Phytopathol. Mediterr. (2004) 43, 95–104

Interactions between *Eutypa lata* and *Trichoderma harzianum*

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Summary. Metabolites produced by three strains of *Trichoderma harzianum* reduced growth of *Eutypa lata in vitro*. Volatile metabolites produced by *T. harzianum* were fungistatic towards both isolates of *E. lata* tested. Growth of some isolates of *E. lata* was inhibited completely by non-volatile metabolites. Infection by *E. lata* was reduced in autoclaved grapevine cane segments co-inoculated with spores of *T. harzianum* and *E. lata*. Scanning electron microscopic examination of gamma-irradiated cane segments and living cuttings inoculated with *T. harzianum* and *E. lata* suggested that antagonism in grapevine wood was mainly by antibiosis. Both the pathogen and the antagonist grew in the xylem vessels and pith parenchyma cells of the wood.

Key words: Eutypa dieback, grapevines, antibiosis, scanning electron microscopy, biocontrol.

Introduction

Eutypa dieback is a destructive canker disease caused by the ascomycete fungus *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul. (syn. *Eutypa armeniacae* Hansf. & M.V. Carter) (Moller and Kasimatis, 1978). The disease causes significant yield loss in grapevines world-wide (Carter, 1991). Yield losses of 30–62% have been reported in California (Munkvold *et al*., 1994), whereas, in South Australia, Wicks and Davies (1999) estimated yield loss of more than Aus$2,800 per hectare in Shiraz, where 47% of the vines were affected by the disease.

Ascospores of *E. lata* enter the xylem vessels of the grapevine via wounds and the fungus then spreads slowly in the wood. Treatment of wounds, such as large pruning cuts, with fungicides or wound sealants is the preferred method of control (Moller and Kasimatis, 1978). Benomyl is effective against *E. lata* (Munkvold and Marois, 1993b), but this fungicide was never registered in Australia as a grapevine wound protectant and the commercial formulation, Benlate®, was withdrawn in 2002. Pruning wounds may remain susceptible to infection for 4 weeks after pruning (Munkvold and Marois, 1994), and chemical treatments may not protect the wound for the entire period of susceptibility. Biological agents capable of colonising pruning wounds may provide better long-term protection than fungicides.

Biological control of *E. lata* on grapevines has been demonstrated using *Fusarium lateritium*, *Cladosporium herbarum* and *Bacillus subtilis* (Carter and Price, 1974; Ferreira *et al*., 1991; Munkvold and Marois, 1993a). However, none of

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these have yet been developed into commercial products. Trichoprotection® products, which contain seven strains of *Trichoderma harzianum,* are registered for control of eutypa dieback of grapevines, armillaria root rot of kiwifruit vines and silver leaf of stone fruit trees in New Zealand. Laboratory investigations of three of the strains of *T. harzianum* in Trichoprotection® products in biological control of *E. lata* are presented in this paper. The aim was to investigate the mechanisms of inhibition of *E. lata* by *T. harzianum* and the interaction between the pathogen and the antagonist in grapevine wood.

Materials and methods

Fungi and plant materials

Trichoderma harzianum strains AG1, AG2 and AG3 were provided by Agrimm Technologies Ltd, New Zealand. *E. lata* isolates M280, M295 and M302 were obtained from M. Cole, Monash University, Australia and isolates CS-Ba.1, CS-Ba.2 and CS-Ba.3 were obtained from Cabernet Sauvignon vines in the Barossa Valley, South Australia in 1999 (S. John, personal communication).

In vitro antibiosis

Inhibition by volatile metabolites

The method of Dennis and Webster (1971b) was used to investigate the ability of the three strains of *T. harzianum* to inhibit the growth of two isolates of *E. lata* by volatile antibiosis. Plugs of *T. harzianum* (8 mm diam.) were placed in the centre of 90 mm Petri dishes containing 20 ml PDA and incubated at 22–25°C in darkness. After 2 days, the bases of fresh plates of PDA inoculated with 8 mm diam. plugs of *E. lata* were inverted over the bases of the plates with the 2-day-old antagonists, taped with Parafilm® and incubated as above for 5 days. Controls consisted of plates of *E. lata* inverted over plates with sterile PDA plugs. There were eight replicates per treatment. The colony diam. of *E. lata* was measured, the diam. of the inoculum plug was subtracted and the data were subjected to analysis of variance (ANOVA).

Inhibition by non-volatile metabolites

The cellophane overlay method of Dennis and Webster (1971a) as modified by Chambers (1993) was used to examine the effect of non-volatile metabolites produced by the three strains of *T. harzianum* on six isolates of *E. lata*. Briefly, sterile uncoated cellophane discs (80 mm diam., Australia Cellophane, Victoria) were placed in 90 mm diam. Petri dishes containing 20 ml PDA. A mycelial plug (8 mm diam.) of the antagonist was placed on the centre of each disc. Sterile plugs of PDA were used for controls. The plates were incubated at 22–25°C in the dark. After 2 days the cellophane and plug of mycelium or agar were replaced by a plug of *E. lata* (8 mm diam.) then plates incubated as above. There were eight to 10 replicates per treatment. The colony diam. of *E. lata* isolates M280 and CS-Ba.1 was measured after 4 days. In a subsequent experiment, the colony diam. of the slow growing isolates M295, M302, CS-Ba.2 and CS-Ba.3 was measured after 6 days. The data were analysed as above. Plugs of *E. lata* which showed no growth after 2 weeks were transferred to fresh PDA and incubated for a further 2 weeks to determine whether effects were fungistatic or fungicidal.

