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SHORT COMMUNICATION

Production of thermotolerant entomopathogenic *Isaria fumosorosea* SFP-198 conidia in corn-corn oil mixture

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Abstract Low thermotolerance of entomopathogenic fungi is a major impediment to long-term storage and effective application of these biopesticides under seasonal high temperatures. The effects of high temperatures on the viability of an entomopathogenic fungus, *Isaria fumosorosea* SFP-198 (KCTC 0499BP), produced on different substrates amended with various additives were explored. Ground corn was found to be superior in producing the most thermotolerant conidia compared to yellow soybean, red kidney bean, and rice in a polyethylene bag production system. Using ground corn mixed with corn oil as a substrate resulted in only 7% reduction in germination compared to ground corn alone (67% reduction) after exposure of conidia to 50°C for 2 h. Corn oil as an additive for ground corn was followed by inorganic salts (KCl and NaCl), carbohydrates (sucrose and dextrin), a sugar alcohol (sorbitol), and plant oils (soybean oil and cotton seed oil) in ability to improve conidial thermotolerance. Unsaturated fatty acids, such as linoleic acid and oleic acid, the main components of corn oil, served as effective additives for conidial thermotolerance in a dosage-dependant manner, possibly explaining the improvement by corn oil. This finding suggests that the corn-corn oil mixture can be used to produce highly thermotolerant SFP-198 conidia and provides the relation of unsaturated fatty acids as substrates with conidial thermotolerance.

Keywords Corn oil · Ground corn · *Isaria fumosorosea* · Thermotolerance · Unsaturated fatty acid

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Introduction

Entomopathogenic fungi, in common with other natural enemies of insects, can be employed to control agricultural pests in a biological control strategy. In the storage and applications of the fungi as biopesticides, however, uncontrollable exposure to high temperature conditions is a usual event, which consequently can reduce the shelf life of those fungi [5, 6, 15, 19]. The minimum shelf life of biopesticides required for successful development is 12–18 months [19]. Nevertheless, currently registered fungal biopesticides, produced in soybeans or rice, show ca. 6–12 months of shelf life at room temperature. The variation of shelf life depends on the sorts of formulation and the natures of commercialized isolates [4].

Physiologically, thermotolerance is closely related to the endogenous accumulation of polyols and trehalose in fungi [9–11, 22, 26], and the alteration of cell wall lipid compositions in yeast and bacteria [7, 8, 23, 29]. Such tolerance can be acquired by careful manipulation of the osmotic pressure of culture media [9, 21, 28] or by supplying materials as substrates for the induction of thermotolerance [3, 14, 24, 25, 27]. In the present work, to produce thermotolerant entomopathogenic *Isaria fumosorosea* SFP-198 (KCTC 0499BP) conidia in a polyethylene bag production

system, effects of high temperature on the germination of SFP-198 conidia produced on different substrates augmented with various additives were investigated. The isolate shows excellent insecticidal activity against greenhouse whiteflies, aphids, and pine gall midges [16, 17]. Briefly, ground corn, selected among two cereal grains and two legumes, was amended with four groups of substrates as follows: (1) two inorganic salts to increase osmotic pressure; (2) two carbohydrates as raw materials for polyols or trehalose; (3) a sugar alcohol as a raw material for polyols; and (4) three plant oils, assuming the oils may affect the composition of lipids in cell walls or the production of polyols within cells. Consequently, a ground corn-corn oil mixture was developed as a production medium. The work was followed up by investigating the influence of unsaturated fatty acids, the main components of corn oil [12], on conidial thermotolerance to provide a plausible explanation for the improvement by corn oil.

Materials and methods

Fungal isolate

I. fumosorosea SFP-198 (KCTC 0499BP) was deposited at the Korean Collection for Type Culture (http://kctc.kribb.re.kr/). The isolate was propagated on Sabouraud dextrose agar medium supplemented with 0.5% (w/v) yeast extract (SDAY, pH 6.0) in petri dishes in a dark place at 25 ± 1°C for 14 days [11].

Solid culture

The abilities of two cereal grains and two legumes to produce thermotolerant conidia were compared. This was followed by the evaluation of ground corns incorporated with various additives concerning their abilities to further improve conidial thermotolerance. First, 100 g of yellow soybean (*Glycine max* L. Merr.), red kidney bean (*Phaseolus vulgaris* L. var. *humilis*), rice (*Oryza sativa* L., Japonica type), or coarse-ground corn (*Zea mays* L.) (all purchased at a local organic food market) was mixed with distilled water (50 ml) in a polyethylene bag (25 × 20 cm) (three bags/treatment). As a subsequent trial, soaked coarse-ground corn (100 g corn + 50 ml distilled water) was augmented with 1 g of an additive such as an inorganic salt [KCl or NaCl (all from Daejung)], a carbohydrate [sucrose or dextrin (all from Daejung)], a sugar alcohol [D-sorbitol (Daejung)], or a plant oil [soybean oil, cotton seed oil, or corn oil (all from Fisher Scientific)] in a polyethylene bag. Lastly, 1, 2, 4, or 8 g of an unsaturated fatty acid, such as linoleic acid or oleic acid (all from Fisher Scientific), or corn oil was added to soaked coarse-ground corn prepared by using the method described above.

