BIOLOGICAL CONTROL OF *PYTHIUM* AND *APHANOMYCES* SPP. BY STRAINS OF *ACTINOPLANES* SPP.

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CHAPTER I

INTRODUCTION

Members of the fungal class oomycetes, such as *Pythium, Phytophthora,* or *Aphanomyces* spp. produce thick-walled resting structures called oospores. Oospores may survive in soils for many years, and often are resistant to chemicals (Hoppe, 1966; Munnecke and Moore, 1969; Stasz and Martin, 1988). These plant pathogens cause diseases with economic impact on numerous crop hosts throughout the world (King and Parke, 1993; Rush and Vaughn, 1993; Van der Plaats-Niterink, 1981).

Generally, diseases caused by *Pythium* spp. are economically more important in Oklahoma than diseases caused by other genera of oomycetes such as *Phytophthora spp.*, which is sometimes a problem in ornamental crops. *Pythium* species that are commonly found in Oklahoma include *P. aphanidermatum* Fritz., *P. arrhenomanes* Drechs., *P. irregulare* Buis., *P. myriotylum Drechs.* and *P. ultimum* Trow. These species causing seed and seedling damping-off and root and fruit rots in several economically important crops of Oklahoma, such as alfalfa, cotton, peanut, ornamental plants, vegetables and wheat. It is hard to assign a specific dollar value to losses in yield and quality attributable to Pythium diseases to Oklahoma's economy. Assuming a 1% loss in yield in a crop industry of $1.2 billion, about $ 12 million per year may be lost due to Pythium induced diseases in Oklahoma. Diseases induced by

Pythium spp., however, are a problem world wide. They are a problem not only in the field, but also in greenhouse crops, including hydroponic systems (Zinnen, 1988) and in postharvest storage.

Diseases caused by *Pythium, Phytophthora,* and *Aphanomyces* spp. are difficult to control on many plant hosts. Cultivars with sufficient genetic resistance to them are not available. Fungicides, such as metalaxyl, are effective against *Pythium* and *Phytophthora* spp. but there is no currently labelled fungicide for use against *Aphanomyces* spp. Fungicides are costly to use, potentially hazardous to applicators, and may not be efficacious or are so for only a limited time (Sanders, 1987; Filonow and Jackson, 1989). Fungicides also increase the risk of environmental pollution. Metalaxyl primarily suppresses mycelial growth and has little effect on oospores. Oospores allow *Pythium* spp to survive inhospitable conditions in soil (Van der Plaats-Niterinck, 1981) and also provide the initial inoculum for disease outbreaks when a new crop is planted (Mitchell, 1978).

Parasitism of one microorganism by another microorganism is very common in nature and may have potential for the biological control of plant diseases. Several microorganisms parasitize oospores (Ayers and Lumsden 1977; Sneh et al., 1977; Sutherland and Lockwood, 1984; Filonow and Lockwood, 1985). Sneh *et al.*, (1977) reported parasitism of *Phytophthora, Pythium* and *Aphanomyces* oospores in soil by a *Pythium* sp., chytridiomycetes, several hyphomycetes, bacteria and *Actinoplanes missouriensis* Couch.

Actinoplanes spp. are members of the actinomycetales in the family

Micromonosporaceae (Goodfellow et al., 1990), which are Gram-positive, non-acid-fast, filamentous bacteria with spores produced in sporangia. *Actinoplanes* spp. release motile spores (zoospores) from sporangia (Couch, 1963). Sporangia have been reported to survive in a desiccated state in soil for several years and still release zoospores when hydrated (Couch, 1963; Higgins et al., 1967). Therefore, they may be capable of long shelf-life as biocontrol agents. In nature, *Actinoplanes* spp. have been isolated from soil types ranging from forest litter to a sandy beach (Makkar and Cross, 1982; Vobis, 1989). *Actinoplanes* spp. have not been reported to cause any animal, human or plant diseases (Lechevalier, 1988), even though the genus is a common soil and litter inhabitant. *Actinoplanes* spp. produce antibiotics (Parenti and Cornelli, 1979) as well as industrial enzymes .

Sneh *et al.* (1977) discovered that oospores incubated in soil were parasitized by *A. missouriensis*. Later, Sutherland and Lockwood (1984) reported *A. missouriensis, A. utahensis,* and *A. philippinesis* to be highly efficient hyperparasites of oospores of *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin *in vitro* and also showed that soybean seed treated with *A. missouriensis* reduced Phytophthora root rot in the greenhouse. Filonow and Lockwood (1985) confirmed control given by *A. missouriensis* and showed that *A. utahensis* also was an effective seed treatment for the control of Phytophthora root rot of soybean. Later, Sutherland and Papavizas (1991) reported that application of *Actinoplanes* spp. suspensions to potting mix did not reduce the incidence of Phytophthora root rot of bell peppers.

Thus, the majority of published works dealing with *Actinoplanes* spp. assessed

their potential for control of *Phytophthora* spp. Little is known of their potential for control of diseases caused by *Pythium* or *Aphanomyces* spp. Moreover, previous methods of *Actinoplanes* spp. application to soil have been by adding suspensions of cultures to soil or by seed treatment. The use of solid carriers, e.g. clay granules for applying *Actinoplanes* spp. to soil has not been tested. Moreover, nothing is known about the factors influencing sporulation of these organisms on solid carriers.

The objectives of this study were:

1. Screen a collection of species and strains of *Actinoplanes* for their parasitism of oospores of *Pythium* spp.
2. Determine factors affecting sporulation of *Actinoplanes* spp. on agar and on solid carriers.
2. Evaluate the strains of *Actinoplanes* spp. for the biological control of *Pythium* spp. and *Aphanomyces cochlioides* in soil.

The written format of this dissertation consists of an introduction, a literature review, a collection of three manuscripts for submission to scientific journals, followed by a summary and statement of conclusions.

CHAPTER II

LITERATURE REVIEW

Oomycetous soilborne fungal pathogens such as *Pythium, Phytophthora, Aphanomyces spp.* cause root diseases that decrease the productivity of most agricultural soils (King and Parke, 1993; Rush and Vaughn, 1993; Van der Plaats-Niterink, 1981). These pathogens cause seed and seedling damping-off and root diseases in a variety of food and fiber crops, and they produce thick-walled resting spores called oospores that may survive in soil for years (Hoppe, 1966). Infested soil thus becomes the source of inoculum for disease outbreaks when a new crop is grown. Mitchell (1978) has reported that the amount of disease produced on a host crop is related to the inoculum density of oospores in soil. Therefore, a reduction in oospore population should result in reduction of disease incidence and/or severity.

At present, however, oospores are difficult to control. Stasz and Martin (1988) showed that oospores of *P. ultimum* are insensitive to many fungicides and are not easily killed by methyl bromide, a commonly used soil fumigant. They have been reported to tolerate heat up to 50 C. Very little genetic resistance is available to *Pythium* and *Aphanomyces* or to many species of *Phytophthora.* Few fungicides are currently available in USA to control *Pythium* and *Phytophthora* spp., and none are registered for *Aphanomyces* spp. Fungicides that are effective against pythiaceous

fungi, e.g. metalaxyl, are costly, increase the risk of environmental pollution, are potentially hazardous to applicators and may not be efficacious (Filonow and Jackson, 1989; Sanders, 1987).

Biological control may offer an alternative or supplement to chemical control of diseases caused by oomycetous fungi. Mechanisms of biological control of oomycetous pathogens have included antibiosis (Bowers and Parke, 1993; Howell and Stipanovic, 1980; 1983; Papavizas, 1985), competition, (Becker and Cook, 1988; Chen et al., 1988) and parasitism of hyphae (Al-Hamdani et al. 1983; Lifshitz et al. 1984; Martin and Hancock, 1987).

Parasitism of one microorganism by an other is a common phenomenon (Barnett and Binder, 1973; Boosalis, 1964). Hyperparasitism is a term used for parasitism of one parasite by another, whereas parasitism of a fungus by another fungus is known as mycoparasitism (Baker, 1987). Hyperparasitism is further subdivided into two subgroups: nectrotrophic, if the host cell is killed by the parasite prior to the utilization of nutrition from the host cell, and biotrophic, if the nature of parasite obtains nutrition from a living host cell, (Barnett, 1958). In nature, parasitism may be a natural biocontrol which may limit populations of soilborne plant pathogens. There has been considerable interest in recent years in introducing natural parasites of plant pathogens into soils for disease control in agriculturally important crops.

Several microorganisms parasitize oospores (Ayers and Lumsden, 1977; Dreschler, 1938; Filonow and Lockwood, 1985; Humble and Lockwood, 1981; Sneh

et al.,1977; Sutherland and Lockwood,1984). Sneh *et al.* (1977) reported parasitism of *Phytophthora, Pythium* and *Aphanomyces spp.* oospores in soil by a *Pythium* sp., chytridiomycetes, several hyphomycetes, bacteria and *Actinoplanes missouriensis* Couch. Oospores infected by *A. missouriensis* carried *Actinoplanes sp.* sporangia that released zoospores (Sneh et al., 1977). Sutherland and Lockwood (1984) reported *A. missouriensis, A. utahensis,* and *A. philippinesis* to be highly efficient hyperparasites of oospores of *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan & Erwin *(Pmg) in vitro.* These authors found that dead oospores were less colonized (31%) than living spores (94%), as determined by vital staining with tetrazolium bromide (MTT). Parasitic efficiency was not affected by the age of *Pmg* oospores or of sporangia of *A. missouriensis* from cultures up to several weeks. In sterilized soil, infested with oospores of *Pmg* and supplemented with sporangia of *A. missouriensis,* parasitism of oospores by *A. missouriensis* was greater at -30 J/kg (-300 mbar) than in saturated soil (0 J/kg). Oospore infection was greater at 15-30 C than below or above this range. Sutherland and Lockwood (1985) also showed that soybean seed treated with *A. missouriensis* reduced Phytophthora root rot in the greenhouse.

Filonow and Lockwood (1985) later evaluated several members of the Micromonosporaceae and the fungus *Hypochytrium catenoides* for control of Phytophthora root rot. Parasites were applied to soybean seed in a carboxymethyl cellulose + starch coating and planted in soil infested with up to 400 oospores of *P. megasperma* f. sp. *glycinea* per g of soil. *H. catenoides* did not provide any control; however, *A. missouriensis* and *A. utahensis* provided significant control. In addition,

Micromonospora sp. and *Amorphosporangium auranticolor,* two other genera of the Micromonosporaceae, also gave effective but inconsistent control. Oospores of only two *Pythium* spp. have been tested for parasitism by a few *Actinoplanes* spp. strains (Sneh et al., 1977; Sutherland and Lockwood, 1984). No work has been done on oospores of *P. arrhenonomanes* Drechs., *P. irregulare* Buis., or *P. myriotylum* Drechs. In addition, except for *A. euteiches*, there is little known about the susceptibility of other *Aphanomyces* spp. oospores to parasitism.

The bacterial family Micromonosporaceae is comprised of four genera, *Actinoplanes, Micromonospora, Pilimelia* and *Dactylosporangium* (Goodfellow et al., 1990). *Actinoplanes* are Gram-positive, non-acid-fast, filamentous bacteria with spores that are produced in sporangia. The genus *Actinoplanes* releases motile spores (zoospores) from sporangia (Couch, 1963; Couch and Bland, 1974). Sporangia have been reported to survive in a desiccated state in soil for several years and still release zoospores when hydrated (Couch, 1963; Higgins et al., 1967). In nature, they are found on plant debris, pollen, etc. in soil and lakes. This attribute may enable them to have long shelf-life as biocontrol agents. *Actinoplanes* spp. have not been reported to cause any animal, human or plant diseases (Lechevalier, 1988), even though the genus is a common soil and litter inhabitant (Makkar and Cross, 1982). They produce antibiotics (Parenti and Cornelli,1979) as well as industrial enzymes.

Zoospores of *Actinoplanes* spp. exhibit chemotaxis to nutrients (Arora, 1986; Palleroni, 1983) and fungal propagules (Arora, 1986), including oospores of *P. ultimum* (Arora, 1987). The capacity to chemolocate and swim to oospores would be

an advantage to a biocontrol agent of oospores. However, sufficient water films may be required by zoospores for movement in soil. Arora (1986) found that a matric potential of ca. -5 J/Kg or greater was needed for zoospores of *A. missouriensis* to move in soil. Zoospore movement in soil also would be influenced by the continuity of water films, soil temperature and their adsorption by colloids.

Species of *Actinoplanes* produce colored colonies on agar under light. Many are orange, e.g. *A. missouriensis,* whereas others are red (*A. italicus),* blue (*A. caeruleus),* etc., which helps in their recognition on agar media. Several media are available for growing Actinoplanes spp., such as colloidal chitin agar (Hsu and Lockwood, 1975) and yeast-extract soluble-starch phosphate agar (Emerson, 1958). Little is known about factors influencing sporulation of *Actinoplanes* spp. Ensign (1978) reviewed sporulation in the actinomycetes, but there is little information specific to *Actinoplanes* spp. Nor is anything known about the sporulation of these organisms on solid carriers suitable for application to soil. Application of *Actinoplanes* spp. for biological control has been by drenching soils with suspensions of hyphae and sporangia or by applying suspensions to seed (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984; Sutherland and Papavizas, 1991).

CHAPTER III

PARASITISM OF OOSPORES OF *PYTHIUM* SPP. BY STRAINS OF *ACTINOPLANES* SPP.

ABSTRACT

Strains of *Actinoplanes* spp. were evaluated for their *in vitro* parasitism of oospores of *Pythium aphanidermatum, P. arrhenomanes, P. irregulare, P. myriotylum* and *P. ultimum.* Oospores of *P. arrhenomanes, P. irregulare* and *P. myriotylum* were identified for the first time as hosts of *Actinoplanes* spp. Newly recorded parasites of oospores of *Pythium* spp. were *A. azureus, A. brasiliensis, A. caeruleus, A. ferrugineus, A. ianthinogenes, A. italicus, A. minutisporangius, A. rectilineatus, A. teichomyceticus, A. utahensis, A. violaceous, A. yunnahensis,* plus 15 strains of *Actinoplanes* yet to be speciated. Parasitized oospores had disorganized cytoplasm and hyphae of *Actinoplanes* sp. emerging from them. Infection of oospores *in vitro* varied from 0% to >90%. Strains also were very active parasites of oospores in sterile soils. When added to nonsterile soils, several strains increased ($P=0.05$) the level of oospore parasitism compared to nonsupplemented soils. Strains of *Actinoplanes* spp. exhibited a host specificity for species of *Pythium in vitro* and in soil. Sporulation of *Actinoplanes* sp. from infected oospores incubated on soil was frequent and more

abundant than that observed *In vitro.*

INTRODUCTION

Soilborne plant pathogens are a limiting factor in the plant productivity of most agricultural soils. Crop productivity is frequently limited by species of *Pythium, Phytophthora, Aphanomyces* and other genera of the fungal class, Oomycetes (Singleton et al. 1992). These worldwide pathogens of diverse food and fiber crops produce thick-walled resting spores, called oospores that may survive for years in soil and which frequently provide the initial inoculum for disease outbreaks when a new crop is planted in infested soil. Since disease incidence in a crop is generally related to the oospore population in soil (Mitchell, 1978), reduction of the oospore population shall result in less disease. Presently, however, oospore populations are difficult to reduce. Stasz and Martin (1988) showed that oospores of *P. ultimum* are insensitive to many fungicides and are not easily killed by methyl bromide, a commonly used soil fumigant. Agents that kill oospores may lessen disease pressure and reduce the need to apply fungicides. There is little genetic resistance to *Pythium* and *Aphanomyces* spp. and to many species of *Phytophthora.* Therefore, the crop productivity of agricultural soils may be increased by agents effective in destroying oospores.

Several microorganisms have been shown to parasitize oospores (Dreschler 1938; Ayers and Lumsden, 1977; Sneh et al., 1977; Humble and Lockwood, 1981; Hsu and Lockwood, 1984; Sutherland and Lockwood, 1984; Filonow and Lockwood, 1985). In 1977, Sneh et al. reported the parasitism of oospores of *Phytophthora, Pythium* and *Aphanomyces* spp. in soil by several fungi and bacteria, including

Actinoplanes missouriensis. *Actinoplanes* spp. belong to the aggregate group, the actinoplanetes, of the family Micromonosporaceae (Goodfellow et al., 1990), in the order Actinomycetales (Vobis, 1989). They are Gram-positive, non-acidfast bacteria which grow by means of nonfragmenting, branched and septate hyphae. *Actinoplanes* spp. and most members of the actinoplanetes produce minute sporangia of various shapes and sizes (3-20 x 6-30 μm), containing several to many sporangiospores which are motile by means of flagella. Hyphal germination of sporangia has not been reported (Palleroni, 1989). *Actinoplanes* spp. are common inhabitants of diverse, natural environments (Makkar and Cross, 1982).

Investigations of *Actinoplanes* and a few related species as potential biocontrol agents of oospores have been made (Sutherland and Lockwood, 1984; Sutherland et al., 1984; Filonow and Lockwood, 1985; Sutherland and Papavizas, 1991). In the genus *Actinoplanes,* only *A. missouriensis, A. philipinensis* and *A. utahensis* have been studied as parasites of oospores and most of these studies have focused on oospores of *Phytophthora* spp. as hosts (Sneh et al., 1976; Sutherland and Lockwood, 1984; Filonow and Lockwood, 1985; Sutherland and Papavizas, 1991). In this paper, we evaluated other species and strains of *Actinoplanes* as parasites and focused on oospores of *Pythium* species, as little was known about their suspectibility to *Actinoplanes* spp.

