Microbiology (2005), **151**, 1691–1695 DOI 10.1099/mic.0.27758-0

Secondary metabolites from endophytic *Streptomyces aureofaciens* CMUAc130 and their antifungal activity

Thongchai Taechowisan,[1] Chunhua Lu,[2] Yuemao Shen[3] and Saisamorn Lumyong[4]

Correspondence
Thongchai Taechowisan
tewson84@hotmail.com

[1]Department of Biology, Faculty of Science, Silpakorn University, Nakorn Pathom 73000, Thailand

[2]School of Life Sciences, Xiamen University, Xiamen 361005, China

[3]Kunming Institute of Botany (KIB), The Chinese Academy of Sciences, Kunming 650204, China

[4]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

Streptomyces aureofaciens CMUAc130 was isolated from the root tissue of *Zingiber officinale* Rosc. (Zingiberaceae). It was an antagonist of *Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively. Evidence for the *in vitro* antibiosis of *S. aureofaciens* CMUAc130 was demonstrated by the zone of fungal-growth inhibition. Microscopic observations showed thickness and bulbous structures at the edges of the inhibited fungal hyphae. The culture filtrate and crude extract from this strain were all inhibitory to tested phytopathogenic fungi. The major active ingredients from the culture filtrate of *S. aureofaciens* CMUAc130 were purified by silica gel-column chromatography and identified to be (i) 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and (ii) 5,7-dimethoxy-4-phenylcoumarin by NMR and mass-spectral data, respectively. Bioassay studies showed that compounds (i) and (ii) had antifungal activities against tested fungi, and their MICs were found to be 120 and 150 μg ml^{-1}, respectively. This is the first report of compounds (i) and (ii) from micro-organisms as active ingredients for the control of phytopathogenic fungi.

Received 9 November 2004
Revised 28 January 2005
Accepted 11 February 2005

INTRODUCTION

Inside the tissue of nearly all healthy plants, there are many endophytic micro-organisms. Endophytes are synergistic to their host and at least some of them are thought to be making returns for the nutrition from the plant by producing special substances, such as secondary metabolites, to prevent the host from being attacked successfully by fungi and pests. During our recent investigations of these endophytes, we isolated a novel endophytic actinomycete from the root tissues of *Zingiber officinale* Rosc. (Zingiberaceae) and it was identified as *Streptomyces aureofaciens* CMUAc130 (Taechowisan & Lumyong, 2003). Here, we report the fermentation, isolation and structure elucidation, as well as the biological activities, of the secondary metabolites that were produced by this strain.

METHODS

Organisms and media. *S. aureofaciens* CMUAc130 was isolated from the root tissues of *Z. officinale* by the surface-sterilization technique (Taechowisan *et al.*, 2003). Identification of the isolate to species level was based on morphological, cultural, physiological and biochemical characteristics and also 16S rRNA gene sequencing, as described by Taechowisan & Lumyong (2003). Solid medium for sporulation of CMUAc130 used in this study was International *Streptomyces* Project medium 4 (ISP-4) and the liquid medium used for fermentation of CMUAc130 was ISP-2 (Shirling & Gottlieb, 1966). Two fungal pathogens (*Colletotrichum musae* and *Fusarium oxysporum*, the causative agents of anthracnose of banana and wilt of wheat, respectively) were used for screening antifungal activity. They were grown on potato dextrose agar (PDA).

***In vitro* assay for antagonism.** An *in vitro* plate-assay technique was developed to test the inhibitory effects of CMUAc130 on the phytopathogenic fungi. Tests for inhibitory activity were made on ISP-2 in Petri dishes. A 0·8 cm diameter ISP-2 agar plug, covered fully with a lawn of CMUAc130, was placed 1·5 cm from the edge of the Petri dish and incubated at 30 °C for 5 days. This was done to allow the culture to be established on the agar surface and to sporulate prior to inoculation of the plates with fungal strains. For each test fungus, a 0·8 cm diameter PDA plug covered with actively growing mycelium was placed about 6 cm from CMUAc130. The inoculated plates were placed in an incubator at 30 °C for 4 days. The inhibition zone was determined by measuring the distance between the fungi and CMUAc130 in dual cultures. Morphology of

fungal mycelium along the edges of the inhibited colonies facing CMUAc130 was examined under a dissecting microscope at 400× magnification.