All polyethylene bags were incubated at 80°C for 1 h in a water bath prior to autoclaving at 121°C for 30 min [13]. A liquid culture as an inoculant was produced in 200-ml SDY broth (pH 6.0) with 1×10^6 conidia/ml of initial density on a rotary shaker (150 rpm) at 25 ± 1°C for 3 days. An inoculum (2 ml) was pipetted into each polyethylene bag. All bags were shaken for ca. 1 min to ensure complete distribution of the inocula throughout the media. Subsequently, all bags were sealed with sterile paper towels for ventilation: a front part of a bag entered into a bottom-cut paper cup (diameter of mouth part, 6.0 cm; height, 8.0 cm), folded out, and the mouth of the cup was covered with two layers of sterile paper towels (Bounty), then secured with a rubber band. After incubation in a dark place at 25 ± 1°C for 3 weeks, all bags were opened and allowed to dry in a small room with a dehumidifier for 2 days. The materials were considered ready for use when moisture content was <5% as follows (mean ± SE, %): mycotized-yellow soybean = 3.0 ± 1.6; -red kidney bean = 2.5 ± 1.1; -rice = 2.8 ± 1.0; and -coarse-ground corn = 2.6 ± 0.9. To measure the production of conidia (number of conidia/g dried mycotized substrate), 10 g of dried mycotized substrate from each bag was mixed with 0.2% (v/v) Tween 80 solution to prepare a 100-ml suspension. The number of conidia was counted using a haemacytometer with a microscope (400×). Conidia powder for a thermotolerance test was collected from mycotized substrates by passing them through a 325-mesh sieve once. Each treatment was replicated three times in an experimental solid culture replicate, and the entire solid culture was repeated twice on different days.

Thermotolerance test

Dried conidia powders were exposed to a high temperature for a given time, and germination rates of the conidia were assessed. Conidia powder (0.1 g) from each bag, held in a capped 15-ml conical tube (three tubes/treatment), was exposed to 50°C for 2 h in an incubator. After incubation, the conidia powder was suspended in 0.2% (v/v) Tween 80 solution and adjusted to ca. 1×10^7 conidia/ml. The Tween 80 solution, used for improving wettability of conidia, showed no adverse effects on the germination of SFP-198 conidia. A sample (20 µl) from each suspension was dropped onto SDAY medium, and all plates were incubated at 20 ± 1°C for 24 h. The germination rate was determined by randomly counting the number of germinated and ungerminated conidia among 100 counts microscopically (400×). A conidium was considered to be germinated if a germ tube had

formed [1]. Unstressed conidia suspension served as a control treatment (0 min). Each treatment was replicated three times in an experimental replicate, and the entire experiment was repeated twice using different batches of conidia on different days.

Data analysis

Data on the number of conidia and germination rates were analyzed by an analysis of variance (ANOVA) and a general linear model (GLM), respectively, considering blocking effects caused by experimental repetition. They were followed by Duncan's multi-range test (MRT) for multiple comparison. All the analyses were conducted using a SPSS ver. 17.0 (SPSS Inc. 2009, Chicago, IL) at the 0.05 (P) level.

Results and discussion

Thermotolerance in ground corn amended with additives

Ground corn was found to be superior to yellow soybean, red kidney bean, and rice in producing the most thermotolerant conidia, showing the greatest production (Table 1). Subsequently, using ground corn supplemented with corn oil as an additive resulted in the greatest further improvement in conidial thermotolerance without a significant decrease in conidial production, as compared to the other treatments including the control (Table 2). The ground corn-corn oil treatment showed the least reduction (6.5%) in conidial germination after the exposure, whereas the control showed 66.8%. The ground corn-KCl and -NaCl treatments

Table 1 Number of conidia (mean ± SE) produced in 100 g of a cereal grain or legume and germination rate (mean ± SE) after exposure of conidia to 50°C for 2 h

Media	No. of conidia/g substrate (mean ± SE)	Germination rate (%) (mean ± SE)	
		Non-exposed	Exposed
Yellow soybean	$1.1 \times 10^9 \pm 1.3 \times 10^8$ c	93.3 ± 0.6 aA	23.0 ± 4.6 bB
Red kidney bean	$0.6 \times 10^9 \pm 0.5 \times 10^8$ d	91.7 ± 2.3 aA	8.3 ± 3.1 cB
Rice	$1.4 \times 10^9 \pm 0.6 \times 10^8$ b	93.0 ± 4.6 aA	7.0 ± 6.0 cB
Ground corn	$2.1 \times 10^9 \pm 1.4 \times 10^8$ a	93.7 ± 3.1 aA	39.3 ± 4.7 aA

