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In vitro Antagonism of *Phytophthora cinnamomi* and *P. citricola* by Isolates of *Trichoderma* spp. and *Gliocladium virens*

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With 4 figures

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Abstract

Three fungi, isolated from soil from which *Phytophthora* was not obtained, were evaluated for antagonism of *Phytophthora* spp. shown to cause root rot of chestnut in South Australia. *Trichoderma hamatum* and *T. pseudokoningii* appeared to inhibit *P. cinnamomi* by mycoparasitism, with evidence of parallel growth and coiling, and both *Trichoderma* spp. and *Gliocladium virens* grew over *P. cinnamomi in vitro*, preventing further growth of this pathogen. Antibiotics produced by young *T. hamatum* cultures and *G. virens* in culture filtrate experiments inhibited growth of *P. cinnamomi* and *P. citricola*, with filtrate from 4-day-old cultures of *G. virens* showing the greatest potential for biocontrol. All three antagonists prevented *P. cinnamomi* and *P. citricola* from causing infection symptoms on micropropagated shoots of chestnut cvs Goldsworthy and Buffalo Queen in an *in vitro* excised shoot bioassay for biocontrol.

Zusammenfassung

In-vitro*-Antagonismus von *Trichoderma* spp.-und *Gliocladium virens*-Isolaten gegenüber *Phytophthora cinnamomi* und *P. citricola

Aus Boden, aus dem keine *Phytophthora*-Arten gewonnen werden konnen, wurden drei Pilzarten isoliert und auf Antagonismus gegenüber *Phytophthora*-Arten geprüft, die in Südaustralien Wurzelfäule bei Kastanien hervorrufen. *Trichoderma hamatum* und *T. pseudokoningii* hemmten *P. cinnamomi* durch Mykoparasitismus, wobei paralleles Wachstum und spiralige Hyphen auftraten. *Trichoderma* spp. und *Gliocladium virens* überwuchsen *P. cinnamomi in vitro* und verhinderten ein weiteres Wachstum des Pathogens. Junge *T. hamatum*-Kulturen und *G. virens* produzierten Antiobiotika, die in Versuchen mit Kulturfiltraten das Wachstum von *P. cinnamomi* und *P. citricola* hemmten. Dabei zeigte das Filtrat 4 d alter *G. virens*-Kulturan das größe Potential für eine biologische Bekämpfung. In einem *in-vitro*-Biotest mit abgetrennten Sproßspitzen verhinderten alle drei Antagonisten, daß *P. cinnamomi* und *P. citricola* Befallssymptome auf *in-vitro*-vermehrten Sproßspitzen der Kastaniensorten Goldsworthy und Buffalo Queen hervorrufen konnten.

Introduction

P. cinnamomi has been shown to cause root rot (ink disease) of chestnut (*Castanea sativa* Miller) in Australia (Washington, 1983). Recently, isolates of *P. cinnamomi* and *P. citricola* were obtained from soil beneath chestnut trees in orchards in the Adelaide Hills, South Australia, and shown to be pathogenic on chestnut (Chambers, 1993). Resistant rootstocks are not available. Consequently, cultural and chemical methods are the only means of preventing and controlling the disease. Fungicides such as metalaxyl, fosetyl-aluminium (Taylor and Washington, 1984), and phosphonate (Pegg et al., 1985; Wicks and Hall, 1990) can prevent disease on woody plants including chestnut. Concerns about their use, however, include the development of resistance to (e.g. Ferrin and Kabashima, 1991) and the expense of repeated applications (e.g. Sivasithamparam, 1991). Thus, there is currently great interest in developing micro-organisms as biocontrol agents of *Phytophthora* root and collar rots and other plant diseases (Baker, 1989). Only a few biocontrol products are available commercially. However, a large number are currently being developed for release (see Baker, 1989).

