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Antagonistic Effects of *Streptomyces* sp. SRM1 on *Colletotrichum musae*

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Abstract: In an attempt to understand the mode of action of this antagonist in nature, the interaction between *Streptomyces* sp. SRM1 and *C. musae* was studied by dual culture on agar plates. Evidence for the antibiosis of *Streptomyces* sp. SRM1 was demonstrated by inhibition zones in *in vitro* plate assay. The crude extract from the culture of *Streptomyces* sp. SRM1 also produced antifungal activity, which showed antagonistic effects against *C. musae* such as swelling, distortion and excessive branching of hyphae and inhibition of spore germination. An indirect method was used to show antagonistic effect of *Streptomyces* sp. SRM1 against *C. musae* in soil. This study suggests the potential of developing *Streptomyces* sp. SRM1 for the biological control of anthracnose disease of banana caused by *C. musae*.

Key words: Anthracnose, antifungal activity, biological control, *Colletotrichum musae*, *Streptomyces* sp. SRM1

INTRODUCTION

Wound anthracnose is the main disease affecting the quality of export banana fruits from many country including Thailand. This postharvest disease is caused by the pathogenic fungus *Colletotrichum musae*. In plantations, *C. musae* conidia contaminate banana fruits during the month after flowering (Chillet *et al*., 2000). These conidia quickly germinate and form melanised appressoria, which are quiescent structures of the pathogen (De Lapeyre de Bellaire *et al*., 2000). These appressoria germinate as the banana ripen and form infected hyphae that colonise the peel and penetrate into the fruit pulp (Chillet *et al*., 2007), leading to the development of anthracnose. Although a rather tedious procedure of injecting the fungicides thiabendazole or imazalil into plantation has been attempted, there are no effective chemical control measures for this disease. Strains of *C. musae* exist which are resistant to thiabendazole (Johanson and Blazquez, 1992). Some microorganisms, including Mycoparasites; *Gliocladium* sp., *Trichoderma* sp., *Pythium* sp., *Verticillium* sp. and *Clonostachys rosea* (Krauss *et al*., 1998), have been reported to be potential antagonists of *C. musae*. These microorganisms can be developed as potential biocontrol agents against *C. musae* by understanding the type of interactions between the fungal pathogen and the control agent (Krauss and Soberanis, 2001). Generally, mechanisms through which microorganisms inhibit fungal pathogens in the rhizosphere are: (i) competition for nutrients, oxygen or space; (ii) parasitism or the physical destruction of fungal cell walls by the action of hydrolytic enzymes produced by the antagonist; (iii) antibiosis or the inhibition of one microorganism by diffusible compound(s) produced by another and/or, by a synergistic combination of these modes of action (Benyagoub *et al*., 1998).

Actinomycetes of the genus *Streptomyces* are well known for their ability to suppress growth of a wide variety of fungal pathogens (Taechowisan *et al*., 2003a; Trejo-Estrada *et al*., 1998a). *Streptomyces* species have been used extensively in the biological control of several phytopathogenic fungi (El-Raheem *et al*., 1995). Many antibiotics produced by actinomycetes have been used directly or have been assumed to be responsible for the biocontrol potential of the producing strain (Smith *et al*., 1990; Trejo-Estrada *et al*., 1998b). *Streptomyces* are also known for their ability to produce fungal cell wall-degrading enzymes such as cellulases, hemicellulases, glucanases and chitinases (Taechowisan *et al*., 2003b; Trejo-Estrada *et al*., 1998b). The role of these hydrolytic enzymes in antifungal activity and biocontrol ability of *Streptomyces* has been investigated (Taechowisan *et al*., 2003b; Valois *et al*., 1996). The earlier studies, an endophytic *Streptomyces aureofaciens* CMUAc130 was shown to exhibit strong *in vitro* antagonism toward several plant pathogenic fungi at least two mechanisms, one was chitinolytic enzyme and

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other was secondary metabolites (Taechowisan *et al*., 2003b; Taechowisan *et al*., 2005). The isolate, referred to as strain SRM1, showed strong antagonism toward *C. musae*. The isolation, selection and identification of strain SRM1, as well as the antagonistic effects of this strain against *C. musae* on hyphal extension and pathogenesis and also spore germination were investigated.

