The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes

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**SUMMARY**

Actinomycetes isolated from carrot rhizosphere were screened for their *in vitro* and *in vivo* antagonism to *Pythium coloratum* Vaartaja, a causal agent of cavity-spot disease of carrots (*Daucus carota* L.). Forty-five streptomycete and non-streptomycete actinomycete isolates were screened for *in vitro* antagonism in a carrot bioassay. Of these, seven which reduced or prevented lesion formation were identified using cultural, morphological, physiological, biochemical and cell wall characteristics as *Streptomyces janthinus*, *S. cinerochromogenes*, *Streptoverticillium netropsis*, *Actinomadura rubra*, *Actinoplanes philippinensis*, *Micromonospora carbonacea*, and *Streptosporangium albidum*. All seven isolates tested produced non-volatile antifungal metabolites, but failed to produce inhibitory volatile compounds. *Actinoplanes philippinensis* and *M. carbonacea* grew epiphytically on the hyphae and oospores of *P. coloratum*. The external surface of the oospores of the pathogen was heavily colonized by both hyperparasites, their hyphae were found to coil tightly around the oospore wall, and frequently caused cytoplasmic collapse of oospores. Sporangia of *A. philippinensis* were often seen to emerge from the colonized hyphae and oospores of *P. coloratum*. None of the other actinomycete isolates showed hyperparasitism.

All seven isolates significantly reduced the incidence of cavity spot in soil artificially infested with the pathogen in the greenhouse. *S. janthinus* and *S. albidum* were the most effective in reducing the disease in inoculated plants. In addition, all the actinomycetes species except *Ac. rubra* and *M. carbonacea*, in the presence or absence of the pathogen, significantly (*P < 0.05*) increased mean fresh root weight compared to the treatment which included *P. coloratum* only. This study shows that these actinomycetes have considerable potential for future use as biocontrol agents of cavity spot under natural field conditions. This is the first report of cavity-spot disease of carrots being controlled by microbial antagonists, and is the first report of non-streptomycete actinomycetes to control a *Pythium* disease.

Key words: Actinomycetes, biological control, carrots, cavity spot, *Pythium coloratum*.

**INTRODUCTION**

Cavity-spot disease affects the carrot industry in many countries of the world (White, 1988; Vivoda et al., 1991; Benard & Punja, 1995; Galati & McKay, 1995) and reduces the quality of carrots so that they become unmarketable, resulting in substantial economic losses. Symptoms appear on carrot roots as small sunken, circular to elliptical or irregularly shaped brown-black lesions or pits often surrounded by a yellow halo, which usually vary from 1 to 10 mm in diameter (White, 1988; Benard & Punja, 1995). Cavity-spot disease is one of the most common and serious diseases of carrots in Western
Australia which is the largest exporter of carrots in the southern hemisphere with c. 29,800 t exported in 1994 (Anon., 1994).

Cavity-spot disease is caused by a variety of species within the genus Pythium (White, 1986, 1988; Vivoda et al., 1991; Benard & Punja, 1995). In Western Australia, P. coloratum and P. sulcatum were isolated from cavity-spot lesions on commercially grown carrots and glasshouse pathogenicity testing implicated them as the causal agents of cavity-spot disease, with P. coloratum being the more severe of the two pathogens (El-Tarabily, Hardy & Sivasithamparam, 1996a).

A variety of methods have been used to control cavity spot. These have included the use of fungicides such as metalaxyl (Lyshol, Semb & Taksdal, 1984; White, 1986, 1988), cultural practices through the use of soil amendment with calcium fertilizers (Perry & Harrison, 1979; Goh & Ali, 1983; Galati & McKay, 1995), crop rotation (Galati & McKay, 1995) and cultivar resistance (White, Dowker & Crowther, 1987; Benard & Punja, 1995; Galati & McKay, 1995).

In Western Australia, the application of lime to carrot-growing soils has been reported to reduce the incidence and severity of cavity spot, and disease reduction is high in soils with a pH > 7 (Galati & McKay, 1995). The application of lime and the reduced disease incidence was associated with an increase in microbial biomass and its activity. This disease suppressiveness was associated with an increase in numbers of aerobic bacteria, fluorescent pseudomonads, Gram-negative bacteria, streptomycete and non-streptomycete actinomycetes in the rhizosphere in comparison to the non-limed soil (El-Tarabily et al., 1996b).

To our knowledge, no biological control measures have been tested for reducing the incidence of cavity spot caused by Pythium species. Therefore, the purpose of this study was to examine the interactions between 43 streptomycete and non-streptomycete actinomycetes isolated and screened for antagonism against P. coloratum in a previous study (El-Tarabily et al., 1996b) and assess their ability to suppress the pathogen in vitro and to reduce the incidence of cavity-spot disease under controlled glasshouse conditions.

**MATERIALS AND METHODS**

**Pathogen**

A highly virulent isolate of *Pythium coloratum* Vaartaja (IMI 366699) previously isolated from cavity-spot lesions on diseased carrots (*Daucus carota* L.) in Western Australia (El-Tarabily et al., 1996a) was used throughout this study. It was grown on potato carrot agar (PCA) (Plaats-Niterink, 1981) at 25 ± 2 °C for 7 d in the dark.