Investigations of interactions between pathogens and potential biocontrol agents have involved the use of *in vitro* pot and field experiments. Possible mechanisms of biocontrol/antagonism demonstrated *in vitro* include mycoparasitism (Dennis and Webster, 1971c) and production of diffusable antibiotics (Dennis and Webster, 1971a) and volatile antibiotics (Dennis and Webster, 1971b). *In vitro* experiments can be useful in elucidating the mechanisms of antagonism of biological control fungi (Duchesne et al., 1989). Mycoparasitism of *P. cinnamomi* hyphae by *T. harzianum* hyphae has been observed *in vitro* (Finlay and McCracken, 1991), and antibiosis in which *T. harzianum* cultures inhibited the growth of *P. parasitica f.* sp. *nicotianae* (= *P. nicotianae*, Hall, 1993) has also been observed (Bell et al., 1982). Smith et al. (1990) initially screened isolates of *Trichoderma* for production of antibiotics active against *P. cactorum* prior to conducting experiments in potting mix. However, other possible mechanisms of control were not investigated. Subsequent greenhouse experiments, in which apple seedlings growing in infested potting mix were inoculated with selected *Trichoderma* and *Gliocladium* spp., demonstrated protection from root rot by some spp.. The work of Roiger and Jeffers (1991) also demonstrated the potential of *Trichoderma* and *Gliocladium* spp. to control *Phytophthora* diseases of woody

hosts. Further investigations into mechanisms of biocontrol of *Phytophthora* spp. by fungi are required.

When soil samples, collected from beneath dead and dying chestnut trees in orchards in the Adelaide Hills, were baited to isolate *Phytophthora* spp., some samples did not yield pathogens. Instead, *Trichoderma hamatum*, *T. pseudokoningii* and *Gliocladium virens* were isolated. The aim of the experiments reported here was, therefore, to investigate the interactions of *T. hamatum*, *T. pseudokoningii* and *G. virens* with *P. cinnamomi* and *P. citricola* to elucidate the mechanisms involved and to assess their ability to control *Phytophthora* infection of micropropagated chestnut shoots.

Materials and Methods

Isolation and culture of fungi

Soil was collected from beneath dead and dying chestnut trees in the Adelaide Hills, South Australia in February, 1991 and *Phytophthora* spp. isolated using the pear baiting method (McIntosh, 1964). Small pieces of tissue were excised aseptically from the margins of firm, dark lesions appearing on the pear; these were placed on 20 ml cornmeal agar (CMA, Oxoid) in 90 mm diameter Petri dishes and incubated at 25°C. *Phytophthora* spp. grew out from these lesions onto CMA after 3–5 days and isolates were identified as *P. cinnamomi* and *P. citricola* on the basis of morphological characteristics (Stamps et al., 1990). *Trichoderma* spp. and *G. virens* were obtained using the above method, from soil samples from which *Phytophthora* spp. could not be obtained. These cultures were maintained on malt extract agar (MEA, Oxoid) at 25°C and identified using morphological characteristics (Rifai, 1969; Bissett, 1991).

Dual culture experiment

An experiment was conducted *in vitro* to observe interactions between colonies of *P. cinnamomi* and the three possible antagonists, based on the method of Dennis and Webster (1971c). *P. cinnamomi* was used because it has previously been reported as a serious pathogen of chestnut (Day, 1938; Crandall et al., 1945; Wicks and Volle, 1976). A sterile piece of porous uncoated Cellophane (80 × 30 mm; Australia Cellophane, Victoria) was placed on the centre of either 20 ml distilled water agar (DWA, 15 g l^{-1} Difco Bi-tek agar) or 1/5 strength M32 (soil mimic) agar (Sivasithamparam et al., 1979) in a 90 mm diameter Petri dish. Agar plugs of *P. cinnamomi* and either *T. hamatum*, *T. pseudokoningii* or *G. virens*, 8 mm in diameter cut from the edge of an actively growing culture, were placed 50 mm apart on the Cellophane. There were 10 replicates of each pathogen × antagonist combination. Plates were sealed with Parafilm and incubated at 25°C in the dark. In order to determine whether growth of the antagonists was stimulated in the presence of *P. cinnamomi*, growth of *T. hamatum*, *T. pseudokoningii* and *G. virens* toward the *P. cinnamomi* cultures was measured daily and compared to control treatments in which plates were inoculated with only *T. hamatum*, *T. pseudokoningii*, *G. virens* or *P. cinnamomi*.

After 4–5 days, when the two fungal colonies had grown together, pieces of Cellophane (5 × 5 mm) supporting the mycelium, were cut from the interaction zone, mounted on slides and stained with lactophenol cotton blue or ammoniacal congo red for light microscopy. Interactions between the fungi were photographed using a Leitz Orthoplan photomicroscope.

