.7 $g \cdot cm^{-3}$, organic matter 2-4%) obtained from Hart, Michigan. It was passed through a 2-mm sieve before use in the greenhouse studies.

Selection in the Field. A 20-year-old asparagus field near Hart, MI was plowed out in the summer of 1989 because of declining yields typical of *Fusarium* infection. The field

was prepared by adding 313.6 kg·ha^{-1} of 20N-4.4P-8.3K fertilizer and disking. Seven brassicas and a control were planted in 9.1 x 18.3-m plots. The brassicas included 'Premier' kale (*B. oleracea* L. *virdis*), 'Rondo' turnip (*B. rapa* L.), 'Global' canola (*B. napus* L. *annua*), 'Glacier' canola (*B. napus* L. *biennis*), yellow mustard (*B. nigra* L.), 'Dwarf Essex' oilseed rape (*B. napus* L. *biennis*), 'Humus' oilseed rape (*B. napus* L. *biennis*)(courtesy of Dr. Dick Auld, University of Idaho) and sorghum-sudangrass (*Sorghum sudanense* (Piper) Stapf.) as the control.

In September 1989, the number of plants and fresh weights were collected from 1 m^2 of each plot. Soil samples (200 g) were collected randomly from the top 10 cm of each plot to determine the microflora populations. The brassicas subsequently were disked and plowed-down, and a rye (*Secale cereale* L.) cover crop was planted for the winter.

In May 1990, the plots were divided in half. One half was used to determine *Fusarium* infection and phytotoxicity, the other half was planted to the same brassica to determine if a second year of cropping would increase disease suppression. Soil samples from each plot were collected again on 3 May. On 19 May the rye was killed with Ranger herbicide (glyphosate, 3378 ml·ha^{-1}). On 31 May 1990, 3 x 3-m indicator plots were established containing 'Syn 4-56' asparagus, 'Pik Red' tomatoes (*Lycopersicum esculentum* L.), 'Market Prize'cabbage (*B. oleracea* var. *capitata* L.), and 'Discover' cucumbers (*Cucumis sativa* L.) for rating of the brassica phytotoxicity.

Three 'Syn 4-56' asparagus seedlings were excavated from each plot on three dates (3 July, 26 July, 20 August) to rate for *Fusarium* infection. The disease rating

scale was 1-5, where 1 = a healthy seedling, 2 = slight infection, 3 = moderate infection, 4 = severe infection, and 5 = dead or dying seedling.

The other half of each plot was rototilled on 30 June and planted with the same brassica which was grown in the previous year. Plant stands and fresh weights were collected from 1 m^2 of each plot in September 1990, before the brassicas were plowed down. The experiment was terminated before the Spring of 1991 based on results from other studies.

The field was returned to commercial asparagus production in the Spring of 1991. In the Fall of 1993, stand counts from two rows in each plot were collected to evaluate any residual effects of the brassicas on the asparagus.

The experimental design was a randomized complete block with five blocks. The asparagus rating data were split over the three sampling times. The data were analyzed by analysis of variance (ANOVA) using MSTAT-C (MSTAT-C, Michigan State University, E. Lansing, MI). The brassica treatments were compared to the control using non-orthogonal F-tests.

Soil Microflora. Soil samples collected from the field in Hart, MI were air-dried and stored at 4C before assay. The samples were diluted serially and spread on plates containing selective media to determine the microflora populations. Media included Komada's for *Fusarium spp.* (Komada, 1975), amended Czepek's Rose Bengal for fungi (Hartung, 1987), Tryptic Soy Agar for bacteria (Martin, 1975). Several media were tested for selective isolation of actinomycetes: chitin agar (Hsu and Lockwood, 1975),

NZ-Amine (Hartung, 1987) and water agar (Tiedje and Dazzo, 1982). The final procedure involved heat-shocking the sample for 2 min at 60C then plating on water agar amended with cycloheximide. Colonies were counted after two days for bacteria, four days for fungi and *Fusarium*, and seven days for actinomycetes. FOA and FM were identified by visible morphology.

The experimental design was a randomized complete block with five blocks and two sampling times (Fall 1989, Spring 1990). The data were analyzed by ANOVA and means were separated by LSD.

Phytotoxicity of Residues. *Brassica* residue (2% of soil dry weight) was combined with unsterilized Spinks loamy sand field soil (2500 g) in the greenhouse (21C day, 10C night) to determine the duration of the phytotoxicity. Treatments included the seven brassicas listed above plus sorghum-sudangrass and poplar excelsior (*Populus alba* L.) as controls. All residues were dried at 60C and ground to pass a 2-mm sieve. The soil was placed in 4-liter clay pots and watered twice a week to maintain soil moisture. Soil samples (130 g) were collected in petri dishes at 0, 2, 4, and 12 weeks and 10 seeds of five bioassay crops were germinated in the soil to determine phytotoxicity. The bioassay crops included: 'Syn 4-56' asparagus, 'Curly' cress (*Lepidium sativum* L.), 'Yorkstar' wheat (*Triticum aestivum* L.), 'Discover' cucumber and 'Market Prize' cabbage. The asparagus seeds were pregerminated on filter paper to assure viable seedlings. The germination percentages were recorded and root lengths were measured with a ruler after

3 days for cucumber, 4 days for wheat, 6 days for cress, and 7 days for asparagus and cabbage.

The experimental design was a randomized complete block with a split over time and four blocks. The root length data were converted to a percentage of the excelsior control for simplicity of interpretation and analyzed by ANOVA. The means were separated by LSD. Asparagus root growth with Sudan and kale was compared to the other brassica using non-orthogonal F-test.

Selection in the Greenhouse. In the Spring of 1990, brassica residue (2% of soil dry weight) was incorporated into pasteurized field soil in 500-ml pots to determine the relative *Fusarium*-inhibition among the species tested. In January the seven brassicas plus sorghum-sudangrass were seeded in the greenhouse. On 20 February, steam-pasteurized Spinks loamy sand soil was inoculated with 1 g of fescue seed inoculum containing FOA or FM and incubated for 5 weeks to allow the *Fusarium* to colonize the soil. On 23 March, dry, ground brassica (12.5 g) or fresh, chopped brassica (2% on dry weight basis) were incorporated and incubated for 5 weeks to allow dissipation of the phytotoxic compounds. Poplar excelsior also was included as a control. On 4 May, a five-week-old asparagus seedling was transplanted into each pot. The seedlings were watered every other day with 100 ml of deionized water and fertilized every 2 weeks with 100 ml of 200 ppm 20N-8.7P-16.6K fertilizer. After 10 weeks, the seedlings were removed from the pots, rinsed and rated for *Fusarium* infection using the 1 to 5 scale. The root lengths were estimated by the line-intersect method (Tennant, 1975).

The experimental design was a completely randomized design with nine brassica treatments, three Fusarium species, fresh versus dry brassica and five replicates. The data were analyzed by ANOVA and LSD. Relationships between the treatments were examined by othogonal contrasts.

Soil samples (10 g) were collected from one replication on three dates (23 March, 4 May, 31 July) to determine if *Fusarium* was present at each stage of the experiment. The samples were air-dried and stored at room temperature. At the end of the experiment, the samples were diluted and plated on Komada's medium (Komada, 1975). The samples from a single *Fusarium* treatment for all brassica treatments were plated at the same time to allow comparison.

Solarization in the Field. During the Summer of 1990 an old asparagus plot with a history of *Fusarium* infection was removed at the Horticultural Teaching and Research Center in East Lansing, MI. The old asparagus crown tissue was removed carefully to reduce the *Fusarium spp.* inoculum. The plot was divided into twelve 4.5 x 5.4-m sections. 'Humus' oilseed rape, which had shown some control of *Fusarium* in other studies, was planted (11.4 $kg \cdot ha^{-1}$) in one-half of the sections on 17 September 1990. The rape reached the five-leaf stage before cold weather halted its growth.

On 17 May 1991, the rape was 60-cm tall and flowering. Stand counts and fresh weights were collected from 1 m^2 of each plot. The rape was cut with a hand scythe and disked into the soil. Selected plots were covered with 6-mil black plastic film to determine if solarization would improve *Fusarium* inhibition. The soil temperature was

measured at 0, 1, 5, 10, and 15-cm depths on 28 June, which was a sunny, clear day (29C air-temperature, 2 pm.). Soil samples (100 g) were collected randomly from the top 10 cm of each plot before and after the plastic treatment to determine FOA and FM populations. After 8 weeks, the plastic was removed and 'Syn 4-56' asparagus seedlings were planted to test for disease. After 6 weeks more (5 September), three seedlings were excavated from each plot and rated for *Fusarium* infection. Root lengths were estimated by the line-intersect method (Tennant, 1975).

The experimental design was a two-factor randomized complete block with two brassica treatments and two plastic-covered treatments and three blocks. The data were analyzed by ANOVA and means were separated by LSD. The brassica treatments were compared to bare soil and the covered treatments were compared to uncovered using orthogonal contrasts.

Extraction of Compounds. Four procedures were utilized for extraction of *Fusarium*-inhibitory compounds from brassicas.

Boiling Water. Brassica residue (10 g) was extracted with boiling water (300 ml) to denature the myrosinase enzymes which cause conversion of glucosinolates to isothiocyanates. The extracts were filtered through Whatman #1 filter paper and lyophilized at 5C. Each extract was spotted at 125, 250, 500, 1000 μg in 10 μl water on PDA plates spread evenly with either FOA or FM. The zone of inhibition was measured after 4 days incubation at 27C.

Acetone-Ethyl Acetate. Frozen 'Humus' rape or fresh mustard flowers and leaves were extracted with acetone. The brassica tissue (200 g) was blended with acetone (1500 ml) for 4 min. The extract was filtered through Whatman #1 filter paper and evaporated under vacuum. The remaining solution was partitioned three times with ethyl acetate (100 ml, 50 ml, 50 ml). The ethyl acetate fraction was dried under vacuum. The water fraction was dried with excess methanol under vacuum. These extracts were spotted at 0, 250, 500 μg in 10 μl of methanol on PDA plates spread evenly with FOA or FM. The zone of inhibition was measured after 4 days incubation at 27C.