**In vitro detection of the antibiotic activity of actinomycete isolates**

In a previous study (El-Tarabily et al., 1996b), 352 out of 817 streptomycete and non-streptomycete actinomycete isolates produced inhibitory compounds active against *P. coloratum* in *vitro* using the dual culture agar method on Hussein’s fish meal extract agar (HFMEA) (El-Tarabily et al., 1996b, c). Out of these, the 45 most inhibitory isolates were tentatively identified to the genus level as *Streptomyces* spp. (12), *Streptovercillum* spp. (6), *Actinoplanes* spp. (8), *Microconispora* spp. (5), *Actinomadura* spp. (9), *Microbispora* spp. (2), and *Streptosporangium* spp. (3). These isolates were chosen for further in vitro and in vivo studies.

**Carrot bioassay to select the most promising antagonists**

A carrot bioassay was developed to assess the ability of the 45 actinomycete isolates to prevent or reduce disease development (lesion formation) in the presence of the pathogen *in vitro*. The carrot bioassay used was a modification of the pathogenicity bioassay method described by Groom & Perry (1985).

The actinomycete isolates were evenly streaked on the entire surface of HFMEA. There were four replicate plates for each actinomycete isolate and the plates were then incubated for 14–20 d at 28 ± 2 °C in the dark to allow for the production and diffusion of metabolites into the agar. HFMEA was used since this medium, unlike others, allows actinomycetes to consistently produce antifungal metabolites. *Pythium coloratum* was also grown in HFMEA by placing an agar disc (5-mm diameter) colonized by *P. coloratum* from the margin of a 5-d-old colony in the middle of the plate for 8 d at 25 ± 2 °C in the dark. After incubation, agar plugs (5 mm diameter discs) were cut from both the actinomycete- and *P. coloratum*-colonized plates using a cork-borer.

Mature, 4-month-old disease-free field grown carrots (cv. Top Pak), from Sun City Farms, Yanchep, 100 km north of Perth, Western Australia were carefully washed and, after removing the foliage, the carrots were then surface-sterilized in 0.5% NaOCl for 3 min and rinsed three times in sterile distilled water. The carrots were placed in plastic trays on sterile paper towels moistened with sterile distilled water. The carrots were inoculated in plastic trays on sterile paper towels moistened with sterile distilled water. The carrots were inoculated by placing the colonized actinomycete and/or *P. coloratum* agar plugs (5-mm diameter discs) in the following combinations onto each carrot: (i) pairing *P. coloratum* and the potential antagonists together, with the latter on the carrot surface (Fig. 1); (ii) the potential antagonist alone, paired with a sterile agar plug above it; (iii) *P. coloratum* alone, with a sterile agar plug below it, and (iv) a sterile non-inoculated agar plug (control) (Fig. 1).
The antagonists were placed onto the carrot surface 2 d before the pathogen plug in order to allow time for the diffusion of antifungal metabolites onto the carrot surface. Since P. coloratum grows rapidly on agar, the presence of a non-colonized agar plug between it and the carrot surface (treatment (iii)) did not affect its pathogenicity. Each carrot was inoculated with the four treatment combinations and each tray contained four carrots with two replicate trays for each isolate. The prepared trays were sealed and covered with aluminium foil and incubated for 4 d under humid conditions at 28 ± 2 °C in the dark. The carrots were sprayed daily with sterile distilled water to prevent desiccation of the plugs. After 4 d, the lesion diameters were measured (length by width) for each inoculation treatment. Lesion diameter was used as a disease index criterion to compare the efficacy of the different isolates.

In order to satisfy Koch's postulates, the discs were removed and any lesions formed were surface-sterilized in 0.5% NaOCl for 3 min, followed by four rinses in sterile distilled water. Sections of the carrot were removed and any lesions formed were surface-sterilized in 0.5% NaOCl for 3 min, followed by four rinses in sterile distilled water. Sections of the carrot were removed and any lesions formed were surface-sterilized in 0.5% NaOCl for 3 min, followed by four rinses in sterile distilled water. Sections of the carrot were removed and any lesions formed were surface-sterilized in 0.5% NaOCl for 3 min, followed by four rinses in sterile distilled water. The carrot was then washed three times with sterile water and then autoclaving in deionized water at 121 °C for 20 min. Each membrane circle was then placed onto the surface of HFMEA, PCA and 1/5 M32 (Sivasithamparam, Parker & Edwards, 1979) in a Petri plate (86-mm diameter). The surface of the agar was allowed to dry for 30 min in a laminar flow cabinet before and after the addition of the membrane. The dialysis membrane overlay technique of Gibbs (1967) involved boiling single-thickness dialysis membrane (type 4531; Union Carbide Corporation, USA) (80-mm diameter), in 0.1 mM ethylenediaminetetra-acetic acid (EDTA), rinsing thoroughly three times with sterile water and then autoclaving in deionized water at 121 °C for 20 min. Each membrane circle was then placed onto the surface of HFMEA, PCA and 1/5 M32 (Sivasithamparam, Parker & Edwards, 1979) in a Petri plate (86-mm diameter). The surface of the agar was allowed to dry for 30 min in a laminar flow cabinet before and after the addition of the membrane. 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ing spores from 7-d-old culture of each isolate on the whole surface of the agar. These cultures were incubated at 28 ± 2°C for 14–20 d in the dark to allow the actinomycetes to grow and to sporulate. Plates of the same media were then inoculated with a 5-mm diameter plug of *P. coloratum*. The lids were removed and the plates containing the *P. coloratum* were inverted over the actinomycete plates. The two plate bases were taped together with a double layer of Parafilm® (American National Can TM, Greenwich, CT 06836, USA). Control plates were prepared in the same way, except that a non-inoculated plate was used instead of a plate containing the antagonist. There were four replicates per treatment. These plates were incubated at 25 ± 2°C in the dark for 4 d. The colony diameter of *P. coloratum* was measured and compared with that of the control. Growth of the pathogen on these plates was assessed after 5 d incubation at 25 ± 2°C.