MATERIALS AND METHODS

Fungal pathogen: Fungal strain used in the *in vitro* antagonism assay was: *Colletotrichum musae*, the causative agents of anthracnose of banana (provided by Dr. Wipornpan Photita, Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand). It was grown on Potato Dextrose Agar (PDA) plates at 30°C and stored on PDA slants at 4°C.

Isolation of actinomycetes: Actinomycetes were isolated from soil samples collected from the environs of Nakorn Pathom, Thailand, during the period April-July 2007. Samples were dried in room temperature for 5 days and then suspended in sterile saline (0.9% NaCl) solution. The serial dilution spread plate technique was used, where dilution 10^{-4} was plated onto humic acid-vitamin (HV) agar (Otoguro *et al*., 2001) containing 100 μg mL^{-1} nystatin and cycloeximide and incubated at 30°C for 1 week. The colonies were inoculated onto International *Streptomyces* Project-2 (ISP-2) medium (Shirling and Gottlieb, 1966) for purification and stock cultures.

Identification of endophytic actinomycetes: The characteristics of the actinomycetes isolates were observed. For morphological characteristics, presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment, sporophore and sporechain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analysed for chemotaxonomic studies (Becker *et al*., 1964; Boone and Pine, 1968).

***In vitro* assay for antagonism:** An *in vitro* plate-assay technique was developed to test the inhibitory effect of actinomycete isolates on *C. musae* growth. Test for inhibitory activity was made on ISP-2 in Petri dishes. A 0.8 cm diameter ISP-2 agar plug, covered fully with a lawn of actinomycete isolate, 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 fungi. In order to investigate the inhibitory effect of actinomycetes on the fungal growth, a 0.8 cm diameter PDA plug covered with activity growing mycelium was placed about 6 cm from actinomycete colony. 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 actinomycete colony in dual cultures.

Extraction of secondary metabolites produced by *Streptomyces* sp. SRM1: Among the 39 isolates of actinomycetes, the isolate SRM1 was found to be the best producer of antifungal substances. This isolate was selected for extraction the secondary metabolites. Spores of *Streptomyces* sp. SRM1 were used to inoculate 250 plates of ISP-2 and incubated for 14 days at 30°C. The culture medium was then cut into small pieces that were extracted with ethyl acetate (3×300 mL). This organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (250 mg).

***In vitro* antagonistic effects of *Streptomyces* sp. SRM1 metabolites on mycelial development and spore germination of *C. musae*:** Crude extract from *Streptomyces* sp. SRM1 was tested for antibiosis using the disk diffusion assay as described by Milici *et al*. (2007). The crude extract was dissolved in methanol (25, 50 and 100 μg mL^{-1}) and 50 μL was applied to sterile (6 mm diameter) paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan), dried and then placed on PDA plate, each plate was then incubated with an agar block (8 mm diameter) containing mycelial mats of the fungi in the center of the plate (the paper discs were 2.2 cm from the fungi). Plates were incubated at 30°C for 72 h and observed for the presence of an inhibition zone. After 72 h, morphological changes on fungal mycelial occurring in the margin of the inhibition zones were examined under a compound microscope at 400x magnification. The experiment was repeated twice.

Fungal spore germination assays were performed according to the procedure described by Lorito *et al*. (1993) with minor variations. A stock suspension of ca. 10^6 conidia of the fungus mL^{-1} in 30% glycerol was prepared and kept at -20°C. Equal volumes (400 μL) of spore suspension, 3x potato dextrose broth and the crude extract (384 μg mL^{-1} in 10% (v/v) methanol) were mixed in sterile microcentrifuge tube. The test solutions were replaced with sterile water in control samples. Tubes were incubated at 30°C for 24 h. A drop of the mixture from each tube was placed on a microscope slide and the percentage of conidial germination was determined from the first 100 spores chosen at random. Data were

transformed into values representing the treatments as a percentage of the control (in which % inhibition of control = 0) by the following equation:

$$I(\%) \frac{1\text{-}St(\%)}{Sc(\%)} \times 100$$

where, I(%) represented the percent inhibition, St(%) represented the percentage of spores germinating in the treatment of interest and Sc(%) represented the percentage of spores germinating in the control. The results of each experiment are reported as the average of three replicates.

Minimum Inhibitory Concentrations (MICs): MICs of crude extract was determined by microbroth dilution methods (Milici *et al*., 2007).