MATERIALS AND METHODS

Fungal hosts. All fungal hosts were isolated in Oklahoma. *Pythium arrhenomanes* Drechs. was isolated from wheat roots. *Pythium aphanidermatum* Fritz., *P. irregulare*

Buis., *P. myriotylum* Drechs., and *P. ultimum* Trow. were isolated from peanut roots or pods. Species identification was according to Van der Plaats-Niterink (1981). Fungi were maintained on corn meal agar and stored as pieces of colonized agar or wheat leaves in sterile water. Fungi were aseptically grown in 250 ml flasks containing 25 ml of clarified V8 broth supplemented with 20 mg cholesterol per L (Filonow and Lockwood, 1985) at 25 C in the dark for 1-2 months prior to harvest. Mycelia were aseptically washed four times with sterile water and comminuted in 50 mM phosphate buffer (pH 6.8) in a Servall Omni Mixer (Ivan Sorvall, Norwalk, CT) for 10 min at a powerstat setting of 75. Macerate was filtered through a 75 μm opening nylon cloth (Nitex^R cloth, Tetko Inc., Elmsford, NY 10523) and the oospores collected in a beaker. Oospores were washed two times by centrifugation (3000 X g) in phosphate buffer for 10 min and concentrated to about 5 x 10^3 oospores/ml as estimated in a hemocytometer. Aseptic technique was followed throughout the procedure. Oospore suspensions were stored in ice until use within 1-3 h.

***Actinoplanes* spp.** Strains of *Actinoplanes* spp. were obtained from J. L. Lockwood (Professor emeritus, Michigan State University) and M. P. Lechevalier (Professor emeritus, Waksman Institute). Several strains have been identified to species and are maintained in the American Type Culture Collection (ATCC; Rockville, MD 20852) whereas others are unknown to species. Those in the ATCC include: *A. azureus* (31157), *A. brasiliensis* (25844), *A. caeruleus* (32937), *A. ferrugineus* (29868), *A. ianthinogenes* (21884), *A. italicus* (27366), *A. missouriensis* (14538), *A. rectilineatus*

(29234), *A. teichomyceticus* (31121), *A. utahensis* (14539), *A. violaceous* (43537), and *A. yunnahensis* (43538). Strains were stored as frozen skimmed milk cultures and routinely maintained on dilute Czapek Dox agar, containing 12 g Czapek Dox broth (Difco, Detroit, MI) plus 17 g agar per L water or on Emerson's YPSS agar (Emerson, 1958) for 14-28 days at 28 C. Agar dishes were flooded with 10 ml of sterile phosphate buffer and sporangia of an *Actinoplanes* sp. aseptically dislodged with a rubber policeman. Sporangia were washed once with phosphate buffer and concentrated by centrifugation (10^4 x g) for 10 min. Sporangial suspensions were adjusted to about 5 x 10^4/ml and were kept at 22-25 C for <4 h prior to use.

Parasitism *in vitro*. One half milliliter each of an oospore and sporangial suspension were mixed together in a well of a sterile plastic tissue culture dish (No. 25820; Corning Glass Works, Corning, NY 14831). There were three replicate wells for each combination of *Actinoplanes* strain and *Pythium* oospore host. After 3 days incubation at 28 C in the dark, the contents of a well were mixed and 2-3 drops were placed on a microscope slide. One hundred oospores per well were microscopically examined at 200-500X magnification for evidence of parasitism. Experiments were conducted over several months, each with 3-5 different strains incubated with 2-5 species of *Pythium.* A strain of *A. missouriensis* or *A. brasiliensis* with reliable degrees of parasitism to all five oospore hosts were included in many experiments as a reference. Experiments were completely randomized.

In separate experiments, selected strains were evaluated for their parasitism to certain oospore hosts at 7 or 14 days to determine if the incubation time drastically

altered the results found at 3 days. These experiments were conducted as previously described. As an aide to assessing parasitism, the viability of oospores after a few *in vitro* experiments was assessed with the vital stain, tetrazolium bromide (MTT) (Sutherland and Cohen, 1983; Sutherland and Lockwood, 1984). One hundred microliter was removed from each replicate well and composited in a sterile plastic well containing 300 μl of 0.1% (w/v) of MTT in distilled water. After incubation at 35 C for 3-5 days, 100 oospores were examined at 500 X mag. Oospores that were shades of red or were light blue were counted as viable. Two hundred to 300 oospores of each *Pythium* spp. were examined.

Parasitism on soils. Suspensions of oospores (5 x 10^3/ml) of *Pythium* spp. and sporangia of *Actinoplanes* spp. (ca. 10^5/ml) were prepared as previously described. Oospores in 0.2 ml of a suspension were vacuum deposited on 1.5 cm x 1.5 cm pieces of nylon cloth (Nitex^R) with 10 μm openings (Lumsden, 1981). Three soils were used: a loamy sand (82% sand, 10% silt, 8% clay; pH 6.2), a loam (47% sand, 32% silt, 21% clay; pH 6.9) and a sandy loam (55% sand, 29% silt, 16% clay; pH 7.0). Soil moisture release curves were determined for each soil. Soil matric potential was adjusted to -1 J/kg (-10 mbar) for all experiments. Soils were used as is (nonsterile) or were autoclaved for 1 h on two successive days (sterile soil). In all experiments 5 g of soil in 9-cm-dia Petri dishes were supplemented with 2 x 10^4 sporangia/g soil of a strain of *Actinoplanes* sp. Soils without added *Actinoplanes* sp. were the controls.

In one set of experiments, a loamy sand soil was supplemented with the

following strains: *A. brasiliensis, A. missouriensis, A. teichomyceticus, A. yunnahensis,* K 30, LLW 211, R 141Y, W 257 or Z 20. Three pieces of nylon cloth bearing oospores of *P. ultimum* were placed on the sterile or nonsterile soil. There were three dishes per treatment. The dishes were completely randomized and incubated in the dark at 28 C in recloseable bags containing moist paper towels. After 14 days of incubation, the pieces of nylon cloth were removed from a dish, the oospores were stained with cotton blue and each cloth piece inverted on a drop of molten (45 C) agar on a slide. After the agar hardened, each cloth was removed and 100 oospores microscopically observed for parasitism.

The susceptibility of all five species of *Pythium* when simultaneously exposed to the same strain of *Actinoplanes* sp. also was assessed. Pieces of nylon cloth each bearing oospores of a different species were placed on supplemented or nonsupplemented loam soil. There was one piece of cloth for each oospore host per dish of soil and three dishes per strain of *Actinoplanes* spp. Treatments were completely randomized. After 14 days of incubation at 28 C, 100 oospores per cloth piece were observed for parasitism as described above.

Parasitism of oospores of *P. aphanidermatum* and *P. ultimum* also was assessed over time on sandy loam soil. Soil was supplemented with *A. brasiliensis, A. teichomyceticus* or W 57. There were 5 pieces of Nitex^{R} bearing oospores in each dish. The dishes were covered, placed inside a large recloseable plastic bag containing wet paper towels and the soils incubated at 28 C in the dark. There were three dishes for each treatment arranged in a completely randomized design. At 3, 7, 14, 28 and

56 days one piece of nylon cloth was removed from each dish, and 100 oospores from each piece microscopically observed for parasitism, as described above.

Statistical analyses. All experiments were repeated. All data were transformed with the arcsin function (Steel and Torrie, 1980). Data from *in vitro* or soil experiments were subjected to a one way or two way analysis of variance using the GLM program of SAS (SAS Institute, Cary, NC). Duncan's Multiple Range test was used to separate means at the $P \leq 0.05$ level of significance. Data from the three-day, *in vitro* experiments were subjected to the FASTCLUS procedure of SAS to aide in selecting strains of *Actinoplanes* spp. for future studies. Transformed means of percent parasitism for each oospore host were sorted into five clusters; however, only clusters with maximum and minimum means are reported. The observed overall R^2 statistic estimates the percent variability in the level of parasitism explained by sorting the means into clusters. Arcsin transforms of percent parasitism of oospores over time on soils was analyzed by linear regression and indicator variables for comparing the intercepts and slopes of regression equations at the $P = \leq 0.05$ level of significance.

RESULTS

Parasitism *in vitro*. Parasitized oospores routinely had a disorganized cytoplasm with hyphae of *Actinoplanes* sp. proliferating from the oospores. Sporangia of *Actinoplanes* sp. often were found on these hyphae (Fig. 1). Breaks in the cell walls of oospores also were observed. Oogonia also were parasitized; however, the susceptibility of oogonia compared to oospores was not determined. Generally, signs of parasitism were not observed until 48-96 h after commencement of experiments,

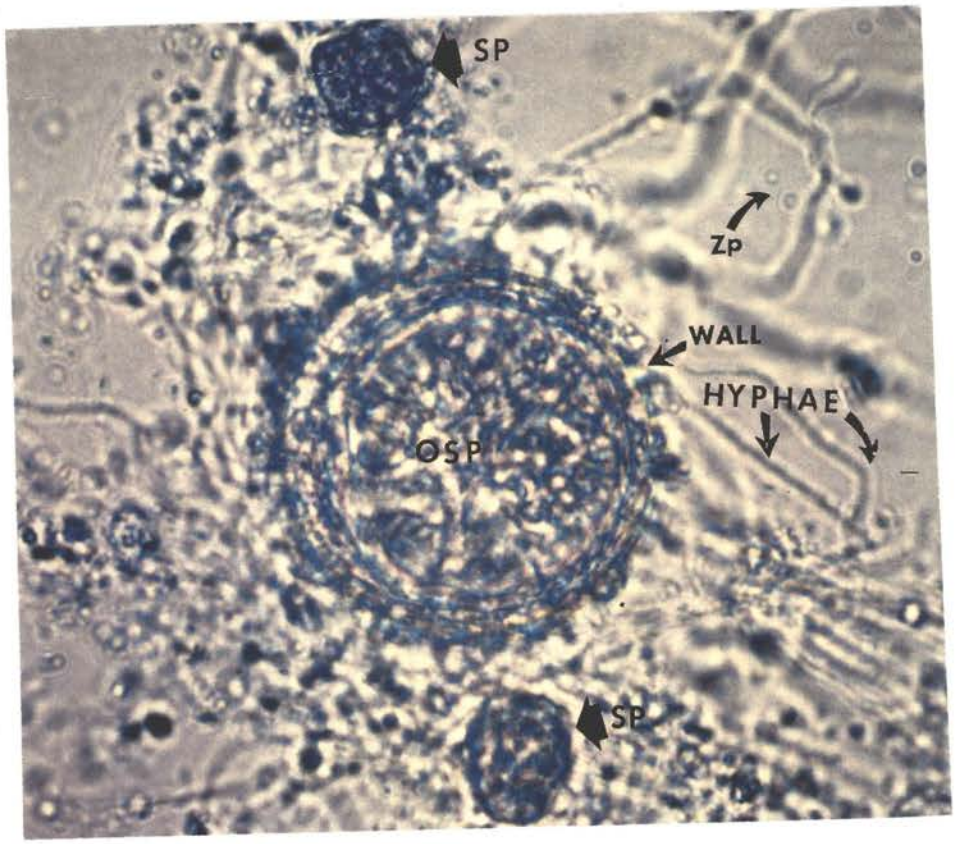

Fig. 1. Oospore of *Pythium ultimum* parasitized by *Actinoplanes brasiliensis* in buffer containing zoospores (Zp) of *A. brasiliensis*. Hyphae and sporangia (Sp) of *A. brasiliensis* emerge from the infected oospore (Osp).

although parasitized oospores were observed at 24 h in a few experiments.

Preliminary assays with freshly prepared suspensions of oospores showed that the MTT method was unsuitable for oospores of *P. arrhenomanes, P. irregulare* or *P. myriotylum.* Uptake of MTT by these latter strains was apparently low in that $\leq$25% of their oospores were viable, as indicated by oospores that were light blue or shades of red (Sutherland and Cohen, 1983). The MTT method was more useful for oospores of *P. aphanidermatum* and *P. ultimum.* Eighty percent to 90% of the oospores of *P. aphanidermatum* and 55% of the oospores of *P. ultimum* in control (nonparasitized) suspensions after staining with MTT were viable. Other oospores in these suspensions were nonviable, as indicated by their clear or occasionally dark purple or black color (Sutherland and Cohen, 1983). Dark purple or black oospores typically comprised $<$5% of an oospore population and had torn cell walls and grossly distorted cytoplasm. However, incubation of the oospores of *P. aphanidermatum* or *P. ultimum* incubated with *Actinoplanes* spp. reduced their oospore viability to 30-50% or 15-35%, respectively.

After 3 days incubation, parasitism of oospores ranged from 0 to 96% (Table 1) and numerous differences ($P \leq .05$) in parasitism were noted between strains for a given oospore host and between oospore hosts for a given strain. For instance, *A. yunnahensis* produced no or little parasitism of the oospores of P. irregulare (0%), *P. aphanidermatum* (9%) or *P. arrhenomanes* (12%), moderate parasitism of *P. myriotylum* (44%) and high parasitism of oospores of *P. ultimum* (96%). *Actinoplanes ferrugineus* and strain Wi 11 generally were the least infective to all oospore hosts

studied (0-14% and 0-6% parasitized oospores, respectively). Strains within a species also differed as to their parasitic spectrum, as shown by the degree of parasitism by strains of *A. missouriensis* (MSU strain, 14538 and E_3-15A). Infectivity of these strains to *P. arrhenomanes* or *P. myriotylum* differed little; however, the strains varied considerably in their parasitism to oospores of *P. aphanidermatum, P. irregulare* or *P. ultimum.*

No strain was highly parasitic (75% or greater) on oospores of all five *Pythium* spp. Indeed, only strain W 309 infected $\geq$50% of all oospore host populations. However, several strains were highly parasitic to one or two oospore hosts, as shown by cluster analysis of the 3-day-parasitism data (Table 2). Five clusters of mean parasitism per oospore host were sorted; however, only strains of *Actinoplanes* spp. in the maximum and minimum clusters are reported. Arcsin transformed means for the cluster of maximum parasitism were 0.80-1.21, whereas means for the cluster of minimum parasitism were 0.04-0.10. The observed overall R^2 was 0.94 or greater, indicating significant differences between the maximum or minimum clusters. Strain W 309 was highly infective to all hosts, except *P. myriotylum* (Table 2). Strains *A. brasiliensis, A. missouriensis,* W 57 and Z 20 were highly infective to two hosts, but not the others, whereas other strains, e.g. P 128, were highly infective of only one host. A few strains also were poor parasites of oospores. For instance, strain Wi 11 was least infective to four or the hosts and P 166 was least infective to three.

Table 1. Percent parasitism of oospores of *Pythium* spp. by strains of *Actinoplanes* spp. in buffer.

Strain	Parasitism %[a]				
	P. aph[b]	P. arr	P. irr	P. myr	P. ult
A. azureus	7 B de	11 B g-m	1 C kl	11 B jk	51 A bcd
A. brasiliensis	55 B a	59 B bc	32 C ef	54 B b-e	77 A abc
A. caeruleus	9 B de	1 C lm	13 B i-l	22 A h-k	11 B f-i
A. ferrugineus	0 C e	5 B i-m	3 BC kl	4 BC k	14 A e-i
A. ianthinogenes	4 B e	27 A efg	33 A ef	30 A g-j	42 A d-g
A. italicus	36 B abc	31 B def	19 B f-j	76 A ab	3 B hi
A. missouriensis (MSU)	20 C b-e	19 A a	34 B ef	53 B c-f	78 A abc
A. missouriensis (14538)	16 D cde	22 D f-j	50 B cd	68 A abc	35 C d-h
A. missouriensis (E_3-15A)	41 B ab	26 BC efg	16 C g-k	62 A d-e	16 C e-i
A. minutisporangius	14 B cde	4 B j-m	3 B kl	41 A e-i	46 A cde
A. rectilineatus	14 B cde	59 A bc	76 A a	31 B f-j	35 B d-h
A. teichomyceticus	21 BC b-e	24 B fgh	4 C kl	65 A a-d	51 A bcd
A. utahensis	12 C cde	78 A a	25 C f-i	50 B c-g	25 C d-i
A. violaceous	25 B b-e	75 A ab	54 A cd	53 A c-f	55 A a-d
A. yunnahensis	9 C de	12 C f-m	0 C l	44 B d-h	96 A abc
K 30	5 B e	18 B f-m	40 A de	49 A c-g	55 A a-d
LLW 211	31 A a-d	11 B g-m	49 A cd	13 B jk	2 B i
P 3	10 B de	24 A fgh	11 B i-l	21 A ijk	26 A d-i
P 114A	12 B cde	1 C klm	61 A bc	14 B jk	10 B ghi
P 128	50 A d	25 A efg	30 A efg	31 A f-j	39 A d-g
P 166	0 C e	2 C klm	11 B i-l	1 C k	16 A e-i
R 141Y	18 B b-e	43 A cde	23 B f-j	50 A c-f	46 A cde
S 21	23 B b-e	20 B f-k	63 A abc	17 B jk	3 B hi
W 13	4 BC e	0 C m	13 BC h-l	16 B jk	31 A d-i
W 57	24 C b-e	4 D j-m	53 B cd	84 A d	82 A ab
W 178	2 C e	20 B f-k	26 A fgh	29 A g-j	17 B e-i
W 257	25 C b-e	19 C f-l	60 B bc	66 AB a-d	84 A d
W 309	50 A d	72 A ab	72 A ab	56 A b-e	82 A ab
Wi 11	0 C e	6 A h-m	1 BC kl	1 BC k	2 B hi
Z 20	55 A a	23 B f-i	77 A a	58 A b-e	55 A a-d

[a]Values are the means of the percent parasitism in 600-1200 oospores in buffer after 3 days incubation at 28 C. Means in a column followed by the same lowercase letter or means in a row followed by the same uppercase letter are not significantly (P=0.05) different from each other according to Duncan's Multiple Range test.