Sequential Extraction. Brassica seeds were extracted sequentially with hexane, ethyl acetate, and methanol. Seeds contained the highest concentration of glucosinolates (Davis, 1988) and glucosinolates (or the degradation product, isothiocyanate) were inhibitory to *Fusarium* (Papavizas and Lewis, 1971). 'Global' canola seeds (200 g) were blended with hexane (1000 ml) for 4 min. The extract was filtered through Whatman #1 filter paper and additional hexane (500 ml) was added to the seeds for 1 h. The second extract was filtered through Whatman #1 filter paper and additional hexane (500 ml) was added to the seeds and allowed to soak for 24 h. The hexane was removed and replaced with ethyl acetate (500 ml), which was removed after 1 and 24 h. The ethyl acetate was replaced with methanol (500 ml), which was removed after 1 and 24 h. All extracts were dried under vacuum and spotted at 0, 250, 500 μg in 10 μl of DMSO on PDA plates spread evenly with FOA or FM. The zone of inhibition was measured after 4 days at 27C.

Soxhlet. Mustard residue (17.2 g) was extracted for 24 h with 250 ml of methanol in a Soxhlet apparatus. Poplar excelsior was extracted as the control. The extracts were dried and tested at 0 and 500 μg in 10 μl of DMSO on PDA plates spread with FOA or FM. The zone of inhibition was measured after 4 days at 27C.

Conversion of Compounds. Some naturally occurring compounds require microbial conversion to become active (Chase, 1990). Since release of isothiocyanate from glucosinolates in brassicas requires myrosinases (Davis, 1988), the inhibition of *Fusarium* may require decomposition of glucosinolates by other soil microorganisms.

A culture of naturally occurring soil microorganisms was isolated from Spinks loamy sand on PDA plates. A suspension of the microorganisms was made by adding 10 ml of saline solution to the plates and stirring with a glass rod.

'Humus' canola residue (2 g) was added to 100 g of sterile, washed sand in 250-ml Erlenmeyer flasks. Sterile water (10 ml) and the microorganism suspension (1 ml) were added to each flask. The control flasks received 11 ml of sterile water. The flasks were incubated at 27C. Samples were extracted at 0, 5, 10, 15 days of incubation with methanol (50 ml x 3 times) and dried under vacuum. The extracts were spotted at 0, 125, 250, 500 μg in 25 μl of methanol on PDA plates spread evenly with FOA or FM. The zone of inhibition was measured after 4 days at 27C.

Results and Discussion

Selection in the Field. Brassicas did not reduce the incidence of disease in asparagus (Appendix A). However, the average rating of all of the brassica treatments combined (2.1) was lower than the sudangrass control (2.3) at the 5% level.

The brassicas yielded from 1.9 (mustard) to 5.7 (turnip) $kg \cdot m^{-2}$ of fresh weight, depending on type of crop (Appendix A). Horricks (1969) found that 7.8 $t \cdot ha^{-1}$ of canola residue reduced the dry weight and plant height of wheat, barley, and oats. However, there were no differences among the toxicity ratings for cabbage, cucumber, and tomato in this study, indicating that phytotoxic compounds were not left in the soil.

After three years, the asparagus field had many missing and stunted plants. However, there were no differences among the stand counts that could be attributed to the brassica treatments.

Soil Microflora. The brassica treatments generally did not affect the populations of FM, total *Fusarium* or total fungi (Appendix B), so the data were combined by sampling time. The decrease in FOA and FM from Fall 1989 to Spring 1990 suggests that *Fusarium* declines over the winter, as one would expect (Table 1).

Although the populations were "low" for all treatments (Stephens et al., 1989), none were completely disease-free. For example, 'Humus' reduced the FM population to zero (Appendix B), but did not eliminate the disease because of the presence of FOA (Appendix A).

Table 1. Influence of time on populations of microflora from Spinks loamy sand field soil.

Time	FM	FOA	Total Fusarium	Total Fungi
Fall 1989	141[a]	167[a]	3903	8547
Spring 1990	38	60	2945	9853

[a]F value significant at $P \leq .05$

'Humus' rape, turnip and 'Dwarf Essex' rape had the lowest combined populations of FOA and FM. This information combined with the Fusarium ratings from the Selection in the Field Study, above, suggested the use of 'Humus' for subsequent research.

No meaningful results are available for the bacteria and actinomycetes counts. The actinomycetes were not plated on the same media, so no comparisons could be made. The bacteria had extreme variability in their counts, suggesting contamination from external sources. Ramirez-Villapudua (1986) found a 16-fold increase in bacteria following cabbage residue. This study can neither confirm nor challenge that result.

Phytotoxicity of Residues. The toxicity of the brassica residues on asparagus varied over time. In general the brassicas inhibited root growth early, but this inhibition dissipated by 12 weeks. Mustard was the least inhibitory at the beginning of the study. After 12 weeks, 'Global' canola and mustard stimulated root growth by 47 and 48%, respectively, over the excelsior control. A interesting comparison was found between sudan and kale, which were similar to the other brassica treatments for the first 4 weeks, but continued to inhibit growth after 12 weeks (Table 2).

Curly cress was considered a sensitive indicator of phytotoxicity, which was demonstrated by the amount of inhibition remaining after 12 weeks (Appendix C). 'Humus' oilseed rape continued to inhibit root growth of cress to 44% of control after 12 weeks. 'Global' canola and mustard stimulated root growth 45 and 65%, respectively, over the excelsior control.

Table 2. Growth of asparagus roots (percentage of the excelsior control) 0, 2, 4, and 12 weeks after incorporation of brassica residues.

Treatments	0, 2, 4 weeks	12 weeks
Sudan, Kale	30.6	68.2
Other Brassicas	44.1	125.6[a]

[a]F values significant at $P \leq .05$

Wheat was less sensitive to the toxicity of brassica. All of the brassicas, except 'Global' canola, inhibited wheat root growth after 2 weeks, but none inhibited after 12 weeks (Appendix C). 'Glacier' canola was the least inhibitory (63%) at the beginning of the experiment. None of the treatments stimulated wheat root growth.

Cucumber was the least sensitive crop to brassica toxicity. Sudan, kale, turnip and 'Global' canola inhibited for just 2 weeks (Appendix C). None of the treatments inhibited after 12 weeks. Mustard was the least inhibitory (58%) at the beginning of the experiment. None of the treatments stimulated cucumber root growth.

Cabbage is sensitive to the compounds produced by other *Brassica spp.* The inhibition was very strong at the beginning, but none remained after 12 weeks (Appendix C). All of the brassicas inhibited cabbage root growth after 2 weeks, but not after 4 weeks. Sudan inhibited root growth for less than 2 weeks.

Mason-Sedun (1987) found that canola inhibited the growth and yield of wheat. Ramirez-Villapudua and Munnecke (1987) found that brassica residues improved the growth of cabbage. This study demonstrates that the phytotoxic effects depend on the subsequent crop and the degree of decomposition of the brassica residue. If conditions are warm and water is not limiting, phytotoxic compounds will dissipate in 4 weeks.

Selection in the Greenhouse. The disease ratings in the greenhouse were highly dependent on the treatments applied. Dry brassica alone increased the disease ratings of asparagus seedlings an average of 66% over the excelsior control (Appendix D). The

dry brassica may have contained *Fusarium spp.* inoculum since it had been grown in an old asparagus field.

Dry turnip, 'Global' canola, 'Glacier' canola, or 'Dwarf Essex' rape residue increased the disease ratings of FOA-inoculated plants by 25% over the excelsior control. All of the brassicas increased the disease ratings of FM-inoculated plants by an average of 73% over the excelsior control. The disease rating with dry sudan was 63% higher for FOA and 43% higher for FM than the excelsior control.

Poplar excelsior reduced the development of FOA or FM infection by 35% over the no residue controls, giving disease ratings of 1.6 and 1.4, respectively. Stephens et al. (1989) considered plants with a rating of 1.5 or less as disease-free. Excelsior may limit disease development by limiting the nitrogen available to *Fusarium spp.* or by improving the WHC of the soil, which will increase the population of microorganisms that can compete with *Fusarium spp.* Brassica residues provide a much richer supply of nutrients than the cellulose/lignan-carbon of excelsior.

The disease ratings for the fresh brassicas were compared with the no residue controls, since fresh excelsior was not tested. Sudan, turnip, or mustard reduced the disease rating of FOA-inoculated plants by 33%. The FM-inoculated plants were not affected by any of the brassica treatments.

The root lengths also were highly dependent on the treatments applied. The brassicas alone did not affect root growth of asparagus, except for dry mustard, which increased by 116% over the excelsior control. 'Global' canola, 'Glacier' canola, mustard, 'Dwarf Essex' rape promoted the growth of FOA-inoculated plants an average

of 67% over the excelsior control. All of the brassicas promoted the root growth of FM-inoculated plants an average of 150% over the excelsior control. Sudan with FOA did not promote root growth, but sudan with FM promoted growth 88%.

The root lengths from the fresh brassicas with FOA were not different than the no residue control, but fresh sudan showed a 48% increase in growth. Fresh 'Glacier' canola with FM promoted root growth by 36%, whereas fresh 'Humus' rape with FM decreased growth by 59% over the no residue control.

Dry 'Dwarf Essex' rape residue with FM produced an average root length of 1396 cm, longer than any of the other brassicas. It also had one of the highest disease ratings (2.8), suggesting that *Fusarium* infection was stimulating root growth to compensate for the disease.

Dilution of the soil samples collected during the greenhouse experiment indicated that *Fusarium* was present at each stage of the experiment (data not shown). *Fusarium* was not present in the control pots until the asparagus seedlings were transplanted, suggesting that it was transferred with the seedlings. FOA was the predominate organism in the control pots.

Solarization in the Field. The disease ratings, root lengths and fresh weights of seedlings grown in brassica-treated and solarized soil were not different than the control (Appendix E). However, root length estimates for seedlings with brassica (461 cm) were shorter than seedlings without brassica (631 cm), indicating a possible phytotoxic effect.

The toxicity should have dissipated from the uncovered plots during the plastic treatment; however, the soil was dry so there was little decomposition.

Soil temperatures were measured under the plastic covers and compared with the bare soil on 28 June 1991. The soil temperature just below the plastic was 48C compared with 39C for the bare soil. At the 1-cm depth the temperature was 37C for both covered and bare soil. At the 5- and 10-cm depths, the covered soil was 5C warmer than the bare soil. At the 15-cm depth, both soils were the same as the ambient temperature of 29C. Ramirez-Villapudua and Munnecke (1987) found reduced disease in cabbage after the soil had been heated to 45C, which would suggest that only the upper layers of soil are warm enough. However, no conclusions can be drawn from the temperatures in this study, since they were sampled on a single date.

Extraction of Compounds. Compounds which inhibit *Fusarium spp.* were not isolated by water or acetone extraction. Acetone extraction of mustard flowers yielded a fraction that was slightly inhibitory at 500 $\mu g \cdot ml^{-1}$. The hexane, ethyl acetate, and methanol extracts of 'Global' canola seeds did not inhibit FOA or FM. No *Fusarium*-inhibitory compounds were extracted by Soxhlet extraction.