Preparation of inoculum and fermentation. A spore suspension of CMUAc130 was prepared in distilled water from cultures grown on ISP-4 medium at 30 °C for 10 days. The suspension was added to ISP-2 broth in each 500 ml Erlenmeyer flask at a rate of 10^8 spores in 100 ml liquid medium. Cultures were kept on a shaker at 120 r.p.m. at 30 °C for 48 h and used as seed stocks. For large production of culture filtrates, CMUAc130 was grown in a modified 3000 ml glass container containing 1500 ml ISP-2 broth, and then with orbital shaking for 5 days under the same conditions. The 5-day-old cultures were filtrated by Whatman paper no. 1 under vacuum. The mycelial mats were washed with distilled water and separated by centrifugation at 5000 r.p.m. for 20 min. The culture filtrate and mycelial mats of CMUAc130 were extracted with ethyl acetate. Both ethyl acetate extracts of liquid filtrate and mycelial mats showed antifungal activity; hence, they were combined.

Fractionation and purification of the compounds. The residue (320 mg) was dissolved in 10 ml methanol and fractionated on a reverse-phase column (Li Chroprep RP-18; Merck) with increasing concentrations of methanol as eluent (50, 70 and 100 %). Fractions were combined on the basis of TLC results and concentrated under vacuum to produce a black gum (120 mg). The black gum was mixed with 2·0 g silica gel (Walk gel). This mixture was then subjected to bioassay-guided separation through a chromatography column (50 × 7·5 cm) over silica gel (50 g) and eluted with chloroform–methanol (100 : 0, 95 : 5, 90 : 10, 85 : 15, 80 : 20, 70 : 30, 60 : 40, 50 : 50 and 0 : 100). About 50 ml per fraction was collected. Purifications were performed by column chromatography over silica gel (finer than 200-mesh). The fractions were combined and made into four pooled fractions on the basis of their TLC results. The pooled fractions, A-1 (eluted with chloroform–methanol from 95 : 5 to 90 : 10), A-2 (eluted with chloroform–methanol from 90 : 10 to 80 : 20), A-3 (eluted with chloroform–methanol from 80 : 20 to 60 : 40) and A-4 (eluted with chloroform–methanol from 60 : 40 to 30 : 70), were screened again for their antifungal activity against *C. musae* and *F. oxysporum* by using the paper-disc method (Rothrock & Gottlieb, 1984). Fractions A-2 and A-3 were most active against these fungi and they showed one major spot with the same R_f value (0·63, chloroform–methanol 20 : 1) in TLC. As they were identical, we combined these fractions and purified them by repeated chromatography over a silica-gel column, followed by recrystallization in a mixture of hexane and chloroform (9 : 1). This yielded active compound (i). Purification of A-1 resulted respectively in compounds (ii), (iii) and (iv). The structures of the active compounds have been identified by using NMR and mass-spectral data.

Structure elucidation of the compounds. The melting point of the compounds was determined on a Buchi-540 melting-point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^{1}H- and ^{13}C-NMR spectra on a Bruker DRX 500 spectrometer and electron impact-MS and fast atom bombardment-MS, respectively, on a Hewlett Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Antifungal-activity assay. Supernatants of CMUAc130 from ISP-2 broth, the extracted fractions or purified compounds were tested for antibiosis against *C. musae* and *F. oxysporum* by using the paper-disc method. Two 8 mm discs of sterile paper (Advantec; Toyo Roshi Kaisha) were soaked respectively in culture filtrate (50 µl), crude extract (50 µl) and each of four purified compounds (0·25 mg in 50 µl). The air-dried discs were placed on a PDA plate. Each plate was then inoculated with an agar block (8 mm diameter) containing mycelial mats of the fungi in the centre of the plate. The paper discs were 2·2 cm from the phytopathogen. Percentage inhibition was obtained 4 days after treatment at 30 °C from the following equation:

Inhibition (%) = [(growth diameter in untreated control − growth diameter in treatment) × 100]/growth diameter in untreated control

Each treatment consisted of three replicates. The experiment was repeated twice.