**Hyperparasitism of hyphae and oospores**

The method of Whipps (1987) was used to examine hyphal parasitism. Briefly, discs (5 mm diameter) cut with a cork borer from the margins of actively growing colonies of *P. coloratum* were transferred to the edge of water agar plates (WA). At the same time, each actinomycete isolate was streak-inoculated opposite the *P. coloratum* colony. *Pythium coloratum* colonized the plate within 36 h, a period considered to be insufficient to allow any antifungal metabolites to diffuse into the agar, before the two colonies came into contact. The plates were incubated in the dark at 28 ± 2°C. Control plates contained *P. coloratum* only. Daily observations were made between 2 and 10 d by cutting an agar plug (5-mm diameter) from the interaction zone, staining with lactophenol cotton blue and examining microscopically for hyphal parasitism.

Oospores of *P. coloratum* were produced aseptically in 250 ml flasks containing 50 ml of potato carrot broth (PCB) supplemented with 20 mg of cholesterol l−1 (Filonow & Lockwood, 1985) at 25 ± 2°C in the dark for 3 wk before harvest. At harvest, the mycelial mats were aseptically washed four times with sterile water and homogenized in 50 mM phosphate buffer (pH 6.8) in an Omni-mixer® (OYMEA) (Williams & Wellington, 1982) for 10 min) in the phosphate buffer and adjusted to about 1 x 10⁸ per ml, using a haemocytometer.

The actinomycetes were grown in oatmeal agar plates supplemented with 01% yeast extract (OMYEA) (Williams & Wellington, 1982) for 10–14 d in the dark at 28 ± 2°C. The spores and mycelium were scraped from the plates and macerated in sterile distilled water in an Omni-mixer at 4000 rpm for 15 min. Spores and mycelial fragments of each actinomycete isolate in sterile distilled water were counted with a haemocytometer and the numbers adjusted to deliver 1 x 10⁸ propagules per ml per plate.

**In vitro parasitism of the oospores was carried out using the method of Sutherland & Lockwood (1984).** Briefly, c. 1 x 10⁴ per ml oospores of *P. coloratum* were impacted onto 47-cm diameter, 0.45 μm pore size polycarbonate membrane-filters (Millipore, Australia Pty. Ltd, N.S.W., Australia), using mild suction through a sterile membrane filter unit. The filters were then inverted over slightly molten 2% WA on Petri plates. After the agar has solidified, the filters were removed leaving the oospores embedded on the agar surface. The 1-ml spore and mycelial suspension of each actinomycete was spread on the agar surface containing the oospores with a glass rod. Plates were incubated at 28 ± 2°C in the dark for 14 d, after which the area containing the oospores was removed from the plate, stained with lactophenol cotton blue and examined microscopically. Hyperparasitism was assessed microscopically at ×40 magnification using light and phase contrast Olympus BH-2 microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Six replicates for each actinomycete isolate were used. Oospores were considered infected when mycelia or spores of the actinomycete colonized and emerged from the oospore, or if actinomycete hyphae had replaced the oospore cytoplasm, or if the cytoplasm was granulated and disorganized.

**Hyperparasitism of hyphae and oospores by the Actinoplanes sp.**

Since the *Actinoplanes* sp. produced motile zoospores, an additional experiment was carried out using the method of Khan et al. (1993). Briefly, 1 ml of each of a *P. coloratum* oospore suspension and *Actinoplanes* (isolate 88) zoospore suspension were mixed together in sterile test-tubes which contained 2 ml of sterile soil extract solution. Soil extract was prepared by heating, but not boiling, 100 g of field soil in 1 l of distilled water for 1 h (Sutherland & Lockwood, 1984). The soil extract was then sterilized by membrane filtration using sterile 0.22 μm Millipore® membranes. There were six replicate tubes. After 8–10 d incubation at 28 ± 2°C in the dark, four drops per tube were microscopically examined at ×40 magnification using phase contrast as described above. For hyphal colonization by the *Actinoplanes*
isolated, the pathogen mycelia were treated as for oospore treatment except that they were not subjected to freezing before immersion in the zoospore suspension of the *Actinoplanes* isolate.