In order to study the interactions using scanning electron microscopy (SEM), agar plugs, as above, were placed 20 mm apart on sterile 0.2 μm Millipore filters on 1/5 strength M32 agar. Preliminary experiments had indicated that these filters maintained their shape better than cellophane during preparation for SEM. After 2 days, when the colonies had grown together, 5–10 mm^2 pieces were cut from the interaction zone and fixed in 2.5% glutaraldehyde in 0.025 M phosphate buffer (pH 7.0) at room temperature for 30 min. Samples were dehydrated through an ethanol series to 100% (each increase of 10% for 10 min), critical-point dried, mounted on stubs and sputter coated with gold. Samples were viewed with a Cambridge S250 SEM, with an accelerating voltage of 20 kV.

Culture filtrate antibiosis

Liquid cultures of *T. hamatum*, *T. pseudokoningii* and *G. virens* were prepared by transferring an 8-mm diameter plug from the margin of a fungal culture on MEA to 50 ml 1/5 strength M32 broth in a sterile 250 ml polycarbonate tub (Disposable Products, South Australia). Cultures were shaken in the dark at 110 r.p.m. at 25°C. The effect of *T. hamatum*, *T. pseudokoningii* and *G. virens* culture filtrates at 2-, 4- and 7-day harvests on growth of *Phytophthora* spp. was assessed. Cultures were harvested by passing them through four layers of muslin cloth, and the filtrate centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was then filter-sterilized through a 0.45 μm Millipore filter. The cell-free culture filtrate was incorporated at 250 ml l^{-1} into 1/5 strength M32 agar cooled after autoclaving. Plugs of either *P. cinnamomi* or *P. citricola*, as before, were placed in the centre of 90-mm diameter Petri dishes containing 20 ml 1/5 strength M32 + culture filtrate agar, and incubated in the dark at 25°C. There were 10 replicates of each pathogen × antagonist combination for each culture harvest. Sterile 1/5 strength M32 broth was incorporated into 1/5 strength M32 agar for controls. Colony diameters were determined as the mean of two measurements at right angles after 7 days.

Volatile antibiosis

To investigate whether antagonists produced volatile antibiotics which inhibited the growth of *Phytophthora* spp., the method of Dennis and Webster (1971b) was used. Plugs of either *T. hamatum*, *T. pseudokoningii* or *G. virens* were placed in the centre of 90-mm diameter Petri dishes containing 20 ml 1/5 strength M32 agar and incubated in the dark at 25°C for 2 days. Fresh plates of 1/5 strength M32 agar were then inoculated with plugs of either *P. cinnamomi* or *P. citricola* and the bases of these plates inverted and taped to the bases of the plates containing the 2-day-old antagonists. Plates were incubated, with *Phytophthora* spp. uppermost, at 25 C in the dark and colony diameters measured after 5 days. Colony diameter was measured at 5 days because by 7 days *Phytophthora* spp. plates had become contaminated by spores of the antagonist. There were 10 replicates for each pathogen × antagonist combination. Controls consisted of *Phytophthora* spp. inverted over plates of 1/5 strength M32 inoculated with sterile plugs of MEA.

Micropropagation of chestnut

Nodal explants of *Castanea sativa* (unknown cv., designated Goldsworthy) and *C. sativa* × *C. crenata* hybrid cv. Buffalo Queen were used for micropropagation. Shoots ca. 20 cm long were excised from glasshouse-grown 3-month-old seedlings and washed, with brushing, in a dilute solution of Tween 20 (Sigma) in DW to increase surface wetting during sterilization. Shoots were surface sterilized with commercial White King bleach (0.024% avail. chlorine) for 15 min, rinsed three times in sterile DW, then soaked in sterile DW for 3 h. Soaking has been found to reduce the amount of black exudate released by shoots into the medium (Chevre et al., 1983). Shoots were then cut into nodal sections of ca. 1 cm and placed in an initiation medium, modified from Vieitez et al. (1986). This consisted of 1/2 strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with all mineral salts at half strength, 30 mg l^{-1} sucrose and 1 mg l^{-1} benzyladenine (BA, Sigma). The pH was adjusted to 5.7 with 1N NaOH. Ten ml was dispensed into 8 × 3 cm polycarbonate universal tubes (Disposable Products, South Australia) and autoclaved for 20 min at 121°C. Uncontaminated explants were transferred to fresh medium 1 week later, to avoid detrimental effects of exudates in the medium.

Shoots were subcultured every 4 weeks to fresh 1/2 MS medium and after three transfers the concentration of BA was reduced to 0.2 mg l^{-1} to reduce the growth of callus at the base of the shoot while maintaining good rates of multiplication. Shoots were grown in a controlled environment room at 25°C, with a 16 h day and under cool white fluorescent lights (ca. 35 μmol m^{-2} s^{-1}).

