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Solid cultures of thrips-pathogenic fungi *Isaria javanica* strains for enhanced conidial productivity and thermotolerance

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ABSTRACT

Entomopathogenic fungi have great potential to control agricultural and horticultural insect pests, however optimizing conidial production systems to demonstrate high productivity and stability still needs additional efforts for successful field application and industrialization. Although many virulent entomopathogenic fungal isolates have been viewed as potential candidates in a laboratory environment, very few of the isolates are being used in practice for application in agricultural fields as commercial products. *I. javanicus* is an entomopathogenic fungus that is parasitic to various diverse coleopteran and lepidopteran insects and thought good candidate as biopesticdes. In this work, the basic characteristics of two entomopathogenic fungi, *I. javanica* FG340 and Pf04, were investigated in morphological examinations, genetic identification, and virulence against *Thrips palmi*, and then the feasibility of various grains substrates for conidial production was assessed, particularly focusing on conidial productivity and thermotolerance. *Isaria javanica* FG340 and Pf04 conidia were solid-cultured on 12 grains for 14 days in a Petri dish. Of the tested Italian millet, perilla seed, millet and barley-based cultures showed high conidial production. The four-grain media yielded $> 1 \times 10^9$ conidia/g of *I. javanica* FG340 and Pf04. Pf04 strain had enhanced thermotolerance up to 45 °C when cultured on Italian millet. In application, it was easy to make a conidial suspension using the cultured grains, and several surfactants were tested to release the conidia. This work suggests several possible inexpensive grain substrates by which to promote conidial production combined with enhanced stability against exposure to high temperature.

Introduction

Entomopathogenic fungi are frequently isolated from soils and insects throughout the world, and have been documented to occur naturally in over 700 host species (Hajek and St. Leger, 1994; Shah and Pell, 2003). These fungi can play an important role in the regulation of their insect host population and are typically harmless to the environment and safe for humans (Zimmermann, 2007a, 2007b). Bio-insecticides based on entomopathogenic fungi such as *Beauveria bassiana* (Bals.-Criv.) Vuill. BotaniGard®, Mycotrol® and Beauverin®, ChongchaeSak® (ERL-836) and *Isaria fumosorosea* (Wise) PreFeRal® and Priority® have been already developed as active ingredients for the microbial control of agricultural and horticultural insect pests and used for integrated pest management (IPM) programs (Castrillo et al., 2010; Kivett, 2015; Lopez et al., 2014; Meyling and Eilenberg, 2007).

The mode of action of these bio-insecticides initiates from the attachment of conidia on the surface of the host (Vega et al., 2012). The spread of conidia in agricultural fields is negatively influenced by environmental conditions such high temperature and ultraviolet radiation which results in fluctuating effectiveness of the control (Fernandes et al., 2008; Rangel et al., 2015). Furthermore, in order to industrialize the fungal bio-insecticides, the economical production and high stability during product distribution are essential for the viability of this approach. In response to this need, to be classified as an effective entomopathogenic fungi for control against insect pests, it is necessary to display virulency against the insect as well as thermotolerance. Unfortunately, it has been difficult to isolate entomopathogenic fungi that possess both advantages (Lee et al., 2015b; Shin et al., 2017).

To accomplish this, previous studies have characterized thermotolerant conidia expressed in differing grain-based solid culture systems

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and indicated that the surface characteristics of the fungal conidia vary with respect to the growth substrates (Carvalho et al., 2004; Kim et al., 2010b; Kim et al., 2011). These previous results suggest that thermotolerant entomopathogenic fungi can be successfully produced on a grain-base solid culture system, and that this technique is an important step to produce large amount of fungal conidia for the development of entomopathogenic fungal granules (Kim et al., 2010a; Lee et al., 2015a; Skinner et al., 2012).

Herein this work, we re-identified entomopathogenic fungi *I. javanica* FG340 and Pf04, which had been investigated as bio-insecticides in Korea (Han et al., 2014; Xie et al., 2016) to make sure if the two isolates are *Isaria* species before the phylogenetic analysis with other isolates within the same species, and investigated their biological performance using microscopic examination and genetic sequencing analysis, and their virulence against melon thrips, *Thrips palmi* Karny in laboratory conditions. This work was conducted to determine optimal grain substrates that efficiently produce a large amount of FG340 and Pf04 conidia and simultaneously maintain high thermotolerance when exposed to high temperature conditions. Additionally, to easily make a conidial suspension using the cultured grains in applications, several surfactants were tested to release the conidia. This work provides basic information on the development of fungal bio-insecticides.

Materials and methods

Entomopathogenic fungi and morphological examination

Entomopathogenic fungi FG340 and Pf04 were obtained from the Agricultural Microbiology Division, National Institute of Agricultural Sciences, RDA, Korea, and used in this study. Fungi were cultured on quarter strength Sabouraud dextrose agar (SDA; Difco™, USA) medium for 10–14 days in the dark. For microscopic examination, conidia and mycelia from 1/4 SDA medium were observed at ×400 on a phase contrast microscope.

Conidia were harvested by 0.03% siloxane solution (Silwet; FarmHannong Co., Ltd. Korea). The conidial suspensions were vigorously agitated,and the conidial concentrations were measured by using a hemocytometer.