The morphological features of the host–hyperparasite interaction of the *Actinoplanes* and *P. coloratum* were further examined by scanning electron microscopy (SEM). Agar blocks (3 mm × 3 mm) containing the oospores of the host and the mycelium hyperparasite were fixed overnight in 3% glutaraldehyde (Sigma) in 0.1 M phosphate buffer, pH 7.0 at 4°C. The samples were then thoroughly washed with the same buffer, postfixed in 2% OsO₄ for 4 h, and dehydrated in a graded ethanol series (Tu, 1973). The agar blocks were then passed through a graded ethanol–amyl acetate series to critical-point dried by the method of Boyde & Wood (1969) using the critical-point drier (Balzers Union, FL-9496 Balzers/Furstentum-Liechtenstein). The specimens were then mounted on aluminium stubs and rotary coated with gold using a sputter coater device (Balzers Union, FL-9496 Balzers/Furstentum-Liechtenstein) and examined in a Phillips XL 20 SEM (Phillips Electron Optics, 5600 MD Eindhoven, The Netherlands).

**Identification of actinomycete isolates to species level**

Identification of the seven actinomycetes to species levels was based on characteristics specific for each species as presented in Bergey’s *Manual of Systematic Bacteriology* (Anon., 1989), based on morphological, cultural, physiological and chemotaxonomical characteristics. No attempt was made to characterize species based on peptidoglycan type, fatty acid pattern, major menaquinone, phospholipid type and molecular percentage of G+C of DNA (Goodfellow, 1989).

The seven were identified as *Streptomyces janthinus* Artamonova and Krasil’nikov in Rautenshtein (Pridham, 1970) (isolate 23), *Streptomyces cinereomagens* Miyairi et al. (isolate 61), *Streptovertebillium netropsis* Baldacci, Farina & Locci (isolate 129), *Actinomadura rubra* Sveshnikova, Makimova & Kudrina (isolate 175), *Micromonospora carbonacea* Luedemann & Brodsky (isolate 74), *Actinoplanes philippinensis* Couch (isolate 88), and *Streptosporangium albicum* Furumai, Ogawa & Okuda (isolate 154).

**Glasshouse trials**

**Production of inoculum of *P. coloratum***

A millet (*Panicum mileaceum* L.) seed-based inoculum was prepared by adding 25 g of seeds and 40 ml of distilled water into 250 ml conical flasks. The flasks were autoclaved at 121°C for 30 min on three consecutive days (Wong, Barbetti & Sivasithamparam, 1984). Under aseptic conditions, the millet seeds were then inoculated with eight agar plugs (6-mm diam.) from the actively growing margins of a *P. coloratum* colony. The flasks were incubated at 25±2°C in the dark for 3 wk and occasionally shaken to ensure uniformity of colonization. Colonized millet seeds which had been autoclaved twice served as the control. Small amounts of the colonized and control millet seeds were plated onto OMYEA before use to confirm to *P. coloratum* was present or absent, respectively.

**Actinomycete inoculum production.** The inoculum for each actinomycete was prepared by placing 50 g of moist wheat bran into 550-ml conical flasks, autoclaving at 121°C for 20 min on three successive occasions as described by Roiger & Jeffers (1991). This mixture was then inoculated aseptically with zoospore suspensions (25 ml) of each actinomycete in 10% glycerol, under aseptic conditions and incubated at 28±2°C in the dark for 4 wk. The flasks were occasionally shaken to ensure uniformity of colonization. Colonized wheat bran which had been autoclaved twice served as the control. Before use, small amounts of the colonized and control wheat bran were suspended in 25 ml of sterile distilled water, and 0.2 ml of this suspension was spread onto OMYEA plates, to confirm that the actinomycete isolates were present or absent.

**Soil and soil characteristics.** A grey non-limed soil collected from Sun City Farms in January (midsummer) 1995 was used in this study. It was air dried, passed through a 1-cm mesh sieve, and steam-pasteurized at 60°C for 30 min immediately before use. The chemical characteristics of the soil were analysed as previously described (El-Tarabily et al., 1996b). The soil characteristics were: pH of 5.1 (in 0.01 M CaCl₂); electrical conductivity 0.64 dS m⁻¹; organic carbon 0.66%; clay 2% the following nutrients (expressed in mg kg⁻¹): bicarbonate-extractable potassium and phosphorus 36.5 and 34 respectively, nitrate-nitrogen 16.5, ammonium-nitrogen 8, sulphate 7.15 iron 146; and the following exchangeable cations (in meq 100 g⁻¹): potassium 0.045, sodium 0.16, calcium 3.2 and magnesium 0.22.