Biocontrol shoot assay

In order to determine whether the three antagonist fungi could reduce lesion development on shoots of micropropagated chestnut incubated with *Phytophthora* spp., a biocontrol shoot assay, modified from the excised shoot assay of Scott et al. (1992), was developed. Plugs of *T. hamatum*, *T. pseudokoningii* or *G. virens* and *P. cinnamomi* or *P. citricola*, taken from actively growing cultures on MEA or CMA, respectively, were placed on opposite sides of 50 ml CMA agar in 250 ml polycarbonate tubs (Disposable Products, South Australia). Inoculated tubs were incubated at 25°C in the dark until the pathogen and potential antagonist had just grown together. Three micropropagated shoots of chestnut cvs Goldsworthy or Buffalo Queen, 2 cm long, were excised from tissue cultures 4 weeks after the last transfer, defoliated and placed upright in the interaction zone between the two fungi. Control shoots were placed in uninoculated tubs, in tubs inoculated only with *P. cinnamomi* or *P. citricola*, and in tubs inoculated only with *T. hamatum*, *T. pseudokoningii* or *G. virens*. Material was incubated in the dark at 25°C for 2 days, then one assessment of lesion length along the shoots was made (after this time *Phytophthora* spp. overwhelmed chestnut shoots). There were three replicate tubs for each antagonist × pathogen × cultivar combination. Fungi were re-isolated from one randomly chosen shoot per tub, by plating a shoot flat on CMA in 90-mm diameter Petri dishes and incubating in the dark at 25°C for 2 days.

Analysis of data

Each *in vitro* experiment was carried out twice, and the results pooled. Analysis of variance was used to compare the effect of the treatments on colony diameter or lesion length and means were compared using the least significant difference (LSD) method. Final colony diameter values from *in vitro* growth inhibition experiments had 8 mm subtracted before means were calculated to give net mycelial growth values.

Results

Dual culture experiment

None of the antagonist fungi grew significantly faster in the presence of *P. cinnamomi* compared to controls (data not shown). The hyphae of *P. cinnamomi* were easily distinguished from the antagonist hyphae, being larger in diameter and more coralloid with abundant hyphal swellings. Growth of *P. cinnamomi* was not inhibited by the antagonists before colonies were overgrown. When fungal colonies grew together, a number of features were noted in the interaction zone. Most commonly, parallel growth of either *T. hamatum*, *T. pseudokoningii* or *G. virens* along *P. cinnamomi* hyphae was observed (Fig. 1A, B, C). Scanning electron micrographs showed that the hyphae of the antagonist were tightly appressed to *P. cinnamomi* hyphae (Fig. 1D); this occurred most frequently with *T. hamatum* and *T. pseudokoningii* hyphae, and less commonly with *G. virens* hyphae. Appressorium-like structures produced by *T. hamatum and T. pseudokoningii* hyphae extending off regions of parallel growth were frequently observed (Fig. 1E); these were never observed in *G. virens* hyphae.

Coiling of *T. hamatum* hyphae around *P. cinnamomi* hyphae was common (Fig. 1F). Large areas of coiling by *T. pseudokoningii* hyphae along single *P. cinnamomi* hyphae were also observed frequently (Fig. 1G). Coiling by *G. virens* hyphae was also observed (Fig. 1H), but was less common than with *Trichoderma* spp.. Following an initial interaction period of ca. 1 day, the antagonist fungus always grew over the *P. cinnamomi* colony and filled the plate, preventing further growth of *P. cinnamomi*.

Culture filtrate antibiosis

Mean colony growth of *Phytophthora* spp. after 7 days on agar medium supplemented with filtrates from 2-, 4- and 7-day old cultures is shown in Fig. 2A and B. Under the conditions used in these experiments, the younger the *T. hamatum* culture, the more the filtrate inhibited growth of both *P. cinnamomi* and *P. citricola*. Filtrate from 2-day-old cultures almost halved the growth of both *Phytophthora* spp. compared to controls. *T. pseudokoningii* filtrate harvested at 2, 4 or 7 days did not inhibit the growth of *P. citricola*, however, the growth of *P. cinnamomi* was significantly inhibited by the 2-day filtrate compared to controls.