Inoculation of autoclaved cane segments

Pathogen-antagonist interactions were examined using methods modified from Rolshausen and Gubler (1998). Bark was removed from 1-year-old canes of cv. Shiraz and the canes cut into 30-mm long segments. Each segment was placed in a McCartney bottle with 3 ml of sterile distilled water (SDW) and autoclaved at 121°C for 20 min. The segments were treated with 25 μl SDW containing 1,000 ascospores or conidia of *E. lata* or *T. harzianum* (AG1), respectively, in the following way. Treatment 1, *E. lata* + *T. harzianum* (AG1); Treatment 2, *T. harzianum* (AG1) only (control); Treatment 3, *E. lata* only (control). Treatment 1 involved application of 25 μl of spore suspension of each fungus at the same time. There were 10 replicates per treatment. The segments were incubated at 23°C for 4 weeks, then surface sterilized in 2.5% NaOCl for 12 min and washed twice in SDW. The segments were then split into 5–10 mm chips, transferred to PDA supplemented with 100 μg ml^{-1} streptomycin sulphate, 50 μg ml^{-1} chlortetracycline and 5 μg ml^{-1} dicloran (EUSM) (Munkvold and Marois, 1993b), and incubated in darkness for 7 days at 23°C. The percentage of segments yielding *E. lata* and *T. harzianum* was determined for each treatment and the results were analysed using the Chi^2 test.

Scanning electron microscopic observation of interactions in wood

One-year-old canes of cv. Shiraz, either sterilized or living, were inoculated with *E. lata* and *T. harzianum*, as described below, and observed using a scanning electron microscope (Philips Field Emission Scanning Electron Microscope XL30). Preliminary observation of un-inoculated canes revealed small, scattered groups of rod-shaped bacteria but no evidence of endophytic fungi.

Inoculation of gamma-irradiated wood

Cane segments (1–1.5 cm long) were sterilized by gamma-irradiation at 25 kGys and placed in 9 mm diam. Petri dishes lined with sterile filter paper discs (80 mm diam.) moistened with SDW. A 5-mm-diam. plug of *E. lata* isolate M280 was applied at one end of each segment and the plates incubated at 23°C in darkness (Mercer and Kirk, 1984). After 4 days, the segments were inoculated at the other end with a plug of *T. harzianum* AG1 (5 mm diam.) and incubated for a further 3 days. The filter paper was moistened with SDW at this stage. Controls comprised segments inoculated at one end with *E. lata* M280 or *T. harzianum* alone. There were three replicates per treatment. After 3 days the segments were split open longitudinally and processed for scanning electron microscopy (SEM).

Inoculation of cuttings grown in the laboratory

Single-node canes of cv. Shiraz were pruned 2–4 cm above the bud and inoculated with 5-mm-diam. plugs of *E. lata* isolate M280 or ascospores from naturally infected wood (1,000 spores in 25 μl SDW). The canes were maintained in water-saturated rockwool pieces (4×4 cm Grodan blocks, Home Hydro, South Australia) on the laboratory bench at 22°C in natural light. After 2 days, the canes that had been inoculated with M280 were inoculated with either a 5-mm-diam. mycelial plug of *T. harzianum* AG1 or with 25 μl SDW containing 1,000 conidia of AG1, and the canes that had been inoculated with ascospores were inoculated with conidia of AG1. The controls were inoculated with mycelial plugs of M280 alone, ascospores of *E. lata* alone, mycelial plugs or conidia of AG1 alone. The inoculum was covered with Parafilm® at each stage. There were three replicates per treatment. The canes were harvested after 2 weeks and 1 cm long segments below the point of inoculation were split open and processed for SEM.

Simultaneous co-inoculation of cuttings grown in the laboratory

Single-node canes of cv. Shiraz were prepared as described above except that the pruning wounds were treated simultaneously with spore suspensions of *E. lata* and *T. harzianum* AG1 in SDW. The controls received either ascospores of *E. lata* or conidia of *T. harzianum* alone. The canes were harvested after 7 days. There were three replicates per treatment. Cane segments (1 cm long) below the point of inoculation were split and processed for SEM.

Scanning electron microscopy

Cane segments (1–1.5 cm long) were fixed overnight in 4% paraformaldehyde; 1.25% glutaraldehyde in phospate buffered saline (PBS); 4% sucrose; pH 7.2. Segments were then washed twice for 30 min in PBS plus 4% sucrose, post-fixed in 1% OsO_4 in PBS for 1 h and dehydrated through a series of 70%, 90%, 95% and 100% ethanol for 20 min three times at each concentration. The third stage of 100% ethanol was followed by an additional hour in 100% ethanol. The segments were then immersed in 100% ethanol: 100% acetone (1:1, v:v) for 15 min and transferred to 100% acetone for another 15 min. The specimens were critical point dried, mounted on metal stubs, coated with gold and palladium and viewed by SEM at an accelerating voltage of 10 kV.