[b]Abbreviations are: *P. aphanidermatum, P. arrhenomanes, P. irregulare, P. myriotylum* and *P. ultimum.*

Table 2. Strains of *Actinoplanes* spp. grouped by cluster analysis for maximum and minimum parasitism of *Pythium* species as hosts

	Parasitism to oospore host				
	Maximum		Minimum		
Host	Strain	Mean	Strain	Mean	R^2
P. aphanidermatum	*A. brasiliensis* P 128 W 309 Z 20	0.80	*A. ferrugineus* K 30 P 166 Wi 11	0.04	0.95
P. arrhenomanes	*A. missouriensis* *A. utahensis* *A. violaceous* W 309 W 13 W 57	1.06	*A. caeruleus* *A. minutisporangius* P 114 A P 166	0.10	0.97
P. irregulare	*A. rectilineatus* W 309 Z 20 *A. teichomyceticus* *A. yunnahensis* Wi 11	1.07	*A. azureus* *A. ferrugineus* *A. minutisporangius*	0.10	0.96
P. myriotylum	*A. italicus* W 57	1.13	P 166 Wi 11	0.04	0.94
P. ultimum	*A. brasiliensis* *A. missouriensis* *A. yunnahensis* W 57 W 257 W 309	1.21	*A. italicus* LLW 211 S 21 Wi 11	0.10	0.97

NOTE: The FASTCLUS procedure of SAS was used. Although five clusters of strains for each host were sorted, only the maximum and minimum clusters and their transformed means are presented above. The R^2 is an estimate of the variability in the level of parasitism explained by the sorting of the means into clusters.

In general, oospores of *P. myriotylum* and *P. ultimum* were the most susceptible to parasitism whereas those of *P. aphanidermatum* and *P. arrhenomanes* were the least. Oospores of *P. irregulare* were intermediate in susceptibility. Mean parasitism by all strains in oospore populations of *P. myriotylum, P. ultimum, P. irregulare, P. arrhenomanes* and *P. aphanidermatum* (Table 1) was 42%, 41%, 32%, 22% and 18%, respectively.

Selected strains showing low parasitism to one or more oospore hosts at 3 days were evaluated for their degree of parasitism to their hosts at 7 or 14 days incubation (data not shown). A few strains showed no appreciable increase in parasitism to any host over time, e.g. P 3, P 166, W 13 and Wi 11. However, most strains exhibited increased parasitism over time, with some showing up to 4 times the incidence of infection on their host at 14 days than at 3 days, e.g. *A. missouriensis* to oospores of *P.arrhenomanes.* The pattern of oospore host susceptibility to strains established after 3 days incubation was not essentially altered after 7 or 14 days of incubation.

Parasitism on soils. Strains of *Actinoplanes* spp. generally parasitized oospores of *P. ultimum* after 3 days incubation on a loamy sand soil. Mean parasitism was 4.6-44.4% on sterile soil and 0-55.1% on nonsterile soil. After 14 days, parasitism was 46.3-75.0% on sterile soil and 24.6-57.2% on nonsterile soil (Table 3). Some strains were highly infective to oospores of *P. ultimum, e.g. A. missouriensis,* K 30 and W 257, whereas others were not, e.g. *A. yunnahensis* and LLW 211. Numerous sporangia of *Actinoplanes* sp. were typically found emanating from parasitized oospores incubated on soils (Fig. 2). Oospores incubated on nonsterile soils not

supplemented with *Actinoplanes* spp. also were parasitized by an indigenous *Actinoplanes* sp. or by a *Streptomyces* sp. (Table 3 and 4; Fig. 3). Oospores parasitized by *Streptomyces* sp. typically had helical hyphae on them and were excluded from parasitism counts. Oospores infected with indigenous *Actinoplanes* sp. had straight hyphae, often bearing globose sporangia proliferating from them. These were included in counts of parasitism.

Supplementation of nonsterile soils with *Actinoplanes* spp. generally increased ($P \leq 0.05$) the parasitism of oospores compared to nonsupplemented soils (Table 3 and 4). A strain of *Actinoplanes* sp. frequently showed a parasitic preference for one or more species of *Pythium* but not others, when exposed simultaneously to all host species (Table 4). Typically, oospores of *P. ultimum* or *P. irregulare* were more susceptible to infection on supplemented nonsterile soil than oospores of *P. aphanidermatum*. Oospores of *P. arrhenomanes* or *P. myriotylum* appeared to be intermediate in their susceptibility.

Parasitism of oospores of *P. aphanidermatum* or *P. ultimum* on a nonsterile sandy loam soil supplemented with *Actinoplanes* sp. generally increased over time (Fig. 3), reaching >75% by 28 days and >90% by 56 days of incubation. Parasitism of oospores of *P. aphanidermatum* or *P. ultimum* by the indigenous *Actinoplanes* sp. in the controls increased over time in nonsterile soil reaching 95% or 79%, respectively after 56 days of incubation.

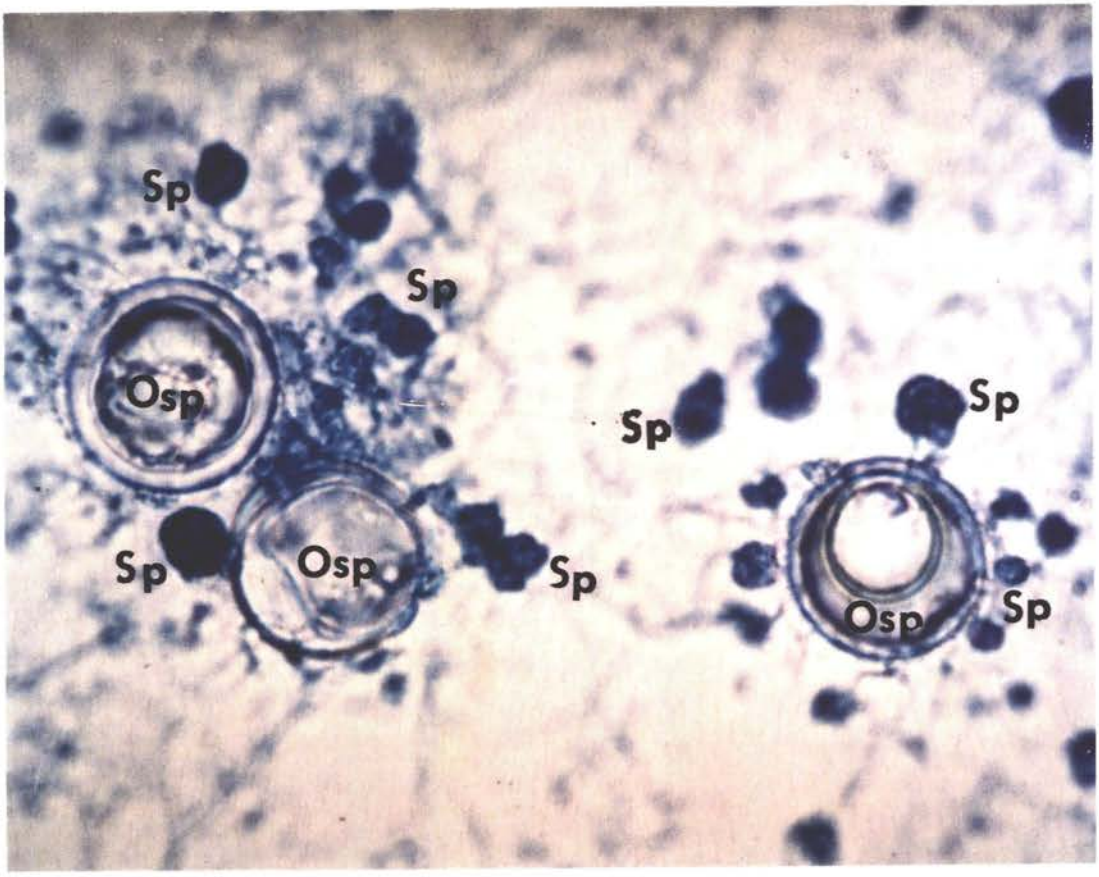

Fig. 2. Parasitism of oospores of *Pythium ultimum* in nonsterile soil supplemented with strain W 57 of *Actinoplanes* spp. Numerous sporangia (Sp) of *Actinoplanes* sp. emanate from the parasitized oospores (Osp).

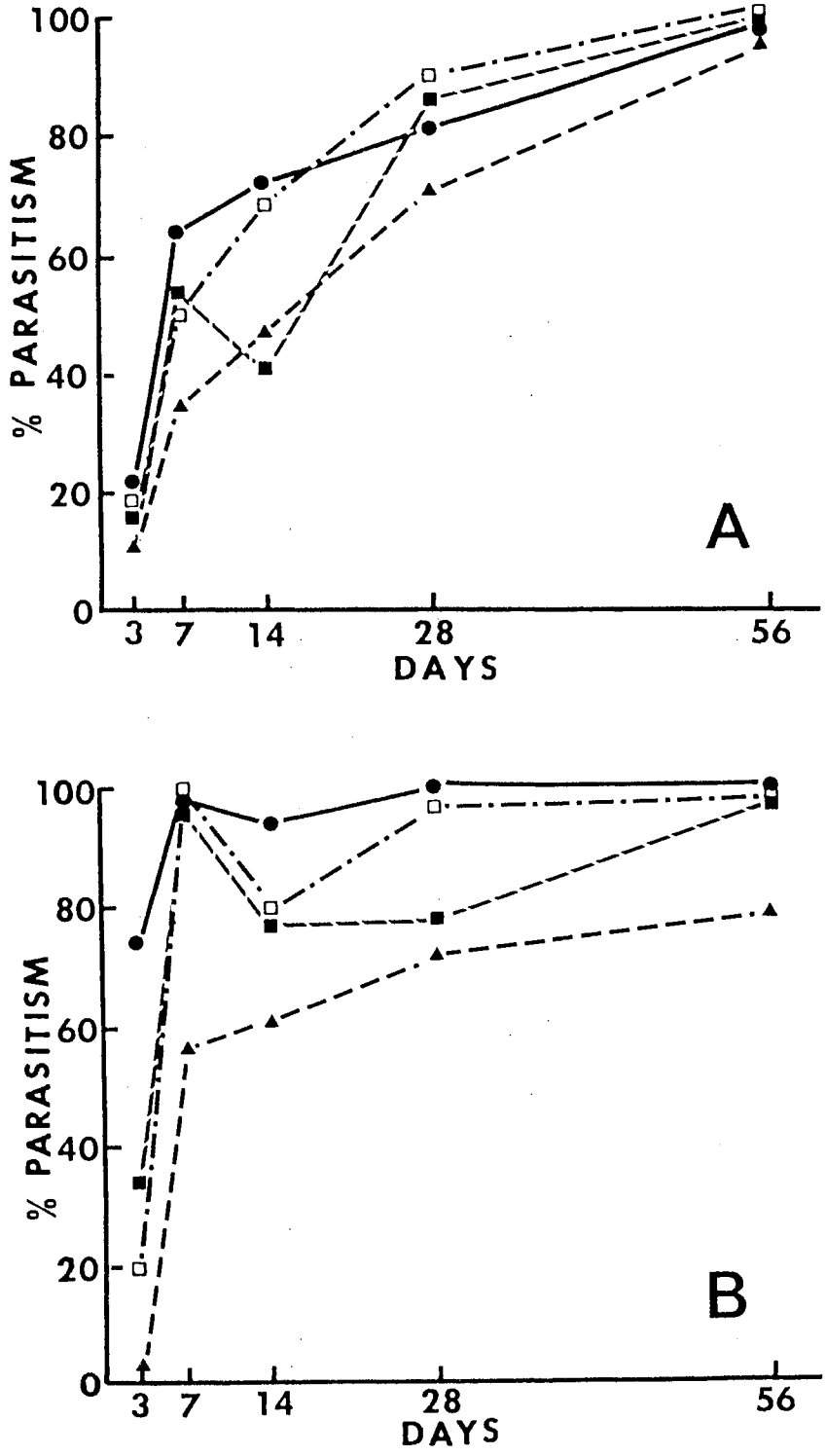

Fig. 3. Percent parasitism over time of oospores of (A) *P. aphanidermatum* or (B) *P. ultimum* on nonsterile soil not supplemented with *Actinoplanes* sp. (▲) or supplemented with *A. brasiliensis* (●), *A. teichomyceticus* (■) or strain W 57 (□).

Table 3. Parasitism of oospores of *P. ultimum* by strains of *Actinoplanes* spp. after 14 days incubation on sterile or nonsterile soil.

Strain	Parasitism %[a]	
	Sterile	Nonsterile
A. brasiliensis	61.8 bc	54.6 ab
A. missouriensis	75.0 a	52.6 ab
A. teichomyceticus	50.0 de	48.7 bc
A. yunnahensis	48.4 de	24.6 d
K 30	64.9 b	57.2 a
LLW 211	46.3 e	28.9 d
R 141 Y	48.4 de	41.6 c
W 257	65.9 b	54.8 ab
Z 20	55.3 c	41.2 c
Control[b]	00.0 f	10.0 e

[a]Means in a column followed by the same letter are not significantly (P = 0.05) different from each other according to the Duncan's Multiple Range test.
[b]No *Actinoplanes* sp. added to soil.

Table 4. Parasitism of oospores of five *Pythium* spp. simultaneously incubated for 14 days on nonsterile soil supplemented with strains of *Actinoplanes* spp.

	Parasitism %[a]				
Strain	P. aphan[c]	P. arrh	P. irreg	P. myr	P. ult
A. brasiliensis	63.3aZ	71.0bZ	78.7aZ	73.3bZ	79.7bZ
A. teichomyceticus	50.3bX	75.0bY	90.0aZ	84.3aZ	83.3bZ
W 57	71.0aY	60.7cY	85.7aZ	86.0aZ	95.0aZ
W 257	40.0bY	67.0cZ	75.0bZ	41.0cY	72.0bZ
Z 20	41.7bX	89.0aZ	77.3bZ	64.7bY	50.3cX
Control[b]	40.0bY	31.3dY	37.0cY	30.7cY	58.3cZ

[a]Means in a column followed by the same lowercase letter or means in a row followed by the same uppercase letter are not significantly (P=0.05) different from each other according to Duncan's Multiple Range test.
[b]No *Actinoplanes* sp. added to soil.
[c]Abbreviations are: *P. aphanidermatum, P. arrhenomanes, P. irregulare, P. myriotylum* and *P. ultimum.*

Analysis of variance showed that the level of parasitism of *P. aphanidermatum* in nonsterile soil supplemented with *Actinoplanes* sp. was greater (P=0.05) than the level in nonsupplemented soil only at 7 or 14 days of incubation (Fig. 3). At 7 days parasitism in all supplemented soils was 50-70% compared to 34% in nonsupplemented soil. At 14 days parasitism on soil supplemented with *A. brasiliensis* or W 57 was greater than that on nonsupplemented soil. Regression analysis, however, showed no significant (P=0.05) differences between the rates of parasitism in supplemented soil compared to that in nonsupplemented soil. For oospores of *P. aphanidermatum* (Fig. 3A) the equations for the arcsin transformation of percent parasitism (P) related to days of incubation (D) were $\underline{P} = 25.31 + 1.03\ \underline{D}$, $\underline{P} = 40.43 + 0.80\ \underline{D}$, $\underline{P} = 34.17 + 1.04\ \underline{D}$ and $\underline{P} = 34.65 + 1.09\ \underline{D}$ in nonsupplemented soil, soil plus *A. brasiliensis,* soil plus *A. teichomyceticus* and soil plus strain W 57, respectively.

Parasitism of oospores of *P. ultimum* was greater ($P \leq 0.05$) on supplemented soils than on nonsupplemented soils at 3, 7 and 56 days of incubation (Fig. 3B). At 14 or 28 days, parasitism on soil with W 57 or *A. teichomyceticus,* respectively were not different than that on nonsupplemented soil. Regression analysis also showed significant differences (P=0.05) between parasitism on supplemented compared to nonsupplemented soils based on intercepts, but not on slopes. For oospores of *P. ultimum* (Fig. 3B) the equations were $\underline{P} = 28.45 + 0.68\ \underline{D}$, $\underline{P} = 71.99 + 0.39\ \underline{D}$, $\underline{P} = 51.12 + 0.70\ \underline{D}$ and $\underline{P} = 54.64 + 0.69\ \underline{D}$ for nonsupplemented soil, soil plus *A. brasiliensis,* soil plus *A. teichomyceticus,* and soil plus strain W 57, respectively.

The incidence of parasitism over time to oospores on sterile soil (data not shown) was less than on nonsterile soil, due to the background parasitism from indigenous *Actinoplanes* spp. Oospores of *P. aphanidermatum* were infected less than oospores of *P. ultimum* on sterile soil. At 56 days of incubation, infection of *P.*

aphanidermatum oospores by the strains was 13.6 - 90%, whereas that for oospores of *P. ultimum* was 79.6 - 100%. No parasitism of oospores occurred on nonsupplemented sterile soil.

Some variation in the level of parasitism on supplemented soils was observed. For instance, at 14 days of incubation (Table 3 & 4; Fig. 3), parasitism to P. ultimum oospores on soils supplemented with *A. brasiliensis* or *A. teichomyceticus* was 55-93% or 49-83%, respectively.

DISCUSSION

Our study expands the host range of *Actinoplanes* spp. to include oospores of *P. arrhenomanes, P. irregulare* and *P. myriotylum.* Prior to our findings only oospores of *P. aphanidermatum* and *P. ultimum* were known to be hosts of *A. missouriensis* (Sutherland and Lockwood, 1984). We also report for the first time many additional species and strains of *Actinoplanes* spp. that were parasitic to oospores of *Pythium* spp. Species newly shown to be parasites of oospores of one or more *Pythium* spp. were *A. azureus, A. brasiliensis, A. caeruleus, A. ferrugineus, A. ianthinogenes, A. italicus, A. multisporangius, A. rectilineatus, A. teichomyceticus, A. utahensis, A. violaceous* and *A. yunnahensis.* In addition, 15 strains of *Actinoplanes* yet to be speciated are reported here for the first time to be parasites of oospores of *Pythium* spp. One strain (Wi 11) did not parasitize oospores of any species tested to any appreciable extent (<7%).

We considered oospores to be parasitized only if microscopic examination showed internal disorganization of the oospore in addition to emergent hyphae of *Actinoplanes* sp. Our observations were similar to those of Sneh et al. (1977) with oospores of *P. megasperma glycinea* parasitized by *A. missouriensis.* Sneh et al. (1977) also concluded that living oospores of *P. megasperma glycinea* were parasitized by *A. missouriensis.* Sutherland and Lockwood (1984) reported that *A.*

missouriensis infected living oospores of *P. megasperma glycinea* far greater (94%) than colonizing nonliving oospores (31%). Our results also support these findings. Apparently healthy oospores of *Pythium* spp., as determined by microscopic examination were parasitized, often extensively, by *Actinoplanes* spp.

