Evaluation of characteristics of *Simplicillium lanosoniveum* on pathogenicity to aphids and *in vitro* antifungal potency against plant pathogenic fungi

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Abstract— *This study aimed to evaluate the aphidicidal and antifungal activities of Simplicillium lanosoniveum in laboratory conditions. S. lanosoniveum isolate Cs0701 shown to be pathogenic to the aphids, Aphis gossypii, Ceratovacuna lanigera and Hysteroneura setariae. The data showed that isolate Cs0701 exudates had greater mycelial growth-inhibiting effects on plant pathogenic fungi, Sclerotium rolfsii, Alternaria brassicicola and Rhizoctonia solani, in cellophane paper antibiosis test. In addition, mycelial growth of Cochliobolus miyabeanus, Curvularia lunata and Fusarium sp. were partially inhibited by isolate Cs0701 exudates. The culture filtrates of isolate Cs0701 were screened for their antifungal activity against the plant pathogenic fungi. The results revealed that A. brassicicola, Cochliobolus miyabeanus and Curvularia lunata conidial germination was inhibited by isolate Cs0701. The culture filtrate was also able to inhibit conidial germination of jasmine orange (Murraya paniculata) powdery mildew, Oidium murrayae. However, plant host range tests showed that isolate Cs0701 was not pathogenic to Pistia stratiotes, Eichhornia crassipes, Lemma perpusilla and Glycine max. Taken together, these findings provide convincing experimental evidence that S. lanosoniveum isolate Cs0701 is biologically active against both aphids and plant pathogenic fungi including powdery mildew. Pot and field trials are necessary to confirm efficacy of S. lanosoniveum against aphids and plant pathogenic fungi.*

Keywords— biological control agent, Simplicillium lanosoniveum, aphidicidal activity, antifungal activity.

I. INTRODUCTION

Salvinia auriculata Aublet (eared salvinia) and *S. molesta* Mitchell (giant salvinia) are two exotic floating ferns sold at local flower markets and aquarium shops. Brown Spot of *S. auriculata* and *S. molesta* caused by *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & Gams 2001 was reported in Taiwan (Chen et al., 2008). Studies of tested isolate of *S. lanosoniveum* grown on potato dextrose agar plates at different temperatures showed that optimum temperature for mycelial growth, conidial germination and sporulation was 25°C (unpublished data). *Simplicillium*, was associated with ticks, nematodes, and scale insects as well as rusts, such as *Aecidium elaeagni-latifoliae*, *Hemileia vastatrix* (coffee rust) and *Uromyces pencanus* (Gams and Zare, 2003; Bischoff and White, 2004; Polar et al., 2005; Baiswar et al., 2014). *S. lanosoniveum* was a mycoparasite of soybean rust caused by *Phakopsora pachyrhizi* in Louisiana and Florida, USA. It was found coiling within sori and around urediniospores and showed a trophic attraction to rust sori, extending from sorus to sorus. The mycophilic and disease-suppressive nature of *S. lanosoniveum* on *P. pachyrhizi*, the soybean rust pathogen was recently documented. However, *S. lanosoniveum* did not cause lesions or necrosis on soybean (Ward et al., 2011; 2012). *S. chinense* F. Liu & L. Cai was reported as pathogens of plant parasitic nematodes (Liu and Cai, 2012; Zhao et al., 2013). *Lecanicillium* and *Simplicillium* (both formerly *Verticillium* spp.) are included in the family Cordycipitaceae, which also includes the anamorphic genera *Beauveria* and *Isaria* (Zare and Gams, 2001; Sung et al., 2007). *Lecanicillium* spp. were reported as pathogens of aphids, scale insects, ticks, nematodes, and whiteflies (Pirali-Kheirabadi et al., 2007; Cuthbertson et al., 2008; Arevalo et al., 2009; Liu et al., 2009).