Results

***In vitro* antibiosis**

Inhibition by volatile metabolites

Volatile metabolites produced by all three strains of *T. harzianum* significantly ($P<0.001$) reduced growth of both isolates of *E. lata* tested (Fig. 1), although there were differences amongst strains of *T. harzianum* ($P<0.001$). For example, the volatile metabolites produced by AG2 were the most effective in reducing the growth of isolate M280, whereas those of AG3 inhibited isolate CS-Ba.1 most strongly. There was no evidence that *E. lata* produced volatile metabolites that inhibited *T. harzianum*.

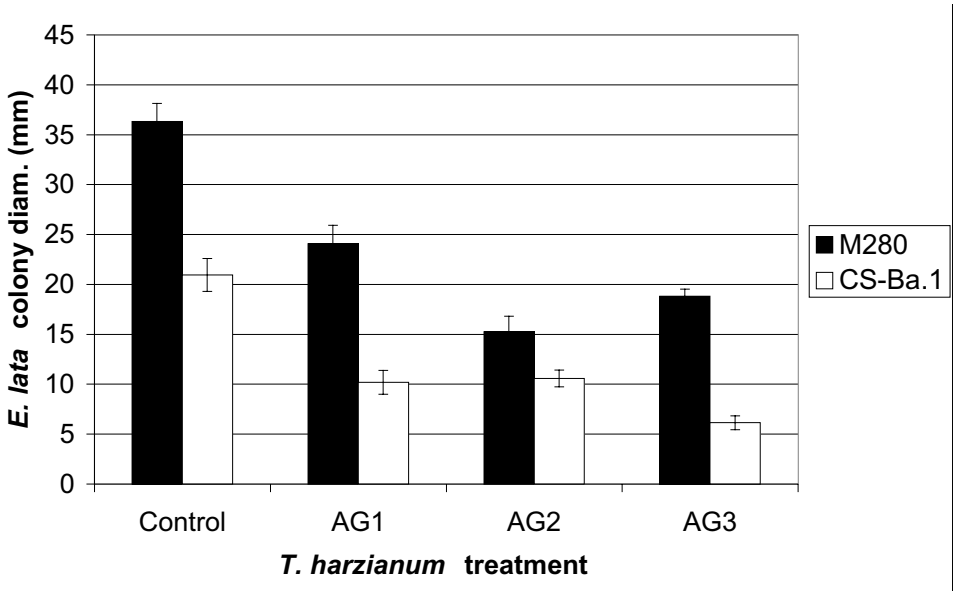


Fig. 1. Inhibition of growth of two isolates of *E. lata* on PDA due to volatile antibiosis by *T. harzianum*. Mycelial plugs of *T. harzianum* were grown on PDA for 2 days in the dark at 22–25°C. Bases of PDA plates inoculated with *E. lata* were inverted over the bases of plates containing the antagonist and incubated for 5 days. Y-axis denotes the average colony diam. (minus the inoculum plug) for eight replicates per treatment. Bars denote standard errors.

Inhibition by non-volatile metabolites

Non-volatile metabolites of all three strains of *T. harzianum* significantly ($P<0.001$) reduced growth of all six isolates of *E. lata* (Fig. 2A and B). All three strains of *T. harzianum* completely inhibited growth of *E. lata* isolates M295 and M302, whereas only *T. harzianum* AG1 completely inhibited isolate CS-Ba3 (Fig. 2B). However, total inhibition of *E. lata* isolates M280, CS-Ba.1 and CS-Ba.2 was not observed. The inoculum plugs of *E. lata* isolates M295 and M302 failed to grow when transferred to fresh PDA, whereas those of isolate CS-Ba.3 produced mycelia when transferred from agar containing non-volatile metabolites of *T. harzianum* AG1 (data not shown).

Interactions in autoclaved cane segments

The pathogen and antagonist were recovered from all of the appropriate control segments cultured on EUSM (Fig. 3). *T. harzianum* was isolated from 90% of co-inoculated canes and *E. lata* from 10% of the canes. This difference was significant ($P<0.001$) according to the Chi^2 test. None of the cane segments yielded both fungi.