PCR and sequence analysis

Fungal genomic DNA was extracted from the hyphae using a modified chemical lysis method (St Leger et al., 2009). The internal transcribed spacer (ITS) regions of the fungi were amplified by PCR using the following primers: ITS1-forward, 5′-TCC GTA GGT GAA CCT GCG G-3′; ITS4-reverse, 5′-TCCTCC GCT TAT TGA TAT GC-3′ (White et al., 1990). A thermal cycler (C-1000, Bio Rad, USA) was preheated to 95 °C for 5 min and then PCR was performed using 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a 10-min final extension at 72 °C. PCR products were analyzed by electrophoresis on 0.8% agarose gels in 1× TBE buffer, gel-purified, direct sequenced using both ITS primers listed above, and blasted against the NCBI database (http://blast.ncbi.nlm.nih.gov/blast) for identification. The representative sequences of the ITS regions of fungi from the study of Luangsa-Ard et al. (2005) were used in phylogenetic analyses (Table S1). Multiple sequence alignments were performed using the MAFFT v7.311 software, and phylogenetic trees were constructed based on the alignment produced by MEGA7 software using the neighbor-joining method. Constructed neighbor-joining trees were subjected to a bootstrap analysis with 1000 replicates, and a distance matrix was obtained from bootstrapped datasets using the maximum composite likelihood method.

Insect rearing and bioassay

The melon thrips, *Thrips palmi* Karny population was reared in a plastic cage (20 × 20 × 30 cm^3) and maintained on cucumber, *Cucumis sativus* L. at 25 °C under 40 ± 10% relative humidity, and a photoperiod of 14:10 h (L:D). For bioassays, the conidial suspension from 1/4 SDA was adjusted to 1×10^7 conidia/ml with 0.03% siloxane solution. Cucumber leaf discs (60 mm diameter) were placed on moistened filter paper in Petri dishes (60 mm diameter), and 3 ml of conidial suspension was sprayed on the leaf discs using a micro sprayer. After 10 min, ten female adult *T. palmi* (up to three days old) were collected and transferred into each dish. The 0.03% siloxane solution was used as control, and the experiment was replicated three times. All dish covers were closed and kept at 25 °C and 40 ± 10% RH with a 16:8 (L:D) regime. Dishes were checked daily to determine the number of dead *T. palmi* until six days after the treatment.

Mass culture on grain media

The fungi were cultured using a grain-based solid culture system. A total of 12 grain types, barley (*Hordeum vulgare* L.), brown rice (*Orysa sartiva* L.), buckwheat (*Fagopyrum esculentum* Moench), cassia seeds (*Senna tora* (L.) Roxb.), Italian millet (*Setaria italic* (L.) P. Beauvois), millet (*Panicum miliaceum* L.), mung beans (*Vigna radiate* (L.) R. Wilczek), oatmeal (*Avena sativa* L.), perilla seed (*Perilla frutescens* (L.) Britton), rice (*Oryza sativa* L.), sesamum (*Sesamum indicum* L.), grain sorghum (*Sorghum bicolor* (L.) Moench) were purchased from local markets and used in the experiments. The grain (100 g) was mixed with 50 ml of distilled water and supplemented with 160 ul of 50% citric acid solution in a polyethylene bag. The bags were pre-heated in a microwave for 5 min, and autoclaved at 121 °C for 15 min. The grain media were transferred to a new 90 mm Petri dish (approximately 10 g/dish) and inoculated with 1 ml of a conidial suspension (1×10^7 conidia/ml with 0.03% siloxane solution). All Petri dishes were incubated at 25 ± 1 °C with a 16:8 (L:D) photoperiod for 14 days. After incubating, the solid cultured conidia were collected with a 0.03% (v/v) siloxane solution and counted using hemocytometer under a microscope. The experiments were repeated three times.

Thermotolerance assay

The cultured fungi from each grain medium were suspended in 0.03% siloxane solution, and then vortexed for 5 min. After vortexing, 1 ml of conidial suspensions (1×10^7 conidia/ml) were transferred to a 1.5 ml microcentrifuge tube and placed in an incubator adjusted at 45 ± 1 °C for 30, 60, 90, and 120 min. Control conidial suspensions were not heat-exposed. After incubation, 10 ul of the conidial suspension from each sample was inoculated on 1/4 SDA medium and incubated at 25 ± 1 °C for 16 h. The relative percent germination was calculated by comparing germination to a control. At least 100 conidia were examined for each treatment in every experiment and assays were repeated three times.

Surfactant assay for conidial release

The surfactant assay followed a previous report by Lee et al. (2016). Eight total surfactants were used in the experiment, six of the surfactants, Silwet L-77, castor oil (CO-2.5), castor oil (CO-12), polyoxyethylene lauryl ether (LE-7), poly (oxyethylene, oxypropylene) glycol block copolymer (PE-61), and polyoxyethylene isotridecyl ether (TDE-3), were obtained from FarmHannong Co., Korea, and the other two surfactants, polyoxyethyelene sorbitan monooleate Tween-20 and Tween-80, were purchased from Sigma-Aldrich Chemical Co, USA. The cultured fungi (2 g) from each grain media were packed in the screen bags (Mesh Bag™, Hana Commerce Co., Seoul, Korea) and then placed in 200 ml of 10% surfactant. After shaking at 150 rpm for 5 min, the number of conidia released into solution was counted by a hemocytometer. No surfactant was used in the negative control. The experiments were repeated three times, and a 10% siloxane solution and sterile water served as positive (with vortexing instead of shaking at