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Running title: Microsclerotia of *Lecanicillium lecanii*

Production of microsclerotia of the fungal entomopathogen *Lecanicillium lecanii* (Hypocreales: Cordycipitaceae) as a biological control agent against soil-dwelling stages of *Frankliniella occidentalis* (Thysanoptera: Thripidae)

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Microsclerotia (MS) production by two isolates of *Lecanicillium lecanii* on various culture media is described, and the efficacy of MS against western flower thrips is evaluated. High concentrations of MS (2.9-3.1×10^5•mL^{-1}) were produced in media with C: N ratios of 7.4:1 and 10.3:1 by isolate SN21. Bioassays using soil-incorporated MS resulted in significant infection and mortality of thrips.

Key words: *Lecanicillium lecanii, Frankliniella occidentalis,* microsclerotia, Fermentation integrated biological pest control

Western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a pest of global significance on a wide range of economically important crops (Kirk and Terry, 2003; Reitz, 2009). Many isolates of *Lecanicillium* spp. (previously known as *Verticillium lecanii*) have been applied as biopesticides for control of *F. occidentalis* (Faria and Wraight, 2007; Ansari et al., 2008). This study describes the formation of microsclerotia (MS) by two isolates of *Lecanicillium lecanii* (Zimm.) Zare & W. Gams, and investigates their activity against *F. occidentalis* in order to provide a basis for development of a biological control technique using *L. lecanii* MS.

A colony of western flower thrips was maintained as described by Ansari et al. (2008). Briefly, about 40-50 adult thrips were introduced into 0.5 L ventilated glass jars containing 3-4 pieces of green bean (*Phaseolus vulgaris* L.) pods at 26 ± 1°C, 60–70% RH and L14:D10 photoperiod. After 12 hours, egg-infested beans were transferred to fresh glass jars. First instars hatched 3 days later and were transferred to fresh glass jars with fresh green bean pods, using a fine camel hair brush. After 3 days, second instars (L2) were collected for experimental use.

The origin and source of the two fungal isolates are as follows: (1) NA1, derived from *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) collected in Nongan, Jilin (2010); (2) SN21, derived from *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) collected in Shenyang, Liaoning (2011). Stocks of isolates NA1 and SN21 were identified as *Lecanicillium lecanii sensu stricto* (Kouvelis et al., 2008) and deposited in the Agricultural Culture Collection of China at the Chinese Academy of Agricultural Sciences (Beijing, China) under accession numbers 30840 and 30841. Both isolates have been under evaluation by our research group as

mycoinsecticides for control of western flower thrips (unpublished data). Each isolate was cultured on agar media and stored in 10% glycerol at -80°C until use.

Liquid culturing, measuring and drying of MS were carried out, as described by Shearer and Jackson (2006) and Jackson and Jaronski (2009), with minor modifications. Briefly, in all liquid culture experiments, an initial concentration of 5×10^6 conidia•ml^{-1} of *L. lecanii* was incubated in medium composed of a nitrogen source (either soybean powder or casamino acids, 10-50 g•L^{-1}), carbon source (glucose, 10-72 g•L^{-1}) and basal salt solution (Jackson and Jaronski, 2009). Cultures were incubated at 25±1°C on rotary shaker incubators (SPH-103B, SHIPING lab equipment Co. Ltd., Shanghai, China) at 150 rpm. Carbon concentration and C: N ratio calculations were based on 40% carbon in glucose, and on 10% carbon and 11% nitrogen in soybean powder (Zabriske et al., 1982) (Table 1). Three replicate flasks for each isolate and media treatment were used, and the entire experiment was repeated twice at different times.

At seven days post-inoculation, all the MS (>50 µm in diameter) in 50 µl of culture broth from each flask were counted using a microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, JAPAN). Fifteen grams of diatomaceous earth (DE) were then added to 300 ml culture broth. The MS-DE mixture was filtered in a circulating water pump (SHZ-Ⅲ, Yarong Biochemical Instrument Factory. Shanghai, China), dried (moisture content ≤5%) in a vacuum dryer (LGJ-10D, Four Rings Scientific Instrument Co., Ltd. Beijing, China), vacuum packed in aluminum foil bags with a vacuum packer (DZ-260, Beijing Jiaode Packaging Machinery Co., Ltd.), and stored at 4°C for later experimental use. The liquid culturing media that produced the most MS for each isolate were used in the following experiments to determine conidial production and the efficacy against western flower thrips of the isolates.

Conidial production was determined for air-dried, MS–DE preparations according to the methods of Jackson and Jaronski (2009). Twenty-five mg of MS–DE formulation was sprinkled onto the surface of a water agar plate, and plates were incubated for eight days at 25°C. Each water agar plate was then flooded with 5 ml of sterile water and the conidia were dislodged from the MS–DE granules using a sterile loop. The concentration of conidia was measured using a hemocytometer under a microscope. To determine the number of conidia that each *L.*

lecanii isolate produced per gram of dried MS–DE preparation, the number of conidia harvested per plate was divided by the weight of the dried MS–DE preparation added to each water agar plate (0.025 g).