Scanning electron microscopy

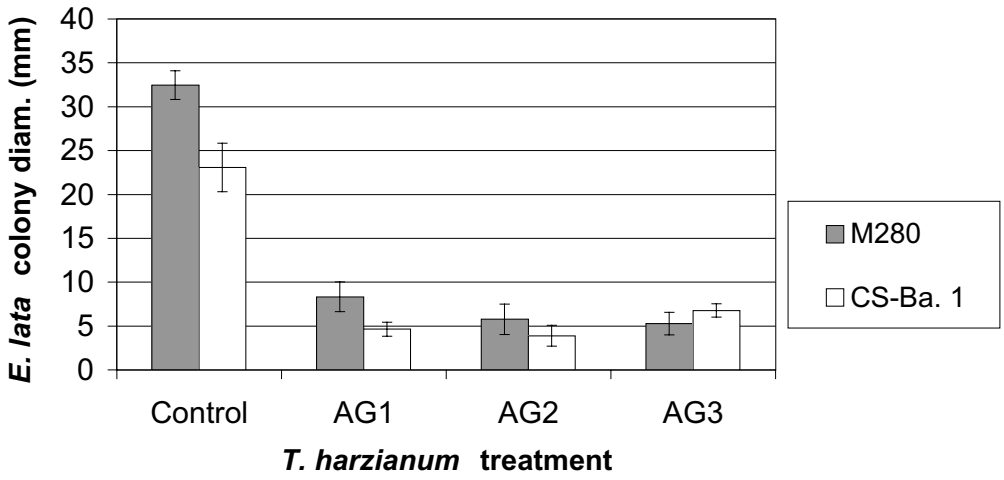
Mycelium of *E. lata* has similar dimensions, septation and branching patterns to that of *T. harzianum*, so the mycelia of the two species could not be distinguished in co-inoculated canes using SEM. Following inoculation with either the pathogen or the antagonist, hyphae grew in the xylem vessels and pith parenchyma cells of the gamma-irradiated canes and the cuttings grown in rockwool.

Interactions in gamma-irradiated wood

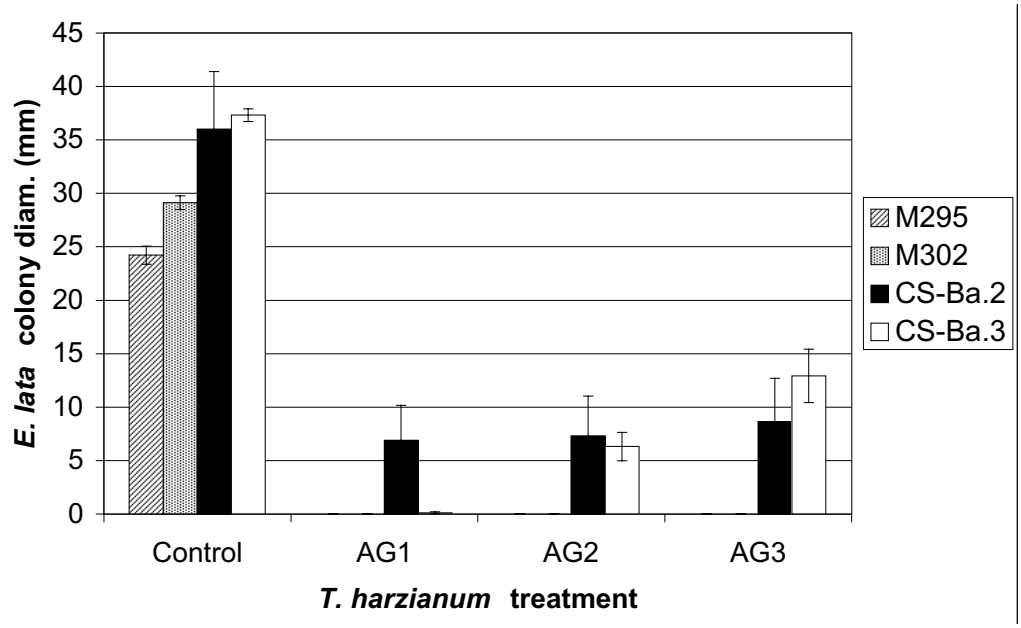
Turgid, healthy hyphae were observed in cane segments inoculated with *E. lata* M280 or *T. harzianum* alone (Fig. 4 A and B). SEM of co-inoculated segments showed hyphae that were flaccid and collapsed (Fig. 4C). Similar observations were made for all three replicates.

Interaction in cuttings grown in the laboratory

Healthy, turgid hyphae were observed in control segments inoculated with either *E. lata* or *T. harzianum*. Loss of turgor and abnormal swelling of hyphae were observed in canes inoculated



A



B

Fig. 2. Inhibition of growth of *E. lata* on PDA by non-volatile antibiosis by *T. harzianum*. Mycelial plugs of *T. harzianum* were grown on cellophane over PDA for 2 days in the dark at 22–25°C. The cellophane and plugs were replaced with plugs of *E. lata* and plates incubated for another 4 or 6 days. Y-axis denotes the average colony diam. (minus the inoculum plug) for eight replicates per treatment. Bars denote standard errors. A. Two isolates of *E. lata* incubated for 4 days on medium containing diffusible metabolites. B. Four isolates of *E. lata* incubated for 6 days on medium containing diffusible metabolites.