Conversion of Compounds. All treatments gave slight inhibition at 500 $\mu g \cdot ml^{-1}$ with or without the microorganisms from soil extract. However, the zones of inhibition quickly became covered with fungal growth. Methanol alone was inhibitory to FOA and FM for a few days, therefore, DMSO was used and the inhibitory fractions were still

inhibitory. It was concluded that non-volatile *Fusarium*-inhibitory compounds were not present in these extracts.

In conclusion, brassica residues may produce volatile compounds which are inhibitory to FOA and FM. They cause a temporary suppression of *Fusarium*, which would have limited benefit to a perennial crop such as asparagus. Brassica residues also are toxic to certain crops, causing suppression of root growth for several weeks. Therefore, growers using brassica cover crops should select subsequent crops carefully.

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CHAPTER 3

INFLUENCE OF ANTIFUNGAL COMPOUNDS FROM A SOIL-BORNE ACTINOMYCETE ON *FUSARIUM SPP.* IN ASPARAGUS

Abstract

Asparagus Decline Syndrome is caused by *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM). Several soil-borne microorganisms have been found inhibitory to Fusarium diseases in other crops. A novel *Streptomyces spp.*(ME2-27-19A) was isolated from asparagus field soil and found inhibitory to *Fusarium spp.* in vitro. Chemical extraction of ME2-27-19A yielded compound(s) that were inhibitory. The inhibitory fraction was purified by vacuum-liquid chromatography and C18 medium-pressure chromatography and found inhibitory at 50 $\mu g \cdot ml^{-1}$. ME2-27-19A extract produced variable control of FOA and FM in vitro, and was phytotoxic at 1000 $\mu g \cdot ml^{-1}$. In soil, ME2-27-19A extract reduced the *Fusarium* population at 100 $\mu g \cdot ml^{-1}$, but also reduce the asparagus shoot length.

Introduction

Fusarium crown and root rot of asparagus (*Asparagus officinalis* L.) are caused by *Fusarium oxysporum* (Schlecht.) emend Snyd. & Hans. f.sp. *asparagi* Cohen (FOA) and *F. moniliforme* (Sheld.) emend Snyd. & Hans. (FM). Effective methods of controlling these diseases of asparagus are not currently available. Resistant varieties, which have proven effective in other vegetable crops (Mace et al., 1981), have not been found in asparagus (Stephens et al., 1989). Chemical treatments with benomyl [methyl 1-(butylcarbamoyl)-1H-benzimidazol-2-ylcarbamate] and captan [N-[(trichloromethyl) thio]-4-cyclohexene-1,2-dicarboximide] produced no positive effects on crown survival or yields over the controls (Lacy, 1979).

However, Smith et al. (1990) found that compounds produced by *Streptomyces griseus* var. *autotrophicus* inhibited *F. oxysporum* f.sp. *asparagi* in vitro and in the greenhouse. Kemira Oy, a European chemical company, has recently started marketing a biofungicide named Mycostop. It is based on *S. griseovirdis* and claimed to be effective against *Fusarium spp.*, although it not registered in the United States. The objective of this study was to determine if compounds extracted from a newly isolated soil microorganism would control *Fusarium spp.* in asparagus.

Materials and Methods

Isolation and culture. Strain ME2-27-19A was one of eight actinomycetes isolated from Spinks loamy sand soil (Psammetitic Hapludalf, sandy, mixed, mesic) collected from an old asparagus field in Oceana County, MI. It was compared with six other actinomycetes

known to have fungicidal activity and it was selected as the most inhibitory to FOA and FM. Biochemical tests were conducted using Biolog GN plates (Biolog, Inc., Hayward, CA). The microorganism was identified tentatively as a *Streptomyces spp.* by Dr. S.K. Mishra (Microbiology Laboratory, Krug International, Houston, TX).

Fermentation. Cultures of ME2-27-19A were grown in 2-liter baffled-bottom Erlenmeyer flasks, containing 400 ml of A-9 medium (peptone 5 g, glucose 10 g, "Brer Rabbit green label" molasses 20 g in 1 liter of distilled water). The inoculated flasks were placed on a rotary shaker at 150 rpm and 27C for 8 days.

Extraction of cells. Microbial cells from 6 liters of fermentation broth were centrifuged (16300 x *g*) after 8 days of growth. The cells were filtered under vacuum to remove excess medium and extracted by homogenizing in methanol:chloroform (MeOH:$CHCl_3$, 1.25 liters, 4:1 v/v) for 10 min. The extract was filtered through a sintered glass funnel under vacuum. The residue from the funnel was homogenized again with 1.25 liters of MeOH:$CHCl_3$ (4:1 v/v) and filtered. The filtrates were combined and dried under vacuum at 40C.

Antifungal/Antibacterial Bioassay. Cultures of *Fusarium oxysporum* f.sp. *asparagi* (MSU-FOA 50) and *Fusarium moniliforme* (MSU-FM F12) were grown on Potato Dextrose Agar (PDA) for 14 days. Cultures of *Aspergillus flavus* and *Candida albicans* were grown on YMG medium (yeast extract 4g, maltose 10g, glucose 4g, agar 12 g, in

1 liter of distilled water). Cultures of *Streptococcus aureus*, *Staphylococcus epidermis*, and *Escherichia coli* were grown on Emmons medium (neopeptone 10 g, glucose 20 g, agar 15 g in 1 liter of distilled water).

Cell or spore suspensions of the test organisms were made by adding 10 ml of sterile saline solution to a culture dish and stirring with a glass rod. The cell concentration was adjusted to 10^6 colony forming units per milliliter (cfu/ml) using a hemacytometer. Bioassy plates were made by spreading evenly 100 μl of the cell suspension in petri dishes containing 20 ml of the appropriate medium. Crude extract of ME2-27-19A was bioassayed by placing 100 μg of extract dissolved in 10 μl of DMSO (dimethyl sulfoxide) in the center of the bioassay plates and incubating at 27C for 4 days. A zone of inhibition characterized by absence of fungal growth was measured.

ME2-27-19A was also bioassayed by adding 500 or 1000 mg in 0.5 ml of DMSO to 100 ml of warm (50C) PDA before pouring the medium into petri dishes. A plugs (5mm) of FOA, FM, *Alternaria solani*, or *Sclerotinia sclerotia* were placed in the center of each petri dish. Radial growth of the fungi was measured daily for 7 days.

Agar Overlay. Crude extract of ME2-27-19A was chromatographed on silica TLC (thin layer chromatography) plates in $CHCl_3$:MeOH (2:1 v/v). The inhibitory bands were identified on the TLC by pouring 10 ml of molten (50C) YMG agar (amended with 6 g of agar in 1 liter of water) containing 10^5 cfu/ml of FOA, FM, *Aspergillus* or *Candida* over the developed TLC plates. The plates were placed in a sterile moisture chamber and incubated at 27C for 4 days.

Purification. The crude extract was purified by vacuum-liquid chromatography (VLC). Crude extract (386 mg), dissolved in MeOH, was loaded on a silica gel column (100 g, 35-75 μm mesh) and eluted with $CHCl_3$:MeOH (1:1 v/v). Twenty fractions (30 ml each) were collected and combined into three major fractions based on similar Rf values from TLC. The silica was eluted with 100% MeOH to remove any remaining compound. The fractions were bioassayed on PDA plates spread with FOA or FM. Zones of inhibition were measured after 4 days at 27C.

A preliminary ^{1}H-NMR spectrum of Fraction 2 was determined on a Varian Gemini 500 MHz spectrometer in deuterated-DMSO (d_6-DMSO).

Minimum Inhibitory Concentration. Fraction 2 from the VLC was bioassayed in test tubes containing liquid Emmons medium to determine the minimum inhibitory concentration. Fraction 2 (2 mg, major band at Rf .32) was dissolved in DMSO (200 μl) and serially diluted in the same solvent. A 20 μl aliquot of each dilution was mixed with 1.96 ml of Emmons medium. The tubes were inoculated with 20 μl of 10^5 cfu/ml solution of FOA or FM. The final concentrations were 0, 20, 30, 40, 50, 100 $\mu g \cdot ml^{-1}$ of Fraction 2 in 2 ml of solution. The inoculated tubes were incubated on the shaker at 27C and the results were recorded after 4 days. The lowest concentration of compound that totally inhibited growth of FOA or FM was recorded as the minimum inhibitory concentration (MIC) for that species.

Separation by C18 chromatography. Separation on C18 TLC plates in MeOH:H_2O (60:40 v/v) indicated that Fraction 2 contained three compounds. Additional crude extract was purified by VLC. Fraction 2 was purified further by dissolving 0.4 g in 4 ml MeOH and injecting it into a medium-pressure C18-column (Chemco Scientific Co., Ltd., Osaka, Japan, 4 x 56 cm, 37-55 μm mesh). Forty fractions (10 ml each) were eluted in MeOH:H_2O (60:40 v/v, 25-30 psi, 2 ml·min^{-1} flow rate) and combined into five major fractions based on similar Rf from C18-TLC. These fractions were bioassayed on PDA plates spread evenly with FOA or FM. Zones of inhibition were measured after 4 days at 27C.

Fraction 3 from the medium-pressure column was further purified on C18 preparative-TLC plates (500 μm thickness) in $CHCl_3$:MeOH (2:1 v/v). The ultra-violet (UV) absorbance of the compound was determined from 800 to 200 nm on a Shimadru (Kyoto, Japan) UV260 spectrometer.

Insecticidal assay. The insecticidal assay was conducted on fourth instar mosquito larvae (*Aedes aegypti*). Mosquito larvae were hatched by placing eggs in 200 ml of degassed, deionized water with a pinch of liver powder. After 2 weeks, five to seven fourth instar larvae in 975 μl of deionized water were placed in 4-ml test tubes. ME2-27-19A crude extract (0, 125, 250, 500, or 1000 μg) in 25 μl of DMSO was placed in the test tubes and left in the dark at room temperature. The numbers of dead larvae were recorded at 2, 4, 6, 12, 24, 48 h. The experiment was repeated with 100 μg each of the VLC and medium-pressure column fractions.

Phytotoxicity. The herbicidal assay was conducted on 'Syn 4-56' asparagus, 'Curly' cress (*Lepidium sativum* L.) and 'Campbell 1327' tomato (*Lycopersicum esculentum* L.) seedlings. The three fractions from VLC were dissolved in MeOH and placed on sterile filter paper in petri dishes at 0, 500, and 1000 μg. The MeOH was allowed to evaporate and 1 ml of sterile water was added to each petri dish. Ten seeds of each bioassay crop were placed in the petri dishes. The petri dishes were sealed with parafilm and incubated at 27C. The root and shoot lengths of the seedlings were measured after 4 days for cress and 14 days for asparagus and tomato.