The fungal pathogen *C. musae* was tested for its response to the crude extract using a Potato Dextrose Agar (PDA) dilution technique. The crude extract (5.12 mg) was dissolved in DMSO (1 mL), then serially diluted two-fold to obtain final concentration ranges of 0.50-256 μg mL^{-1} in PDA. The medium (5 mL) was added to a 5 cm diameter Petri dish. An 8 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. The experiment was repeated twice.

Direct observation of the antagonistic effects of strain SRM1 on spore germination of *C. musae* in soil environment: A buried slide technique (Stevenson, 1956) was used. About 50 g of soil (pH = 7.6) was sieved, air-dried and sterilized in 250 mL plastic bags by autoclaving at 121°C for 30 min. A 5 mL spore suspension of strain SRM1 containing 3×10^8 colony forming units mL^{-1} was prepared from ISP-2 plates. Spores were washed thoroughly with sterile distilled water, inoculated into the sterile soil and incubated at 30°C for 7 days. A 10 mL spore suspension of *C. musae* containing 2×10^6 spores mL^{-1} was mixed in 100 mL of sterile 1.8% molten agar and coated on sterile microscope glass slides by dipping them. When the agar layer had set, slides were carefully inserted vertically into plastic bags containing sterile soil alone (control) or strain SRM1-inoculated soil and incubated at 30°C. At the end of each specified incubation period (2, 4 and 7 days), the slides were removed carefully and examined immediately under a compound microscope at 400X magnification. Each observation period involved analysis of two plastic bags for each treatment. Each plastic bag contained three spore-coated glass slides. After the second day of incubation, the number of spores that germinated and formed germ tubes on the control and test slides was compared visually under a light microscope. The percentage of spore germination was calculated in 30 microscopic fields.

RESULTS AND DISCUSSION

Thirty-nine strains were isolated and classified into *Streptomycete* -like strains by colony and morphological observation. All isolates were tested for the *in vitro* antagonistic assay against *C. musae*. Six isolates (J72, KJ5, NC1, NC2, SRM1 and WTA2) inhibited the growth of *C. musae*. The inhibition zones of this tested fungi was over 10 mm when cultured dually with these six isolates for 4 days at 30°C. One isolate, SRM1, showed strong antagonism towards *C. musae*.

Morphological observation of 10 day old culture of SRM1 revealed that sporophores to be monopodially branched and flexuous, producing open spirals of oval-shaped spores (1×1.5 μm) with smooth surfaces. The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was white changing to ash-grey with yellow soluble pigment occasionally discernable. Based on results in morphological observation as well as on the presence of LL-diaminopimelic acid in the whole-cell extract, the isolate SRM1 was identified as belong to the genus *Streptomyces* (Fig. 1).

Since, the isolate SRM1 producing the most active substance, it was cultured in the large scale to get more culture and its secondary metabolites were extracted by ethyl acetate. The crude extract of SRM1 at a concentration of 1.25, 2.5 and 5 μg $disc^{-1}$ produced inhibition zones with a diameter of about 12-28 mm on paper disc assay (Fig. 2a). When observed under a dissecting microscope, fungal mycelium along the edges of the colonies facing the crude extract of SRM1 appeared thickened, with bulbous-like formations along the ends (Fig. 2b, c). On the control disc, however, fungal mycelium showed regular, radial growth. The different concentration of the crude extract showed efficacy in suppressing *C. musae*. The minimum concentration of the crude extract for inhibition in hyphal extension of *C. musae* was 25 μg mL^{-1}. The crude extract of SRM1, at a concentration 128 μg mL^{-1} completely inhibited spore germination of *C. musae* (Table 1). After 24 h of incubation, spore germination of *C. musae* was reduced when increasing the concentration of the crude extract, in contrast, a high degree of germination occurred in both experiments when spores were mixed with either distilled water or with 10%