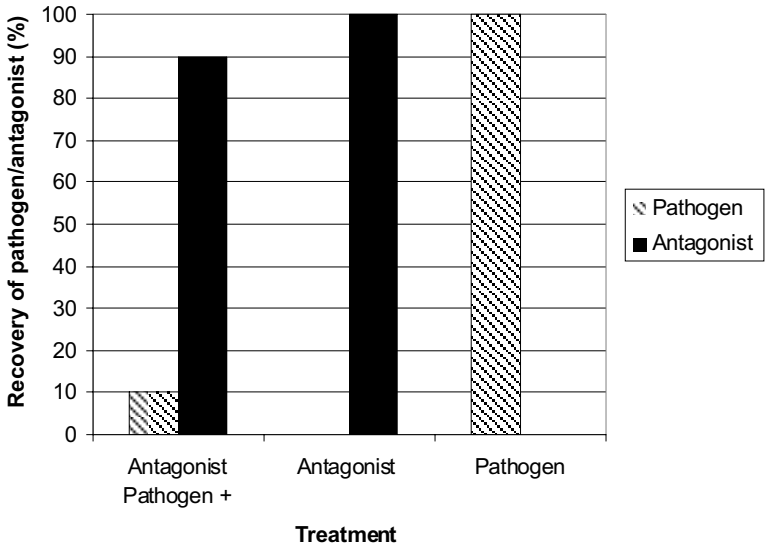


Fig. 3. Colonisation of autoclaved segments of Shiraz canes by *E. lata* 4 weeks after simulataneous co-inoculation with 1,000 spores of *E. lata* and *T. harzianum* AG1. There were 10 replicates per treatment.

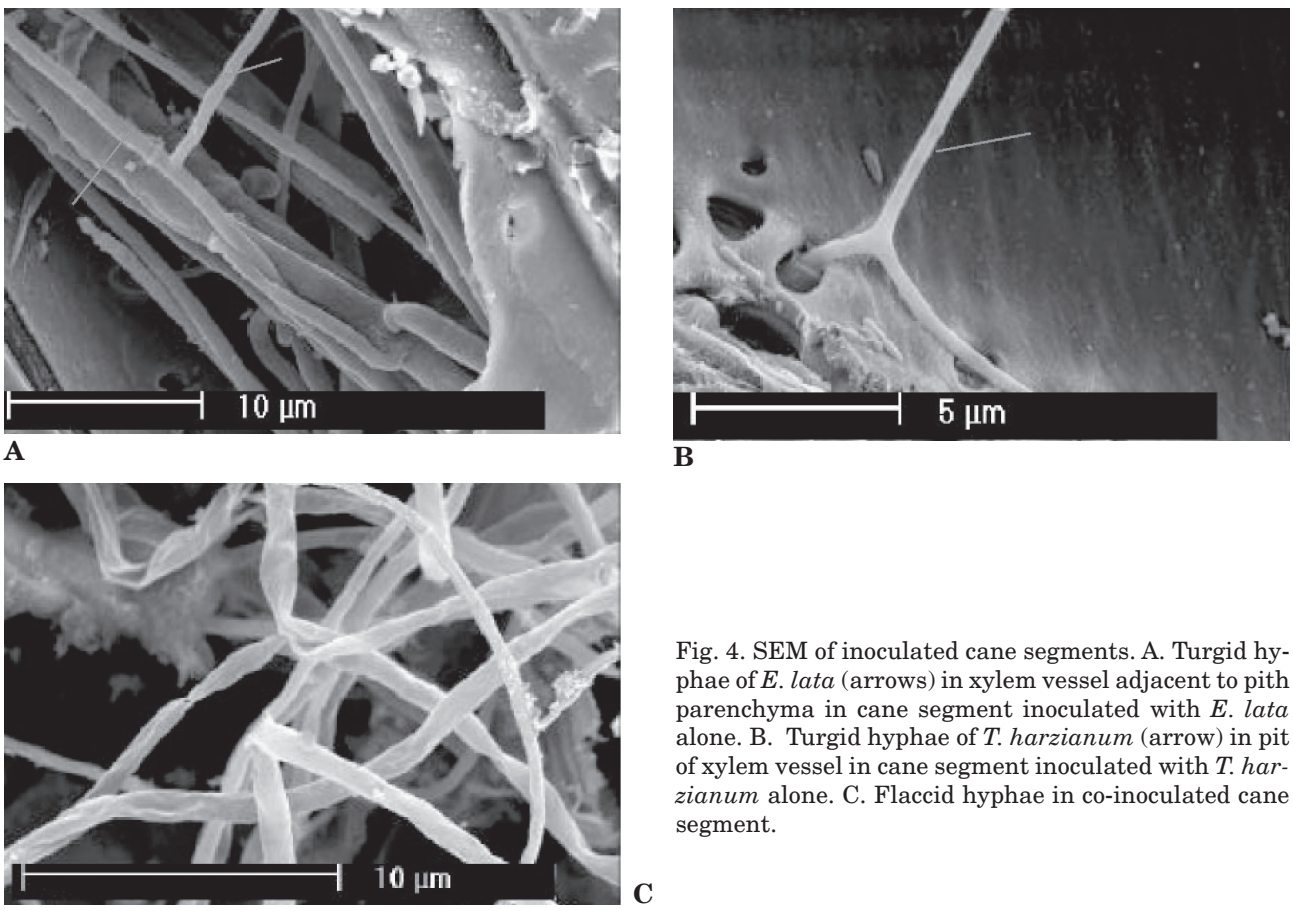


Fig. 4. SEM of inoculated cane segments. A. Turgid hyphae of *E. lata* (arrows) in xylem vessel adjacent to pith parenchyma in cane segment inoculated with *E. lata* alone. B. Turgid hyphae of *T. harzianum* (arrow) in pit of xylem vessel in cane segment inoculated with *T. harzianum* alone. C. Flaccid hyphae in co-inoculated cane segment.

with mycelial plugs of *E. lata* M280 and 2 days later with spores of *T. harzianum* (Fig. 5 A and B). Collapsed and shrivelled hyphae were observed in the canes co-inoculated with mycelial plugs of both pathogen and antagonist (Fig. 5C). Abnormal swelling and collapse of hyphae were observed in the canes co-inoculated with spores of the pathogen and antagonist (Fig. 5D). Furthermore, parallel growth and winding or coiling of hyphae were observed in this material.