The experimental design was a randomized complete block design with three blocks. The data were analyzed by analysis of variance (ANOVA) using MSTAT-C (MSTAT-C, Michigan State University, E. Lansing, MI). The means were separated by LSD and relationship between treatments were analyzed by linear contrasts.

In Vitro Assay with Asparagus and Fusarium. The effects of ME2-27-19A extract on FOA and FM in asparagus were determined by incorporating crude extract into 20 ml of partially cooled (50C) Hoaglands agar (Hoagland and Arnon, 1938). The final concentration of the extract was 0, 100, 200, 1000 $\mu g \cdot ml^{-1}$ in 20 ml of agar. Asparagus seedlings were surface-disinfected by acetone infusion (Damicone et al., 1981) and germinated on water agar. Germinated seedlings were transferred to the test tubes and grown in a growth chamber under 16-h days with 15C night temperature and 25C day temperature. After 3 weeks, as the seedlings were initiating lateral roots, 0.1 ml of 10^7 cfu/ml solution of FOA or FM was added. The seedlings were grown for an

additional 5 weeks and rated for Fusarium infection on a scale of 1-5, where 1 = healthy seedling, 2 = slighty infected, 3 = moderately infected, 4 = severely infected, 5 = dead or dying seedling. The root and shoot dry weights also were measured.

The experimental design was a randomized complete block design with four blocks. The data were analyzed by ANOVA using MSTAT-C. The means were separated by LSD and the relationship between extract concentrations was analyzed by linear contrasts.

Assay of ME2-27-19A Extract in Sterile Soil. ME2-27-19A extract was incorporated into sterilized Spinks loamy sand soil to determine the effects of ME2-27-19A on asparagus and FOA or FM in soil. Since purification required several steps and yielded only a minute amount of pure compound, it was decided an active ingredient method should be used. A known amount of crude extract (2g) was separated by VLC and medium-pressure column as before. The active ingredient content was calculated from the extraction procedure as follows: 2 g of crude extract separated by VLC yielded 0.8125 g of Fraction 2. Separation of Fraction 2 in the medium-pressure C18 column yielded 0.0402 g of Fraction 3. Purification of Fraction 3 by C18 preparative-TLC yielded 0.0260 g of pure compound, or 13 $mg \cdot g^{-1}$ of crude extract.

Fusarium inoculum was prepared by adding a plug of FOA or FM to autoclaved fescue seed (*Festuca rubra* L.). The fungi were grown for 3 weeks with mixing each day. The seed was then air-dried and ground to pass a 2-mm sieve in a Wiley mill.

Asparagus seeds were surface-disinfected with 0.5% NaOCl (20% bleach) for 20 min, followed by infusion with benomyl (2.5 g) in acetone (100 ml) for 18 hr (Damicone et al., 1981). The seeds were rinsed with acetone, followed by sterile deionized water and dried on a sterilized clay tile (Stephens and Elmer, 1988). The seeds were germinated of 0.6% water agar.

The soil was Spinks loamy sand soil (Psammetitic Hapludalf, sandy, mixed, mesic, pH 6.4, CEC 2 meq·100 g^{-1}, BD 1.4-1.7 g·cm^{-3}, OM 2-4%) and was autoclaved on three consecutive days. Crude extract was incorporated into dry soil to give 0, 100, 250, 500, 1000 μg a.i./g of soil. Benomyl (50% a.i., wettable powder) was incorporated at the same active ingredient concentrations as the control. Fescue seed inoculum of FOA or FM were added to give 10^6 cfu/ml. Germinated asparagus seedlings were transferred to the treated soil and 1 ml of filter-sterilized, 100 ppm 20N-8.7P-16.6K fertilizer solution was added. The seedlings were grown in the growth chamber for 10 weeks and rated for Fusarium infection on the 1-5 scale. The shoot lengths, number of shoots, shoot dry weights and root dry weights also were measured.

The *Fusarium* population was counted by placing 10 g soil in 100 ml sterile saline solution and diluting serially. The 10^{-2} dilutions were plated on Komada's medium (Komada 1975) and the number of colonies were counted after 4 days. Similarly, bacterial counts were conducted by plating the 10^{-6} dilution on PDA.

The experimental design was a randomized complete block with five blocks. Only four blocks were sampled for the microflora populations because of the time

required to sample each block. All data were analyzed by ANOVA and means were separated by LSD. The extract concentrations were compared using orthogonal F-tests.

Results and Discussion

The wild type of ME2-27-19A produced 1 $g \cdot liter^{-1}$ of crude extract in A-9 medium after 8 days of fermentation. In the GN Biolog plates, ME2-27-19A used Tween 40, Tween 80, αD-glucose, methyl pyruvate, D-gluconate, D-glucuronate, and asparagine as carbon sources. The crude extract inhibited growth of *Aspergillus flavus*, *Candida albicans*, *Fusarium oxysporum* f.sp. *asparagi*, *Fusarium moniliforme*, *Gleosporium spp.*, and *Staphylococcus epidermidis* at 100 $\mu g \cdot ml^{-1}$. The inhibition of FOA and FM was similar to that of nystatin, another microbially produced antibiotic (Hazen and Brown, 1951).

Agar Overlay. Zones of inhibition remained over the bands at Rf .10, .65, and .68 on the agar overlay TLC plates. All three bands fluoresced under 366 nm UV-light. The band at Rf .10 was more stable when left exposed on silica TLC plates, and was selected for further purification.

Purification. Fractions 2 and 3 from the VLC were inhibitory to FOA and FM in vitro. The fraction that was eluted with 100% MeOH from the VLC column reacted with ninhydrin, indicating an amino-group. This fraction had caused much difficulty in earlier attempts to purify the compound because it would not remain soluble in MeOH or $CHCl_3$.

The preliminary ^{1}H-NMR experiment with VLC Fraction 2 in deuterated-DMSO revealed that it did not contain an aromatic moiety (Appendix J). The signal at δ6.25 appeared as a multiplet suggested that it contained olifinic bonds. The group of signals between 4.2-5.1 ppm suggested the presence of sugar or aliphatic protons attached to oxygenated carbon. The doublet at δ5.2 revealed the anomeric proton of a hexose moiety. The multiplets from 3.0-4.0 ppm indicated the presence of aliphatic or amino protons. Since the compound was not pure, additional spectral work will be carried out later.

Minimum Inhibitory Concentration. The minimum inhibitory concentration of the Fraction 2 from the VLC was 50 $\mu g \cdot ml^{-1}$ for FOA and FM. Smith et al (1990) found the MIC of faeriefungin was 12.5 $\mu g \cdot ml^{-1}$ for FOA.

Separation by C18 chromatography. Fraction 3 from the C18 medium-pressure column was the most inhibitory to FOA and FM. Further purification by C18 preparative-TLC was limited by the solubility of Fraction 3. It was soluble at 30 $mg \cdot ml^{-1}$ in MeOH, but insoluble in H_2O. On C18 preparative-TLC plates, it formed a precipitate which prevented the solvent system from moving. It finally was separated by loading just 21 mg on a 20 x 20 C18 plate (500 μm). The UV spectrum revealed absorption maxima at 349, 331, and 316 nm (Appendix I) and were indicative of one or more chromophores in the purified compound.

Insecticidal assay. The crude extract of ME2-27-19A killed 95% of the mosquito larvae at 125 $\mu g \cdot ml^{-1}$ after 12 h. However, the insecticidal fractions from the VLC were relatively nonpolar and different from the fraction that inhibited *Fusarium*, as demonstrated by the lack of insecticidal activity in the purified C18 fractions. Since insecticidal compounds were not of direct interest in this project, the compounds were not isolated.

Phytotoxicity. The seedling assay for toxicity showed no differences in the root elongation of asparagus or tomato (Table 1). However, the root length of curly cress was decreased 42% by VLC Fraction 3. The shoot length of asparagus was more than doubled by 1000 $\mu g \cdot ml^{-1}$ of VLC Fraction 3. The shoot length of tomato increased 21% with 1000 $\mu g \cdot ml^{-1}$ of VLC Fraction 1. Curly cress showed the least variation of these indicator plants.

In Vitro Assay with Asparagus and Fusarium. Although asparagus seedlings showed no inhibition from ME2-27-19A crude extract on filter paper, seedlings placed in Hoagland's agar containing 1000 $\mu g \cdot ml^{-1}$ of crude extract failed to grow. When these seedlings were transferred to fresh Hoagland's agar, they resumed normal growth.

Asparagus seedlings grown in Hoagland's agar had higher disease ratings when inoculated with FOA or FM than the control, as expected (Table 2). ME2-27-19A extract had no effect, except for inhibiting the asparagus at 1000 $\mu g \cdot ml^{-1}$, so the data were combined to compare the effects of *Fusarium*.

Table 1. Phytotoxicity from VLC fractions of ME2-27-19A crude extract.

Fraction	Concn. ($\mu g \cdot ml^{-1}$)	Root Length (cm)	Shoot Length (cm)	ASPARAGUS
1	0	2.7	0.73	
	500	1.9	0.99	
	1000	2.2	0.98	
2	0	2.4	0.77	
	500	3.2	1.27	
	1000	3.4	1.23	
3	0	1.4	0.69	
	500	2.5	0.95	
	1000	1.8	1.60	
$LSD_{.05}$ (interaction)		ns	ns	

Fraction	Concn. ($\mu g \cdot ml^{-1}$)	Root Length (cm)	Shoot Length (cm)	CURLY CRESS
1	0	1.9	1.6	
	500	1.5	1.4	
	1000	1.7	1.8	
2	0	1.9	1.6	
	500	2.0	1.8	
	1000	1.8	1.7	
3	0	1.9	1.6	
	500	1.4	1.6	
	1000	1.1	1.6	
$LSD_{.05}$ (interaction)		0.5	ns	

Fraction	Concn. ($\mu g \cdot ml^{-1}$)	Root Length (cm)	Shoot Length (cm)	TOMATO
1	0	1.2	3.3	
	500	1.2	3.4	
	1000	1.3	4.0	
2	0	1.0	3.6	
	500	1.1	3.6	
	1000	0.9	3.6	
3	0	1.0	2.9	
	500	1.2	3.2	
	1000	0.9	3.2	
$LSD_{.05}$ (interaction)		ns	0.6	

[z]Means represent the average of 3 replications.