The effect of *G. virens* culture filtrate on *Phytophthora* spp. colony growth varied with time of harvest, with 4-day filtrate completely inhibiting the growth of *P. citricola*, and strongly inhibiting growth of *P. cinnamomi* compared to controls. *G. virens* filtrate harvested at 2 and 7 days also significantly reduced *P. citricola* growth compared to controls, but to a lesser extent, with less inhibition by the older filtrate. *G. virens* cultures harvested at 2 and 7 days caused changes in *P. cinnamomi* colony morphology. Sparse, aerial mycelial growth was commonly observed and hyphae were more vesicular in appearance. No marked effects on the colony morphology of *P. citricola* were observed.

Volatile antibiosis

Mean colony growth of *P. cinnamomi* and *P. citricola* in the volatile antibiosis experiment is shown in Fig. 3. *T. hamatum* and *G. virens* had little effect on the growth of either *P. cinnamomi* or *P. citricola*. Exposure to *T. pseudokoningii*, however, significantly inhibited both *Phytophthora* spp.; colony growth of *P. cinnamomi* was less than half that of controls (ca. 5 mm and 16 mm, respectively) and just over half that of controls for *P. citricola* (ca. 28 mm and 47 mm, respectively).

Biocontrol shoot assay

After 2 days, lesions had developed along almost the entire length of shoots of chestnut cvs Goldsworthy and Buffalo Queen incubated with only *P. cinnamomi* or *P. citricola*, whereas shoots incubated with the antagonist alone had slight basal discolouration only. Likewise, shoots incubated with the pathogen and one of the three antagonists developed only minor lesions (Fig. 4). No lesions developed on shoots incubated on uninoculated CMA. There was no significant difference between lesions on shoots caused by *P. cinnamomi* or *P. citricola*. However, in all cases, the antagonist significantly reduced lesion development ($P < 0.01$). There was no significant difference between cvs in lesion lengths on shoots inoculated with the pathogen and antagonists. Only the antagonist fungi were re-isolated from shoots incubated in the presence of the antagonist with or without *Phytophthora* spp., whereas in control treatments in which shoots were incubated only with *P. cinnamomi* or *P. citricola*, only *Phytophthora* spp. were isolated.

Discussion

Both *Trichoderma* spp. inhibited the *Phytophthora* spp. apparently by direct antagonism with minor inhibition by antibiosis. *Trichoderma* spp. are known to be mycoparasitic on a range of fungi. Dennis and Webster (1971c) found that two isolates of *T. viride* penetrated hyphae of *P. cactorum* and *P. erythroseptica*, and prevented further growth of these *Phytophthora* spp. by growing over the colony. Parasitism by *T. hamatum* hyphae was observed by Chet et al. (1981) to control hyphal growth of *Pythium* spp. and *Rhizoctonia solani*. Parasitism was thought to be accompanied by the production of lytic enzymes rather than antibiosis. Coiling and penetration of hyphae of *R. solani*