Healthy, turgid hyphae were observed in all canes inoculated with *T. harzianum* or *E. lata* alone (Fig. 6A and B), whereas abnormal swelling and collapse of hyphae were observed in the canes inoculated with both fungi simultaneously (Fig. 6C and D).

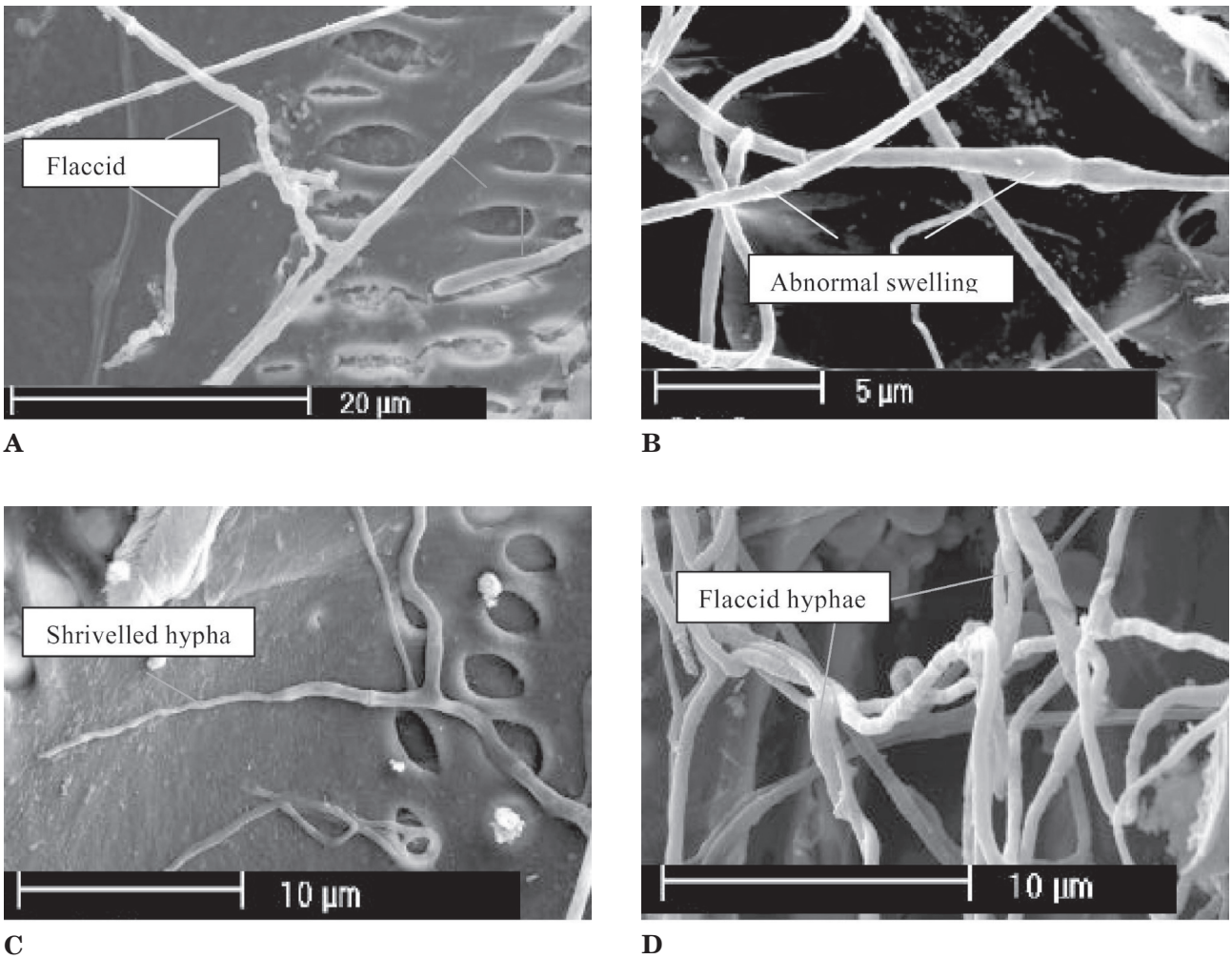


Figure 5. SEM of cuttings inoculated with *E. lata* and 2 days later with *T. harzianum*. A. Flaccid hyphae in cutting co-inoculated with mycelial plugs. B. Abnormal swelling of hyphae in cutting co-inoculated with mycelial plugs. C. Shrivelled hypha in cutting co-inoculated with mycelial plugs. D. Loss of turgidity and parallel growth of hyphae in vessel of cutting co-inoculated with spores.