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important crops, especially in the subtropical and tropical regions. Microbial antagonists are widely used for the biocontrol of fungal plant diseases due to their perceived increased level of safety and minimal environmental impacts. Fungal biological control agents have several mechanisms of action including mycoparasitism, production of antibiotics or enzymes, competition for nutrients and the induction of plant defense responses that allow them to control pathogens (Brimner and Boland, 2003). The development of new biocontrol products against plant diseases required the process involves screening of high numbers of candidate antagonists and to fulfill many different requirements (Köhl et al., 2011).

S. lanosoniveum has recently been discovered as a dual pathogen of aquatic ferns, *Salvinia* spp., and soybean rust, *P. pachyrhizi* (Chen et al., 2008; Ward et al., 2012). The objectives of this study were to examine the pathogenicity of *S. lanosoniveum* to plants and aphids and the in vitro antifungal activity of *S. lanosoniveum* culture filtrates on plant pathogenic fungi.

II. MATERIAL AND METHOD

2.1 Inoculum preparation

S. lanosoniveum isolate Cs0701 isolated from brown spot of *S. auriculata* was described previously (Chen et al., 2008) and maintained in our laboratory. The culture of isolate Cs0701 was grown on potato dextrose agar (PDA; Difco Laboratories Michigan, USA) at 25°C with diurnal light (12 hrs). The spores were then collected from a 14-d-old culture with sterile distilled water containing 0.01% Tween 20 (v/v). The concentrations of the spore suspensions were determined in a hemacytometer and adjusted to 10^6 spores per ml. It was used as the inoculum for pathogenicity test.

2.2 Pathogenicity test of *S. lanosoniveum* on plants

Three aquatic plants, *Lemma perpusilla* Torr., *Pistia stratiotes* L., and *Eichhornia crassipes* (Mart.) Solms and vegetable soybean (*Glycine max* (L.) Merr., cvs. Kaohsiung No. 8 and Kouki) were used to investigate the pathogenicity of *S. lanosoniveum*. Aquatic plants were floated in 1000-ml rectangular plastic basins filled with 500 ml of tap water. Five leaves of each 24-d-old plant of vegetable soybeans were cultivated on 5 L pot (four plants per pot) filled with pasteurized sandy loam soil.

A spore suspension of isolate Cs0701 (10^6 spores per ml) was sprayed onto tested plants. All treatments, including controls misted with sterile water containing 0.01% Tween 20 (v/v), were replicated three times. The plastic basins and pots were covered with plastic bags for 1-d then removed plastic bags and placed in a growth chamber maintained at 25°C with 12-h fluorescent light cycles. Disease severity was rated daily for up to 7-d using a disease scale of 0 to 4, where 0 = leaves without symptoms; 1 = 1-10% of leaf area showing symptoms; 2 = 11-25% of leaf area showing symptoms; 3 = 26-50% of leaf area showing symptoms; and 4 = over 51% of leaf area showing symptoms.

2.3 Pathogenicity test of *S. lanosoniveum* on aphids

The rusty plum aphids, *Hysteroneura setariae* (Thomas) (Hemiptera: Aphididae) collected from infested *Eleusine indica* (L.) Gaertn. were used to investigate the pathogenicity of *S. lanosoniveum*. Aphids were fed on leaves attached to detached stems of *E. indica* that were inserted into a 100-ml flask filled with 75 ml of tap water. Fifty aphids on detached leaves and stems uniformly distributed to each flask. A spore suspension of isolate Cs0701 (10^6 spores per ml) was sprayed onto tested aphids. All treatments, including controls misted with sterile water containing 0.01% Tween 20 (v/v), were replicated three times. The flasks were covered with plastic bags and placed in a growth chamber maintained at 25°C with 12-h fluorescent light cycles for 5-d. The dead aphids were counted and then placed in moist Petri dishes for observation of pathogen sporulation in order to determine whether the death of larvae was due to infection of pathogen. The mortality of insects was calculated by percentage of infected dead aphids.