MICs. Purified compounds (i) and (ii) were assayed on PDA in Petri dishes to determine the MICs of these compounds against *C. musae* (Elson *et al.*, 1994). The purified compound (2 mg) was dissolved in DMSO (200 µl), diluted serially in the same solvent and added to PDA at 48 °C. The medium (5 ml) was added to a 5 cm diameter Petri dish. The final concentrations were 0, 1, 5, 10, 30, 60, 90, 120, 150 and 180 µg ml^{-1}. A 5 mm diameter plug of the fungi, removed from the margin of a 4-day-old colony on PDA, was placed 1·5 cm from the edge of the plate. Linear growth of the fungi at 30 °C was recorded 2 days after treatment. Each treatment consisted of three replicates. Percentage inhibition was obtained from the equation described above. The experiment was repeated twice.

Data analysis. Data from the antifungal-activity assay and MICs of CMUAc130 were analysed by SPSS for Windows 11.01. Means of treatments for each experiment were compared by using Duncan's multiple-range test ($P \leqslant 0{\cdot}05$).

RESULTS

Results of the dual cultures showed that *S. aureofaciens* CMUAc130 was inhibitory to the growth of *C. musae* and *F. oxysporum*. The inhibition zone of these tested fungi was over 20 mm when they were cultured dually with *S. aureofaciens* CMUAc130 for 4 days at 30 °C (Fig. 1a). When observed under a dissecting microscope, fungal mycelium along the edges of the colonies facing CMUAc130 appeared thickened, with bulbous-like formations along the ends (Fig. 1b, c). On the control plate, however, fungal mycelium showed regular, radial growth.

The crude extract (120 mg) of *S. aureofaciens* CMUAc130 was subjected to silica gel-column chromatography and four pooled fractions, A-1, A-2, A-3 and A-4, were obtained. All fractions were tested for their activity. Fractions A-1 and A-4 did not reduce growth of *C. musae* or *F. oxysporum*. Fractions A-2 and A-3 were markedly effective in reducing 43 and 57 % growth of *C. musae* and 38 and 52 % growth of *F. oxysporum*, respectively, indicating that they contained active ingredients. These active fractions were then subjected to repeated chromatography, followed by recrystallization in a mixture of hexane and chloroform (9 : 1), and then designated compound (i) (17 mg). Purification of A-1 resulted in compounds (ii) (11 mg), (iii) (14 mg) and (iv) (11 mg).

Structure elucidation of compound (i)

5,7-Dimethoxy-4-*p*-methoxylphenylcoumarin ($C_{18}H_{16}O_5$; Fig. 2) was a white, amorphous powder: melting point, 151–152 °C (EtOH); UV λ_{max} (MeOH) nm (log ε): 250 (4·07), 325 (4·29); ^{1}H NMR ($CDCl_3$): δ7·20 (2H, *d*, J = 8·5 Hz, H-2′, H-6′), 6·87 (2H, *d*, J = 8·5 Hz, H-3′,