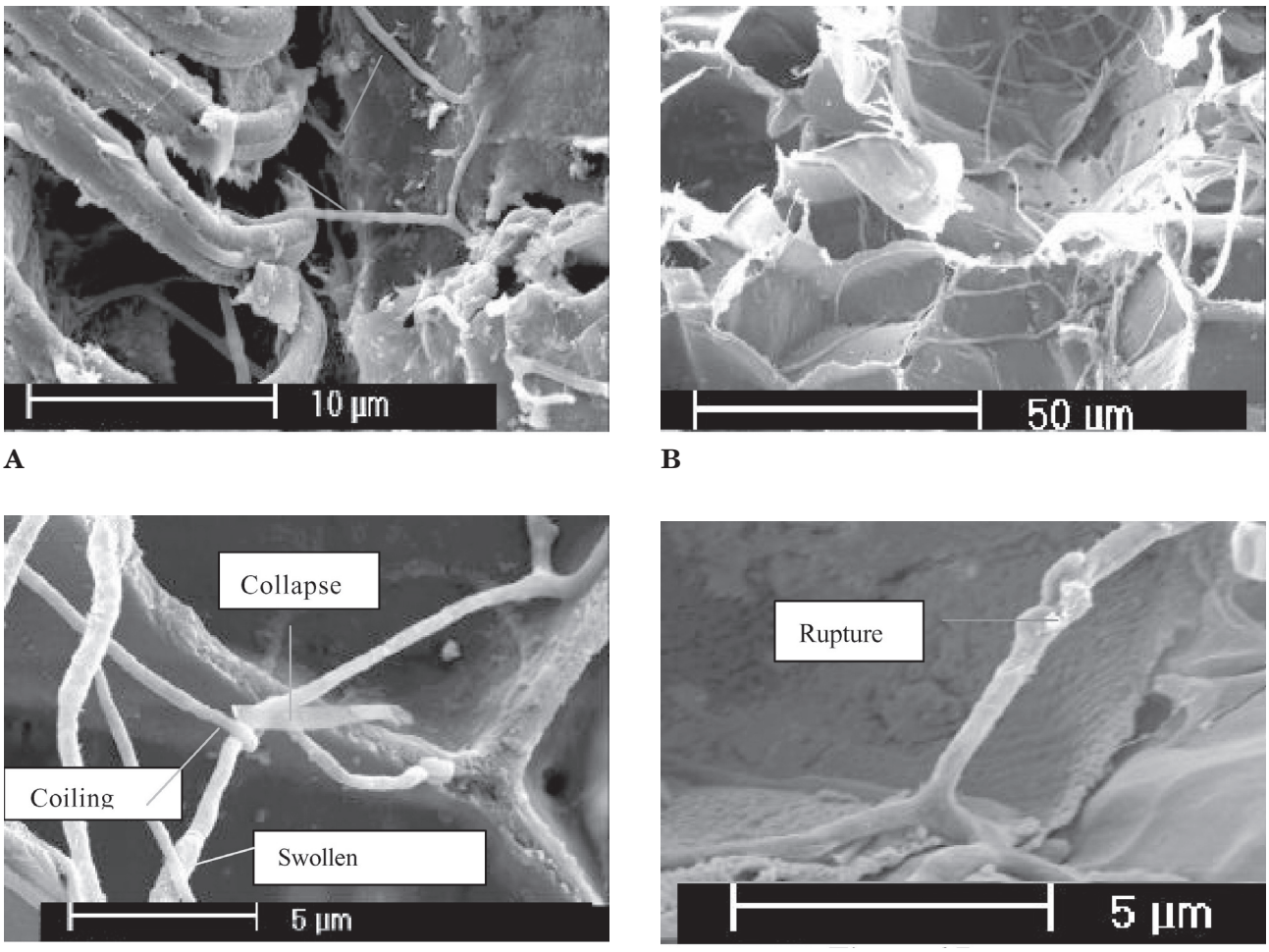


Fig. 6. SEM of cuttings simultaneously inoculated with spores of *E. lata* and *T. harzianum*. Arrows indicate healthy hyphae. A. Turgid hyphae in cutting treated with *E. lata* alone. B. Healthy hyphae of *T. harzianum* in pith parenchyma in cutting treated with *T. harzianum* alone. C. Coiling, abnormal swelling and collapse of hyphae in co-inoculated cutting. D. Rupture and collapse of hyphae in co-inoculated cutting.

Discussion

Mycelial growth of *E. lata* on PDA was inhibited by volatile and non-volatile metabolites produced by all three strains of *T. harzianum*. Volatile metabolites produced by *Trichoderma* spp. have been shown to inhibit a range of fungi (Dennis and Webster 1971a; Ghisalberti and Sivasithamparam, 1991; Chambers and Scott, 1995). The strains used in the present study produced a coconut odour, which has previously been characterized as 6-n-pentyl-2H-pyran-2-one (Claydon *et al*., 1987), and reported to inhibit fungi such as *Rhizoctonia solani* (Dennis and Webster, 1971a), *Botrytis cinerea* and *Ophiostoma ulmi* (Merlier *et al*., 1984).