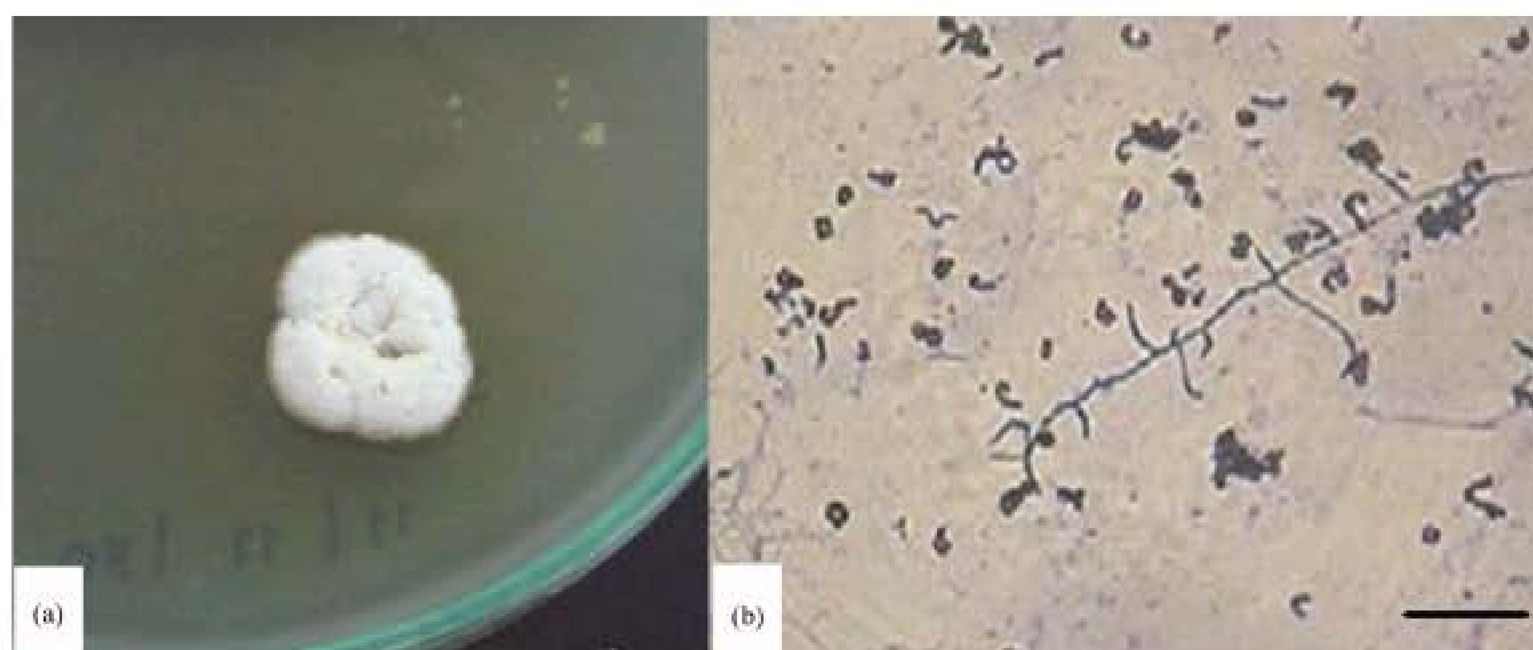

Fig. 1: Morphology of *Streptomyces* sp. SRM1 colony (a) and mycelium (b). A white colony , changing to orange with faint yellowish soluble pigment. An open spirals, spores spherical to-oval-shaped under light microscopy observation. Bar = 10 μm