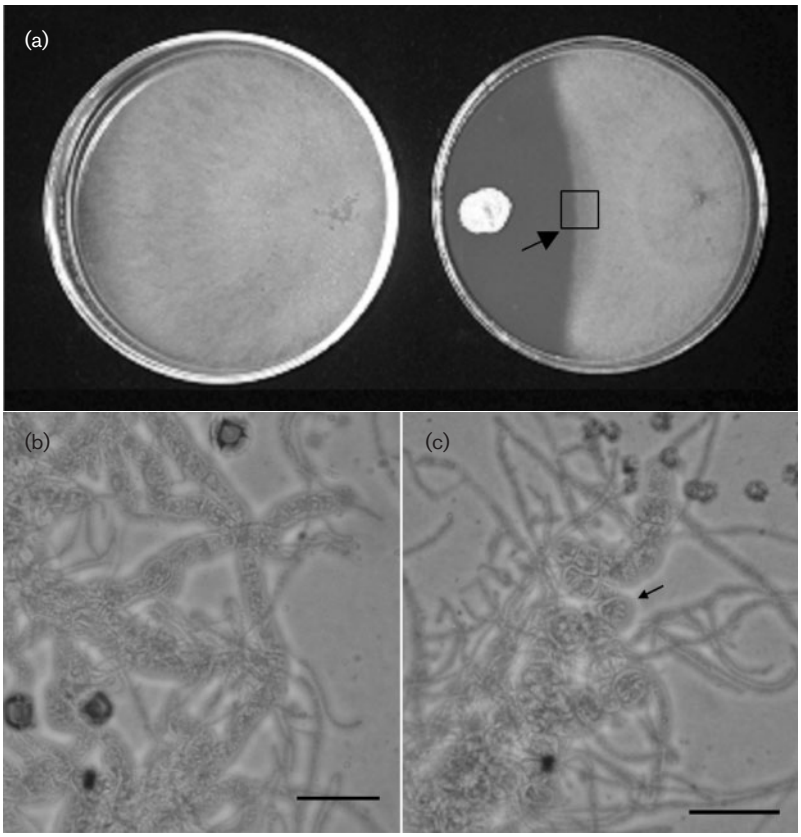

Fig. 1. Antagonism of *S. aureofaciens* CMUAc130 to *C. musae* on ISP-2 medium. (a) *In vitro* plate assay after 4 days paired incubation (arrow shows the source of fungal-mycelium samples for microscopic studies). (b) Hyphal ends of *C. musae*. (c) Hyphae showing thickened and bulbous structures (arrow) at the edges of the inhibited fungal colonies in the paired-culture plate. Bars, 20 μm.

H-5′), 6·50 (1H, *d*, *J*=2·5 Hz, H-8), 6·22 (1H, *d*, *J*=2·5 Hz, H-6), 5·96 (1H, *s*, H-3), 3·83 (6H, *s*, OMe-7, OMe-4′), 3·46 (3H, *s*, OMe-5); IR ν_{max} ($CHCl_3$) cm^{-1}: 1710, 1610, 1595, 1510, 1158, 1111, 1052, 952, 872, 860, 830; MS *m*/*z* (relative intensities): 312 $[M]^+$ (80), 284 $[M–CO]^+$ (100), 269 $[M–MeCO]^+$ (37), 241 $[M–43–CO]^+$ (2).

Structure elucidation of compound (ii)

5,7-Dimethoxy-4-phenylcoumarin ($C_{17}H_{14}O_5$; Fig. 2) was a white, amorphous powder: melting point 214–215 °C (MeOH); UV λ_{max} (MeOH) nm (log ε): 256 (4·04), 324 (4·22);); UV λ_{max} (sodium methoxide) nm: 256, 368; ^1H NMR (CD_3COCD_3): δ8·50 (1H, *s*, exchangeable D_2O, OH-4′), 7·14 (2H, *d*, *J*=8·5 Hz, H-2′, H-6′), 6·82 (2H, *d*, *J*=8·5 Hz, H-3′, H-5′), 6·51 (1H, *d*, *J*=2·5 Hz, H-8), 6·37 (1H, *d*, *J*=2·5 Hz, H-6), 5·80 (1H, *s*, H-3), 3·91 (3H, *s*, OMe-7), 3·53 (3H, *s*, OMe-5); $\Delta\delta = \delta C_5D_5N - \delta CD_3COCD_3$ =H-2′+H-6′ (+0·19), H-3′+H-5′ (+0·32), H-8 (+0·11), H-6 (+0·01), H-3 (+0·38) OMe-7 (−0·16), OMe-5 (−0·23); IR ν_{max} ($CHCl_3$) cm^{-1}: 1708, 1612, 1598, 1512, 1159, 1112, 1054, 952, 870, 860, 832; MS *m*/*z* (relative intensities): 298 $[M]^+$ (100), 270 $[M–CO]^+$ (82), 255 $[M–MeCO]^+$ (29), 227 $[M–43–CO]^+$ (15).

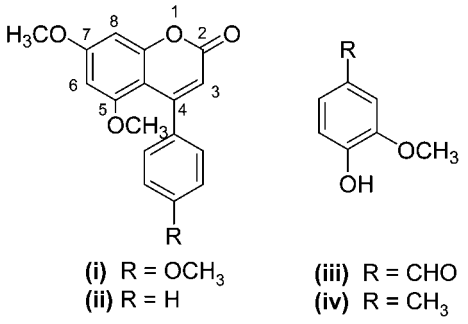

Fig. 2. Chemical structures of 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin (i), 5,7-dimethoxy-4-phenylcoumarin (ii), vanillin (iii) and 3-methoxy-4-hydroxytoluene (iv).

Compounds (iii) (14 mg) and (iv) (11 mg), obtained from A-1, were identified as vanillin and 3-methoxy-4-hydroxytoluene (Fig. 2) by comparison with their authentic samples.