**Soil infestation.** Wheat bran colonized with each actinomycete was thoroughly dispersed through the soil by mixing in a cement mixer (0.5% weight of colonized wheat bran-based inoculum/weight of air dry steam-pasteurised soil) 2 wk before adding the pathogen inoculum and sowing. The drained pots were watered twice a week. After 2 wk, for the treatments which included the pathogen alone or the pathogen in combination with an antagonist, millet seeds infested with *P. coloratum* were thoroughly dispersed through the soil (0.5% (w/w)) (weight of colonized millet seeds/weight of air dry steam-
pasteurized soil) by mixing in a cement mixer. This inoculum density had previously been shown to be the optimum level required to cause cavity-spot disease under glasshouse conditions (El-Tarabily et al., 1996a).


Immediately after adding the pathogen inoculum, carrot seeds (cv. Primo F1; Vilmorin Seeds, France) were disinfected by initial immersion in 70% ethanol for 1 min, and then in diluted NaOCl (0.5% available chlorine) for 2 min. They were then rinsed five times with sterile distilled water and planted into the pots. The variety Primo was used since it is a highly susceptible cv. grown in Western Australia (Galati & McKay, 1995). The seed (10 per pot) were placed into 20-cm diameter pots (Smith and Nephew, Australia) filled with 8 kg of soil containing the required soil treatments. The seeds were sown to a depth of 0.5 cm in each pot and when emergence was complete (c. 10 d) the seedlings were thinned to five per pot. Each treatment was replicated seven times with five plants per replicate, in fully randomized blocks, and the experiment was repeated once. The pots were placed in an evaporatively cooled glasshouse and maintained at 25 ± 5 °C. The free-draining pots were watered twice a day to field capacity and were fertilized weekly with a liquid fertilizer which contained K$_2$HPO$_4$ (2.48 g), CH$_3$N$_2$O (5.48 g), KNO$_3$ (5.94 g), CaCl$_2$ (2.01 g), MgSO$_4$ (4.45 g), CuSO$_4$ (0.0099 g) and ZnMoO$_4$ (0.0013 g) in 301 of distilled water.

**Disease assessment.** Carrots were harvested 16 wk after commencement of the experiment. The leaves were removed and the carrots were washed, weighted and examined for cavity-spot lesions. Disease incidence was expressed in three ways: (i) as number of cavity-spot lesions per carrot; (ii) as number of cavity-spot lesions per cm$^2$ of carrot root; and (iii) as a disease index rated on a 1–5 scale, where 1 = no visible cavities, 2 = from one to five cavities, each < 3 mm diam., 3 = from six to ten cavities < 3 mm diam., 4 = from 11 to 20 cavities < 3 mm diam. or up to two cavities > 10 mm diam., and 5 = more than 20 cavities < 3 mm, or up to three cavities > 20 mm diam.

In order to satisfy Koch's postulates, cavity-spot lesions were dissected from 50 randomly selected carrots and surface-sterilized in 0.5% NaOCl for 3 min, followed by four rinses in sterile distilled water. Sections of the lesions were cut and air-dried, plated on P$_2$ARP and incubated at 25 °C (± 2 °C) in the dark for 4 d. Colonies were transferred to PCA to encourage formation of sexual structures and to grass-blade cultures (Plaats-Niterink, 1981) to induce sporangial formation, zoospore discharge and sexual reproductive structures in order to confirm the identification.

**Statistical analysis**

Analysis of variance was carried out using Superanova® (Abacus Concepts, Inc., Berkeley, California, USA) to evaluate the effect of actinomycetes on lesion formation in the carrot bioassay, production of volatile, non-volatile antifungal metabolites, cavity-spot development and on fresh weight of carrot root in the glasshouse trials. Significant differences between means were determined by Duncan's New Multiple Range Test at $P = 0.05$.

**Results**

**Carrot bioassay**

Out of the 45 isolates screened, five prevented lesion formation. These were: S. janthinus, S. cinerochromogenes, Strep. netropsis, Ac. rubra, and Strep. albifidum. These isolates, when paired with the pathogen on the carrot surface (treatment (i)) complete inhibited the pathogen and no lesions were formed compared with the treatment which consisted of the pathogen plug alone (treatment (iii)). Lesions produced by the pathogen were large brownish-black, round to elliptical, water-soaked and depressed with clearly defined edges (Fig. 1b). This clearly indicated that the antagonist and/or its antifungal metabolites(s) in the agar plug was able to inhibit the pathogen and prevent it from producing lesions on the carrot surface. In addition, P. coloratum was not re-isolated from the healthy carrot tissue beneath these treatments. Of the remaining 40, some reduced lesion development to varying degrees, whilst others failed to reduce lesion formation. Of these, A. philippinensis and M. carbonacea were chosen for further studies since they significantly ($P < 0.05$) reduced lesion development compared to the pathogen treatment alone (Table 1). *Pythium coloratum* was recovered from lesion tissue. None of the actinomycetes tested alone (treatment (ii)) appeared to have any detrimental effects on carrots and none caused any lesions.