The volatile metabolites produced by *T. harzianum* inhibited, but did not prevent, mycelial growth of *E. lata*. In contrast, the non-volatile metabolites had a fungicidal effect on some isolates of *E. lata* and a fungistatic effect on others. The degree of inhibition by the three strains of *T. harzianum* varied with the different isolates of *E. lata* (Fig. 1, 2A and B). The strains of *T. harzianum* may have produced different antibiotics or enzymes (Kullnig *et al*., 2000) or the same compounds at var-

ying concentrations. Strains belonging to the same species can differ physiologically in their production of metabolites (Moubasher, 1963; Dennis and Webster, 1971a, 1971b). For example, Mercer and Kirk (1984) demonstrated variation in the antagonistic activity of three isolates of *T. viride* towards *Chondrostereum purpureum*; of the three isolates of *T. viride* tested, one strongly inhibited, one slightly inhibited and the other stimulated mycelial growth of the pathogen. Also, the isolates of *E. lata*, a genetically diverse fungus (Péros *et al*., 1999), may have differed in sensitivity to the metabolites, in accordance with previous reports that isolates of a pathogen may differ in sensitivity to antagonists (Gibbs, 1967; Mazzola *et al*., 1995).

In our experiments designed to assess non-volatile antibiosis, it was assumed that the inhibition of *E. lata* was caused by antibiotics or enzymes of *T. harzianum* which had diffused through the cellophane into the PDA. Nutrient depletion by *T. harzianum* was considered unlikely to account for the inhibition of *E. lata* as PDA is rich in nutrients and incubation was for only 2 days (Dennis and Webster, 1971a). The cellophane overlay method has also been used to demonstrate inhibition of mycelial growth of *Fomes annosus, Fusarium oxysporum* and *Phytophthora* spp. by non-volatile metabolites produced by *Trichoderma* spp. (Dennis and Webster, 1971a; Chambers, 1993; Etebarian *et al*., 2000).

Trichoderma harzianum reduced the recovery of *E. lata* from autoclaved cane segments when the two fungi were applied simultaneously as spores. *T. harzianum* alone was re-isolated from nine of the 10 co-inoculated segments, whereas only *E. lata* was isolated from the remaining segment. This suggests that *T. harzianum* did not colonise that single cane segment, hence, *E. lata* was able to establish.

A criticism of using autoclaved wood is that growth of *T. harzianum* and *E. lata* may be promoted by the high moisture content of the wood and the lack of competition from indigenous microflora. Munkvold and Marois (1994) reported that the microflora of fresh grapevine pruning wounds ranged from 10^2–10^3 colony forming units (cfu) immediately after pruning to 10^6 cfu 3–28 days later. Furthermore, since the wood was killed by sterilization, natural resistance reactions, such as accumulation of lignin, suberin and phenolics and formation of tyloses, would not have taken place (Schmidt *et al*., 2001). Nevertheless, tests on sterilized, generally autoclaved, wood have proved valuable in the selection of biological agents for the control of wood pathogens (Munkvold and Marois, 1993a; Schmidt *et al*., 2001).

SEM allowed the observation of the effect of the interaction between *T. harzianum* AG1 and *E. lata* M280 in wood. It was evident that co-inoculation with the two fungi resulted in changes to the integrity of the hyphae. Whereas hyphae in wood inoculated with either fungus alone were healthy, some of those in co-inoculated wood were collapsed, shrivelled and showed abnormal swellings. The similar morphology of *T. harzianum* and *E. lata* prevented the determination of whether one or both of the fungi were affected in this way. However, taking into account the evidence presented here of the inhibition of growth of *T. harzianum* by *E. lata in vitro*, it seems likely that *T. harzianum* affected *E. lata* adversely in the wood. This hypothesis is supported by the failure of *E. lata* to grow from nine of 10 autoclaved wood segments that had been inoculated with both fungi *in vitro*, whereas *E. lata* grew from all of the control segments cultured on EUSM.

Parallel growth and coiling were observed occasionally in the co-inoculated cuttings. These features may indicate mycoparasitic activity, as reported previously for *Trichoderma* spp. (Elad *et al*., 1983). However, there was no evidence of mycoparasitism when interaction zones of *E. lata* isolates M280 and CS-Ba.1 cultured with *T. harzianum* on PDA were observed by light microscopy (S. John, unpublished). This may reflect differences in the culture environment. Nutrient stress is thought to be necessary for the expression of some cell wall degrading enzymes involved in mycoparasitism by *Trichoderma* spp. (Lorito, 1998). The conditions in wood segments may have been conducive for parasitic interactions between *T. harzianum* and *E. lata*.

In summary, the three representative strains of *T. harzianum* from Trichoprotection® products inhibited the growth of *E. lata* by antibiosis *in vitro*. Also, AG1 limited colonisation of grapevine wood by a virulent isolate of the pathogen and there were indications of damage to the integrity of hyphae in wood. These observations suggest that *T. harzianum* has potential in the biological control of *E. lata* on grapevines. Further research on the use of *T. harzianum* to protect pruning wounds on grapevine in the glasshouse and in the vineyard is in progress.

Acknowledgements

We thank the University of Adelaide and Agrimm Technologies Ltd for supporting this research, M. Cole, Monash University for supplying *E. lata* isolates M280, M295 and M302, and L. Waterhouse, Adelaide Microscopy, for assistance with SEM.

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Accepted for publication: December 3, 2003