Results with vital staining also suggested that living oospores of *P. aphanidermatum* and *P. ultimum* were parasitized and killed by *Actinoplanes* spp. Results with *P. arrhenomanes, P. irregulare* or *P. myriotylum* were not as conclusive due to the lower percentage ($\leq$25%) of these oospores in nonparasitized populations that were viable according to the MTT method. Nevertheless, considering the >75% parasitism of these species by some strains of *Actinoplanes* spp., it is reasonable to expect that living oospores of these species also were parasitized. In other studies (Khan and Filonow unpublished) addition of various strains of *Actinoplanes* spp. to sand infested with predetermined densities of *P. irregulare* oospores reduced (P=0.05) the densities to 10-50% of their original levels.

Our study suggests that strains of *Actinoplanes* spp. exhibit a host specificity for species within the genus *Pythium.* Evidence to support host specificity is based on the spectrum of parasitism exhibited *in vitro* (Table 1) and the separation of these data by cluster analysis (Table 2) to demonstrate for each species of *Pythium,* a particular cluster of strains showing maximum parasitism differentiated from those showing minimum parasitism. Host specificity also was observed in soil studies. Parasitism by some strains in soil was significantly (P=0.05) less for oospores of *P. aphanidermatum* than for oospores of *P. ultimum* (Table 4), and some strains were highly parasitic to oospores of certain species of *Pythium* whereas others were not (Table 3 and 4).

To our knowledge, this is the first report of a specificity among strains of *Actinoplanes* spp. as hosts of *Pythium* oospore. However, in a study of the host range

of several parasites of oospores, Sutherland and Lockwood (1984) reported that *A. missouriensis* parasitized oospores of different genera according to the following: *Pythium* < *Aphanomyces* < *Phytophthora.* In their study (Sutherland and Lockwood, 1984), the level of parasitism to *P. aphanidermatum* by *A. missouriensis* was not significantly (P=0.05) different than that to *P. ultimum.* Their results were contrary to ours which used the same strain (*A. missouriensis* (MSU)), but which found oospores of *P. aphanidermatum* to be significantly (P=0.05) less susceptible than oospores of *P. ultimum* (Table 1).

Chemotaxis might account for the host specificity by strains of *Actinoplanes* spp. that we found. Arora (1987) reviewed motility and chemotaxis in the Actinoplanaceae and reported that *A. missouriensis* and *A. utahensis* were chemotactically attracted to oospores of *P. ultimum* in soil. Our own observations (Khan et al. 1992) also suggest that zoospores of some strains of *Actinoplanes* spp. may be attracted *in vitro* to oospores of *P. arrhenomanes* and *P. ultimum.* The role of chemotaxis in determining *Actinoplanes* strain-oospore host specificity warrants study.

Other mechanisms may play a role in determining *Actinoplanes* strain-oospore host specificity, such as lectin binding phenomena that have been implicated in mycoparasitic relationships (Barak et al. 1986; Nelson et al. 1986; Chet, 1989). However, nothing is known regarding the presence of cell surface receptors on oospores or their possible role in binding microbial parasites. Differences may exist in the production of degradative enzymes by strains of *Actinoplanes* spp. such as those found in the degradation of fungal host hyphae by *T. harzianum* (Elad et al. 1982; Ridout and Coley-Smith, 1988). Cellulose and other β-glucans are major constituents of oospore cell walls (Bartnicki-Garcia and Wang, 1983); however, the production of cell-wall-degrading enzymes by *Actinoplanes spp.* has not been studied.

Transmission electron microscopy of the cell walls of oospores of *P.*

megasperma glycinea parasitized by *A. missouriensis* suggested mechanical invasion of the oospores (Sutherland et al. 1984). Penetration of oospores by *Actinoplanes* spp. may be affected by the thickness and/or chemical composition of the oospore cell wall. Additional ultrastructural studies would be helpful in determining how *Actinoplanes* spp. penetrate *Pythium* spp. oospores.

Strain selection is an important criteria in developing a biological control agent. Cluster analysis suggested several strains, e.g. *A. brasiliensis,* W 57 and W 309, as candidates for further evaluation. Since agricultural soils may harbor more than one pathogenic species of *Pythium,* strain W 309 may be particularly valuable, as it was highly parasitic to four *Pythium* spp. Some strains of *Actinoplanes* spp. were very effective parasites of oospores on sterile or nonsterile soil, a finding which supports their further evaluation as biocontrol agents for soil application.

Soils used in this study differed in texture and pH. In addition, oospores of *Pythium* spp. were parasitized by indigenous *Actinoplanes* spp. in the nonsterile soils. This "background" parasitism was expected since *Actinoplanes* spp. are common inhabitants of soil (Makkar and Cross, 1982; Palleroni, 1989). The level of background parasitism in the soils varied from 10% to >90% depending on the soil, the host species, and the duration of incubation. Nevertheless, the overall level of parasitism of oospores in most experiments was significantly ($P=0.05$) increased by supplementing the soils with certain strains of *Actinoplanes* spp.

Little is known of the population densities of *Actinoplanes* spp. in agricultural soils, or of the environmental factors that influence these densities. Some soils may harbor high populations and/or highly infective strains of *Actinoplanes* spp., whereas others may not. Soils may contain more than one pathogenic species of *Pythium.* Supplementation of soils with sufficient populations and with appropriate strains may enhance naturally occurring biological control. Our preliminary results (Khan and

Filonow unpublished) support this approach. In addition, *Actinoplanes* strains may be useful additions to greenhouse potting mixes, in which natural populations of *Actinoplanes* spp. may be low or absent.

The effects of environmental factors on the parasitic activity of *Actinoplanes* spp. in soils need further study. Parasitism of *P. megasperma glycinea* oospores by *A. missouriensis* was greatest in soil at 0 J/kg matric potential and 20-30 C (Sutherland and Lockwood, 1984). We used -1 J/kg matric potential for soils and 28 C in all our experiments to optimize conditions for the selection of strains based on parasitic activity. In other work (Khan et al. 1993), we have observed biological control activity by strains of *Actinoplanes* spp. in field soil at 18-26 C. Sutherland and Lockwood (1984) reported that *A. missouriensis* was an active parasite in soil as dry as -1500 J/kg and at 15 C. At $\leq$10 C parasitism of oospores was $<10\%$.

Profuse production of sporangia of *Actinoplanes* spp. on parasitized oospores of *Pythium* spp. was commonly observed in soil, whereas such sporulation was less frequent *in vitro*. Factors that may account for this effect need to be determined. The production of secondary inoculum via sporulation from an infected fungal host would be an advantageous attribute to a microbial parasite in biocontrol.

CHAPTER IV

A CLAY GRANULE METHOD FOR APPLYING BIOCONTROL INOCULUM OF *ACTINOPLANES SPP.* TO SOIL

ABSTRACT

Strains of *Actinoplanes* spp. grew and sporulated best at 30 C on dilute Czapek-Dox agar (pH 7) under low light ($\mu E/m^2/sec$) or in the dark. Continuous fluorescent light exposure of 150, 32 or 4 $\mu E/m^2/sec$ generally inhibited growth and sporulation of strains. On agar at pH 5, growth and sporulation was nearly completely suppressed, regardless of temperature. Growth and sporulation were typically inhibited at 10 C, regardless of agar pH compared to responses at 20 or 30 C. After 3 wk, strains did not sporulate on rice hulls or perlite treated with dilute Czapek-Dox broth plus 0.1% w/v peptone and incubated in the dark at 28-30 C. Montmorillonite clay granules (350-500 μm dia) treated with this nutrient and incubated under the same conditions supported extensive sporulation of strains W 57, W 257, ATCC 25844 (*A. brasiliensis*) and NRRL 16254 (*A. yunnahensis*), but not strain ATCC 31121 (*A. teichomyceticous*). Inoculum densities of strains on granules treated with sucrose or starch were 10^9-10^{10} cfu/g granule compared to 10^7-10^8 cfu/g for granules treated with other carbon sources. A 100-fold decrease in inoculum densities on granules occurred

after two month storage at 5-35 C. Thereafter, densities changed little during an additional 2 mo. Four-month-old granules in buffer after 24 h released motile zoospores of *Actinoplanes* sp. Populations of oospores of *Pythium ultimum* incubated in these suspensions were 81-82% parasitized by *Actinoplanes* sp. after 7 days. Clay granules may have potential as means for delivering *Actinoplanes* sp. to soil for biocontrol evaluations.

INTRODUCTION

Many species of the oomycetous fungi, *Aphanomyces, Pythium* and *Phytophthora* spp. cause seed and seedling damping-off and root rot of several economically important plant hosts (Erwin et al. 1983; Pfender, 1984; Schneider, 1978; Van der Plaats-Niterink, 1981). Sufficient genetic resistance to these pathogens is lacking in most economically important plant hosts. There is no fungicide currently registered in the USA that is effective against *Aphanomyces* spp. (Rush and Vaughn, 1993). Metalaxyl and other oomycete-specific fungicides may be used to control Pythium and Phytophthora incited diseases; however, resistance to these fungicides may be a problem (Bower and Coffey, 1985; Sanders, 1987). Fungicides may pose a hazard to the environment and may not be cost effective. The oospores of some oomyceteous pathogens are known to survive in soil for years (Hoppe, 1966; Lumsden and Ayers, 1975) and may be difficult to kill. For instance, thick-walled oospores of *P. ultimum* are not readily affected by chemicals and heat (Stasz and Martin, 1988). Since the disease potential in soil is typically related to the density of

oospores in soil (Mitchell, 1978), destruction of oospores should reduce the amount of disease in infested soil.

Destruction of oospores by microbial parasites may be a means for controlling soilborne diseases caused by oomycetous fungi. Sneh et al. (1977) reported that *Actinoplanes missouriensis* Couch., a Gram-positive, filamentous, sporangiate bacterium (Vobis, 1989) was a parasite of the oospores of *Phytophthora megasperma glycinea (Pmg)* Kuan & Erwin. Sutherland and Lockwood (1984) increased the host list of *Actinoplanes* spp. to include *P. aphanidermatum* Fritz., *P. ultimum* Trow., and *Aphanomyces euteiches* Drechs. Recently, Khan et al. (1993) showed that oospores of *P. arrhenomanes* Drechs., *P. irregulare* Buis., and *P. myriotylum* Drechs., also were hosts. Twelve additional species of *Actinoplanes* were also identified as new parasites of *Pythium* spp. oospores (Khan et al., 1993). Several of these strains were shown to be highly parasitic, exhibiting > 80% infection of *Pythium* spp. oospores.

Actinoplanes spp. have been applied to seed or soil for purpose of biocontrol (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984; Sutherland and Papavizas, 1991). Soybean seed has been treated with a suspension of hyphae and sporangia in water (Sutherland and Lockwood, 1984) or in a solution of carboxymethylcellulose and starch in water (Filonow and Lockwood, 1985). *Actinoplanes* spp. also have been applied in water as a drench to soil or as a rootdip (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984; Sutherland and Papavizas, 1991). There is no work regarding the use of solid carriers for the delivery of *Actinoplanes* spp. inoculum to soil. We investigated some factors that influence the

growth and sporulation of *Actinoplanes* spp. and we report the development of a clay granule method for applying biocontrol inoculum of *Actinoplanes* spp. to soil.

MATERIALS AND METHODS

Growth and sporulation of *Actinoplanes* spp. on agar.

Effect of culture media. *Actinoplanes* spp. were stored in glycerol at -70 C and routinely maintained on dilute Czapek-Dox agar (dCDA) (12g Czapek-Dox Broth (Difco, Inc. Detroit, MI, USA)+ 18g agar/L) at 28 C. Growth and sporulation of strains K 30, W 257, W 57, W178, Z 20, ATCC 25844 (*A. brasiliensis*), *A. missouriensis* (LL-E_3-15 A), ATCC 31121 (*A. teichomyceticus*) and NRRL 16254 (*A. yunnahensis*) were evaluated on the following culture media: (1) minimal salt medium (M3; Rowbotham and Cross, 1977), (2) M3 plus 1% (w/v) fructose (M3+F), (3) potato dextrose agar (PDA), (4) 1/3 dilution of CDA (dCDA), (5) chitin agar (CA) (Hsu and Lockwood, 1975), and (6) yeast extract-phosphate-soluble starch agar (YPSS) (Emerson, 1958). Potato dextrose broth, Czapek-Dox broth, and yeast extract were purchased from Difco, Inc. (Detroit, MI). All media were prepared with 1.8% (w/v) agar. Petri dishes (9 cm dia) of agar media were spread inoculated with a 0.2 ml of a suspension of *Actinoplanes* sp. in 50 mM K^+ phosphate buffer (pH 6.8). The dishes were sealed with Parafilm^R. There were 3 dishes per medium per strain. Dishes were incubated at 28 C in the dark. After 2 wk the extent of colony growth was visually estimated with the unaided eye, whereas the extent of sporulation was estimated with a microscope at 80X using a rating of 0-4, where 0=no hyphal growth or sporulation and 4=>75% of the agar surface with growth or >75% of the colony

surface area with sporangia.

Effects of pH and temperature. The pH of the dCDA was adjusted from 5-8 by adding sterile dilute NaOH or HCl to the cooled medium before pouring into dishes. Suspensions of strains 25844 or K 30 were spread uniformly over the entire agar surface on the dishes. Dishes at each pH level were incubated in the dark at 10, 20, or 30 C. After two weeks the extent of hyphal growth and sporulation were estimated as described above. There were 3 dishes per strain per pH per temperature treatment.

Effects of light intensity. Dishes of dCDA inoculated with 25844 or W 257 were exposed to constant light (150 $\mu E/m^2/sec$) at 25-28 C under fluorescent (General Electric F40CW Cool white) lights for two weeks. Inoculated dishes covered with aluminum foil to exclude light were incubated alongside those exposed. Inoculated dCDA dishes in an incubation chamber were also exposed to fluorescent light intensities of 32, 4, or 1 $\mu E/m^2/sec$ by varying the distance of the dishes from the light source. As controls, companion dishes were covered in aluminum foil and placed alongside those exposed to light. Dishes were incubated for 2 wk in incubators (Percival, Boone, Iowa) set at 10 C, 20 C or 30 C. There were 3 dishes per strain per light intensity per temperature treatment. To assess the effect of light duration on growth and sporulation, three dishes of dCDA inoculated with strain 25844 or W 257 were exposed to 1-32 $\mu E/m^2/sec$ of light for 30 min per day. Companion dishes covered with foil and incubated under the light were controls. Dishes were incubated at 25-28 C for 2 wk prior to assessing growth and sporulation. In all experiments, hyphal growth and sporulation were estimated as described above.

Sporulation of *Actinoplanes* spp. on soild carriers.

Preparation and inoculation of carriers. Shake cultures of strains W 57, W 257, 25844, 16254 and 31121 were grown in 200 ml of YPSS broth in 2-L flasks in the dark at 28 C for 2-4 wk. The cultures were aseptically harvested, washed 3X with sterile water, macerated in an Omni mixer (50% power, 30 sec) (Dupont, Inc. Wilmington, DE), and used to inoculate the following solid carriers; rice hulls, perlite, and clay granules. The rice hulls and perlite were used as is. Montmorillonite clay was obtained by grinding Ultra Premium Cat Litter^{R} (Wal-Mart, Inc. Bentonville, AR) to pass a 30 mesh sieve and collecting it on a 45 mesh sieve. The granules were washed to remove fines and dried at 70 C for 48 h. Particle size of the granules was 350-500 μm dia.

Four g of solid carrier in 9-cm dia. glass Petri dishes were autoclaved at 135 C for 3 h, and aseptically saturated with 6 ml dCD broth (pH 7) plus 0.1% w/v peptone as an additional nitrogen source (dCDB+P). The carriers were aseptically dried at 70 C for 48 h, cooled and inoculated with 5 ml of a suspension of an *Actinoplanes* sp. strain. The dishes were sealed in Parafilm^{R}. Dishes were incubated in the dark at 28 C for up to 3 weeks. Periodically, the dishes were examined for the extent of sporulation using a microscope (80X).

In some experiments, sucrose, the carbon source in dilute Czapek-Dox broth was replaced (w/w) by soluble starch, mannitol, maltose, mannose or glucose to determine which was the best carbon source for sporulation on clay granules.

Survival of *Actinoplanes* spp. on clay granules. Clay granules bearing sporangia of *Actinoplanes* sp. were aseptically air-dried in a laminar flow hood for 48 h, composited in a plastic bag, and stored at 23-25 C until use in other experiments. Five g portions of the air-dried granules were transferred to glass vials and incubated at 5, 20 and 35 C. Periodically for up to 4 months, the inoculum density of *Actinoplanes* sp. on granules (cfu/g of granules) from the vials was determined by serial dilutions of triplicate portions of 0.1 g granules in 10 ml of phosphate buffer on each of 5 dishes of chitin agar (Hsu and Lockwood, 1975). Colonies were counted after 7 days incubation at 28 C in the dark with one 30 min exposure to 32 $\mu E/m^2/sec$ of light per day.

The infection potential of strains on four-month-old granules was determined by floating 1 cm X 1 cm pieces of nylon cloth (15 μm openings; Tetko, Inc. Elmsford, NY) bearing oospores of *P. ultimum* (Khan et al., 1993) on 10 ml of phosphate buffer (pH 7) containing 1.0 g of granules at 28 C in the dark for 7 days. There were 3 cloth pieces per strain. Oospores on cloth pieces floated on buffer containing granules without *Actinoplanes* sp. were the control. Cloth pieces were removed, the oospores stained in cotton blue and inverted on molten agar on a glass slide. After cooling, the cloth was removed and oospores in the agar examined for parasitism (Khan et al. 1993). Zoospore motility in the granule suspensions was assessed after 24 h of incubation by examining 50 μl drops on a glass slide at 800X magnification.

All experiments were repeated one or more time. Results presented herein are

from representative experiments.

RESULTS

Growth and sporulation of *Actinoplanes* spp. on agar.