**Dialysis membrane overlay technique**

Growth of *P. coloratum* was significantly ($P < 0.05$) inhibited by each of the seven actinomycetes grown on the three agar media overlaid with dialysis membrane before inoculation of the pathogen com-
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pared with the control treatment (Table 1). Colonies of *P. coloratum* on control plates reached the plate margin within 36–48 h. All actinomycete isolates, except for *A. philippinensis*, and *M. carbonacea*, completely inhibited the pathogen on the three media tested (Table 1) whereas for the *A. philippinensis* and *M. carbonacea* isolates, the degree of inhibition was greatest on the nutrient rich HFMEA and least on 1/5 M32.

The non-volatile metabolites of *S. janthinus*, *S. cinerochromogenes*, *Strep. netropsis*, *Ac. rubra* and *Strepto. albidum* were found to have fungicidal activity, since *P. coloratum* did not grow from the plugs once transferred from the treatment plates to fresh medium in the absence of antifungal metabolite(s). The metabolites of *A. philippinensis*, and *M. carbonacea* were moderately fungistatic since *P. coloratum* grew from the plugs once transferred to a fresh plate (Table 1).

**Ability of actinomycetes to produce volatile compounds**

All seven actinomycete isolates failed to produce any volatile compounds when grown on any of the three media used. The growth of *P. coloratum* was not affected and there were no visible effects on colony morphology (Table 1).

**Hyperparasitism**

There was no evidence of any extensive coiling, penetration or degradation of *P. coloratum* hyphae or oospores by *S. janthinus*, *S. cinerochromogenes*, *Strep. netropsis*, *Ac. rubra* and *Strepto. albidum*. In contrast, for *A. philippinensis* and *M. carbonacea* there was evidence of hyperparasitism. The external surface of the oospores of the pathogen was heavily colonized by both hyperparasites; their hyphae were found to coil tightly around the oospores wall, and frequently caused cytoplasmic collapse of oospores.

The fine hyphae of *A. philippinensis* (0.5 μm diam.) were c. 20–25 % the diameter of the host fungus and could be easily differentiated using the light microscope (Figs 2a, b; 3a, c, 4a, b). *A. philippinensis* grew epiphytically on the hyphae (Fig. 2a, b) and oospores (Figs 3a–d; 4a, b) of *P. coloratum*. The sporangia and hyphae of *A. philippinensis* were also found to colonize *P. coloratum* hyphae and oospores (Figs 2b; 3b–d). Parasitized oospores frequently had disorganized cytoplasm with hyphae of *A. philippinensis* proliferating from the oospores (Figs 3a, c, d; 4a, b). Sporangia of *A. philippinensis* often appeared to emerge from these hyphae (Fig. 2b) and from colonized oospores (Fig 3b–d). Zoospores of *A. philippinensis* were often observed within the sporangia which were borne on long thin sporangiophores (Fig. 3d).
Figure 2. Germination of zoospores of *Actinoplanes philippinensis* and subsequent growth of hyphae (a, b) and sporangia (arrows) (b) of the antagonist on and around hyphae of *Pythium coloratum* when the pathogen hyphae were suspended in soil extract containing *A. philippinensis* zoospores at 28 °C for 6 d (× 400, scale bar = 10 μm).

Glasshouse trials

The application of all seven actinomycetes significantly (*P* < 0.05) reduced cavity-spot incidence compared to the treatment which contained the pathogen alone (treatment 1) (Table 2). Although cavity-spot lesions were observed in all treatments (except the uninfected control and actinomycetes alone), there were significant (*P* < 0.05) reductions in the disease index, the number of lesions per carrot and the number of lesions per cm² of carrot root between carrots grown in each of the actinomycete + pathogen infested soil treatments and the pathogen infested soil only. *S. janthinus* (treatment 2) was the most aggressive antagonist, followed by *Streptosporangium albicum* (treatment 8) (Table 2). *Pythium coloratum* was recovered on PgARP from cavity-spot lesions in all infested treatments. No visible symptoms were observed in the shoot system.

The actinomycetes alone in the absence of the pathogen did not produce any harmful effects on seed germination and subsequent plant growth. Treatments with all the actinomycetes species in the presence or absence of the pathogen (except *Ac. rubra* in the presence or absence of the pathogen and *M. carbonacea* in the presence of the pathogen only) significantly (*P* < 0.05) increased mean root weight compared with *P. coloratum* treatment alone (Table 2).

**DISCUSSION**

This study is the first record of cavity spot disease of carrots being controlled by microbial antagonists, of non-streptomycete actinomycetes controlling a *Pythium* disease, and of *Streptosporangium* controlling a soil-borne plant pathogen. The mechanism involved in disease reduction appears to be antibiosis for *S. janthinus, S. cinerochromogenes, Strep. netropsis, M. carbonacea, Ac. rubra, A. philippinensis,* and *Strepto. albicum.* *A. philippinensis* and *M. carbonacea* were capable of parasitizing *P. coloratum* oospores in addition to producing antifungal metabolites. However, other factors may also have been involved in the reduction of cavity-spot incidence.
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This study therefore clearly indicates the potential of streptomycete and non-streptomycete actinomycetes as potential biological control agent of *P. coloratum* in Western Australia. It also indicates the importance of the *in vitro* bioassay for the screening of potential antagonists before their screening in *in vivo* trials. Similar bioassays should always be used in conjunction with the dual culture and the dialysis membrane overlay techniques when screening for biocontrol agents, but are expected to give a better indication of what might occur under field conditions. Such *in vitro* assays using plant material are valuable as they allow a rapid screening of large numbers of antagonists in the presence or absence of the pathogen to give a more realistic picture than using the conventional agar antagonism methods.
The results, however, should be confirmed by glasshouse experiments.