Table 2. Effects of FOA and FM on asparagus seedlings in vitro.

FUS	Disease Rating[zy]	Root Dry Wt (mg)[z]	Shoot Dry Wt. (mg)[z]
O	1.3	5.5	17.6
FOA	2.8	4.8	18.3
FM	3.5	4.8	18.6
$LSD_{.05}$	1.3	ns	ns

[z]Means represent the average of 4 replications.
[y]Fusarium infection rating based on the scale of 1-5, where
1 = healthy seedling, 2 = slightly infected, 3 = moderately infected,
4 = severely infected, 5 = dead seedling.

ME2-27-19A alone (200 $\mu g \cdot ml^{-1}$) caused a browning effect on the asparagus roots which increased the disease rating. FM-inoculated seedlings with 100 $\mu g \cdot ml^{-1}$ of ME2-27-19A extract had a disease rating of 4.3, while the FM-inoculated seedlings with 200 $\mu g \cdot ml^{-1}$ of extract had a rating of 3.7.

Assay of ME2-27-19A Extract in Sterile Soil. ME2-27-19A extract reduced the population of FOA and FM at 1000 $\mu g \cdot g^{-1}$ in sterilized field soil (Table 3). It also reduced the shoot length by 92% and the number of shoots by 82% at 1000 $\mu g \cdot ml^{-1}$. Bacterial populations were not affected by either ME2-27-19A or benomyl.

Benomyl reduced the FM population to zero at 100 $\mu g \cdot g^{-1}$, but required 1000 $\mu g \cdot g^{-1}$ to eliminate FOA (Appendix G). Benomyl increased the number of shoots by 70%, suggesting that it removed apical dominance. Manning and Vardaro (1977) found a similar increase in shoot number from benomyl treatments. Benomyl has not been used for control of FOA and FM in asparagus because it produced no positive effects on yield over the control (Lacy, 1979).

ME2-27-19A does not compare favorably with benomyl for control of FOA or FM. While ME2-27-19A crude extract demonstrated some activity against FOA and FM, the purified compound was not sufficiently active to warrant further testing in the greenhouse or field. Although ME2-27-19A was not effective against FOA or FM, this paper establishes a protocol which may be used for future attempts to control these pathogens.

Table 3. Effects of ME2-27-19A crude extract on asparagus seedlings inoculated with FOA or FM in sterilized field soil.

CMPD	Concn ($\mu g \cdot ml^{-1}$)	FUS	Fusarium Pop.	Bacteria Pop. (log cfu)	Number of Shoots (log cfu)	Shoot Length (cm)	Shoot Rating
19A	0	0	1.5[z]	7.2[z]	2.6[yw]	9.9[yw]	1.6[yx]
	100		4.5	8.2	2.0	5.5	4.4
	250		0.0	8.2	1.2	4.4	3.6
	500		0.0	7.6	1.0	2.1	3.2
	1000		0.0	7.6	0.6	0.7	3.4
	0	FOA	5.9	7.6	1.6	4.8	5.0
19A	100		5.5	7.7	1.2	3.3	5.0
	250		5.4	7.6	0.8	2.4	5.0
	500		5.5	7.8	0.8	1.8	4.8
	1000		1.5	7.3	0.4	1.0	4.2
	0	FM	5.6	7.9	2.0	7.5	4.6
19A	100		5.7	7.4	1.2	3.2	5.0
	250		4.1	7.3	1.0	2.4	5.0
	500		2.8	7.7	0.4	1.1	4.8
	1000		2.7	7.8	0.4	0.7	4.6
$LSD_{.05}$(interact.)			2.4	1.3	0.76	1.8	1.2

[z]Means represent the average of 4 replicates.
[y]Means represent the average of 5 replicates.
[x]Shoot rating based on a scale of 1-5, where
1 = healthy seedling, 2 = slightly stunted, 3 = moderately stunted,
4 = severely stunted or diseased, 5 = dead seedling.
[w]Linear F-value significant at $P \leq .01$

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CHAPTER 4

INFLUENCE OF A NOVEL SOIL-BORNE ACTINOMYCETE ON *FUSARIUM* DISEASES IN ASPARAGUS

Abstract

Asparagus decline syndrome is caused by *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM). Several soil-borne microorganisms have been found to inhibit Fusarium diseases in other crops. A novel *Streptomyces spp.* (ME2-27-19A) was isolated from asparagus field soil and found inhibitory to FOA and FM in vitro. ME2-27-19A cells, applied as a root-dip in vitro, reduced the disease incidence in asparagus. In soil, ME2-27-19A decreased the number of root lesions caused by *Fusarium spp.*, but also reduced the root length of the asparagus. ME2-27-19A cells colonize the entire root system in sterile system, but not at sufficient concentration to inhibit FOA and FM. Maltodextrin was a satisfactory carrier for ME2-27-19A and other biological control agents.

Introduction

Effective methods are not currently available for control of *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM) in asparagus (*Asparagus officinalis* L.). Resistant varieties, which have been effective in other vegetable crops (Mace et al., 1981), have not been found in asparagus (Stephens et al., 1989). Crown-dip treatments with benomyl [methyl 1-(butylcarbamoyl)-1H-benzimidazol-2-ylcarbamate] and captan [N-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide]produced no positive effects on crown survival or yields of asparagus (Lacy, 1979).

However, Cassini et al. (1985) employed an antagonistic microorganism, *Pseudomonas fluorescens*, to reduce *Fusarium oxysporum* infection in asparagus. Smith et al. (1990) found *Streptomyces griseus* var. *autotrophicus* was antagonistic to FOA in vitro and limited disease severity in asparagus seedlings in the greenhouse. Kemira Oy, a European chemical company, recently started marketing a biofungicide under the name Mycostop (Lahdenpera et al., 1990). It was based on *Streptomyces griseovirdis* and claimed to be effective against *Fusarium spp.* The objective of this study was to determine if a newly isolated actinomycete could control *Fusarium spp.* in asparagus.

Materials and Methods

Microorganisms. Cultures of the pathogens (MSU-FOA 50 and MSU-FM F12) were grown on potato dextrose agar (PDA) for 14 days. Spore suspensions were made by adding 10 ml of sterile saline solution to the petri dishes and stirring with a sterile glass

rod (Stephens et al., 1985). The spore concentration was adjusted to 10^6 colony forming units per milliliter (cfu/ml) using a hemacytometer.

Fescue-seed inocula of FOA and FM were prepared by the method described by Elmer (1987). Red fescue seed (*Festuca rubra* L.)(200 g) was combined with 100 ml water and autoclaved on three consecutive days. The seed was inoculated with a plug (5mm) of FOA or FM grown on PDA. The cultures were shaken daily for 3 weeks to assure uniform growth of the fungi. The colonized seed was air-dried and ground to pass a 2-mm sieve in a Wiley mill.

The *Fusarium*-antagonistic actinomyces strain ME2-27-19A was isolated from Spinks loamy sand soil (Psammetitic Hapludalf, sandy, mixed, mesic) collected from an old asparagus field in Oceana County, MI. The organism was identified tentatively as a *Streptomyces spp.* by Dr. S.K. Mishra (Microbiology Laboratory, Krug International, Houston, TX). Preliminary tests showed a zone of inhibition when applied to PDA plates spread with *Fusarium* inoculum.

ME2-27-19A was grown in 500-ml baffled-bottom flasks containing 100 ml of A-9 medium (peptone 4 g, dextrose 10 g, "Brer Rabbit green label" molasses 20 g in 1 liter of distilled water). The flasks were incubated on a rotary shaker at 150 rpm and 27C for 8 days.

ME2-27-19A spore suspensions were prepared by adding 10 ml of sterile saline to 8-day-old PDA plates and adjusting with a hemacytometer.

Plants. The asparagus cultivar Syn 4-56 (Nourse Farms Inc., South Deerfield, MA) was used in all experiments. Seeds were surface-disinfected with 0.5% NaOCl (20% bleach) for 20 min, followed by infusion with benomyl (2.5g) in acetone (100ml) for 18-20 h (Damicone et al., 1981). Seeds were rinsed with acetone, followed by sterile, deionized water and dried on a sterilized clay tile (Stephens and Elmer, 1988). The seeds were germinated on 0.6% water agar. For in vitro experiments, germinated seedlings were transferred to 20 ml of Hoagland's nutrient agar (Hoagland and Arnon, 1938; amended with 6 g agar/liter). For greenhouse experiments, the seedlings were transferred to 96-cell (5 x 28 x 53 cm) plastic trays containing sterilized Spinks loamy sand soil.

In Vitro Agar Assay. Two methods were compared for introducing the antagonist: root-dip or agar-surface inoculation. Smith et al. (1990) used root dips to apply *S. griseus* to asparagus seedlings. The root-dip treatments were achieved by dipping germinated seeds in saline suspensions of ME2-27-19A (0, 10^7, 10^9 cfu/ml) before transferring them to Hoagland's agar. Surface-inoculated treatments were achieved by pipetting 0.1 ml of ME2-27-19A spore suspension (0, 10^7, 10^9 cfu/ml) into the test tubes before transferring the seedlings. Both treatments were incubated 2 weeks before inoculation with 0.1 ml of FOA or FM spore suspension (10^7 cfu/ml). The inoculated seedlings were incubated for an additional 5 weeks with a 16-h photoperiod (25C day, 15C night). The seedlings were rated for disease infection on a scale of 1-5, where 1 = healthy seedling, 2 = slightly infected, 3 = moderately infected, 4 = severely infected, and 5 = dead seedling. The shoot and root dry weights were measured.

The experiment was repeated four times. In the last experiment, 0.1 ml of YMG medium (yeast extract 4 g, maltose 10 g, glucose 4 g, in 1 liter of distilled water) was added to each test tube to give the antagonist additional nutrients.

The experimental design was a randomized complete block with five blocks. The data were analyzed by analysis of variance (ANOVA) using MSTAT-C (MSTAT-C, Michigan State University, E. Lansing, MI) and means were separated by LSD. The results were examined by linear contrasts for the actinomycete treatment.

Greenhouse Assay. ME2-27-19A was applied to asparagus seedlings in the greenhouse to determine its effects on FOA and FM in soil. Spinks loamy sand (Psammetitic Hapludalf, sandy, mixed, mesic, pH 6.4, CEC 2 meq/100 g, BD 1.4-1.7 $g \cdot cm^{-3}$,OM 2-4%) was passed through a 2-mm sieve and autoclaved on three consecutive days. Dry soil was inoculated with FOA or FM on fescue seed (2.5 g/500 ml soil) placed in 500-ml clay pots and watered.