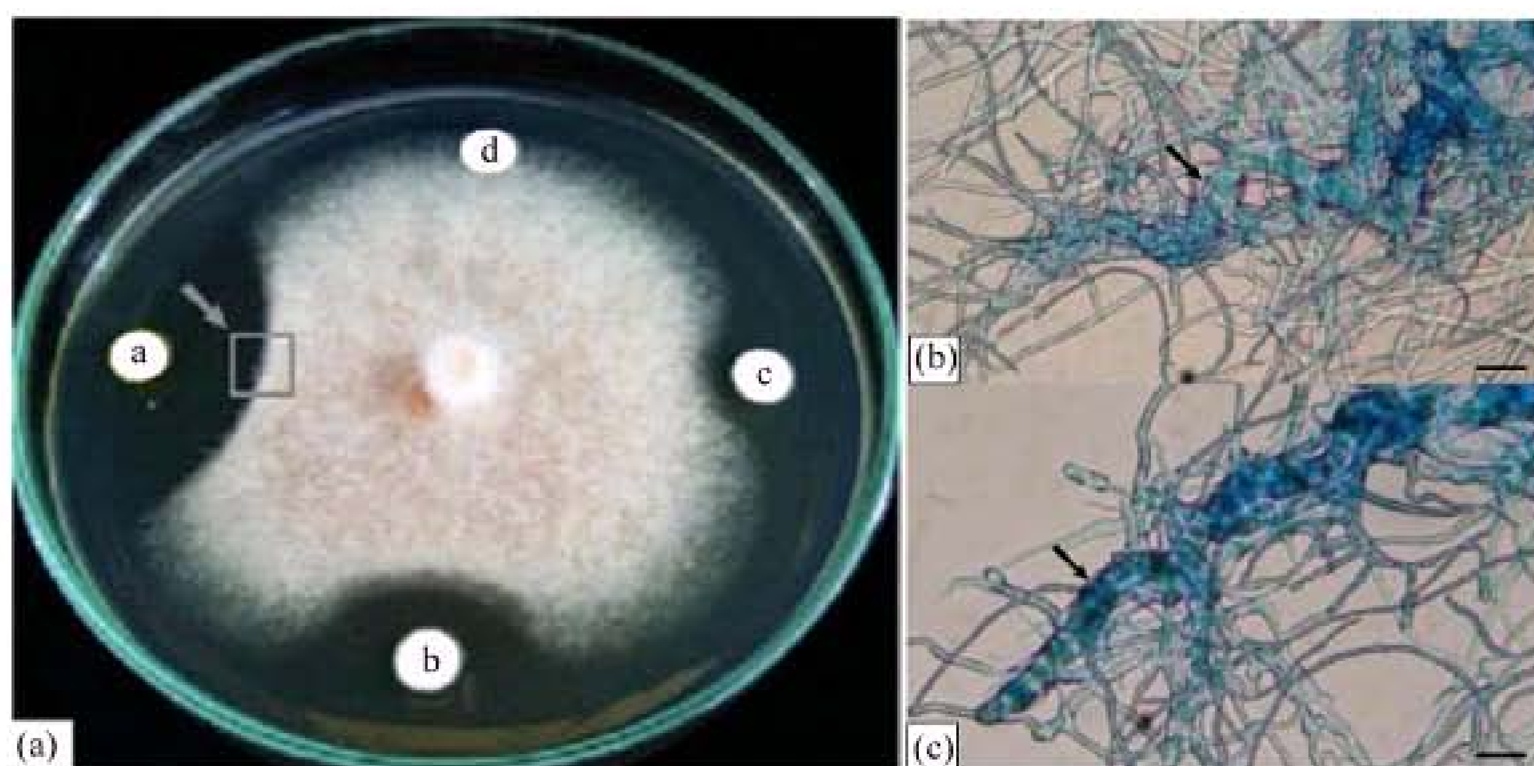

Fig. 2: Antagonism of the crude extract of *Streptomyces* sp. SRM1 to *Colletotrichum musae* on ISP-2 medium. (a) *In vitro* plate assay after 72 h incubation at difference concentration, a = 5, b = 2.5, c = 1.25 μg disc^{-1} and d = control (arrow shows the source of fungal-mycelium samples for microscopic studies). (b and c) Hyphae showing thickened and bulbous structures (arrow) at the edges of the inhibited fungal colonies in the *in vitro* antagonistic assay. Bar = 10 μm

Table 1: Effect of crude extract of *Streptomyces* sp. SRM1 on spore germination inhibition of *Colletotrichum musae*

Concentration of the crude extract (μg mL^{-1})	Mean percentage of spore germination inhibition after 24 h
128	100.00±0.0
64	95.45±3.36
32	82.47±11.24
16	50.61±5.72
Control (solvent)	43.38±3.65
Distilled water	41.53±5.11

(v/v) methanol. The presence of solvent in the crude extract sample did not have any negative effect on spore germination when compared with distilled water (Fig. 3).