Effect of culture media. *Actinoplanes brasiliensis* grew and sporulated well on all the medium (Table 5), whereas all other strains exhibited different degrees of growth or sporulation depending on the medium. Strain W 178 grew well on most media, but did not sporulate on any medium. Typically, dCDA, CA and YPSS promoted extensive colony growth of nearly all strains, whereas growth of some strains, e.g *A. utahensis* and W 257 were restricted on PDA or M3 agar. Sporulation of several strains was much reduced on PDA compared to the other media. Over all, we found that dCDA was convenient to prepare and promoted abundant growth and sporulation of nearly all strains of *Actinoplanes* spp. tested.

Effect of temperature and pH. Temperature and pH interacted to affect growth and sporulation (Fig. 4). At 10-30 C growth and sporulation were inhibited at pH 5. At pH 6 these responses were inhibited at 10 or 20 C, but at 30 C growth and sporulation markedly increased. Extensive growth and sporulation were found at pH 7 at 20 or 30 C, but not at 10 C. At pH 8 growth increased when temperature increased from 20 to 30 C, but sporulation decreased.

Effect of light intensity. Continuous exposure to 150 $\mu E/m^2/sec$ of fluorescent light for 14 days inhibited growth and sporulation of all *Actinoplanes* spp. Typically, colony growth on agar exposed to continuous light had only a few large brightly colored colonies with no or little sporulation. Dishes kept in the dark, however, had

profuse growth and sporulation, but colony color was less vivid than in the light (Fig. 5) Moreover, continuous exposure of strains to less intense light (4 or 32 $\mu E/m^2/sec$) also reduced sporulation (Fig. 6). The effect was most pronounced at 10 C. A light intensity of 1 $\mu E/m^2/sec$ did not reduce sporulation of the strains compared to no-light controls at 30 C or 20 C; however, at 10 C no sporulation was observed. At 10 C or 20 C and light intensities of 32 or 4 $\mu E/m^2/sec$, colony growth of strains was reduced compared to controls whereas at 30 C colony growth was not different. Colony colors of strains at 4 or 32 $\mu E/m^2/sec$ typically were more vivid than the subdued colony colors found in strains incubated in the dark or exposed to 1 $\mu E/m^2/sec$. A 30 min exposure per day to light (1-32 $\mu E/m^2/sec$) did not reduce growth and sporulation and maintained colony color brightness.

Sporulation of *Actinoplanes* spp. on solid carriers.

Extent of sporulation. Strains of *Actinoplanes* spp. did not sporulate on rice hulls and sporulation on perlite was sparse, covering less than 2% of the top surface of perlite. Sporulation on clay granules treated with dCDB+P was more extensive (Fig. 7). Strains 25844, 16254, W 57 and W 257 consistently sporulated on clay granules, covering 20-50% of the top surface area in a dish. Strain 31121 sporulated sparsely (<2%) or not at all on clay granules. Inoculum densities of strains 25844, 16254 W 57 and W 257 ranged from 10^7 to 10^{10} cfu/g granules. Starch or sucrose promoted greater sporulation of strain 25844 or B 16254 on clay granules than other carbon sources (Table 6).

Survival of strains on clay granules. Inoculum densities (cfu/g granules)

Table 5. Effect of different media on growth and sporulation of *Actinoplanes* spp.

Species or strain	dCDA[a]		PDA		CA		M3		M3+F		YPSS	
	G	S	G	S	G	S	G	S	G	S	G	S
A.brasiliensis	4.0	4.0	4.0	4.0	4.0	4.0	3.5	4.0	4.0	3.0	4.0	3.3
A.teichomyceticus	4.0	4.0	3.7	0.6	4.0	3.7	1.5	1.0	1.0	4.0	3.3	2.5
A.missouriensis	4.0	2.8	1.0	0.0	4.0	3.3	3.7	3.7	3.5	4.0	4.0	3.5
A.utahensis	4.0	4.0	0.6	2.0	4.0	4.0	2.5	4.0	3.7	2.3	4.0	2.5
K 30	4.0	4.0	0.0	0.0	4.0	1.3	3.0	3.3	1.5	0.0	4.0	2.5
W 257	4.0	3.3	1.3	0.3	2.8	1.1	1.0	3.7	3.5	0.0	4.0	2.3
W 57	4.0	4.0	2.1	0.0	4.0	3.1	3.1	2.0	2.1	0.0	4.0	3.1
W 178	4.0	1.5	2.6	0.3	4.0	0.0	4.0	0.6	1.5	0.0	4.0	0.0
Z 20	4.0	4.0	4.0	3.4	4.0	3.8	0.5	0.4	0.0	0.0	4.0	4.0

[a] Media tested were: dCDA= Dilute Czapek-Dox Agar, PDA= Potato Dextrose Agar, CA= Chitin Agar, M3= M3 media, M3+F= M3 media with 1% w/v fructose, and YPSS= Yeast extract-phosphate-soluble starch medium. The extent of hyphal growth (G) was visually estimated with the unaided eye and sporulation (S) was microscopically (80X) estimated after 14 days using a rating of 0-4, where 0= no growth and 4=>75% of the agar surface area with growth or colony surface area with sporulation.

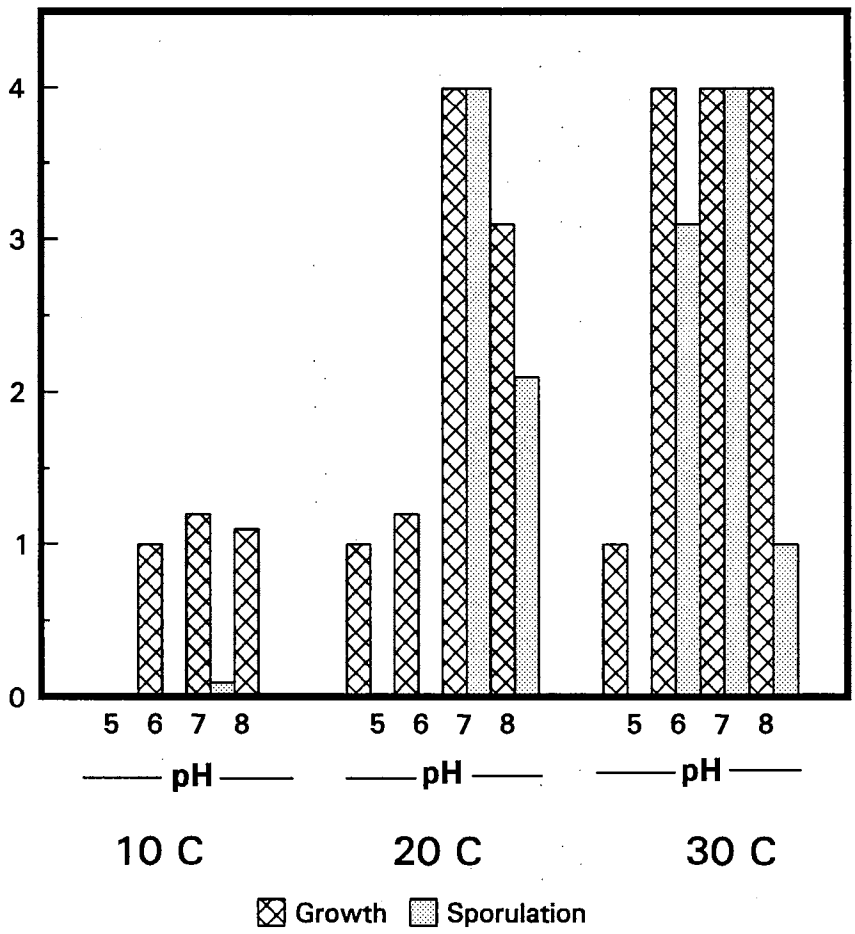

Fig. 4. Effect of different temperatures and pH on growth and sporulation of *Actinoplanes* sp. strain W 257.

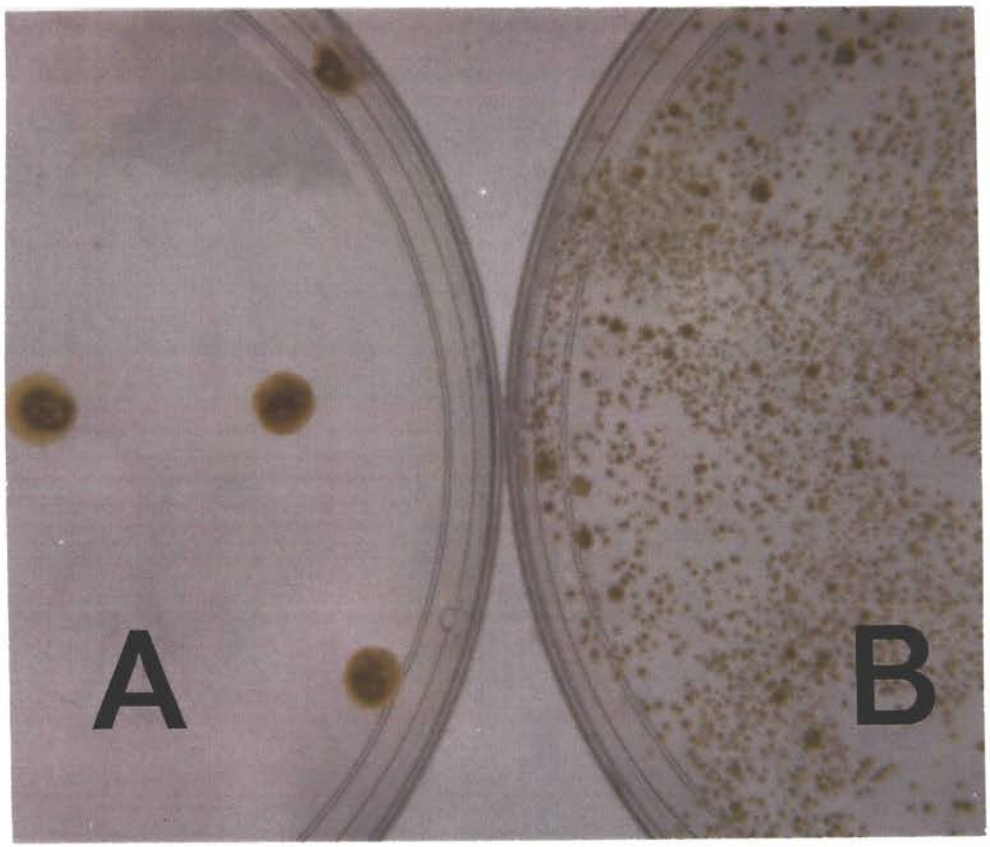

Fig. 5. Effect of light on growth and sporulation of *Actinoplanes* sp. strain W 257 after 14 days exposure at (A) 150 $\mu E/m^2/sec$ and (B) in darkness.

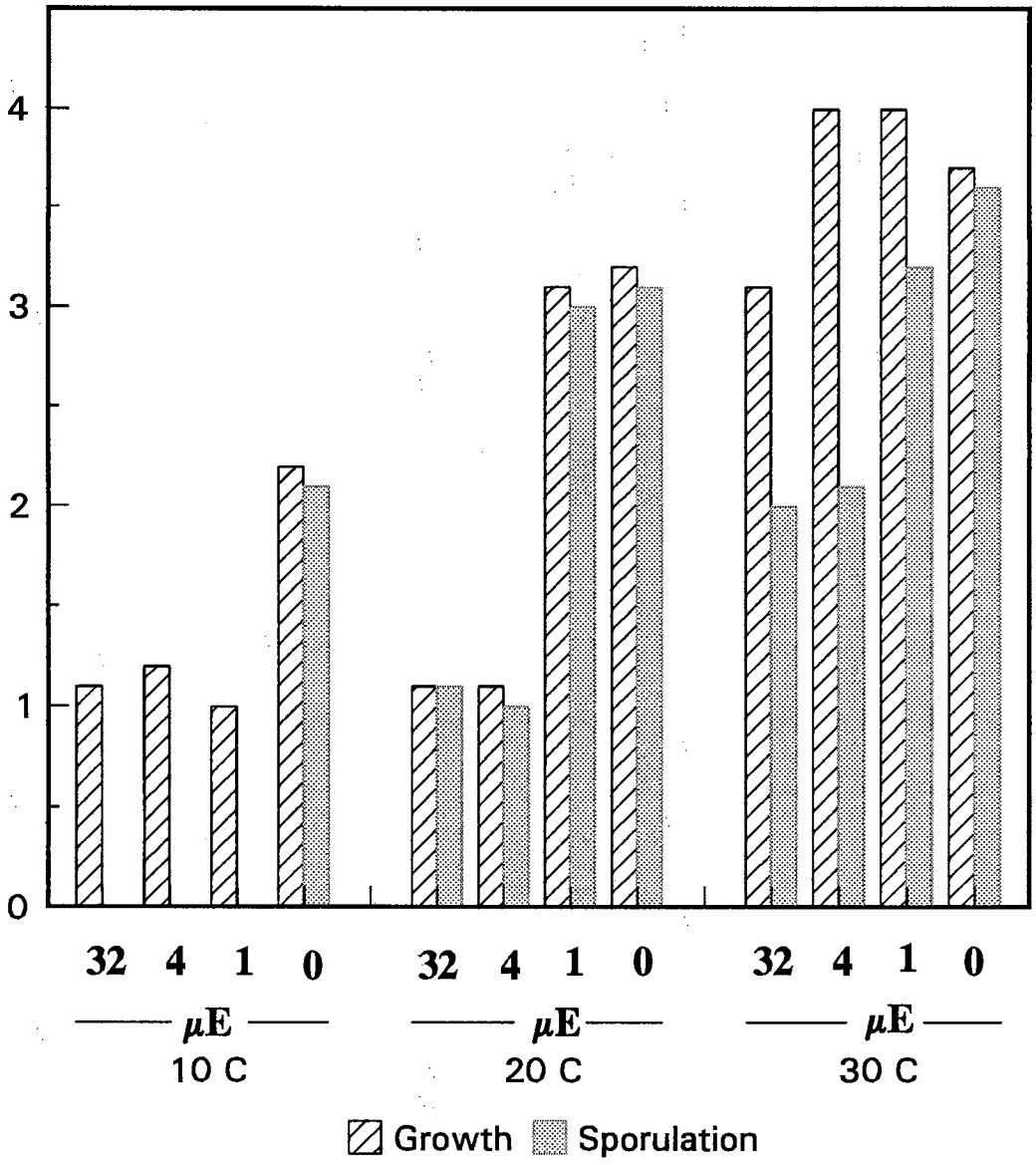

Fig. 6. Growth and sporulation of strain W 257 of *Actinoplanes* sp. after 14 days incubation on agar at 10, 20 or 30 C exposed to continuous fluorescent light intensities (32, 4 or 1μE/m^2/sec). Growth and sporulation were assessed using an index of 0-4, where 0= no growth or sporulation and 4=>75% of the agar surface with growth or >75% of the colony surface with sporulation.

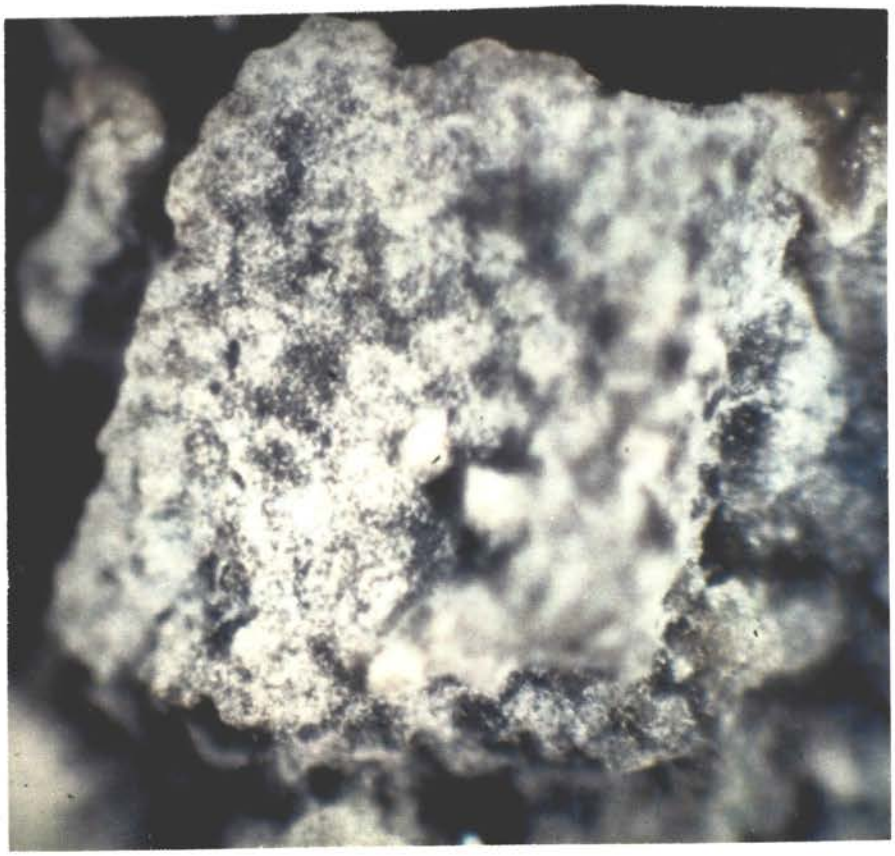

Fig. 7. Sporulation of *A. brasiliensis* on a clay granule. Sporangia appear as white powder.

Table 6. Inoculum densities of strains of *Actinoplanes* spp. from clay granules treated with various carbon sources[a].

Carbon Source	cfu/g granule[b]	
	25844	16254
Sucrose	$6.5X10^9$	$6.5X10^9$
Starch	$1.4X10^{10}$	$1.6X10^{10}$
Glucose	$5.6X10^7$	$4.2X10^7$
Mannitol	$3.7X10^8$	$4.2X10^8$
Maltose	$2.0X10^8$	$8.0X10^7$
Mannose	$1.8X10^7$	$3.6X10^7$

[a]Four g of clay granules were aseptically saturated with 6 ml of dilute Czapek-Dox broth containing 0.01 g of sucrose/ml or sucrose was replaced with the same w/v of the other carbon sources.
[b]Means of three 0.1 g samples. Several dilutions of each sample were plated on each of 5 chitin agar plates.