ME2-27-19A inoculum was prepared by centrifuging (16300 x *g* for 10 min) 900 ml of cell culture and resuspending the cells in 90 ml of fresh A-9 medium. The cells were homogenized for 2 min to produce a uniform, thick liquid. Ten-week-old asparagus seedlings were washed to remove the soil from their roots, inspected for abscence of Fusarium symptoms, and sorted by weight. The seedlings were dipped in the ME2-27-19A inoculum (0, 10^6, 10^8 cfu/ml) and immediately transplanted into the soil. The seedlings were fertilized once a week with 100 ml of 200 ppm 20N-8.7P-16.6K fertilizer.

After 10 weeks, the root-ball was sliced into 2-cm sections and samples were collected to determine distribution of ME2-27-19A and *Fusarium* in the soil and in the rhizosphere. For the soil, 10 g from each section was placed in 100 ml of saline. For the roots, 1 g from each section was placed in 10 ml of sterile saline solution to determine the rhizosphere microflora. The root samples were placed in an ultrasonic water bath for 2 min to dislodge the rhizosphere organisms. The root and soil solutions were diluted serially and plated on selective media. The *Fusarium* population was counted on Komada's medium (Komada, 1975). The actinomycete population was counted on chitin agar (colloidal chitin 2g, agar 15 g, cycloheximide 45 mg in 1 liter of distilled water). The colloidal chitin was prepared by the method of Lingappa and Lockwood (1962). ME2-27-19A was counted after 2 days and FOA or FM were counted after 4 days.

After plating the solutions, the number of Fusarium lesions were counted for each root system and root lengths were estimated by the line-intersect method (Tennant, 1975). The number of lesions per meter of root was calculated from the number of lesions and the root length. The disease incidence of the roots was rated on the 1-5 scale. The root and shoot dry weights also were measured.

The experimental design was a two-factor randomized complete block with five replicates. The greenhouse experiment was repeated with 20-week-old asparagus seedlings. The data were analyzed by ANOVA and means were separated by LSD. The treatments were analyzed for linear contrasts.

Video-Imaging. The root lengths of the plants from the second greenhouse experiment were estimated by both line-intersect method and video-imaging (Smucker 1989). During video-imaging, the root lengths are divided into groups based on root width. One advantage for asparagus research is that fleshy (> 1mm) and fibrous (< 1mm) root lengths may be determined without the labor of physically separating them.

The roots were washed and stored in a solution of 15% methanol at 4C. Malachite green (20 $\mu g \cdot ml^{-1}$) was added to stain the roots 24 h before imaging. The roots were spread evenly in a 45 x 60-cm glass tray and video-recorded with a robotic camera system. The images were processed by the Root Image Processing Laboratory (Michigan State University, E. Lansing) on a Viacom computer system.

Root-Colonizing Ability. The ability of ME2-27-19A to colonize the rhizosphere of asparagus was determined by the method of Misaghi (1990). Germinated seeds were transferred to petri dishes containing water agar (Difco-Bacto agar 6 $g \cdot liter^{-1}$). ME2-27-19A cell suspension (2 μl) was placed 2 mm below root tips. The roots were inclined and allowed to grow through the cell suspension. The seedlings were observed daily with a 100X microscope. In a second experiment, a plug of FOA or FM was place at the edge of the petri dish to observe the interaction between ME2-27-19A, *Fusarium* and asparagus.

Maltodextrin. Kemira Oy markets a powdered *Streptomyces* preparation under the name Mycostop (Lahdenpera et al., 1990). Preliminary work was conducted on formulation of the antagonist to enhance colonization, viability and covenience of application.

A spore suspension of ME2-27-19A was made by adding 10 ml of sterile maltodextrin solution (10%) to a PDA plate containing 8-day-old ME2-27-19A colonies and stirring with a glass rod. The spore suspension was transferred to a sterile scintillation vial and lyophilized at 4C and 20 mtorr. Samples were dissolved and plated after 0, 5 and 12 months to determine viability.

Results and Discussion

In Vitro Agar Assay. The disease ratings for seedlings treated with FOA or FM were always higher than the no-*Fusarium* control, as expected. In a preliminary experiment the disease rating did not change, suggesting that the concentrations of antagonist (0, 10^2, 10^4, 10^6 cfu/ml) were too low (data not shown).

In the first experiment, the highest concentration of the actinomycete was 10^8 cfu/ml. However, the disease ratings for the high actinomycete concentration were not different than the antagonist-free control. Since there were no differences among the actinomycete concentrations, the data were combined for presentation (Table 1).

In the second experiment, the disease rating and the root dry weight for the FOA-inoculated seedlings improved as the concentration of surface-applied antagonist increased (Appendix H). However, the disease rating still did not decrease to the level of the Fusarium-free seedlings. The root and shoot dry weights were reduced 37 and 42%,

Table 1. Effects of FOA or FM on asparagus seedlings in vitro after root-dip (dip) or surface-application (drench) of ME2-27-19A.

Time	Fusarium spp	Disease Rating (Dip)[zy]	Disease Rating (Drench)	Drench Root Dry Wt. (mg)	Drench Shoot Dry Wt. (mg)
1	O	1.0	1.0	no data	no data
	FOA	2.4	2.4		
	FM	2.9	3.0		
$LSD_{.05}$		0.8	0.9		
2	O	1.0	1.0	4.4	16.5
	FOA	3.4	3.9	2.6	9.1
	FM	3.0	3.5	2.9	9.9
$LSD_{.05}$		1.1	1.2	1.7	7.6
3	0		1.0	4.2	19.1
	FOA		3.7	3.4	13.5
	FM		2.9	3.3	14.9
$LSD_{.05}$			1.3	2.0	4.9

[z]Means represent the average of four blocks and three actinomycete treatments
[y]Disease rating based on 1-5 scale, where 1 = healthy seedling, 2 = slightly infected, 3 = moderately infected, 4 = severely infected, 5 = dead seedling

respectively, by the *Fusarium* treatments. The antagonist alone decreased the dry weight of asparagus shoots an average of 49%. The disease ratings from the root-dip treatments were not different at any concentration, so the root-dip treatment was discontinued after the second experiment.

In the third experiment, the disease rating of the FOA- and FM-inoculated seedlings showed no difference over the range of antagonist concentrations (Appendix H) and were combined for presentation (Table 1). The root dry weight of the asparagus seedlings decreased an average of 60% for FOA and 53% for FM as the antagonist concentration increased. The antagonist alone decreased shoot dry weight an average of 40% (Appendix H).

Addition of YMG to the growth medium had no effect on the disease rating of the *Fusarium*-treated plants (Table 2). However, the shoot dry weights for the FM-treated seedlings were 64% higher at the high actinomycete concentration, and might have improved even more if additional YMG had been added. On the other hand, actinomycete populations are normally 10^4 cfu/g in natural soil and the concentration of the highest treatment was five-fold higher than a natural population. Therefore, any beneficial effect would not likely be sustained for long.

Greenhouse Assay. In the first greenhouse study, the disease ratings for FOA- and FM-inoculated plants were 100 and 187% higher, respectively, than the non-inoculated control (Table 3). The disease rating for the FOA-treated plants increased 66% as the concentration of ME2-27-19A increased. The shoot dry weights were 57% lower for

Table 2. Effects of ME2-27-19A supplemented with YMG on asparagus inoculated with FOA or FM in vitro.

ACT conc	FUS	Disease Rating[zy]	Root Dry Wt. (mg)	Shoot Dry Wt. (mg)
0	0	2.0	4.4	12.6
10^6		1.8	3.1	13.0
10^8		2.3	3.3	16.4
0	FOA	2.5	3.4	9.3
10^6		3.0	2.0	8.2
10^8		2.3	2.7	11.9
0	FM	3.5	2.9	7.0
10^6		3.8	2.2	10.1
10^8		3.0	3.8	11.5
$LSD_{.05}$ (interaction)		1.0	2.2	4.4

[z]Means represent the average of 4 replicates.
[y]Fusarium disease rating based of the scale of 1-5 where
1 = healthy seedling, 2 = slightly infected, 3 = moderately infected
4 = severely infected, 5 = dead seedling.

Table 3. Effects of ME2-27-19A on asparagus inoculate with FOA and FM in sterilized field soil in the greenhouse.

Time	ACT conc	FUS spp	Disease Rating[z,y]	Root Lesions/ meter	Shoot Dry Wt. (mg)	Root Dry wt. (mg)
1	0	0	1.4	0.70	43.7	112.0
	10^6		1.4	0.68	43.0	102.7
	10^8		1.8	1.16	67.0	110.0
	0	FOA	2.4	7.47	23.2	57.2
	10^6		2.8	9.93	19.9	41.2
	10^8		4.0	22.62	22.7	51.8
	0	FM	4.0	27.11	9.8	31.3
	10^6		4.4	23.87	12.9	37.0
	10^8		4.4	22.01	13.8	33.9
$LSD_{.05}$(interaction)			0.9	10.60	13.9	24.1
2	0	0	1.8	3.08	939	3380
	10^6		1.8	2.01	927	3532
	10^8		1.6	2.29	893	3075
	0	FOA	2.8	8.27	1000	2421
	10^6		3.0	9.45	680	2258
	10^8		2.4	5.81	671	1669
	0	FM	2.4	6.53	1131	3510
	10^6		2.2	6.21	671	1669
	10^8		2.4	6.54	888	2263
$LSD_{.05}$(interaction)			0.8	3.76	ns	1185

[z]Means represent the average of 5 replicates.
[y]Fusarium disease rating based of the scale of 1-5 where
1 = healthy seedling, 2 = slightly infected, 3 = moderately infected
4 = severely infected, 5 = dead seedling.

FOA and 76% lower for FM. The root dry weights were 54% lower for FOA and 68%lower for FM than the control. The number of lesions per meter of root for the FOA-inoculated plants increased 169% as the ME2-27-19A concentration increased, a possible synergistic effect.

The counts of the soil microorganisms were so variable that conclusions could not be drawn, except that both ME2-27-19A and FOA or FM were present in the respective treatments. ME2-27-19A produced distinctive growth on chitin agar, allowing it to be distinguished from other bacteria. This medium also may be useful for identifying ME2-27-19A in natural soil or for isolating other actinomycetes.