Results from the buried slide technique showed that the spores of *C. musae* on glass slides buried in uninoculated sterile soil germinated normally and the germ tubes developed into long hyphal strands. By the 7th day, healthy and extensive hyphal growth with sporulation was evident on the control slides (Fig. 4). However, in the sterile soil in which the isolate SRM1 was grown, fungal spore germination was reduced compared to the control slides. The percentage of spore germination was observed 27.62 and 18.24% in the percent of SRM1 on the second and fourth day while there are no observation of fungal spore germination in the present of SRM1 on the seventh day. A lytic effect caused by SRM1 was first

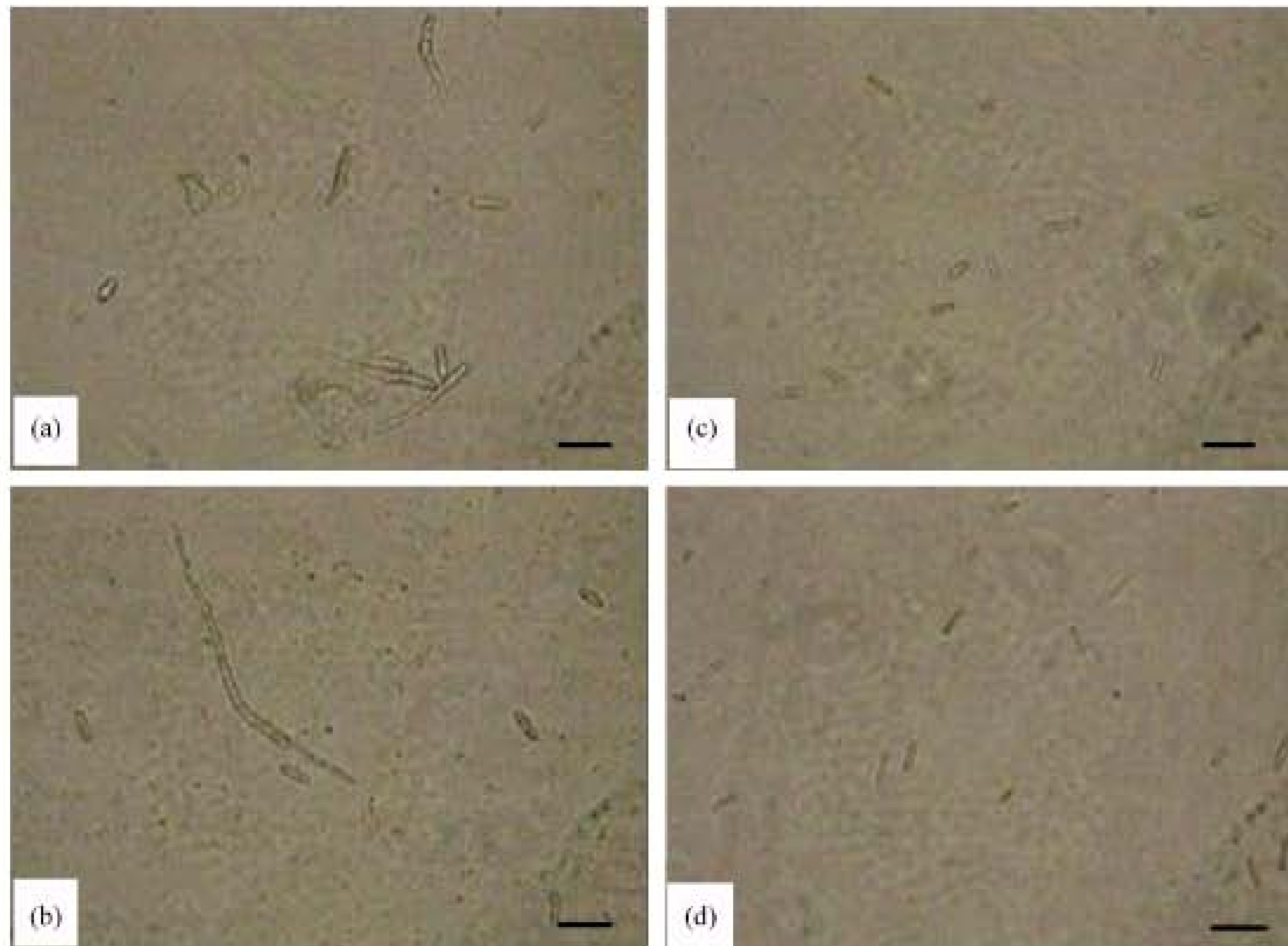

Fig. 3: Spore germination inhibition after incubation at 30°C for 24 h with: (a) 20 mL sterile distilled water, (b) 10% (v/v) methanol, (c) 64 μg mL^{-1} of crude extract and (d) 128 μg mL^{-1} of crude extract. Bar = 10 μm