In dual agar culture, all isolates inhibited the pathogen, whilst in the carrot bioassay some isolates completely prevented lesion development, others reduced the size of lesions or had no effect at all. Those actinomycetes which failed to reduce lesion diameter in the carrot bioassay indicated that the production of antifungal metabolites in agar does not necessarily mean that this effect will be repeated on plant material (Cook & Baker, 1983). The reduction or prevention of lesion development by certain actinomycete strains used in the present study was associated with antibiosis as well as other mechanisms such as hyperparasitism. None of the actinomycetes tested appeared to have any detrimental effect on carrots.

Non-volatile antifungal metabolites, produced by some species of micro-organisms known to be antagonistic to certain soil-borne pathogens, have been extensively studied by means of dialysis-membrane overlay technique (Gibbs, 1967; Whipps, 1987; Simon & Sivasithamparam, 1988). In the present study, all isolates except for *M. carbonacea* and *A. philippinensis* completely inhibited the growth of *P. coloratum* after 7 d of growth on the three media tested. An impoverished medium (1/5 M32) (Sivasithamparam *et al.*, 1979) was used since it is likely to induce the microbes to produce antibiotics as secondary metabolites in response to nutrient depletion (Cook & Baker, 1983). This medium was developed to simulate the nutrient status equivalent to that of a natural West Australian agricultural soil (Sivasithamparam *et al.*, 1979). As our results indicate that the actinomycete isolates tested produced non-volatile compounds on 1/5 M32, it is possible that these compounds could also be produced in soil which might be nutrient impoverished. The transfer of the inhibited *P. coloratum* plugs to fresh media and their subsequent inability to grow indicated a possible fungicidal effect of the metabolites produced by five of the actinomycete isolates tested. The HFMEA medium appears to be suitable for the screening of potential actinomycete antagonists using the dual culture technique.

Whipps (1987) screened many fungal antagonists against a range of soil-borne plant pathogens using water agar, soil-extract agar and PDA, and reported that the medium used had significant effects on the morphology and growth rates of the pathogens, and on the production of volatile and non-volatile antibiotics produced by the antagonists and the responses of the pathogens to these antibiotics. He also reported that the medium also affected competition and mycelial interactions between the pathogen and the antagonists. In our study, we observed no differences in the inhibition rates among five of the seven actinomycete antagonists tested on the three media. This observation differs from that of

<table>
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<tr>
<th>Disease index</th>
<th>Mean number of lesions per ear of carrot</th>
<th>Mean number of lesions per carrot</th>
<th>Mean root weight (g)</th>
</tr>
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<tr>
<td>4.88 f</td>
<td>1.24 b f</td>
<td>3.44 b f</td>
<td></td>
</tr>
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<td>5.80 a f</td>
<td>1.21 b f</td>
<td>3.66 b f</td>
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<td>5.17 a f</td>
<td>1.25 b f</td>
<td>3.62 b f</td>
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</tr>
<tr>
<td>5.35 c f</td>
<td>1.20 b f</td>
<td>3.64 b f</td>
<td></td>
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<tr>
<td>5.36 c f</td>
<td>1.20 b f</td>
<td>3.69 b f</td>
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Plants were harvested after 16 wk. * Values with the same letter within a column are not significantly (P > 0.05) different according to Duncan's New Multiple Range Test.
Whipps (1987), who used the same dialysis membrane overlay technique (with fungal antagonists and found that PDA (a rich medium) gave greatest inhibition of growth compared to poorer media (water agar and soil extract agar). The difference between our study and that of Whipps (1987) might be a result of the ability of the actinomycete isolates, in contrast to fungi, to produce antibiotics on both nutrient-rich and poor media. On the other hand, the A. philippinensis and M. carbonacea isolates were influenced by substrate and medium composition. Both these isolates were more inhibitory on the rich HFMMA than on the nutrient poor PCA or 1/5 M32. These results agree with those of Whipps (1987).

In the present study, oospores of P. coloratum were identified for the first time as hosts of A. philippinensis and M. carbonacea. There are many reports of parasitism of oospores in vitro by a range of actinomycete genera, these include A. missouriensis Couch parasitising oospores of Aphanomyces cochlioides Drechsler, A. euteiches Drechsler, Pythium aphanothecatum (Edson) Fitzp., P. ultimum Trow, Phytophthora citrophthora (R. E. Sm. & E. H. Sm.) Leonian and Ph. megasperma Drechsler f. sp. glycinea Kuan and Erwin (Sutherland & Lockwood, 1984). They also reported that A. philippinensis, A. utahensis Couch, Ampullariella regularis Couch, Spirillospora albida Couch, and Micromonaspora sp. parasitized oospores of Ph. megasperma f. sp. glycinea in vitro. Oospores of many Pythium spp. including P. aphanothecatum, P. arrenomanes Drechsler, P. irregularure Buisman, P. myriotylum Drechsler and P. ultimum were reported to be parasitized by a wide range of Actinoplanes spp. (Khan et al., 1993).