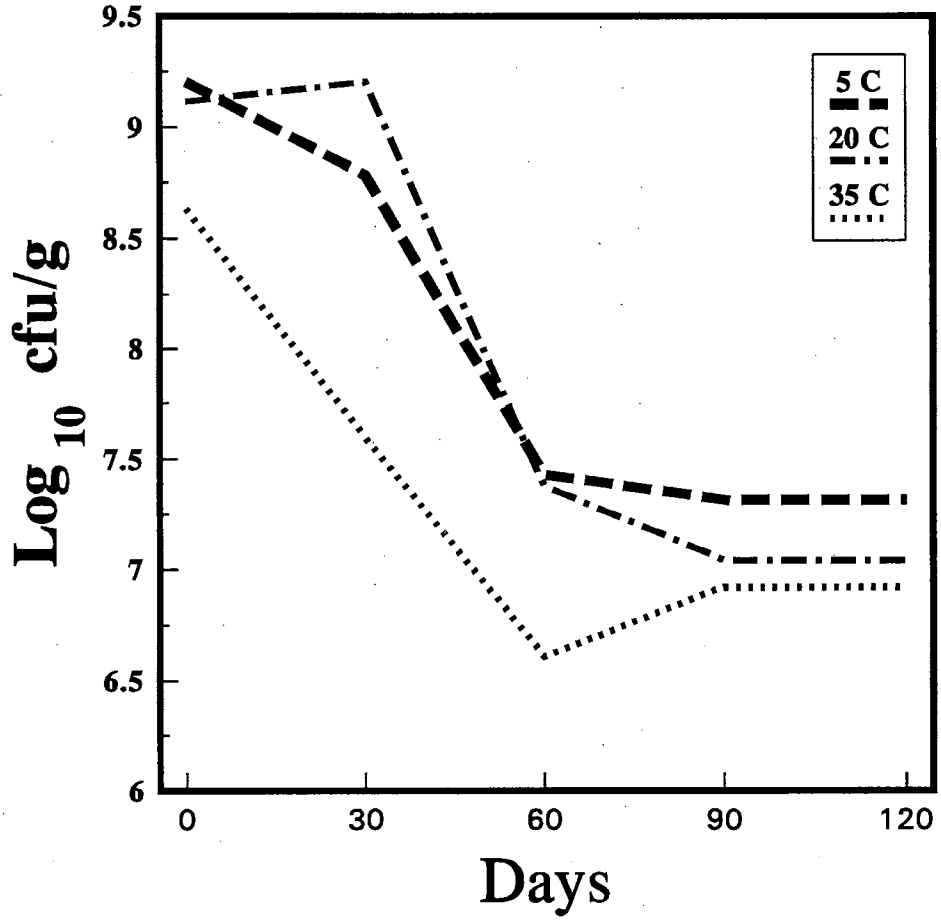

Fig. 8. Survival of *Actinoplanes brasiliensis* on granules during long-term storage at different temperatures.

of strains were generally reduced after long-term storage of granules (Fig. 8). Densities from 60-day-old granules were typically 100-fold less than the initial densities prior to storage (Day 0). Densities from 90 or 120-day-old granules were little changed compared to those from 60-day-old granules. Storage of granules at 35 C caused the greatest decline in densities. The dynamics of inoculum density decline were similar for granules stored at 5 C and 20 C.

After 24 h of incubation, motile zoospores of *Actinoplanes* sp. were observed in buffer containing 4-month-old granules of strain 25844 or W 57. After 7 days incubation, 81-82% of the oospores of *P. ultimum* in buffer containing granules of strain 25844 or W 57 were parasitized, whereas oospores in buffer containing noninoculated granules were not parasitized.

DISCUSSION

Actinoplanes spp. may have potential for the biocontrol of soilborne diseases caused by oomycetous fungi (Filonow and Lockwood, 1985; Khan et al. 1993; Sutherland and Lockwood, 1984). Application of biocontrol agents to soil or potting mix should be convenient and amenable with the delivery of other inputs, such as seed, fertilizer and pesticides. We report here a clay granule delivery system for applying biocontrol inoculum of *Actinoplanes* sp. The montmorillonite clay granules are a size (350-500 μm dia) similar to many granules, e.g. Ridomil 5G^R (metalaxyl granules; Ciba Inc. Greensboro, NC) and may be applied by hopperbox or other methods for granule delivery.

Rice hulls and perlite were not suitable as carriers because our strains of

Actinoplanes spp. sporulated little or not at all on them. Rice hulls are a biological material and may contain a chemical inhibitor of sporulation; however, perlite is generally considered to be biologically inert and less likely to be inhibitory. The dCDB+P medium that coated the perlite and other carriers was buffered to pH 7.0, a pH determined in our agar experiments to favor sporulation. After incubation for several weeks, the pH of all carriers (as a suspension in 20 ml of water) decreased to 6.2-6.5. This pH range did not adversely affect sporulation on clay granules; therefore, pH may not be a factor in the lower sporulation on perlite.

In addition to pH, temperature was an important factor influencing growth and sporulation of *Actinoplanes* spp. Cool temperature (10 C) severely restricted growth and sporulation in agar. Most likely, similar responses will occur in soil, but little is known regarding the effect of temperature on *Actinoplanes* spp. growth and sporulation in soil. A temperature of 28-30 C promoted sporulation of most *Actinoplanes* spp. strains in our collection. We routinely maintain all cultures at 28 C, since a few strains that are poor sporulators grew better at 28 C than 30 C.

Continuous light of 4 $\mu E/m^2/sec$ or greater inhibited sporulation of *Actinoplanes* spp. and as intensity increased sporulation typically decreased. To our knowledge, this is the first report of this effect. Light is a necessary stimulus for the production of colony pigmentation in *Actinoplanes* spp. and other related organisms (Dominas, 1968), and is an important attribute in the identification of these bacteria. For inducing sporulation on carriers, however, exposure to light should be prevented or minimized. A level of 1 $\mu E/m^2/sec$ permitted extensive sporulation of all strains

tested, while maintaining colony pigmentation on agar.

Clay granules of *Actinoplanes* spp. in water produced populations of 10^7-10^{10} cfu/g of granule when dilutions were plated on agar. A 1% w/w granule application to soil would produce 10^5-10^8 cfu/g soil of *Actinoplanes* spp. which is a reasonable density for microbial biocontrol; however, this rate would be prohibitive even if applied in furrow or as a band over the row. An application rate of 0.01% w/w to soil would most likely be more attractive to growers; therefore, improvements in sporulation are needed, perhaps to 10^{11} or 10^{12} cfu/g granules.

The results of our study suggested that the shelf-life of granules of *Actinoplanes* spp. may be limited, since inoculum densities produced from granules declined 100-fold within two months of storage at 20 C. Storage at 5 C did not suppress this inoculum density decline and storage at 35 C increased it. Nevertheless, after this initial decline, densities were relatively stable for 2 more months. Possibly, improvements in formulation of the granules may reduce this initial decline phase. Also, zoospores of strains 25844 and W 57 released from four-month-old granules were still motile and retained their high infectivity of oospores, suggesting that although densities may decline, the virulence of the strains was not appreciably reduced by long-term storage.

In summary, knowledge gleaned from our agar experiments has enabled us to develop a clay granule system for convenient delivery of *Actinoplanes* spp. to soil or potting mix. Granular application should facilitate evaluations of *Actinoplanes* spp. for their biocontrol effectiveness against oomycetous soilborne plant pathogens.

CHAPTER V

STRAINS OF *ACTINOPLANES* SPP. FOR THE BIOLOGICAL CONTROL OF *PYTHIUM* SPP. OR *APHANOMYCES COCHLIOIDES* IN SOIL.

ABSTRACT

Actinoplanes brasiliensis (ATCC 25844), *A. teichomyceticus* (ATCC 31121), *A. yunnahensis* (NRRL 16254) and strains K 30, W 57, W 257 and Z 20 of *Actinoplanes* spp. are known parasites of oospores. In the laboratory, soil infested with 10^3 oospores of *Pythium irregulare* per g was treated with sporangial suspensions of strains. Only W 57, W 257 or 25844 reduced ($P \leq 0.05$) root rot severity and increased ($P \leq 0.05$) peanut seedling emergence when compared to the control. Inoculum density of *P. irregulare* in sand determined by dilution plating was reduced by all of the strains. In the laboratory, strain 25844 consistently improved plant stand and/or reduced root rot severity of table beets when applied on clay granules at 10% or 5% w/w to soils infested with 750-2000 oospores of *P. ultimum*/g. Granular rates of 25844 at 1% or less were ineffective. Strains W 57 or W 257 were generally not effective at any rate. Strains 25844 and W 57 protected table beets from *P. ultimum* in field plots (10^3 oospores/g) at the 5% rate, but not at 0.1%. In the laboratory, with soil infested with 10^3 oospores of *Aphanomyces*

cochlioides per g, emergence of sugar beets was increased when strains W 57 and W 257 were applied at the 5% rate, but only W 57 reduced root rot. A 5% rate of W 57 or W 257 increased emergence and reduced root rot ($P \leq 0.1$) of sugar beet in plots infested with 500 oospores of *A. cochlioides*/g. The 0.1% level was not effective. Seed treatment (10^7-10^8 cfu/seed) was evaluated in the laboratory. In soil infested with *P. aphanidermatum* strain 25844 improved ($P \leq 0.05$) the stand of garden pea compared to controls, whereas, in soil infested with *P. ultimum*, K 30 improved the stand of cotton seedlings. Results of our studies suggest that strains 25844, W 57 or W 257 may have potential as biocontrol agents against *Pythium* and *Aphanomyces* species.

INTRODUCTION

Soilborne root diseases caused by *Pythium* spp. (Stanghellini and Burr, 1972; Van der Plaats-Niterink, 1981) and *Aphanomyces* spp. (King and Parke, 1993; Rush and Vaughn, 1993; Schneider, 1978) result in severe economic losses world-wide and are difficult to control. Sufficient genetic resistance to these pathogens is lacking in most economically important plant hosts. There is no fungicide currently registered in the USA that is effective against *Aphanomyces* spp. (Rush and Vaughn, 1993). Metalaxyl and other oomycete-specific fungicides may be used to control Pythium root rot; however, resistance to these fungicides may be a problem (Bower, and Coffey, 1985; Sanders, 1987). Fungicides may pose a hazard to the environment and may not be cost effective. Oospores produced by *P. ultimum* are recalcitrant to

chemical attack (Stasz and Martin, 1988), survive in soils for years (Hoppe, 1966; Lumsden and Ayers, 1975; Stanghellini and Burr, 1972), and may cause long-term disease problems.

Destruction of oospores by microbial parasites may be a means for controlling soilborne diseases caused by oomycetous fungi. Several microorganisms have been shown to parasitize oospores (Ayers and Lumsden, 1977; Dreschler, 1938; Filonow and Lockwood, 1985; Hsu and Lockwood, 1984; Humble and Lockwood, 1981; Khan et al. 1993; Sneh et al. 1977; Sutherland and Lockwood, 1984). Sneh et al. (1977) reported that *Actinoplanes missouriensis*, a Gram-positive, filamentous, sporangiate bacterium in the actinomycetales (Vobis, 1989) was a parasite of the oospores of *Phytophthora megasperma glycinea (Pmg)*. Sutherland and Lockwood (1984) increased the host list of *Actinoplanes* spp. to include *P. aphanidermatum, P. ultimum* and *Aphanomyces euteiches*. Recently, Khan et al. (1993) showed that oospores of *P. arrhenomanes, P. irregulare* and *P. myriotylum* also were hosts. Twelve additional species of *Actinoplanes* were also identified as new parasites of *Pythium* spp. oospores (Khan et al. 1993). Several of these strains were shown to be highly parasitic, exhibiting $>80\%$ infection of *Pythium* spp. oospores.

Strains of *Actinoplanes* spp. have been applied to seed or soil for purposes of biocontrol. In greenhouse trials, soybean seed treated with *A. missouriensis* and planted in *Pmg* infested soil produced plants with reduced root rot (Sutherland and Lockwood, 1984). Filonow and Lockwood (1985) corroborated this finding and showed that *A. utahensis* also was effective in increasing soybean stand and reducing

root rot severity incited by *Pmg*. Conversely, *A. missouriensis* and *A. utahensis* were ineffective as sporangial suspensions for control of root rot of pepper caused by *P. capsici* in potting mix (Sutherland and Papavizas, 1991).

Nothing is known about the potential of *Actinoplanes* spp. as biocontrol agents against Pythium and Aphanomyces incited root diseases. In the present study, we evaluated several strains of *Actinoplanes* spp. that we had previously identified to be efficient parasites of *Pythium* spp. oospores (Khan et al. 1993) for their efficacy to reduce root diseases of caused by *Pythium* spp. and *A. cochlioides*. Finally, we report a system for delivery of *Actinoplanes* sp. strains in the form of sporangia borne on clay granules to soil.

MATERIAL AND METHODS

Pathogen infested soil and sand. *Pythium aphanidermatum* Fritz., and *P. irregulare* Buis. were isolated from peanut pods and *Pythium ultimum* Trow. was isolated from soil. An isolate of *A. cochlioides* Drechs. was provided by C.M. Rush (Texas Agricultural Experiment Station, Bushland). Cultures of *Pythium* spp. were maintained on cornmeal agar and *A. cochlioides* was maintained on oatmeal agar (Schneider, 1978). Fungi were grown in 150 ml corn meal broth supplemented with 20 mg cholesterol/L for *Pythium* spp. (Khan et al. 1993) or 150 ml of oat meal broth (Schneider, 1978) for *A. cochlioides* in autoclavable plastic pans (15 cm X 25 cm X 5 cm) with sealable lids. The lids had a small hole plugged with foam to allow air diffusion inside the pans. Cultures were incubated at 25 C in the dark for 4-6 wk. They were harvested, washed 4 times with sterile water and comminuted in water in a

Servall Omni mixer (Dupont, Inc. Wilmington, Del.) at a powerstat setting of 50 for 4 min. The macerate was filtered through nylon cloth with 75-μm openings (Nitex^R; Tetko Inc., Elmsford, NY) into a beaker. Oospores were washed two times by centrifugation (4500X*g*) for 5 min. Oospores from several pans were composited and the oospore density/ml of the suspension estimated in a hemocytometer. Oospores were mixed by machine tumbling into a Norge loam soil (44% sand, 32% silt and 21% clay; pH 6.9) or a Meno fine sandy loam (74% sand, 8% silt and 18% clay; pH 6.2). Soils had oospore densities of 500-1000/g for *A. cochlioides* and 750-2000/g for *Pythium* spp. Oospores of *P. irregulare* (5000/g) also were mixed into water-washed, autoclaved (2h) sea sand (50 mesh; Sargent-Welch, Skokie, IL). Infested soils or sand were stored at 22-25 C for up to 2 wk prior to use.

Actinoplanes spp. and methods of application. *Actinoplanes* spp. were stored in glycerol at -70 C and routinely maintained on dilute Czapek-Dox agar (dCDA) (12g Difco Czapek-Dox Broth + 18g agar/L) or yeast-extract-phosphate-soluble-starch agar (YPSS)(Emerson, 1958). The following strains were evaluated for their biocontrol efficacy: K 30, W 57, W 257, Z 20, ATCC 25844 (*A. brasiliensis*), ATCC 31121 (*A. teichomyceticus*) or NRRL 16254 (*A. yunnahensis*). Petri dishes (9 cm dia.) of dCDA were inoculated with strains and incubated at 28 C in the dark for one month. Sporangia were harvested and suspensions prepared as described elsewhere (Khan et al. 1993). Aqueous suspensions contained $5X10^5$ sporangia/ml of water and were used to inoculate oospore-infested sand. Shake cultures of strains were grown in 200 ml of YPSS in 2-L flasks for 2-4 wk. The cultures were aseptically harvested, washed two

times, macerated in a Omni mixer (50% power, 30 sec), and used to inoculate granules or treat seed.

Montmorillonite clay granules (Ultra Premium Cat Litter, Wal-Mart, Inc.) were ground to pass a 30 mesh sieve, collected on a 45 mesh sieve, washed with tap water to remove fines and dried at 70 C for 48 h. Granules were placed in 9-cm glass Petri dishes (4g/dish) and autoclaved at 135 C for 3h. Dishes of cool granules were aseptically treated with 6 ml of dilute Czapek-Dox Broth (12 g Difco Czapek-Dox broth/L) supplemented with 0.1% (w/v) peptone. Granules in covered dishes were dried at 70 C for 48h, cooled and aseptically inoculated with 5 ml of a macerated suspension of *Actinoplanes* sp., and incubated at 28 C in the dark for 2-3 wk. Granules of a strain were aseptically dried in a laminar flow hood for 24 h and composited in a plastic bag for storage at room temperature (23-25 C) until use. Inoculum density of *Actinoplanes* sp. on granules (cfu/g of granules) was determined by serial dilutions of 0.1 g granules in water on chitin agar (Hsu and Lockwood, 1975). Densities were variable and ranged from 2.5 X 10^6 to 6.6 X 10^8 cfu/g for early lots of granules and 4.3X10^8 to 7.6X10^{10} cfu/g for later lots.

Strains K 30, W 57, W 257 and 25844 of *Actinoplanes* spp. were evaluated as seed treatments. A 25 ml macerated suspension of a strain was centrifuged for 5 min at 4,500 X *g*, and the pellet was mixed with 5 ml of 1% (w/v) carboxymethylcellulose (CMC) plus 1% (w/v) of soluble starch (SS) (Filonow and Lockwood, 1985). Seed of garden pea (*Pisum sativum* L., cv. Little Marvel) and cotton (*Gossypium hirsutum* L., cv. Paymaster HS-26) were coated with this mixture, dried 4h in a laminar flow hood

and planted within 2 h of drying. Populations of strains on seed were 10^6-10^8 cfu/seed as determined by plating dilutions of seed shaken in 50 mM K^+ phosphate buffer (pH 6.8) on chitin agar (Hsu and Lockwood, 1975).