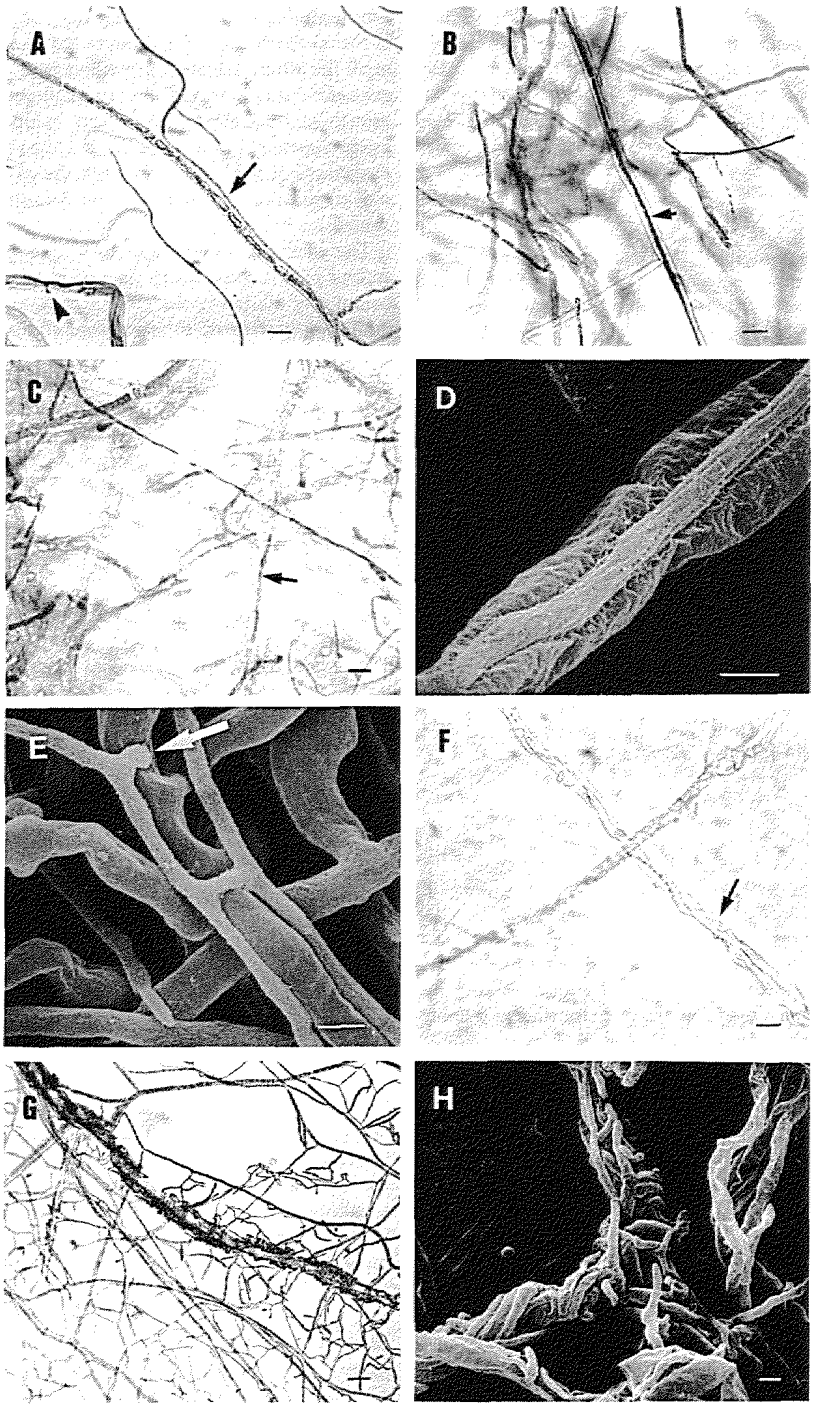

Fig. 1 Light and scanning electron micrographs of hyphal interactions of *T. hamatum*, *T. pseudokoningii* and *G. virens* with *P. cinnamomi* hyphae in dual culture

Fig. 1A–C: light micrographs of hyphae stained with lactophenol cotton blue. Bars = 20 μm. A: parallel growth of *T. hamatum* hypha (a) and *P. cinnamomi* (p) hypha (arrowed). Appressorium-like structure indicated by arrowhead. B: parallel growth of *T. pseudokoningii* hypha (a) and *P. cinnamomi* hypha (arrowed). C: parallel growth of *G. virens* (a) hypha on *P. cinnamomi* (p) hypha (arrowed). D, E: scanning electron micrographs. Bars = 10 μm. D: *T. pseudokoningii* (a) hypha tightly appressed to *P. cinnamomi* (p) hypha. E: *T. hamatum* (a) hypha on *P. cinnamomi* (p) hypha. Appressorium-like structure (arrowed) in region of parallel growth. F, G: light micrographs of hyphae stained with lactophenol cotton blue. F: *T. hamatum* (a) hypha coiling around *P. cinnamomi* (p) hypha (arrowed). Bar = 20 μm.G: *T. pseudokoningii* (a) hyphae tightly coiled around *P. cinnamomi* (p) hypha. Bar = 4 μm. H: scanning electron micrograph of *G. virens* (a) hypha coiled tightly around *P. cinnamomi* (p) hypha. Bar = 10 μm