In our study, hyperparasitism could be an important mechanism of biocontrol of P. coloratum by the actinomycetes, especially the non-streptomycete actinomycetes. As in many other instances of hyperparasitism, it is a component of antagonism rather than the sole mechanism of biocontrol. For example, with Gliocladium spp. it has been proved that antibiotics and hyperparasitism function together, the antibiotic destroying the integrity of the host cell-wall which enable the hyperparasite (Gliocladium sp.) to penetrate otherwise resistant fungal cells (Di Pietro et al., 1993).

Of the actinomycete species evaluated in this study, little is known about their ability to control Pythium species. However, against other pathogens Strept. netropsis has been shown to control Verticillium wilt in cotton (Askarova & Khashimova, 1970; Polyarnichko, 1975). Actinomadura sp. were reported to control root rot of snapdragon caused by Phytophthora cinnamomi Rands (You, Sivasitharaman & Kurböke, 1996). Micromonaspora carbonacea was previously shown to reduce root rot disease of Banksia grandis L. caused by Ph. cinnamomi (El-Tarabily et al., 1996c). Micromonaspora globosa Kripps was used to control gram wilt caused by Fusarium udum Butler (Upadhayay & Rai, 1987). In the USA, A. missouriensis, A. utahensis, Ampullariella antarcticolor Couch, Gliocladium aphanothecatum and Micromonaspora spp. were reported to reduce Ph. megasperma f. sp. glycinea root-rot of soy-bean (Filonow & Lockwood, 1985). Khan, Filonow & Singleton (1994) evaluated some strains of Actinoplanes spp. for their efficacy to reduce soil-borne disease caused by oomycetes fungi including P. ultimum, P. aphanothecatum and A. cochlioides.

In the present study, the actinomycete inoculum was introduced to the soil 2 wk before adding the pathogen and sowing the carrot seeds. This incubation period may aid the establishment of the introduced actinomycetes, or enable them to multiply in the soil or to activate the mechanism(s) of antagonism, as suggested by Rothrock & Gottlieb (1984). This incubation period might, besides allowing the antagonists to establish themselves in soil, facilitate the hyperparasitism of P. coloratum oospores, inhibition of oospores germination and sporangial production, and/or production of antibiotics that interfere with the chemotactic activities of the zoospores. On the other hand, since these actinomycetes were isolated from the carrot rhizosphere they might not become active until they are stimulated by root exudates. It is noteworthy that P. coloratum can attack carrots at any stage of development after 3 wk (El-Tarabily, Hardy & Sivasithapharam, 1997), and this therefore implies that the antagonists may need to be established in the soil before the pathogen.

Of the seven actinomycetes, A. philippinensis should be the most attractive agent to control the pathogen as it naturally produces sporangia and zoospores under conditions which are considered to be very conducive for the saprophytic activity of P. coloratum. However, the results of our pot trials indicate that S. janthinus and Strept. albium were superior to A. philippinensis. It is still possible, however, that in irrigated carrot crops in Western Australia, where soil saturation following irrigation from overhead sprinklers is not uncommon, A. philippinensis could perform better.

In addition to the disease suppression, we also observed that the selected actinomycetes significantly stimulated root weight compared with the untreated control and with the treatment which included the pathogen alone (Table 2). This was observed for all combinations of actinomycetes with or without P. coloratum, except for Ac. rubra with and without the pathogen and for M. carbonacea in the presence of the pathogen only. This is the first record of S. janthinus, S. cinerochromogenes, Strept. netropsis, Strepto. albium, and A. philippinensis promoting plant growth. This observed increase in plant growth supports other observations where actinomycetes have been reported to stimulate plant
growth in the presence of plant pathogens (Turhan, 1981; Filonow & Lockwood, 1985; Mishra et al., 1987; Tahvonen & Avikainen, 1990; Yuan & Crawford, 1995; El-Tarabily et al., 1996a).

It is interesting that the presence of the pathogen did not diminish the growth promotion effect evident in the plants exposed to the actinomycetes. This indicates that the parasitic activity of \( P. \) coloratum was independent of the biological activity of actinomycetes. This again could be an indication that the actinomycetes were producing growth factors and were active in the rhizosphere or within the carrot tissues and were not competing for the same sites as the pathogen for their respective activities.

This study has shown that certain actinomycetes can reduce the cavity-spot disease of carrots caused by \( P. \) coloratum under the conditions employed in the current investigation. This work was carried out under controlled glasshouse conditions with steam-pasteurised soil and, although this is a practical procedure, steam-pasteurization could drastically affect these results. Pasteurized soil was used with the aim of disinfecting the soil of resident fungal pathogens. The effect, however, on the tested treatments of the soil microflora affected by pasteurization is not known. Field trials will be carried out in Western Australia to evaluate their potential as biocontrol agents.

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