Biocontrol experiments in the laboratory. Initial experiments were conducted with small lots of the loamy sand soil infested with 10^3 oospores/g of *P. irregulare*. Small lots of soil permitted the harvest of sufficient sporangia from strains such as 16254 that sporulated less than others. Styrofoam cups were filled with 150 cc of autoclaved (2 h) vermiculite that was overlayed with 20 g of infested soil. Five ml of a $4X10^4$ sporangia/ml suspension of a strain was pipetted evenly over the soil. Nontreated soil was the control. There were 5-15 cups per strain. Cups plus several wet paper towels were placed inside a plastic pan which was enclosed in a large plastic bag. After incubation at 25-27 C for 7 days, the cups were removed from the bag and one peanut (*Arachis hypogaea* L. cv. Pronto) seed was placed on the soil and covered with ca. 30 cc of vermiculite. Cups were completely randomized under high intensity, metal halide lamps (ca. 250 $\mu E/m^2/sec$; 8h day/16h dark). At 24 days after planting (DAP) emerged seedlings were counted and roots were examined for necrosis, using an index of 1-5, where 1= no necrosis and 5=>75% of the root with necrosis.

The effect of *Actinoplanes* spp. on the inoculum density of *P. irregulare* in oospore-infested sand was assessed. Two ml of a $5X10^4$ sporangia/ml suspension of a strain was mixed into 2 g of autoclaved sand infested with $5X10^3$ oospores of *P. irregulare* in sterile glass Petri dishes (6 cm dia). Three dishes per strain were sealed with Parafilm^R, placed inside plastic bags containing wet paper towels to minimize

drying of the sand, and incubated at 25 C. After 7 and 20 days, 0.5 g of sand were transferred to 4.5 ml of sterile 0.1% w/v agar in water in sterile screw-capped glass vials. These were hand-shaken for one min and a 1/10 dilution of these were prepared. Dilutions (10^{-1} and 10^{-2}) were each plated on five dishes of a medium selective for pythiaceous fungi (Lewis and Filonow, 1990) and incubated at 25 C. Sand (ca. one g) remaining in each glass dish was suspended in 5 ml of water in a glass vial, handshaken for one minute and the sand allowed to settle for one minute after which the liquid was filtered through nylon cloth (45 μm openings) into a centrifuge tube. The liquid was centrifuged at 4500X *g* for 5 min., and 4 ml of the supernatant was suctioned out. The remaining liquid was shaken and 20 μl or 50 μl drops examined for incidence of parasitized oospores at 200 or 500X under a microscope (Khan et al. 1993).

The efficacy of clay granules for delivering biocontrol inoculum was evaluated. Air-dry granules at 0.1-10% w/w were mixed into infested soil in plastic bags prior to filling 100 cc plastic cups. Soil in cups were kept moist for 7 days to encourage parasitism of oospores by *Actinoplanes* sp. prior to planting table beet (*Beta vulgaris L.* cv. Detroit Red) in *Pythium* sp. infested soil or sugar beet (*Beta vulgaris L.* cv. Monitor VSH 23) in soil infested with *A. cochlioides*. Noninfested soil was the controls. There were 5 beet seeds per cup and 5 cups per treatment arranged in a randomized complete block. Cups were watered every two days and were incubated at 25-27 C under light (8 h per day) for 24-28 days. Seedlings were counted and root rot assessed as described above.

For seed treatments, cotton seeds coated with strains were planted in 100 g of soil infested with 10^3 oospore/g of *P. ultimum* in 100 cc plastic cups. Treated pea seed were planted into 100g of *P. aphanidermatum* infested soil (10^3 oospores/g). Nontreated seed and seed treated with CMC+SS were the controls. There was one pea or one cotton seed planted per cup and 20 cups per treatment arranged in a randomized complete block. Cups were watered every other day, and incubated at 24-27 C under light (8 h per day). After 4 weeks, stand counts were taken and root rot severity rated as described above.

Biocontrol experiments in the field. Plots were at the Plant Pathology Farm, Stillwater, OK in a field of Norge loam soil, described above. There were 5-20 rows spaced 30 cm apart per plot. Holes (6 cm dia X 7 cm deep, ca. 200 cc) were made in rows and were located with rings of clear plastic which fit into the hole and stood ca. 1-2 cm above the soil surface. Holes were filled with sieved (<2mm) field soil sp. obtained from the plot area and infested with 500-1000 oospores/g soil. Infested field soil was supplemented with granules to the desired concentration (0.1-5% w/w granules/soil) and 200 cc of soil was dispensed into each hole. Granules without *Actinoplanes* sp. at 1% w/w or metalaxyl (1.3 kg a.i./ha; Ciba Inc. Greensboro, NC) were controls. There was one treatment per hole per row arranged in a randomized complete block. Plots were kept moist by irrigating every other day with ca. 0.5 cm of water except after rain. Soils were incubated for 7-10 days prior to planting seeds of table beet (cv. Detroit Red) in *P. ultimum* infested soil or sugar beet (cv. Monitor VSH 23) in soil infested with *A. cochlioides*. Plant stand was recorded at 28 DAP

prior to uprooting, washing and evaluating for root rot severity, as described above.

An experiment was conducted to assess the effect of *Actinoplanes* sp. inoculum density in soil on biocontrol efficacy. Granules bearing sporangia of strain 25844 were mixed into *P. ultimum* infested soil (10^3 oospores/g) at 0.001%, 0.01%, 0.1% or 1% w/w, yielding 10^5, 10^6, 10^7, or 10^8 cfu/g soil, respectively. There was one inoculum density treatment per hole per row and 20 rows per treatment. Noninfested soil treated with 1% w/w nontreated granules were the control. After 14 days, one bean seed *(Phaseoulous vulgaris* L. cv. Bountiful) was planted per hole. Stand counts were taken at harvest and root rot measured, as described above.

Statistical analysis. Except for the experiment assessing the effects of inoculum densities of strain 25844 on disease, all experiments were repeated one or more times. Stand counts and root severity data were arcsin transformed or not prior to an analysis of variance and means were separated by the LSD test at $P \leq 0.05$ (Steel and Torrie, 1980). Results with transformed data were the same as nontransformed data; therefore, results from nontransformed data are given.

RESULTS

Biocontrol of *Pythium* spp. Sporangial suspensions of strains 25844, W 57 and W 257 applied to *P. irregulare*-infested soil in cups increased emergence and reduced root rot, whereas strains 16254, 31121 and Z 20 did not (Table 7). Strains 25844, W 57 and W 257, therefore, were selected for further testing.

Inoculum densities of *P. irregulare* determined by dilution plating from untreated oospore-infested sand were 40-46% of the potential population (5000

Table 7. Effect of strains of *Actinoplanes* spp. on control of seedling disease of peanut caused by *Pythium irregulare*[a].

Strain	Emergence[b] %	Root rot index[c] (1-5)
16254	20.0	3.6
25844	100.0	1.4
31121	33.3	2.6
W 57	66.7	1.6
W 257	80.0	1.6
Z 20	40.0	2.8
None	20.0	3.9
LSD($P \leq 0.05$)	32.6	2.1

[a] Values are means of 15 cups planted with one seed each. Cups contained 150 cc of vermiculite overlayed on top with 20 g of autoclaved soil infested with 10^3 oospores of *P. irregulare*/g. Five ml of a suspension ($4x10^4$ sporangia/ml) of *Actinoplanes* spp. were added to the soil. Seven days later one peanut seed (cv. Pronto) was placed on the soil and covered with vermiculite. Infested soil not treated with sporangia was the control.

[b] emergence at 24 days after planting expressed as a percent of 15 planted seeds.

[c] Root rot severity was assessed with an index of 1-5, where 1=no root rot, and 5=>75% of the root with necrosis.

Table 8. Effect of strains of *Actinoplanes* spp. on inoculum density of *P. irregulare* in sand[a].

Treatment	*P. irregulare*[b] (p/g)		Reduction %	
	7 days	20 days	7 days	20 days
16254	1868	1668	19.9	26.8
25844	1250	1210	46.4	46.9
31121	1714	1522	26.5	33.2
K 30	1583	1333	32.1	41.4
W 57	1192	1449	48.9	36.4
W 257	1685	556	27.7	75.6
None	2332	2278		
LSD($P \leq 0.05$)	513	314		

[a] Values represent means of 3 dishes/strain. Two ml of a suspension of *Actinoplanes* sp. sporangia ($5x10^4$/ml) was applied to two g of sand infested with $5x10^3$ oospore/g of *P.irregulare.*

[b] Inoculum density was determined by plating serial dilutions on a selective medium.

oospores/g). Nevertheless, densities in sand treated with *Actinoplanes* spp. strains were typically lower than in nontreated sand (Table 8). Recovery of oospores by wet sieving from sand was low. Nevertheless, parasitized oospores were not recovered from nontreated sand, whereas they were always recovered from oospore-infested sand treated with *Actinoplanes* spp.

In laboratory experiments with clay granules, strain 25844 applied at 5% w/w to soil infested with 10^3 oospores/g of *P. ultimum* increased stands of table beets 2-fold and reduced root rot compared to control (Fig. 9). At the time of planting, populations of *Actinoplanes* spp. in soils treated with strain 25844 were $8X10^7$ cfu/g. In soil infested with 2000 oospores/g of *P. ultimum*, the 10% w/w rate of 25844 ($5X10^8$ cfu/g soil) increased table beet emergence to 40% compared to 20% in the nontreated control. Granules of 25844 applied at 1% w/w ($2X10^7$ cfu/g) to soil infested with 10^3 oospores/g of *P. ultimum* increased table beet stand 2.5 fold compared to the nontreated soil control in one experiment but no increase in stand was noted in a second experiment.

Strain W 57 and W 257 were not efficacious in *P. ultimum* infested soil at any granular rate in laboratory experiments, except for W 257 which was effective at the 5% granular rate in one experiment (Fig. 9).

Emergence of pea seed treated with strains 25844, B 16254, K 30, W 57 or W 257 was 4-fold to 8-fold greater than untreated seed in soil infested with 10^3 oospores/g of *P. aphanidermatum* (Table 9) . However, CMC+SS treated seed also showed greater emergence than untreated seed. When compared to emergence of

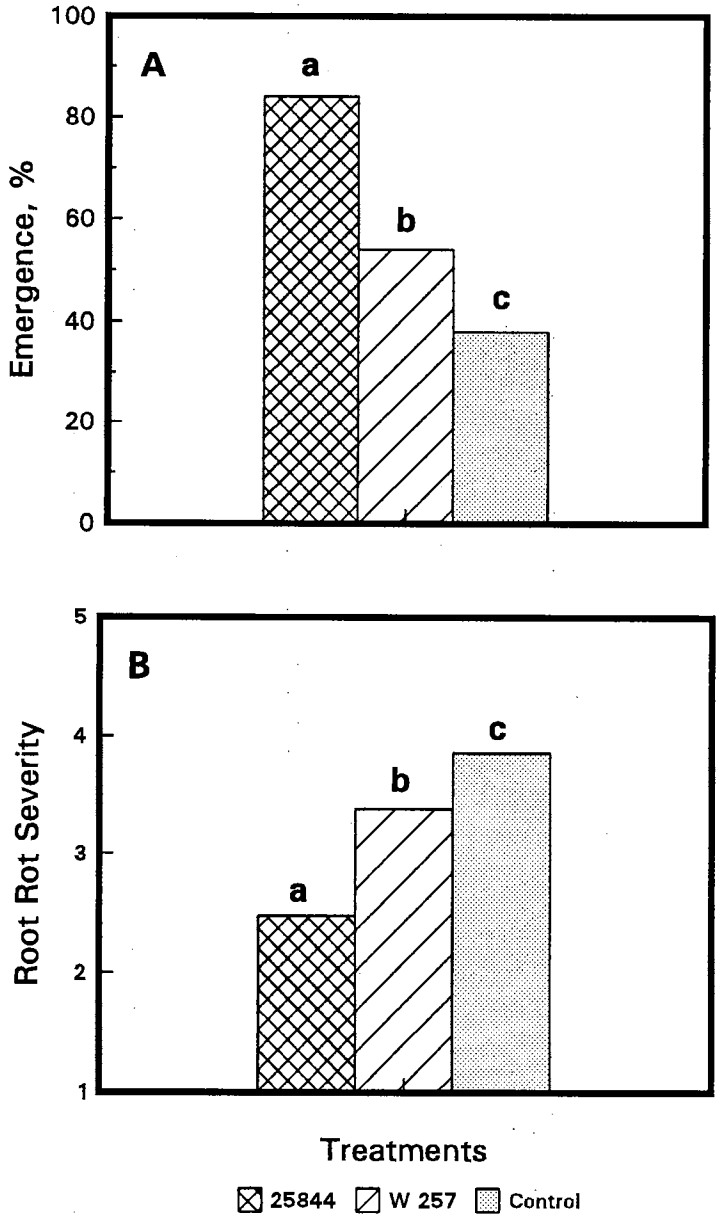

Fig.9. Emergence (A) and root rot severity (B) of table beet seedlings at 28 days in the laboratory after planting in cups of soil infested with 10^3 oospores of *P. ultimum* per g and treating with 5% w/w of granules bearing sporangia of *Actinoplanes* spp. The control was infested soil that received 5% w/w nontreated granules. Seed were planted 14 days after granule application. Root rot severity was rated using a 1-5 index, where 1 = healthy root with no necrosis and 5 = > 75% of the root with necrosis. Values are means of 5 cups, each planted with 5 seeds. Means with the same letter are not different at $P \leq 0.05$.

Table 9. Effects of seed treatments with strains of *Actinoplanes* spp. on control of damping-off of pea seedlings caused by *P. aphanidermatum*[a].

Strain	Emergence,[b] (%)	Root rot index[c] (1-5)
16254	52	3.1
25844	81	2.0
K 30	52	3.1
W 57	43	3.3
W 257	43	3.3
Nontreated	10	4.1
CMC+SS only	48	4.1
LSD($P \leq 0.05$)	29	1.2

[a] Values are means of 20 cups per treatment planted with 1 seed each. Cups contained 200 g of nonsterile soil supplemented with 10^3 oospores/g. Seeds (cv. Little Marvel) were treated with suspensions of strains of *Actinoplanes* spp. in carboxymethylcellulose (CMC) plus starch (SS) at 10^7-10^8 cfu/seed.

[b] Emergence at 24 days after planting.

[c] Root rot severity was assessed using an index of 1-5, where 1=no root rot, and 5=>75% of the roots with necrosis.

CMC+SS treated seed, only strain 25844 treated seed showed greater emergence. In another experiment, treated cotton seed were planted in soil that has been infested with 10^3 oospores/g of *P. ultimum*. Only strain K 30 at 10^8 cfu/seed improved emergence (35%) when compared to emergence (20%) of the CMC+SS treated seed. In this test, emergence of seed treated with 25844 was 3% compared to 20% for the control.

Strains applied on granules at 5% w/w to holes in the field also improved the stand of table beets in *P. ultimum* infested-soil. Emergence of table beets in soil infested with 10^3 oospores/g was increased by strain 25844 and W 57, but not W 257 when compared to the nontreated control (Fig. 10). However, only 25844 reduced root rot severity compared to the nontreated control. Improvement in emergence and reduction in root rot severity by 25844 and W 257 were better than that given by metalaxyl. In this experiment, inoculum densities of *Actinoplanes* spp. in soil prior to planting were $4X10^7$-$7X10^7$ cfu/g. In a second experiment with soil in holes infested with 750 oospores/g of *P. ultimum*, only strain 25844 increased ($P \leq 0.10$) table beet emergence (72%) compared to the nontreated control (40%). Strains 25844, W 57 and W 257 also were applied at 0.1% (w/w) granules to soil in holes, yielding populations of 4 x 10^4-10^6 cfu/g soil depending on the strain. None of these strains were effective at this rate against *P. ultimum*.

An experiment assessed the importance of biocontrol effectiveness to strain density in soil. Emergence of beans in *P. ultimum* infested-soil treated with granules of 25844 at 1% w/w ($2X10^8$ cfu/g soil) was 100% and less than in nontreated soil

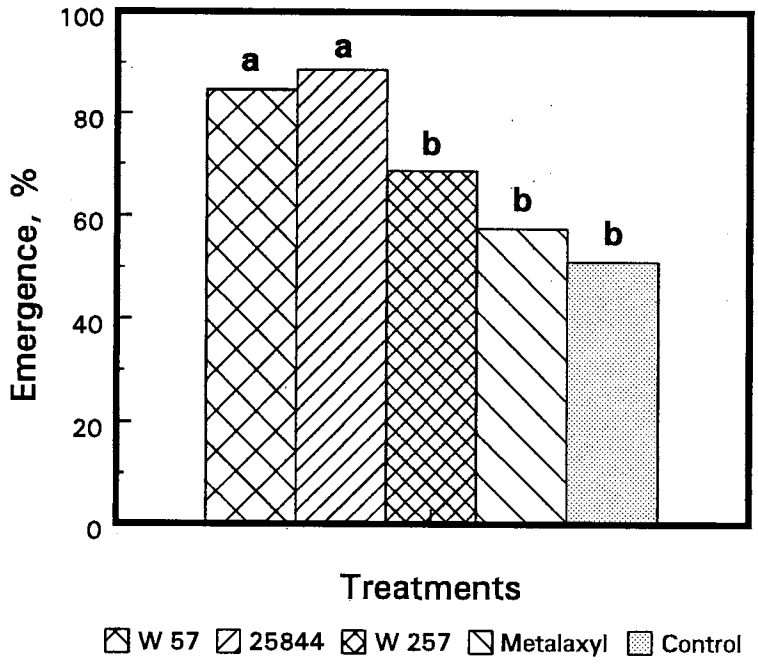

Fig. 10. Emergence at 24 days after planting of table beet seedlings in field plots infested with 10^3 oospores of *P. ultimum* per g and treated at 5% w/w with granules bearing sporangia of strains 25844, W 57 or W 257. Controls were infested plots treated with metalaxyl (1.3 kg a.i./ha) or 5% w/w nontreated granules. Seed were planted 10 days after granule application. Values are means of 20 replicates, with one plant each. Means with the same letter are not different at $P \leq 0.05$.