The detached leaf method was also used for treatment of other aphids with conidial suspension. Plant leaves were sterilized with aqueous solution of sodium hypochlorite (0.5%, v/v), washed three times with distilled water and then air dried. The leaves were placed on 1.5% agar in 90 × 20 mm^2 plastic Petri dishes. A batch of 30 adult aphids (2-d-old each) was settled on each leaf, 1-d before treatment. Melon and cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae) on taro, *Colocasia esculenta* (L.) Schott, and cucumber, *Cucumis sativus* L., and sugarcane woolly aphid, *Ceratovacuna lanigera* Zehntner (Homoptera: Pemphigidae) on sugarcane, *Saccharum officinarum* L., were used to investigate the pathogenicity of *S. lanosoniveum* using the inoculation method as described above.

2.4 Effect of *S. lanosoniveum* exudates released on solid media on mycelial growth of plant pathogenic fungi

The tested fungal isolates for antagonistic activity of *S. lanosoniveum* were isolated from diseased plants previously in our laboratory. These plant pathogenic fungi were: *Alternaria brassicicola* (Schwein.) Wiltshire (brassica dark leaf spot), *Cochliobolus miyabeanus* (Ito & Kurib.) Drechsler ex Dastur (rice brown spot), *Curvularia lunata* (Wakker) Boedijn (black kernel of rice), *Fusarium* sp. (fruit rots of melon), *Geotrichum* sp. (fruit sour rot of litchi), *Rhizoctonia solani* J. G. Kühn (damping-off in seedlings), and *Sclerotium rolfsii* Sacc. (southern blight). The culture of these plant pathogenic fungi and *S.*

lanosoniveum isolate Cs0701 was grown on PDA at 25°C with diurnal light (12 hrs). A mycelial disc (5 mm diam.) of a 5-d-old culture of these isolates was used as the inoculum for mycelial growth.

Using the cellophane paper antibiosis method, an agar disc (5 mm diam.) containing isolate Cs0701 was transferred aseptically on the center of a cellophane paper (5 × 5 cm) which was overlaid on the surface of a PDA plate. Forty-eight hours after incubation, the cellophane paper was removed, and the medium was assayed for the inhibitory activity against mycelial growth of tested fungi. The inoculum of tested fungal species was transferred aseptically on the same place as isolate Cs0701 pre-inoculation. The mycelial growth of tested fungal species was recorded after a 3-d incubation period at 25°C. Plates prepared without pre-inoculation of isolate Cs0701 acted as the control. Each treatment was replicated 3 times. Growth inhibition rate (%) = (mean radius of control – mean radius of plate with exudates) / mean radius of control × 100 (Song and Ji, 2006).

2.5 Effect of *S. lanosoniveum* culture filtrates on conidial germination of plant pathogenic fungi

The cultures of tested plant pathogenic fungi, *A. brassicicola*, *C. miyabeanus*, *C. lunata*, and *Geotrichum* sp., were grown on PDA at 25°C with diurnal light (12 hrs). The conidia were then collected from a 14-d-old culture with sterile distilled water containing 0.01% Tween 20 (v/v). The concentration of the conidial suspension was adjusted to 10^6 conidia per ml based on hemacytometer counts and used as the inoculum for conidial germination.

Mycelial disc of isolate Cs0701 was grown in Erlenmeyer flasks (100 ml) containing 50 ml of potato dextrose broth (PDB; Difco Laboratories Michigan, USA) for 7 d at 25°C on a rotary shaker (140 rpm). The culture medium was filtered through a Whatman No. 1 filter paper and sterilized twice, by filtration through a 0.45 μm and then 0.22 μm Millipore membrane. The culture filtrates were screened for antifungal activity against the plant pathogenic fungi. Fifteen μl culture filtrate of isolate Cs0701 was mixed with an equal volume of conidial suspension of tested plant pathogenic fungi and drops on a cavity glass slide. Tested plant pathogenic fungi had grown in the presence of 15 μl sterilized distilled water was used as the control. Cavity glass slides with conidia were kept moist by placing them on L-shaped glass rods on moistened paper towels in Petri dishes (90 mm) sealed with parafilm and then incubated at 25°C (0L: 24D). All treatments were replicated three times. Two hundred conidia per slide were examined under a light microscope, and the germination rate was calculated after 15-hr incubation. Inhibition rate of conidial germination (%) = (mean germination rate of control – mean germination rate of plate with culture filtrates) / mean germination rate of control × 100.