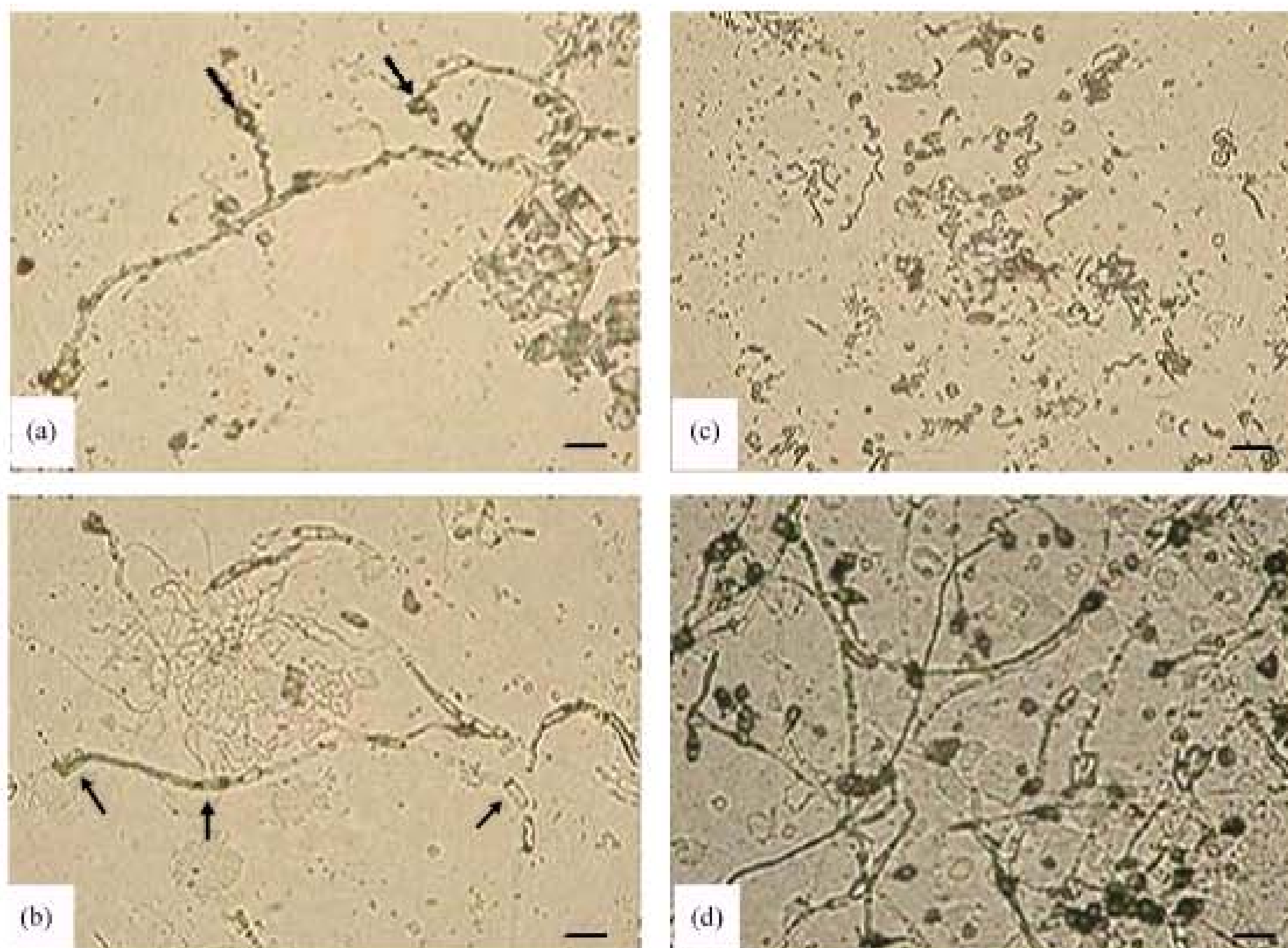

Fig. 4: Hyphal development of *Colletotrichum musae* in SRM1-inoculated control soil (x400). (a) Early stages of hyphal distortion and lysis (arrowed) after 2 days in soil culture. (b) Distorted and swellings (arrow) of hyphae after 4 days in soil culture. (c) Massive lysis of hyphae after 7 days in soil culture, hyphae is less while the spore of SRM1 is abundant. (d) Healthy hyphal growth with abundant sporulation after 7 days in uninoculated control soil. Bar = 10 μm

detected in the fungal hyphae after 2 days of incubation. Hyphal distortion, like swelling or bulbous growth, was also observed. By the fourth day, distortion and lysis of the hyphae were more frequently noted.