In the second greenhouse experiment, the concentration of ME2-27-19A was slightly lower and the plants were several weeks older, making comparison between the two experiments difficult. The ratings for *Fusarium*-inoculated plants averaged 50% higher than control, while the lesions/meter were two-fold higher. The root dry weights decreased 36% for FOA and 25% for FM. The root dry weight of FM-inoculated seedlings without ME2-27-19A was not different from the non-inoculated control. However, the dry weight of the FM-inoculated plants with ME2-27-19A were 33 to 50% lower than the control. The counts of the microflora showed no differences between the sections, suggesting that ME2-27-19A and *Fusarium* were not limited to one section.

Video Imaging. Video imaging of the asparagus roots revealed that the line-intersect method underestimated the root lengths by 67% (Table 4). There were no differences in the fleshy root lengths, however, the fibrous root lengths were lower with FOA and

FM treatments. The fleshy root length of FM-treated seedlings decreased as the concentration of ME2-27-19A increased.

Root Colonizing Ability. The study of root-colonizing ability revealed that ME2-27-19A cells colonized the full length of the root in a sterile system, but were not sufficient to keep the pathogen from invading its host plants.

Maltodextrin. Maltodextrin powder provided a method for storing and applying ME2-27-19A. At the beginning of the experiment, it had a concentration of 1.8×10^7 cfu/g. After 5 months, it had decreased by 47% (9.6×10^6 cfu/g). After 1 year, it had decreased to 1% of the original concentration (1.5×10^5 cfu/g), which would limit its effectiveness. Maltodextrin has potential as a formulating agent for this actinomycete, although its shelf-life is limited, which is common with biofungicides.

In conclusion, ME2-27-19A may be one of the organisms which contribute to the *Fusarium*-suppressiveness of soils, but it does not provide sufficient control to be used as a biological control agent for *Fusarium spp.* in asparagus.

Table 4. Video imaging data from asparagus roots grown in sterilized field soil with ME2-27-19A and FOA or FM.

ACT conc	FUS spp	Fibrous (<1mm) Root Length (m)	Fleshy (>1mm) Root Length (m)	Fibrous/ Fleshy Ratio	Estimated Total Root Length	
					Video (m)	Line Intersect (m)
0	0	49.1	13.2	3.8	62.3	21.8
10^6		46.6	14.0	3.3	60.7	21.0
10^8		50.5	12.1	4.2	62.5	21.1
0	FOA	31.1	10.8	2.9	41.9	13.4
10^6		27.7	9.2	3.2	36.9	10.5
10^8		24.1	8.3	3.1	32.4	10.5
0	FM	45.6	12.7	3.8	58.3	21.9
10^6		37.3	10.7	3.5	48.0	15.2
10^8		32.1	8.8	3.7	40.8	12.9
$LSD_{.05}$		14.0	3.7	0.5	17.3	7.0

[z]Means represent the average of 5 replicates.

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SUMMARY AND CONCLUSIONS

Asparagus decline syndrome presents some difficult challenges. Asparagus is a deep rooted, perennial crop, so temporary controls of *Fusarium spp.* have limited effect. Unlike most other commercial crops, asparagus has no natural resistance to FOA and FM. In addition, asparagus produces autotoxic compounds that predispose it to infection by FOA and FM.

This dissertation examined two biological agents which have been identified as possible controls for *Fusarium spp.* Brassica residues have been used to control *Fusarium oxysporum* f.sp. *conglutinans* in cabbage and *Aphanomyces* root rot in pea. However, the effective compounds were volatile, which would have limited influence on FOA or FM in asparagus. Brassica residues also are phytotoxic which would prevent intercropping with asparagus or other sensitive crops. Solarization was not effective for reducing Fusarium inoculum.

Several soil-borne microorganisms have been isolated that compete with *F. oxysporum* or reduce infection in susceptible plants. ME2-27-19A may be one of the many organisms competing with *Fusarium spp.* for the limited supply nutrients, but its inhibitory compounds were not effective at eliminating FOA or FM.

Introducing microorganisms into the soil can be expensive because of the excessive quantities of inoculum or partial sterilization required to overcome the natural

population. Yet, introduction of microorganisms may be necessary when a suitable antagonist is not present.

While the biological agents examined here were not effective at controlling FOA or FM, the basic hypothesis remains valid: to modify the soil environment so that asparagus and the naturally suppressive microorganisms have an advantage. Practices which may maintain asparagus health include liming heavily at planting, preventing drought stress, and preventing mechanical damage.

APPENDICES

APPENDIX A

Effects of brassica cover crops on disease rating and phytotoxicity in the field.

Treatment	Asparagus Disease Rating[zy]	Phytotoxicity[w] Tomato[x]	Cucumber[x]	Cabbage[x]	Brassica Stand Count[w] (plants/m^2)	Brassica Fresh Weight[w] (kg·m^{-2})	1993 Asp. Stand Count (plants/ row)[w]
Sudan	2.3	2.4	2.4	2.0	13	2.3	23.3
Kale	1.9	1.8	1.8	2.0	104	4.6	24.3
Turnip	2.0	3.0	2.2	2.3	104	5.7	23.3
'Global'	2.1	2.0	2.4	2.3	87	3.9	26.5
'Glacier'	2.2	2.2	2.0	1.8	116	5.2	25.1
Mustard	2.1	2.8	2.0	1.8	175	1.9	25.5
'Dwf Essex'	2.1	3.0	2.6	2.8	101	4.0	24.3
'Humus'	2.0	2.4	1.6	1.8	142	4.2	24.6
$LSD_{.05}$	ns	ns	ns	ns	46	1.1	ns

[z]Means represent the average of 3 subsamples from 5 replicates. There were no significant differences for the three sampling dates, so the data were combined for presentation.

[y]Fusarium infection rating based on the scale of 1-5, where
1 = healthy seedling (0-10 lesions), 2 = slightly infected (10-30 lesions), 3 = moderate infected (30-40 lesions), 4 = severely infected (40+ lesions), 5 = dead seedling.

[x]Phytotoxicity ratings based on the scale of 1-4, where
1 = green foliage with a dense canopy, 2 = green foliage, but sparse canopy, 3 = chlorotic foliage, 4 = dead or dying foliage, or poor plant stand.

[w]Means represent the average of 5 replicates.

APPENDIX B

Effects of brassica cover crops on microflora populations in field soil (in cfu/g).

Time	Treatment	FM[z]	FOA	Total Fusarium	Total Fungi
Fall 1989	sudan	230	120	4080	9060
	kale	180	140	4230	9000
	turnip	80	110	3090	7160
	'Global'	130	100	4980	10238
	'Glacier'	130	130	3850	6800
	mustard	60	200	4110	7380
	'Dwf Essex'	140	230	3070	7030
	'Humus'	180	310	3810	11710
	$LSD_{.05}$	ns	ns	ns	ns
Spring 1990	sudan	40	100	3070	8160
	kale	70	60	3210	16240
	turnip	40	30	3070	10980
	'Global'	20	80	2700	7850
	'Glacier'	40	50	3360	8830
	mustard	50	90	2470	8570
	'Dwf Essex'	40	30	1990	9030
	'Humus'	0	40	3690	9170
	$LSD_{.05}$	ns	ns	ns	ns

[z]Means represent the average of 5 replicates.

APPENDIX C

Growth as a percentage (%) of excelsior control for asparagus, cress, wheat, cucumber or cabbage roots following incorporation of brassica residues (2% of soil dry weight).

	Asparagus			
Treatment	0 weeks	2 weeks	4 weeks	12 weeks
sudan	5.3	40.7	50.2	66.4
kale	0.8	44.8	41.8	70.1
turnip	59.1	32.8	63.8	114.3
'Global' canola	5.5	44.0	29.6	147.7
'Glacier' canola	15.4	28.9	44.8	116.8
mustard	67.8	82.5	93.6	148.9
'Dwf Essex' rape	56.3	30.8	61.2	108.1
'Humus' rape	0.0	28.8	49.9	117.7

$LSD_{.05}$ (treatment within time) = 44.1

	Curly Cress			
sudan	4.2	27.1	53.4	99.2
kale	1.4	24.1	69.2	101.6
turnip	7.3	38.5	71.4	66.8
'Global' canola	7.2	34.4	42.4	145.2
'Glacier' canola	3.2	23.7	40.9	78.3
mustard	7.7	32.7	82.8	165.2
'Dwf Essex' rape	3.0	60.6	108.3	81.9
'Humus' rape	0.0	16.8	60.0	44.3

$LSD_{.05}$ (treatment within time) = 76.6

	Wheat			
sudan	26.5	81.3	78.3	123.6
kale	41.6	53.9	79.9	98.5
turnip	33.3	59.5	85.1	135.5
'Global' canola	24.3	75.8	71.2	118.3
'Glacier' canola	62.8	57.6	78.9	106.2
mustard	27.7	47.5	86.8	112.0
'Dwf Essex' rape	36.0	49.0	81.3	103.3
'Humus' rape	24.8	42.0	86.4	102.9

$LSD_{.05}$ (treatment within time) = 47.8

APPENDIX C (continued)

Treatment	Cucumber 0 weeks	2 weeks	4 weeks	12 weeks
sudan	9.8	90.8	107.2	90.8
kale	17.8	86.8	96.6	85.4
turnip	15.0	71.9	105.1	95.8
'Global' canola	37.9	76.4	84.1	92.2
'Glacier' canola	22.0	67.6	108.8	90.2
mustard	58.8	63.7	98.2	94.6
'Dwf Essex' rape	23.7	65.7	105.7	96.0
'Humus' rape	6.9	58.4	96.5	103.4

$LSD_{.05}$ (treatment within time) = 30.5

	Cabbage			
sudan	1.5	75.2	86.1	106.1
kale	5.8	53.8	88.1	89.2
turnip	14.7	41.0	81.7	100.3
'Global'canola	0.0	42.2	87.9	106.6
'Glacier' canola	2.3	15.6	81.9	98.6
mustard	4.9	32.3	82.2	107.0
'Dwf Essex' rape	11.6	15.7	96.2	95.0
'Humus' rape	1.2	37.0	93.1	91.9

$LSD_{.05}$ (treatment within time) = 33.1

APPENDIX D

Ratings of Fusarium infection of asparagus seedlings following incorporation of fresh or dry brassica residues (2% of soil dry weight) in the greenhouse.

Treatment	Dry Residue +FOA	Dry Residue +FM	Dry Residue +O	Fresh Residue +FOA	Fresh Residue +FM
sudan	2.6	2.0	1.4	1.6	2.6
kale	2.0	2.6	1.8	2.0	2.2
turnip	2.4	2.4	1.8	1.6	2.2
'Global' canola	2.2	2.2	2.0	2.4	2.6
'Glacier' canola	2.2	2.2	1.2	2.4	1.8
mustard	2.0	2.4	2.0	1.6	2.0
'Dwf Essex' rape	2.2	2.8	1.6	2.2	2.0
'Humus' rape	1.8	2.4	1.2	2.0	2.2
excelsior	1.6	1.4	1.0		
no residue	2.4	2.2			

$LSD_{.05} = 0.65$

Root growth (cm) of asparagus seedlings following incorporation of brassica residues (2% of soil dry weight) in FOA- or FM-inoculated soil.