The entomopathogenic efficacies of the MS–DE mixtures of the *L. lecanii* isolates were assessed according to the methods of Ansari et al. (2008), with minor modifications. Briefly, in 250-ml plastic pots, dried MS–DE mixtures that had been stored for either 1 or 90 days were uniformly mixed into plant growth media, composed of vermiculite, clay loam, and nutrition soil in a 2:1:1 proportion, to yield a final concentration of 100 μg MS–DE•100 g^{-1} media. One ventilation hole (6 cm diameter) was made in the lid of each pot and covered with nylon gauze (64 μm pore size). A 5×4 cm section of a yellow sticky trap with thrips attractant added (Lei et al., Chinese patent no. 200910090398.1) was attached to the inner part of the lid to trap adult thrips. Ample water was added to pots (effluent water was observed from the bottom of pots) every 6 days. Controls consisted of growing media, water and no MS-DE.

One week after the first watering of the MS-mixed medium, a small piece of bean pod (2 cm length) was placed on the media surface in each pot as a source of food for thrips, and twenty L2 thrips were added to each pot. Pots were kept in a constant temperature room (25±1°C, 60–70%R.H., and L14:D10 photoperiod). Experiments were replicated five times for each isolate and the whole experiment was conducted twice.

Emerging adults were attracted to and then adhered to the sticky traps. On the eighth day after the first thrips adult emergence, sticky traps were removed from pots, and adults trapped on sticky cards were incubated separately at 25°C in Petri dishes lined with moist filter paper. At ten days post-incubation, thrips were examined microscopically, and the numbers of fungi-infested and healthy adults were recorded.

For each study (MS production and efficacy), data from the repeated experiments were pooled for statistical analysis. In the efficacy study, mortality data were normalized using the arcsine square root transformation before further analysis. SPSS (version 11.0) software was used for ANOVA followed by Duncan's multiple range tests to detect statistical differences among treatments. Significance was set at the 5% probability value.

Both isolates produced fewer and less uniform MS with casamino acids as the nitrogen source than with soybean powder as the nitrogen source (data not shown). With soybean powder

as the nitrogen source, the yield of *L. lecanii* MS varied among culture media for each isolate (Table 1). For isolate NA1, the highest MS concentrations were produced in both carbon-poor (5 g•L^{-1}) media with C: N ratios of 4.5:1 and carbon-rich (25 g•L^{-1}) media with C: N ratios ranging from 4.5:1 to 10.3:1; lower MS concentrations were produced in other culture media (F=13.29; df=5, 12; P<0.001). For isolate SN21, the highest MS concentrations were produced in carbon-rich (29.2 and 31.6 g•L^{-1}) liquid media with C: N ratios of 7.4:1 and 10.3:1 respectively, and significantly lower MS concentrations were produced in other culture media (F=17.412; df= 5, 12; P<0.001). Conidia production by air-dried, MS–DE preparations of *L. lecanii* was 87.3×10^5 conidia•g^{-1} for isolate NA1 and 87.3×10^5 and 142.6×10^5 conidia•g^{-1} for isolate SN21.

MS-DE treated soils produced significantly greater mean mortalities of western flower thrips than control soils, but there were no significant differences in mortality between the isolates (storage for 1 day: 98%, 100% and 0% for NA1, SN21 and control, respectively; F=4574.129, df=2,6, P<0.001; storage for 90 days: 72%, 84% and 0% for NA1, SN21, and control, respectively; F=95.588, df=2,6, P<0.001).

Our results indicate that MS production by *L. lecanii* isolates NA1 and SN21 is affected by liquid culture conditions and that MS of both isolates significantly reduce *F. occidentalis* survival through the production of infective conidia. In our study, the use of casamino acids was not conducive to MS formation by *L. lecanii,* which is in contrast to results of studies of MS formation by other fungal species, including *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore (Jackson and Schisler, 1995)*, Mycoleptodiscus terrestris* (Gerd.) Ostaz. (Shearer and Jackson, 2006), and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Jackson and Jaronski, 2009). Furthermore, the highest MS concentrations by the two isolates in our study, NA1 and SN21, were achieved with different carbon content and C: N ratios in the liquid media. Thus, it is apparent that environmental conditions required for formation of MS varies among fungal species and even among isolates of a particular species. The MS of both isolates proved to be highly virulent to western flower thrips, even after 90 days of storage. Both isolates produced greater mortality than observed in previous studies (Vestergaard et al. 1995; Gouli et al. 2009), although differences in thrips species and development stages used in experiments cannot be discounted.

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192 **Table 1. Comparison of MS production by isolates of *L. lecanii* in various liquid culture media[a]**

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Isolate	Culture Media				Microsclerotia
	C ($g \bullet L^{-1}$)	C:N	Glucose ($g \bullet L^{-1}$)	Soybean powder ($g \bullet L^{-1}$)	(mean MS× $10^4 \bullet mL^{-1}$)
NA1	5	4.5:1	10	10	16.9a
	5.8	7.4:1	12.8	7.2	6.8bc
	6.4	10.3:1	14.4	5.6	5.8c
	25	4.5:1	50	50	12.6ab
	29.2	7.4:1	64	36	10.8abc
	31.6	10.3:1	72	28	14.6a
SN21	5	4.5:1	10	10	6.4c
	5.8	7.4:1	12.8	7.2	12.0b
	6.4	10.3:1	14.4	5.6	8.1bc
	25	4.5:1	50	50	9.3bc
	29.2	7.4:1	64	36	29.3a
	31.6	10.3:1	72	28	31.2a

194 [a] For each isolate, mean values followed by the different letters are significantly different using

195 Duncan's multiple range test ($\alpha = 0.05$).

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