The effect of lyophilized liquid culture filtrates of *S. lanosoniveum* on conidial germination of *Oidium murrayae* Hosag. & Br., powdery mildew of jasmine orange (*Murraya paniculata* Jack.) was conducted on plain glass slides following the slide spore-germination method mentioned above except using 2.5% cellulose acetate in acetone instead of distilled water. Glass slides were coated with cellulose acetate film by spreading 200 μl of 2.5% cellulose acetate in acetone (Zaracovitis, 1964; Chu et al., 2006) which containing lyophilized liquid culture filtrates (0.01%) over a 75-by-25-mm area at the center of a slide and evaporating the acetone in a fume hood. Conidia of *O. murrayae* were added by gently tapping diseased leaves of jasmine orange held with a forceps approximately 30 cm above the slides, and slides were kept moist as described above. Germination was recorded after incubation at 25ºC for 24 h, and 200 spores were counted in each of three replicates. The inhibition rate of conidial germination was calculated as described above.

2.6 Statistical analysis

The data were subjected to analyses of variance (ANOVA). The treatment mean values were compared by the use of Fisher's least significant difference test. Values of $P < 0.05$ were considered significant.

III. RESULTS AND DISCUSSION

3.1 Pathogenicity test of *S. lanosoniveum* on plants and aphids

After 7-d of incubation, no symptoms developed on inoculated aquatic plants, *Lemma perpusilla*, *Pistia stratiotes*, and *Eichhornia crassipes* and vegetable soybean (cvs. Kaohsiung No. 8 and Kouki), while control of 4 tested plants remained symptomless. This is consistent with recent results showing that *S. lanosoniveum* did not cause lesions or necrosis on soybean in either coinoculated treatments or *Simplicillium*-only controls (Ward et al., 2012). *S. lanosoniveum* was previously reported to be the causal agent of brown spot on the aquatic ferns *Salvinia auriculata* and *Salvinia molesta* in Taiwan (Chen et al., 2008). Furthermore, Dong et al. (2011, 2014) reported an endophytic fungus, *S. lanosoniveum* var. *tianjinienss* Q. L. Dong, was isolated from *Chroococcus* (a genus of cyanobacteria). Yu et al. (2013) confirmed *S. lanosoniveum* was one of

alkaloids-producing fungal endophytes isolated from wild *Sophora alopecuroides* in Ningxia, China. However, no symptoms were observed on *P. stratiotes*, *E. crassipes*, *L. perpusilla* and *G. max* that have been inoculated with isolate Cs0701. Thus pathogenicity test on host plant of *S. lanosoniveum* showed that it may have a very narrow host range. However, in addition to the ability of *S. lanosoniveum* to cause brown spot on *Salvinia auriculata* and *S. molesta*, further a broad assessment of possible plant host range is required.

After spraying spore suspension of isolate Cs0701 (1×10^6 conidia/ml), 86.33% of the rusty plum aphids, *Hysteroneura setariae*, were infected within 5-d, whereas the control aphids remained symptomless (Fig. 1). *S. lanosoniveum* isolate Cs0701 had high virulence against the aphids, *Aphis gossypii* and *Ceratovacuna lanigera*, while control remained symptomless (Table 1). Isolates recovered from inoculated aphids showed the same morphological characteristics as the original isolates thus completing Koch's postulates. Aphids are serious insect pests in greenhouses crops, especially cucumber, pepper, and tomato, all over the world. They multiply rapidly and transmit plant viruses. The inadequate usage of pesticides has resulted in insecticide resistance. Fungal pathogens of aphids were safer and natural alternatives to pesticides (Kim et al., 2001). Biological control, including the use of entomopathogenic fungi, is an emerging strategy used for controlling aphids. *Lecanicillium* spp. have been mass-produced, and considered as biocontrol agents against some insect pests. Fadayivata et al. (2014) suggests that *L. longisporum* has high virulence against the aphids *Sipha maydis* and *Metopolophium dirhodum*. The *S. lanosoniveum* isolate Cs0701 was shown to be pathogenic to the aphids. Therefore, a broad assessment of pathogenicity of *S. lanosoniveum* on other aphids needs to be further studied. These results suggest the potential of a dual role for *S. lanosoniveum* as microbial control agents against aphids and the soybean rust pathogen, *P. pachyrhizi* (Ward et al., 2012).