Treatment	Dry Residue +FOA	Dry Residue +FM	Dry Residue +O	Fresh Residue +FOA	Fresh Residue +FM
sudan	431	749	626	759	543
kale	550	1031	610	369	361
turnip	541	717	522	357	575
'Global'canola	799	1031	280	305	551
'Glacier' canola	761	974	525	514	833
mustard	682	1065	893	415	410
'Dwf Essex' rape	774	1396	482	644	489
'Humus' rape	523	783	416	306	252
excelsior	451	399	414		
no residue	513	613			

LSD.05= 346

APPENDIX E

Effects of 'Humus' cover crop and solarization on the disease rating and growth of asparagus in the field.

Treatments	Disease Rating[zy]	Root Length (cm)[y]	Seedling Fresh Weight (g)[y]
No brassica, No cover	2.4	593.2	5.1
No brassica, Cover	2.7	669.3	3.6
Brassica, Cover	2.7	480.7	3.3
Brassica, No cover	2.7	442.2	4.9
$LSD_{.05}$	ns	ns	ns

[z]Fusarium infection rating based on the scale of 1-5, where
1 = healthy seedling, 2 = slightly infected, 3 = moderately infected,
4 = severely infected, 5 = dead seedling.
[y]Means represent the average of 6 subsamples from 3 replicates.

APPENDIX F

Effects of ME2-27-19A crude extract on asparagus seedlings inoculated with FOA or FM in vitro.

FUS	ACT Concn $\mu g \cdot ml^{-1}$	Disease Rating[zy]	Root Dry Wt (mg)[z]	Shoot Dry Wt. (mg)[z]
O	0	1.0	6.7	19.4
	100	1.3	4.4	16.9
	200	1.5	5.3	16.6
FOA	0	2.5	4.9	18.9
	100	2.7	4.6	13.4
	200	3.3	4.8	22.7
FM	0	2.7	5.0	19.0
	100	4.3	3.4	16.5
	200	3.7	6.0	20.2
$LSD_{.05}$		1.3	2.5	8.0

[z]Means represent the average of 4 replications.
[y]Fusarium infection rating based on the scale of 1-5, where
1 = healthy seedling, 2 = slightly infected, 3 = moderately infected,
4 = severely infected, 5 = dead seedling.

APPENDIX G

Effects of benomyl on asparagus seedlings inoculated with FOA or FM in sterilized field soil.

CMPD	Concn ($\mu g \cdot ml^{-1}$)	FUS	Fusarium Pop. (log cfu)	Bacteria Pop. (log cfu)	Number of Shoots	Shoot Length (cm)	Shoot Rating
	0	0	1.5[z]	7.2[z]	2.6[y]	9.9[y]	1.6 [yx]
Ben	100		0.0	7.5	3.6	8.6	1.6
	250		0.0	8.0	2.2	9.1	1.6
	500		0.0	7.7	3.2	7.5	2.0
	1000		0.0	7.5	3.8	6.2	2.6
	0	FOA	5.9	7.6	1.6	4.8	5.0
Ben	100		2.4	8.2	3.2	10.1	2.4
	250		1.6	7.9	3.0	10.4	2.2
	500		1.0	8.0	4.4	7.7	3.0
	1000		0.0	5.7	3.2	5.9	2.6
	0	FM	5.6	7.9	2.0	7.5	4.6
Ben	100		0.0	8.0	3.6	5.9	2.8
	250		0.0	8.0	4.2	6.2	2.6
	500		0.0	7.6	4.0	6.1	2.4
	1000		0.0	7.9	2.8	7.6	2.4

[z]Means represent the average of 4 replicates.
[y]Means represent the average of 5 replicates.
[x]Shoot rating based on a scale of 1-5, where
1 = healthy seedling, 2 = slightly stunted, 3 = moderately stunted, 4 = severely stunted or diseased, 5 = dead seedling.

APPENDIX H

Effects of ME2-27-19A applied as a root-dip (dip) or surface-application (drench) on asparagus inoculated with FOA or FM in vitro.

Time	ACT concn	FUS spp	Disease Rating (Dip)[z,y]	Disease Rating (Drench)	Drench Root Dry Wt. (mg)	Drench Shoot Dry Wt. (mg)
1	0	0	1.0	1.0		
	10^6		1.0	1.0	no data	no data
	10^8		1.0	1.0		
	0	FOA	2.5	2.8		
	10^6		2.3	2.0		
	10^8		2.3	2.3		
	0	FM	2.8	3.0		
	10^6		2.8	3.0		
	10^8		3.0	3.0		
$LSD_{.05}$(interaction)			0.8	0.9		
2	0	0	1.0	1.0	5.3	24.6
	10^6		1.0	1.0	3.9	10.4
	10^8		1.0	1.0	4.0	14.6
	0	FOA	4.0	4.7[x]	1.5	11.1
	10^6		2.8	4.3	3.7	8.4
	10^8		3.5	2.7	2.8	7.8
	0	FM	3.3	3.7[x]	3.0	10.3
	10^6		3.3	3.7	2.3	9.8
	10^8		2.5	3.0	3.3	9.7
$LSD_{.05}$(interaction)			1.1	1.2	1.7	7.6

[z]Means represent the average of 4 replicates.

[y]Disease rating based on scale of 1-5, where 1 = healthy seedling, 2 = slightly infected, 3 = moderately infected, 4 = severely infected, 5 = dead seedling.

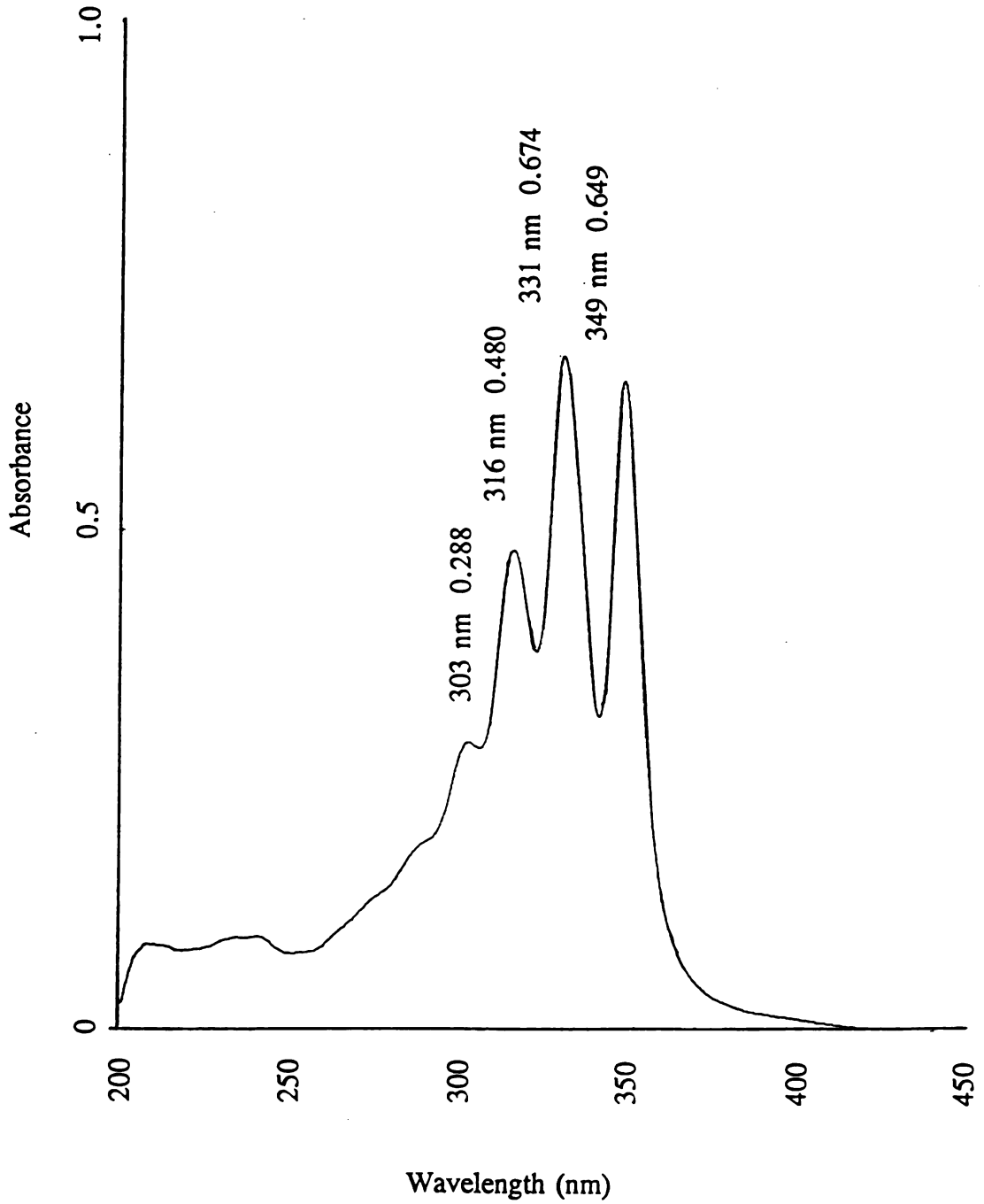
[x]Linear F-test significant at $P \leq .05$

APPENDIX H (continued)

Time	ACT concn	FUS spp	Disease Rating (Dip)[zy]	Disease Rating (Drench)	Drench Root Dry Wt. (mg)	Drench Shoot Dry Wt. (mg)
3	0	0		1.0	6.0[x]	19.2
	10^6			1.0	3.1	19.3
	10^8			1.0	3.6	18.9
	0	FOA		4.0	5.3[x]	12.7
	10^6			4.0	2.7	14.7
	10^8			3.0	2.1	13.1
	0	FM		3.0	4.5[x]	14.5
	10^6			2.8	3.4	17.1
	10^8			3.0	2.1	13.0
$LSD_{.05}$(interaction)				1.3	2.0	4.9

APPENDIX I

UV-spectrum of Fusarium-inhibitory compound from ME2-27-19A (0.23 mg) in methanol (10 ml).



APPENDIX J

^{1}H-NMR spectrum (500 MHz) of Fusarium- inhibitory compound from ME2-27-19A in d_6-DMSO.