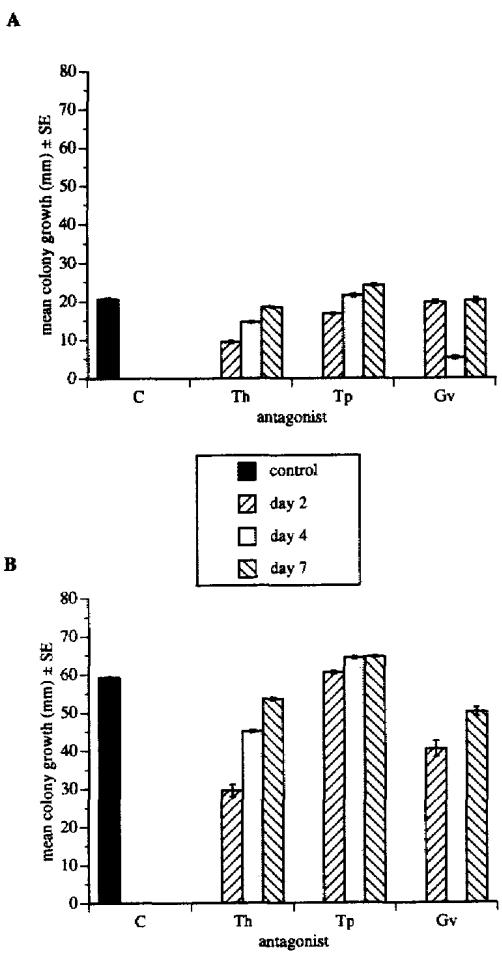

Fig. 2 Growth of *P. cinnamomi* or *P. citricola* after 7 days on 1/5 strength M32 culture medium containing filtrate from 2-, 4- and 7-day old cultures of *T. hamatum*, *T. pseudokoningii* and *G. virens*. Bars represent SE. Least significant difference = 2.517, $P < 0.001$, n = 10. c = control; Th = *T. hamatum*; Tp = *T. pseudokoningii*; Gv = *G. virens*. A: *P. cinnamomi*. B: *P. citricola*

and *S. rolfsii* by *T. hamatum* and *T. harzianum*, and appressorium-like structures and hooks, similar to those reported here, were observed by Elad et al. (1983) using the SEM. When these hooks were peeled off, hyphal entry points were observed. Chet et al. (1981) also observed formation of appressorium-like structures, by *T. hamatum* hyphae on *Pythium* spp. and *Rhizoctonia solani* hyphae. Entry of antagonist hyphae into *Phytophthora* spp. hyphae was not observed using the light microscope here. However, entry points into hyphae of *P. cinnamomi* may have been beneath appressorium-like structures observed using the SEM.

Sivan et al. (1984) noted that culture filtrate of *T. harzianum* strongly inhibited growth of *Pythium aphanidermatum* in culture, whereas *T. hamatum* filtrates caused only minor inhibition in growth. *T. hamatum* did not appear to produce non-volatile antibiotics. The results presented here show that culture filtrate of all three antagonists significantly inhibited growth of both *Phytophthora* spp.. Generally, the younger the antagonist culture, the more the filtrate inhibited pathogen growth,

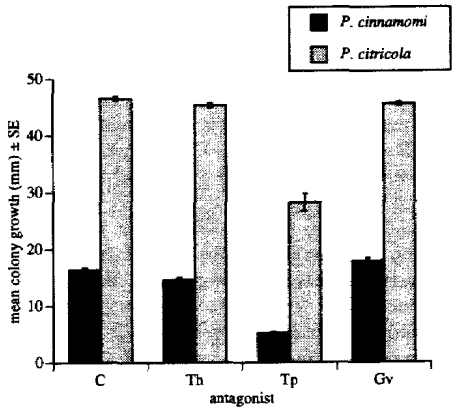

Fig. 3 Growth of *P. cinnamomi* and *P. citricola* after 5 days on 1/5 strength M32 culture medium inverted over cultures of possible antagonists to test for inhibition by production of volatile antibiotics. Bars represent SE. LSD = 2.41, $P < 0.001$, n = 10. Th = *T. hamatum*; Tp = *T. pseudokoningii*; Gv = *G. virens*

although a 4-day-old filtrate of *G. virens* inhibited the growth of *Phytophthora* spp. most strongly. Filtrate from actively growing mycelium (i.e. younger than 4 days old) inhibited colony growth strongly, whereas old mycelium (7-day-old), yielded filtrates which had little or no effect in inhibiting the pathogen. The antagonist fungus is likely to have been degenerating at this stage, with hyphal lysis releasing cell contents into the medium, providing additional nutrients for pathogen growth. Of the three antagonists screened, *G. virens* caused the greatest inhibition of both species of *Phytophthora*. Colony growth of both *Phytophthora* spp. was strongly or completely inhibited in the