The antifungal activity of different fractions from culture broth of *S. aureofaciens* CMUAc130 was evaluated by using *C. musae* and *F. oxysporum*. The paper-disc assay method indicated that compound (i) was most effective at inhibiting the growth of *C. musae* and *F. oxysporum* (Table 1). The same percentage of inhibition was detected for compound (ii). Both the culture filtrate of *S. aureofaciens* CMUAc130 and its crude extract as the control also showed efficacy at suppressing *C. musae* and *F. oxysporum*. However, vanillin and 3-methoxy-4-hydroxytoluene extracted from culture broth of *S. aureofaciens* CMUAc130 showed weak inhibition of fungal growth.

Table 1. Fungal-growth inhibition of tested substances by paper-disc assay

Results, the means of two replicates, were obtained 4 days after treatment at 30 °C.

Substance	Growth inhibition (%)	
	C. musae	*F. oxysporum*
Compound:		
(i)*	66	72
(ii)*	66	72
(iii)*	32	35
(iv)*	26	30
Crude extract*	82	88
Culture filtrate†	58	69

*At the amount of 0·25 mg in 50 µl on paper discs soaked with tested substances.

†At the amount of 50 µl 5-day culture broth.

The concentrations of compounds (i) and (ii) were highly correlated (R^2, 0·93 and 0·95, respectively) with the inhibition of mycelial growth of *C. musae* in PDA plates (Fig. 3). The relationship equation between concentration of compound (i) (x) and inhibition percentage of mycelial growth of *C. musae* (y) was $y=663{\cdot}94x+1{\cdot}6096$, whilst that for compound (ii) was $y=657{\cdot}69x+0{\cdot}3133$. The minimum concentrations of compounds (i) and (ii) for inhibition of *C. musae* were 120 and 150 µg ml^{-1}, respectively. Mycelial growth of *C. musae* was inhibited completely by 150–180 µg ml^{-1}.

DISCUSSION

The use of antagonistic micro-organisms, such as endophytic *Streptomyces* species, is an ideal method of controlling plant diseases (El-Shanshoury *et al.*, 1996; Trejo-Estrada *et al.*, 1998; Yuan & Crawford, 1995). A commercial product containing *Streptomyces griseoviridis*, Mycostop, was applied through the irrigation system to control important plant pathogens, such as *F. oxysporum*, *Botrytis cinera* and *Alternaria brassicicola* (Tahvonen, 1982a, b, 1993; Tuomi *et al.*, 2001). However, seeds treated with *S. griseoviridis* were not effective in the control of *C. musae* of banana. Recently, we reported that *S. aureofaciens* CMUAc130 was as effective as the fungicide nystatin when tested with the whole-plate diffusion method. This study revealed that 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin isolated from the ethyl acetate extract of the ferment broth of *S. aureofaciens* CMUAc130 inhibited growth of the phytopathogenic fungi.

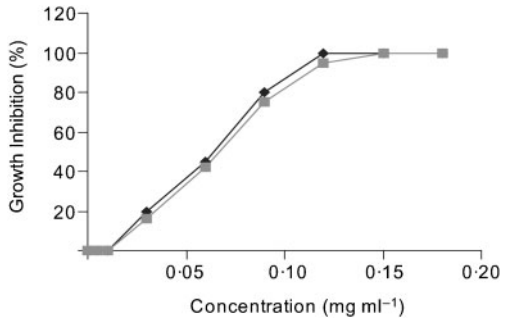

Fig. 3. Inhibition of growth of *C. musae* by different concentrations of compounds (i) (◆) and (ii) (■).

Previous reports indicated that 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin was produced by numerous species of plants, including *Hintonia latiflora* (Mata *et al.*, 1990), *Exostema caribaeum* (Mata *et al.*, 1988) and *Coutarea hexandra* (Delle Monache *et al.*, 1983), but nothing is known about its activity. Our study is the first in which 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin from culture filtrates of an endophytic *Streptomyces* species was isolated from the root tissue of *Z. officinale*. It further proved that 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin or 5,7-dimethoxy-4-phenylcoumarin was one of the antifungal components, as evidenced by the fact that the culture filtrate, crude extract, pure 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin from *S. aureofaciens* CMUAc130 were all inhibitory to *C. musae* and *F. oxysporum*. In our study, hyphae of *C. musae* treated with culture filtrates of *S. aureofaciens* CMUAc130, pure 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin showed signs of necrosis and fractures when examined under a scanning electron microscope (data not shown). This suggests that 4-arylcoumarins from *S. aureofaciens* CMUAc130 may be related to hyphal collapse of *C. musae*. The results of this study conclude that 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin is a major ingredient in the culture filtrate of *S. aureofaciens* CMUAc130 and it may play an important role in the inhibition of the damping-off pathogen *C. musae* and *F. oxysporum*.