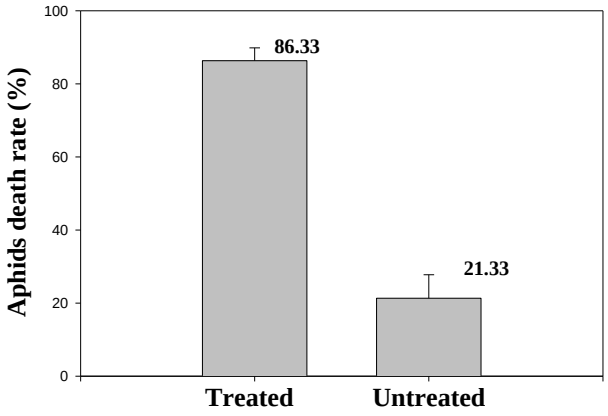

FIG. 1. PATHOGENICITY TEST OF *SIMPLICILLIUM LANOSONIVEUM* ISOLATE CS0701 ON RUSTY PLUM APHIDS (*HYSTERONEURA SETARIAE*) 5 DAYS AFTER INOCULATION.

3.2 Inhibition of mycelial growth by *S. lanosoniveum* exudates

Using the cellophane paper antibiosis method, five plant pathogenic fungi were cultivated up to 3-d in media pre-inoculated with *S. lanosoniveum*. In this study, *S. lanosoniveum* exudates showed varied antifungal potentials when tested. Mycelial growth of *S. rolfsii*, *A. brassicicola* and *R. solani* was inhibited between 70% - 80% compared to control medium (Table 2). *S. lanosoniveum* exudates inhibited the mycelial growth of *C. miyabeanus*, *C. lunata* and *Fusarium* sp. between 30% - 50%, while the exudates had no effect on the mycelial growth of *Geotrichum* sp.

TABLE 1

DISEASE DEVELOPMENT OF THE MELON OR COTTON APHID, *APHIS GOSSYPII* ON DETACHED TARO AND CUCUMBER LEAVES, AND SUGARCANE WOOLLY APHID, *CERATOVACUNA LANIGERA*, ON DETACHED SUGARCANE LEAVES, TWO DAYS AFTER INOCULATION WITH *SIMPLICILLIUM LANOSONIVEUM* ISOLATE CS0701

Pathogen	Mortality (%)[1]		
	A. gossypii on taro	*A. gossypii* on cucumber	*C. lanigera* on sugarcane
S. lanosoniveum	93.0 a	99.0 a	98.5 a
Un-inoculated	5.5 b	20.5 b	36.5 b

[1] ***The percentages of mortality in each horizontal row with different letters are significantly different according to Fisher's least significant difference (P = 0.05).***

TABLE 2
THE ANTAGONISTIC ACTIVITY OF *SIMPLICILLIUM LANOSONIVEUM* ISOLATE CS0701 CULTURE EXUDATES ON PLANT PATHOGENIC FUNGI USING THE CELLOPHANE PAPER ANTIBIOSIS METHOD

Plant pathogenic fungi	Mycelial growth inhibition (%)[1]
Sclerotium rolfsii	80.28 ± 1.61
Alternaria brassicicola	74.85 ± 4.09
Rhizoctonia solani	72.57 ± 6.09
Cochliobolus miyabeanus	49.98 ± 13.29
Fusarium sp.	47.62 ± 1.47
Curvularia lunata	33.51 ± 3.03
Geotrichum sp.	12.81 ± 11.1

[1] The values indicate means ± standard deviation of 3 replicates.