All of actinomycetes isolated in this study belong to the *Streptomycete*-like group. The predominance of *Streptomycetes* in actinomycete population in soil sample is distributed at a high frequency. This could also be due to the fact that no special pretreatment techniques were adapted to enhance the isolation of rare actinomycetes for example; HV agar was amended with antibacterial agents was used in conjunction with physical (dry heating) and chemical (phenol, chlorhexidine gluconate, benzethonium chloride and γ-collidine) pretreatments to achieve the highly selective isolation of the *Micromonospora*, *Microbispora*, *Streptosporangium*, *Dactylosporangium* and *Actinoplanes* (Hayakawa *et al*., 1991a-c).

In the *in vitro* antagonistic assay showed promising results for the use of streptomycetes as antifungal agents against phytopathogenic fungi. Similar results have been reported previously in actinomycetes screening studies (Crawford *et al*., 1993). It was noted that some actinomycetes which inhibited pathogens on agar plate also did so, in soil (Fravel, 1988). Thus, *in vitro* assays appear to be useful in identifying which actinomycete antagonists might function in soil.

The isolate SRM1 was selected for one trait of ten associated with biocontrol agents, its ability to produce the secondary metabolites active against *C. musae*. In the *in vitro* assay, preinoculation of the isolate SRM1 was done to allow growth and spore germination of the culture prior to inoculation with *C. musae*. Thus, the antagonism between the isolate SRM1 and the *C. musae* may have involved production of secondary metabolites in agar. The *in vitro* studies further indicated that culture of the isolation SRM1 in agar medium produced the secondary metabolites that showed clear zones of inhibition against *C. musae* and induced morphological changes such as swollen and distorted germ tubes that branched more freely than normal conidia. The secondary metabolites from the crude extract also inhibited spore germination and hyphal development of *C. musae*. The isolate SRM1 also showed the inhibitory effects on spore gemination and hyphal lysis of *C. musae* in soil by the buried slide technique. These results indicated that using the secondary metabolites of SRM1 could be controlling fungal growth and might be a promising method of biocontrol of some plant pathogens.

Antibiosis is particularly considered to provide an advantage in biological disease control because compounds mediating antibiosis can diffuse rapidly in nature and direct contact between the antagonist and pathogen is nor necessary (Hajlaou *et al*., 1994). Antibiotics have been implicated in the antagonism of fungi by actinomycetes. These are many reports related to antibiotic substances that induced malformations such as stunting, distortion, swelling, hyphal protuberances or the highly branched appearance of fungal germ tubes (Gunji *et al*., 1983). Previously, a strain of *Streptomyces hygroscopicus* var. *geldanus* produced geldanamycin in culture and in soil was able to biocontrol diseases caused by *Rhizoctonia solani* (Rothrock and Gottlieb, 1984) and other studies, a strain of *Streptomyces violaceusniger* that produced at least three antifungal compounds has been reported to suppress damping off of lettuce caused by *Pythium ultimum* (Trejo-Estrada *et al*., 1998b). On the other hand, *Streptomyces* are also known for their ability to cause lysis of fungal hyphae by producing chitinases and glucanases (Taechowisan *et al*., 2003b; Valois *et al*., 1996).

This study demonstrated that antibiosis mediated by diffusible metabolites was involved in the antagonism of strain SRM1 against *C. musae* in sterile soil. However, further studies are needed to determine whether inhibition of fungal spore germination and hyphal swelling, distortion and lysis will be detected when *C. musae* is placed in strain SRM1-inoculated nonsterile soils. In addition further studies are needed to characterize the antifungal substances of strain SRM1, for example bioactive compound(s) and hydrolytic enzyme production. These studies will be useful in the manipulation and development of strain SRM1 as a potential biological control agent for anthracnose of banana caused by *C. musae*.

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