As stated in several reports, *Streptomyces* activity in plants not only protects against pathogens, but the metabolic products of *Streptomyces* also influence plant growth and physiology (Katznelson & Cole, 1965; Mishra *et al.*, 1987). As 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin has been isolated from numerous plants, but not from members of the Zingiberaceae, further investigations are necessary to determine the relationship between 5,7-dimethoxy-4-*p*-methoxylphenylcoumarin and this plant family. Also, its cytotoxic activity against human cell lines should be studied.

ACKNOWLEDGEMENTS

This work was partially supported by the Faculty of Science, Silpakorn University, Nakorn Pathom, Thailand. The authors are grateful to Mr Y.-N. He and Ms H.-L. Liang in KIB for measuring NMR and MS data, respectively.

REFERENCES

Delle Monache, G., Botta, B., Neto, A. S. & Lima, R. A. (1983). 4-Arylcoumarins from *Coutarea hexandra*. *Phytochemistry* **22**, 1657–1658.

El-Shanshoury, A. E. R., El-Sououd, S. M. A., Awadalla, O. A. & El-Bandy, N. B. (1996). Effects of *Streptomyces corchorusii*, *Streptomyces mutabilis*, pendimethalin, and metribuzin on the control of bacterial and *Fusarium* wilt of tomato. *Can J Bot* **74**, 1016–1022.

Elson, M. K., Kelly, J. F. & Nair, M. G. (1994). Influence of antifungal compounds from a soil-borne actinomycete on *Fusarium* spp. in asparagus. *J Chem Ecol* **20**, 2835–2846.

Katznelson, H. & Cole, S. E. (1965). Production of gibberellin-like substances by bacteria and actinomycetes. *Can J Microbiol* **11**, 733–741.

Mata, R., Calzada, F. & Garcia, M. R. (1988). Chemical studies on Mexican plants used in traditional medicine. VI. Additional new 4-phenylcoumarins from *Exostema caribaeum*. *J Nat Prod* **51**, 851–856.

Mata, R., del Rayo Camacho, M., Cervera, E., Bye, R. & Linares, E. (1990). Secondary metabolites from *Hintonia latiflora*. *Phytochemistry* **29**, 2037–2040.

Mishra, S. K., Taft, W. H., Putnam, A. R. & Ries, S. K. (1987). Plant growth regulatory metabolites from novel actinomycetes. *J Plant Growth Regul* **6**, 75–83.

Rothrock, C. S. & Gottlieb, D. (1984). Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia solani* in soil. *Can J Microbiol* **30**, 1440–1447.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.

Taechowisan, T. & Lumyong, S. (2003). Activity of endophytic actinomycetes from roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi. *Ann Microbiol* **53**, 291–298.

Taechowisan, T., Peberdy, J. F. & Lumyong, S. (2003). Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J Microbiol Biotechnol* **19**, 381–385.

Tahvonen, R. (1982a). Preliminary experiments in the use of *Streptomyces* spp. isolated from peat in the biological control of soil and seed-borne diseases in peat culture. *J Agric Sci Finl* **54**, 357–369.

Tahvonen, R. (1982b). The suppressiveness of Finnish light coloured *Sphagnum* peat. *J Agric Sci Finl* **54**, 345–356.

Tahvonen, R. (1993). The disease suppressiveness of light-coloured sphagnum peat and biocontrol of plant diseases with *Streptomyces* sp. *Acta Hortic* **342**, 37–42.

Trejo-Estrada, S. R., Sepulveda, I. R. & Crawford, D. L. (1998). *In vitro* and *in vivo* antagonism of *Streptomyces violaceusniger* YCED9 against fungal pathogens of turfgrass. *World J Microbiol Biotechnol* **14**, 865–872.

Tuomi, T., Heino, M., Nordstrom, K. & Laakso, S. (2001). Fiber fractions from processing of barley in production and conservation of a biologic control agent. *Appl Biochem Biotechnol* **94**, 135–145.

Yuan, W. M. & Crawford, D. L. (1995). Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. *Appl Environ Microbiol* **61**, 3119–3128.