3.3 Inhibition of conidial germination by *S. lanosoniveum* culture filtrates

The culture filtrates of *S. lanosoniveum* isolate Cs0701 were screened for antifungal activity against the conidial germination of plant pathogenic fungi. The conidial germination inhibition of isolate Cs0701 on *A. brassicicola*, *C. miyabeanus* and *C. lunata* (62.67-84.33%) were observed (Table 3). The data presented here indicate that the antifungal potential of extracellular metabolites from *S. lanosoniveum* against some plant pathogenic fungi. These results in our study are in agreement with the findings that compounds produced by antagonistic fungi have potential antifungal activities against plant pathogens (Mercier and Manker 2005; Koitabashi 2005).

TABLE 3
THE EFFECT OF *SIMPLICILLIUM LANOSONIVEUM* ISOLATE CS0701 CULTURE FILTRATE ON CONIDIAL GERMINATION OF PLANT PATHOGENIC FUNGI

Strain	Conidial germination inhibition (%)[1]			
	Alternaria brassicicola	***Cochliobolus miyabeanus***	***Curvularia lunata***	***Geotrichum* sp.**
S. lanosoniveum isolate Cs0701	84.33±3.51	66±2.65	62.67±3.21	64±1.73

[1] The values indicate means ± standard deviation of 3 replicates.

Oidium murrayae was used as the test organism because of its high germination rate. Eighteen Chinese herbs contained substances in their aqueous extracts inhibitory to conidial germination of *O. murrayae* (Chu et al., 2006). The culture filtrate was also able to inhibit conidial germination of *O. murrayae* (inhibition rate 74.62%) (Fig. 2). Elkot and Derbalah (2011) also suggested the possible use of the culture filtrates of *Epicoccum nigrum*, *E. minitans*, *Trichoderma harzianum* and *T. viride* as alternative to fungicides for powdery mildew control in squash. Dong et al. (2011) demonstrated that strain DT06 of *S. lanosoniveum* produced bioactive metabolites (with the highest yield on Sabouraud's medium), exhibiting strong antimicrobial activity against Gram positive bacteria, and the biosynthesis of which was partly associated with cell growth. Therefore, further studies are needed to assess the isolation, purification, and structure elucidation of the active antifungal metabolites produced by *S. lanosoniveum* isolate Cs0701.

Kim et al. (2007) demonstrated *Lecanicillium* species had high virulence against the aphids and suppressed development of cucumber powdery mildew caused by *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff, (2000) (= *Sphaerotheca fuliginea* (Schlechtend.: Fr.) Pollacci). *Lecanicillium* spp. had the potential of a dual role for as microbial control agents against aphids and powdery mildew. Overall, the results from this study indicate that *S. lanosoniveum* is a promising biocontrol agent and a potent producer of antifungal compounds. Further pot and field experiments need to be conducted to determine whether the effectiveness of *S. lanosoniveum* for controlling aphids and plant pathogenic fungi including powdery mildew.

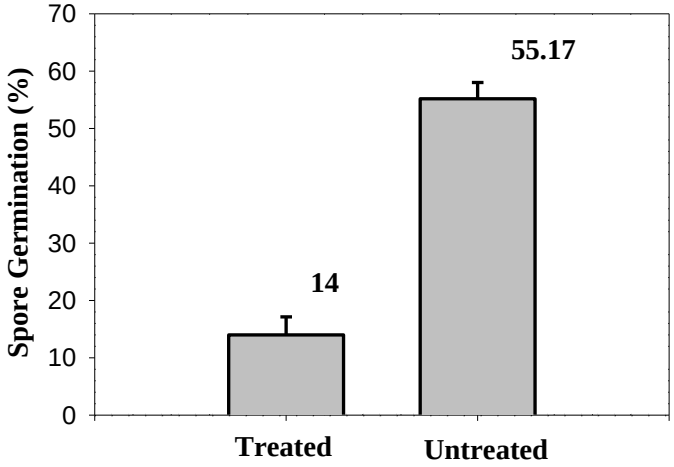

FIG.2. EFFECT OF CULTURE EXTRACT OF *SIMPLICILLIUM LANOSONIVEUM* ISOLATE CS0701 ON CONIDIAL GERMINATION OF POWDERY MILDEW OF JASMINE ORANGE (*MURRAYA PANICULATA*), *OIDIUM MURRAYAE*.

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