(82%). Rates of strain 25844 from 0.001% ($9X10^4$ cfu/g soil) to 0.1 % ($3X10^7$ cfu/g soil) of 25844 did not increase bean emergence compared to the nontreated control.

Biocontrol of *Aphanomyces cochlioides*. In the laboratory, a 5% granular rate of strain 25844 generally improved sugar beet emergence in soil infested with 10^3 oospores/g of *A. cochlioides* (Fig. 11). Emergence was increased 8-9 fold compared to the control in one experiment (Fig. 11) and 2.5 fold by strain 25844 in another experiment. However, there was no increase in a third experiment. Densities of *Actinoplanes* spp. in treated soils were 2-$4X10^7$ cfu/g. Strains W 57, W 257 and 31121 increased sugar beet emergence compared to the control in one experiment (Fig. 11) but not in two others. Root rot severity was reduced by 25844 in one experiment (Fig. 11) and in another (3.6 compared to 4.5 for the control), but not in a third experiment.

In the laboratory, strains on granules applied at 1% and 0.1% w/w to soils infested with 10^3/g oospores of *A. cochlioides* did not increase sugar beet emergence or reduce root rot on seedlings. However, in an experiment in which the inoculum density of *A. cochlioides* had been reduced to 500 oospores/g soil, only the 1% rate of 25844 increased sugar beet emergence to 44% compared to the control (17%).

In two field tests the 5% granular rate of strain 25844 ($4X10^7$ cfu/g soil) was not efficacious against *A. cochlioides*, whereas strains W 57 and W 257 ($5X10^8$ and $4X10^7$ cfu/g, respectively) increased emergence (Fig. 12). At a rate of 0.1%, however, none of the strains were effective.

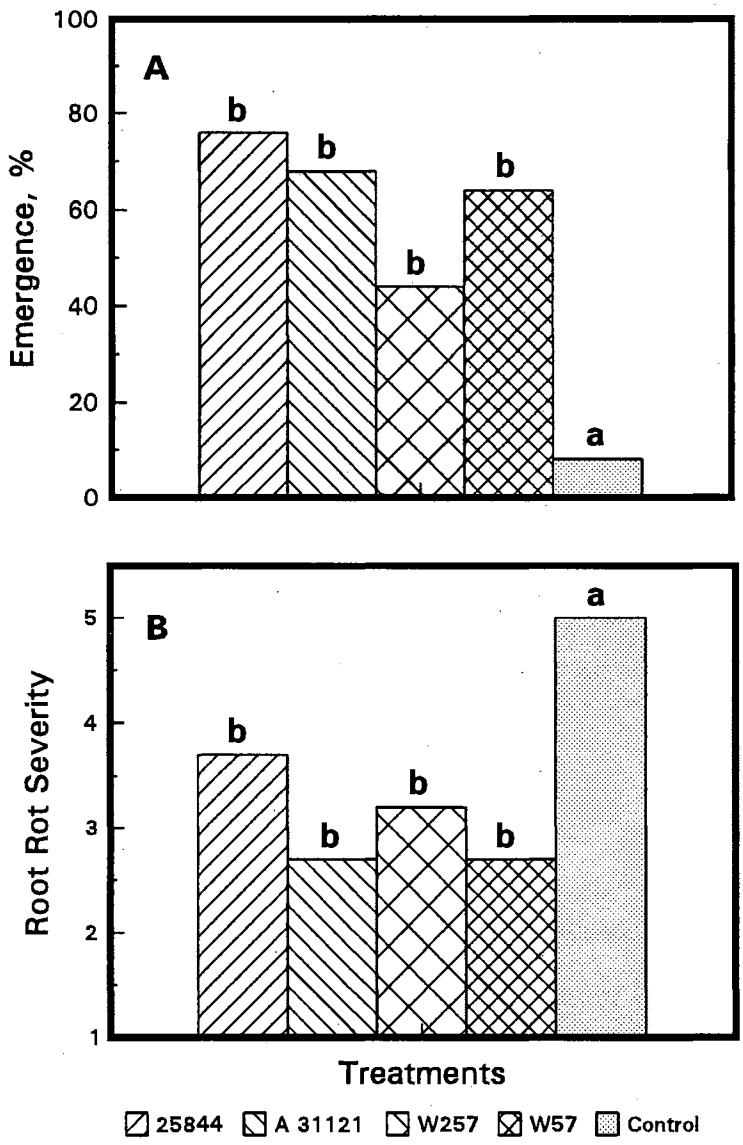

Fig. 11. Emergence (A) and root rot severity (B) of sugar beet seedlings at 28 days in the laboratory after planting in cups of soil infested with 10^3 oospores of *A. cochlioides* per g and treated with 5% w/w of granules bearing sporangia of *Actinoplanes* spp. strains. The control was infested soil that received 5% w/w nontreated granules. Seed were planted 7 days after granule application. Root rot severity was rated using a 1-5 index, where 1 = healthy root with no necrosis, and 5 = >75% of the root with necrosis. Values are means of 7 cups with five seeds each. Means with the same letter are not different at $P \leq 0.05$.

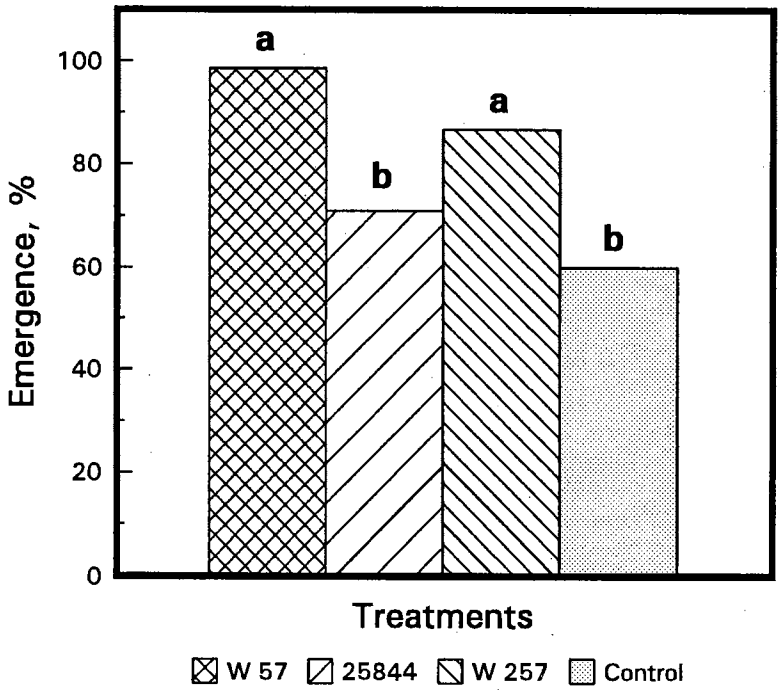

Fig. 12. Emergence of sugar beet seedlings at 28 days after planting in field plots infested with 500 oospores of *A. cochlioides*/ g and treated with 5% w/w of granules bearing sporangia of *Actinoplanes* spp. strains. The control was infested soil that received 5% w/w nontreated granules. Seed were planted 7 days after granule application. Values are means of 10 replicates with 5 seeds each. Means with the same letter are not different at $P \leq 0.05$.

DISCUSSION

Our study shows for the first time that some strains of *Actinoplanes* spp. have promise as biocontrol agents against phytopathogenic *Pythium* spp. and *A. cochlioides* in soil. Previous attempts to use *Actinoplanes* spp. as biocontrol agents have been directed against *Phytophthora* spp. (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984; Sutherland and Papavizas, 1991). In particular, we newly report that strain ATCC 25844 (*A. brasiliensis*) and strains W 57 and W 257 of *Actinoplanes* spp. exhibit biological control activity, supporting the suggestion (Khan et al. 1993) that these strains may have potential for use in the field based on their efficient parasitism of *P. ultimum* oospores in soils. Prior to our findings only *A. missouriensis* (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984) and *A. utahensis* (Filonow and Lockwood, 1985) had been shown to reduce plant disease caused by an oomycetous pathogen (*P. megasperma* f. sp. *glycinea*).

Others found that *A. missouriensis, A. utahensis* and *A. philippinensis* did not protect pepper seedlings against *P. capsici* (Sutherland and Papavizas, 1991). These workers applied *Actinoplanes* spp. to soil at 10^2-10^3 cfu/ml as drenches comprised of macerated mycelial suspensions in water or as root dips comprised of 10^3 sporangia per ml of water. These inoculum levels may have been too low for satisfactory control. Our work showed that $< 10^7$ cfu/g soil of *Actinoplanes* spp. applied to soil prior to planting did not provide control. However, densities of 10^7-10^8 cfu/g were frequently effective depending on the strain, the pathogen and the location (laboratory or field) of the experiment.

Our study also shows that results from laboratory tests of biocontrol agents do not necessarily predict their performance in field tests. Strain W 57 and W 257 were effective against *P. ultimum* in laboratory tests but not in the field. In addition, strain 25844 was effective against *A. cochlioides* in the laboratory, but was typically ineffective in the field. Possibly, differences in the microbial environments in contained soil compared to bulk soil in the field may account for these results.

The use of clay granules bearing sporangia of *Actinoplanes* spp. is the first report of this method for applying *Actinoplanes* spp. biocontrol inoculum to soil. Prior to this method, sporangia were applied in a water suspension as a drench or root dip (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984; Sutherland and Papavizas, 1991) or as a suspension of hyphae and sporangia applied to seed (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984). Application of clay granules bearing *Actinoplanes* sp. in our experiments was convenient and easy and this method warrants further development. Improvement in sporulation on granules is needed. Consistent large batch production of inoculum at 10^{10} or 10^{11} cfu/g of granules would enable lower granular rates to be applied. Possibly, an application rate of 0.01% w/w or lower to soil in the field or potting mix in the green house may be possible with improvement in sporulation.

Parasitism of oospores was most likely the mechanism of biocontrol operative in soil treated with sporangia of *Actinoplanes* spp. The strains that we evaluated had previously been identified as parasites of *Pythium* spp. oospores in soil (Khan et al. 1993). Also, sporangia of strains 25844, W 57 and W 257 parasitized oospores of *A.*

cochlioides in buffer (Khan and Filonow, unpublished). During the development of the clay granule delivery method, we found that strains on granules in buffer released motile zoospores of *Actinoplanes* spp. that parasitized oospores of *P. ultimum* (Khan and Filonow, unpublished). In addition, the decline in the recovery of *P. irregulare* from oospore-infested sand treated with *Actinoplanes* spp. suggests that the oospores were parasitized. Recovery of parasitized oospores from treated sand, but not from nontreated sand supports the conclusion that populations of *P. irregulare* oospores were attenuated by parasitism.

Our successful results in experiments using seed coated with *Actinoplanes* sp. corroborated the findings of others (Filonow and Lockwood, 1985; Sutherland and Lockwood, 1984). The results suggest that another mechanism of biocontrol may be used by strains of *Actinoplanes* spp. Strains used for seed treatment were grown in shake culture. It is our experience that shake culture does not favor sporulation in the strains that we worked with. Prior to planting, microscopic examination of coated seed did not reveal sporangia, although some may have formed from hyphae while coatings were drying. Sporangia also may be quickly formed on seed in soil and release zoospores that would parasitize nearby oospores. Possibly, strains of *Actinoplanes* spp. may produce antifungal compounds (Parenti and Coronelli, 1979). Nevertheless, seed coating also is a convenient method of application in which efficacy may be improved by further research.

The effects of soil environmental factors on hyperparasites has been reviewed (Adams, 1990; Baker, 1987). However, little is known regarding the effects of factors

such as soil texture, matric potential and temperature on the biocontrol efficacy and survival of *Actinoplanes* spp. In a laboratory study using autoclaved soil, Sutherland and Lockwood (1984) found that parasitism of *Pmg* oospores was greatest in saturated soil (0 J/kg) at 20-30 C. The influence of these factors and others, e.g. soil pH on the biocontrol efficacy and survival of *Actinoplanes* spp. strains in natural soils in the field needs to be investigated.

The timing of granule application also needs to be studied. Most of our experiments were done after waiting 7 days prior to planting to allow time for parasitism of oospores. One experiment (Fig. 10) was done after waiting 10 days and another (Fig. 9) after 14 days. Perhaps strains of *Actinoplanes* spp. would be more efficacious when applied several weeks prior to planting. It is also not known if *Actinoplanes* spp. would be efficacious on granules applied at planting. Although it is generally accepted that microbial parasites need time to find and invade their host, a strain of *Actinoplanes* spp. applied in abundant density on granules at planting may quickly kill sufficient numbers of oospores to reduce disease. Since the target of *Actinoplanes* spp. is the oospore, periodic application of granules to a field with a history of severe root disease caused by *Pythium* or *Aphanomyces* spp. may eventually decrease the disease potential of the field. In this regard, *Actinoplanes* spp. may have value in conjunction with oomycete-specific fungicides or with other biocontrol agents (Howell, 1982; Loper, 1988) that typically suppress the hyphal phase of oomycetous fungi.

In summary, the results presented herein suggest that strains 25844, W 57 and

W 257 of *Actinoplanes* spp. have promise in the biological control of some oomycetous fungi. These strains should be given further study. Perhaps other genera of the actinoplanetes should also be examined for biocontrol potential as well.

SUMMARY AND CONCLUSIONS

Strains of *Actinoplanes* spp. have promise for the biological control of soil borne diseases of plants caused by *Pythium* spp. and *Aphanomyces cochlioides*. Results from the first objective showed that in the laboratory, several strains of *Actinoplanes* spp. parasitized oospores of *Pythium aphanidermatum, P. arrhenomanes, P. irregulare, P. myriotylum* and *P. ultimum in vitro* (Chapter III). Parasitized oospores had disorganized cytoplasms and hyphae of *Actinoplanes* sp. emerging from them. Oospores of *P. arrhenomanes, P. irregulare* and *P. myriotylum* were identified for the first time as hosts of *Actinoplanes* spp. Incidence of oospore infection after 3 days incubation varied from 0-90%. Newly recorded parasites of oospores of *Pythium* spp. in buffer were *A. azureus, A. brasiliensis, A. caeruleus, A. ferrugineus, A. ianthinogenes, A. italicus, A. minutisporangius, A. rectilineatus, A. teichomyceticus, A. utahensis, A. violaceous, A. yunnahensis,* plus 15 strains of *Actinoplanes* yet to be speciated. Cluster analysis of the data from evaluations *in vitro* suggested several strains as candidates for further evaluation in soil. When added to nonsterile soils, several strains increased the incidence of oospore parasitism compared to nonsupplemented soils. Strains of *Actinoplanes* spp. appeared to exhibit a host specificity for species of *Pythium* in buffer and in soil. Profuse sporulation of sporangia of *Actinoplanes* sp. on parasitized oospores was more common in soil than

in buffer. Factors that account for this effect need to be determined, since the production of secondary inoculum via sporulation from infected oospores may be advantageous to the use of *Actinoplanes* spp. in biological control.

Others have applied *Actinoplanes* spp. as water suspensions of hyphae and sporangia to soil, roots or seed for the biological control of Phytophthora root rot of soybean or pepper. Results from the second objective of this dissertation (Chapter IV) enabled the development of a clay granule system for delivering biocontrol inoculum of *Actinoplanes* sp. to soil or potting mix. Results of agar experiments showed that a culture medium of dilute Czapek-Dox broth (at 1/3 the recommended sucrose concentration (w/v)) at pH 7 and incubation at 28-30 C in the dark or light ($1\mu E/m^2/sec$) promoted extensive growth and sporulation of several strains of *Actinoplanes* spp. Although light may be necessary for colony pigmentation in *Actinoplanes* spp., light intensities of 4-150 $\mu E/m^2/sec$ suppressed growth and sporulation. Rice hulls or perlite were not suitable as carriers of *Actinoplanes* spp. because strains sporulated little or not at all on them. Strains W 57, W 257, 25844, 16254, but not 31121 sporulated extensively on autoclaved montmorillonite clay granules (350-500 μm dia.) treated with a solution (pH 7) of dilute Czapek-Dox broth plus 0.1% peptone (w/v) and incubated in the dark or light (1 $\mu E/m^2/sec$) for 3 wk. Some strains had inoculum densities of 10^8-10^{10} cfu/g of air-dried granules. These densities on granules declined 100-fold after two months storage at 5, 20 or 35 C. Thereafter, inoculum densities changed little during an additional two months. Zoospores of *Actinoplanes* spp. released from 4-month-old granules in buffer were

highly infective of *P. ultimum* oospores, suggesting that although inoculum densities on granules may decline with storage, the virulence of strains may not.

Results from objective III (Chapter V) showed that some strains of *Actinoplanes* spp. were effective biological control agents for reducing plant disease in soil caused by *Pythium* spp. or *A. cochlioides*. In the laboratory strain 25844 increased the stand and/or reduced root rot severity of table beets when applied on clay granules at 10% or 5% w/w to soils infested with 750-2000 oospores of *P. ultimum*/g. Strains 25844 and W 57 also protected tables beets from *P. ultimum* in field plots at the 5% rate. In the laboratory, granules of W 57 or W 257 at 5% rate applied to soil infested with 10^3 oospores of *A. cochlioides* increased the emergence of sugar beets. In field plots infested with 500 oospore/g soil a 5% rate of W 57 or W 257 also increased sugar beet emergence. Granular rates of 1% or less typically were not effective, except where the inoculum densities of strains in soil were 10^8 cfu/g or higher. Seed treatment (10^7-10^8 cfu/seed) showed some promise for increasing plant stands in soil infested with *Pythium* spp.

Findings from this work suggest that strains 25844, W 57 or W 257 may have promise for the biological control of *Pythium* and *Aphanomyces* spp. in soil. These strains should be given further study to assess their benefit to field crops and perhaps to the floriculture and bedding plants industry. Improvements in sporulation on granules are needed to enable lower granular rates to be applied. Timing of granular application also needs to be investigated. Use of strains as seed coatings should be studied further. The effects of environmental factors such as temperature and matric

potential on biocontrol efficacy and strain persistence in soils need study.

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