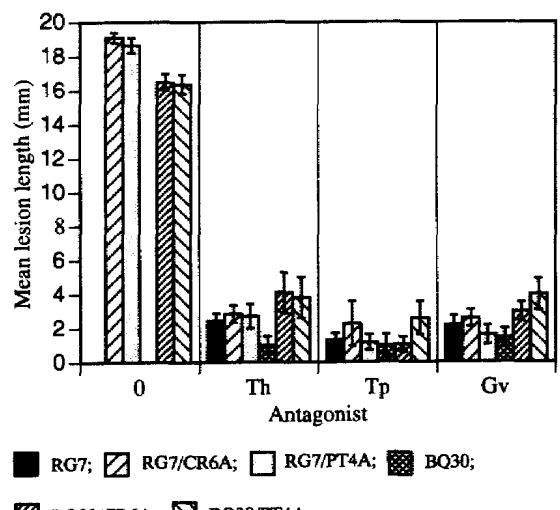

Fig. 4 Mean lesion length along micropropagated shoots of chestnut cvs Goldsworthy (RG7) and Buffalo Queen (BQ30) incubated for 2 days with co-cultures of *P. cinnamomi* (CR6A) or *P. citricola* (PT4A) and *T. hamatum* (Th), *T. pseudokoningii* (Tp), or *G. virens* (Gv) on CMA in polycarbonate tubs at 25°C in the dark. Controls were shoots incubated on uninoculated CMA, or inoculated with either the antagonist or pathogen alone. Bars represent SE. Least significant difference = 3.27 (calculated with n = the minimum number of replicates, therefore is the most conservative value), $P < 0.01$, n = >9

culture filtrate experiment, whereas hyphal coiling was rare and no appresoria were observed in dual culture with *P. cinnamomi*. This lack of physical interaction suggests that *G. virens* inhibits *P. cinnamomi* by antibiosis rather than by mycoparasitism.

Filtrates need to be analysed for the presence of antibiotics, especially gliovirin and gliotoxin (Howell and Stipanovic, 1983, 1984; Wilcox et al., 1992), which may be of greatest use in biocontrol of *Phytophthora* spp..

Dennis and Webster (1971b) identified a volatile antibiotic produced by *Trichoderma* sp. which inhibited growth of *Rhizoctonia solani* and other test fungi with activity being related to a 'coconut' odour. This antibiotic activity and odour have been characterized as 6-n-pentyl-2H-pyran-2-one, with pentenyl pyrone as a minor component (Claydon et al., 1987). Isocyanide compounds, produced by *Trichoderma* spp., have also been grouped by Ghisalberti and Sivasithamparam (1991) into this category of volatile metabolites. Although *T. pseudokoningii* was found to produce volatile antibiotics in the present study no coconut odour was detected nor was identification of antibiotics attempted. Lynch (1987) recognized the potential of volatile antibiotics as fungicides for use as soil drenches; they would leave no harmful residues and would not be toxic to workers. The identity and potential of volatile antibiotics produced here by *T. pseudokonngii*, which significantly inhibited both species of *Phytophthora*, needs to be further examined.

In the dual culture experiments, all three isolates of *Trichoderma* and *Gliocladium* overgrew mycelium of *P. cinnamomi*, preventing further growth. Bell et al. (1982) found that most isolates of *Trichoderma*, screened *in vitro* against *P. parasitica* f. sp. *nicotianae*, completely overgrew the pathogen and covered the entire surface of V8 agar medium. When discs from two of these co-cultures were used to inoculate 'Hicks'-type tobacco seedlings, by placing a mycelial plug against the base of the stem of each plant, no disease developed after 12 days. In the biocontrol shoot assay developed in this study, all three antagonists reduced lesion development by *P. cinnamomi* and *P. citricola* on chestnut shoots in comparison with controls. The three antagonist fungi grew over the *Phytophthora* spp. mycelium, as in dual culture experiments, inhibiting the growth of *Phytophthora* spp. and preventing *Phytophthora* spp. from causing lesions on chestnut shoots. The biocontrol shoot assay could, therefore, be used as a rapid screening method for potential biocontrol agents before verification of biocontrol capabilities in soil experiments.

The results of dual culture, antibiosis experiments and the biocontrol shoot bioassay, indicated that *T. hamatum*, *T. pseudokoningii* and *G. virens* have potential as biocontrol agents of chestnut root rot. The *in vitro* experiments reported here also indicated the possible mode of action of each antagonist. Smith et al. (1990) also used preliminary *in vitro* experiments to screen cultures of *Trichoderma* spp. for biocontrol potential against *P. cactorum*. Pot and field experiments are necessary to evaluate the potential of these isolates for controlling *Phytophthora* root rot of chestnut.

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