Means followed by the same lower case letter in columns or by the same upper case letters in rows do not significantly differ by the Duncan's MRT test ($P = 0.05$). No significant differences were observed over the experimental repetitions (no. of conidia: $P = 0.037$, germination rates: $P < 0.001$)

Table 2 Number of conidia (mean ± SE) produced in 100 g of ground corn amended with 1 g of an additive and germination rate (mean ± SE) after exposure of conidia to 50°C for 2 h

Additive	No. of conidia/g substrate (mean ± SE)	Germination rate (%) (mean ± SE)	
		Non-exposed	Exposed
Inorganic salt			
KCl	$8.5 \times 10^8 \pm 1.0 \times 10^8$ bc	97.3 ± 3.1 aA	82.6 ± 4.0 bB
NaCl	$8.0 \times 10^8 \pm 1.2 \times 10^8$ bc	96.1 ± 1.2 aA	71.1 ± 5.6 cB
Carbohydrate			
Sucrose	$3.9 \times 10^8 \pm 8.2 \times 10^7$ d	95.2 ± 3.3 aA	69.4 ± 9.3 cB
Dextrin	$6.4 \times 10^8 \pm 7.3 \times 10^7$ c	97.3 ± 1.8 aA	46.8 ± 4.8 dB
Sugar alcohol			
Sorbitol	$6.4 \times 10^8 \pm 7.3 \times 10^7$ c	98.8 ± 1.2 aA	67.0 ± 9.3 cB
Plant oil			
Soybean oil	$9.1 \times 10^8 \pm 8.2 \times 10^7$ b	98.9 ± 0.6 aA	65.5 ± 6.9 cB
Cotton seed oil	$8.9 \times 10^8 \pm 8.9 \times 10^7$ b	96.8 ± 2.3 aA	50.3 ± 6.7 dB
Corn oil	$1.3 \times 10^9 \pm 1.2 \times 10^8$ a	97.7 ± 1.5 aA	91.2 ± 2.3 aB
Control			
No additive	$1.6 \times 10^9 \pm 1.6 \times 10^8$ a	98.3 ± 1.0 aA	31.5 ± 5.4 eb

Means followed by the same lower case letter in columns or by the same upper case letters in rows do not significantly differ by the Duncan's MRT test ($P = 0.05$). No significant differences were observed over the experimental repetitions (no. of conidia: $P = 0.041$, germination rates: $P < 0.001$)

also showed less reduction in germination, with 14.7 and 25.0%, respectively, than the control. It possibly suggests the relationship of osmotic pressure or water potential of the medium with conidial thermotolerance, as described above. Adding two carbohydrates, such as sucrose and dextrin, and a sugar alcohol (D-sorbitol) showed, respectively, 25.8, 50.5, and 31.8% reduction in germination. A possible association of the carbohydrates and the sugar alcohol with the accumulation of trehalose or polyols in fungal cells may be the background of the improvement, but this needs further investigation. Adding soybean oil and cotton seed oil into ground corn showed 33.4 and 46.5% reduction in germination, respectively. These results were significantly worse than that of the ground corn-corn oil treatment (6.5%). This probably suggests the different characters of the three plant oils in influencing conidial thermotolerance.

Thermotolerance in ground corn amended with unsaturated fatty acids

The ground corn-unsaturated fatty acid treatments increased conidial thermotolerance in a dosage-dependent manner (Table 3). It possibly provides a clue about the further improvement in the corn-corn oil treatment. Overall, the more linoleic acid or oleic acid was added to ground corn, the better the thermotolerance of conidia, but the less conidial production. The contribution of linoleic acid to conidial thermotolerance was more significant than that of oleic acid at the same dosages.

Unsaturated fatty acids, the main components of corn oil, may be involved in the production of highly thermotolerant conidia, given the roles of unsaturated fatty acids in protecting cells from thermal stress. Different levels of conidial thermotolerance among the three plant oil treatments based on ground corn may be explained by the percentages of the two unsaturated fatty acids in the three plant oils. The percentage of the unsaturated fatty acids in corn oil is greater than those in the other oils. In detail, corn oil, soybean oil, and cotton seed oil are composed of ca. 87% (28% mono-unsaturated and 59% poly-unsaturated), 79% (21% mono-unsaturated and 58% poly-unsaturated), and 70% (18% mono-unsaturated and 52% poly-unsaturated) unsaturated fatty acids, respectively [2, 18, 20], though their percentages are dependent on the varieties of source plants.

In conclusion, a ground corn-corn oil mixture as a medium was developed to produce highly thermotolerant *I. fumosorosea* SFP-198 conidia in the polyethylene bag production system. These results also suggest that unsaturated fatty acids in corn oil are involved in the improved conidial thermotolerance. From an industrial perspective, the developed medium may be superior to current mass production media including soybeans or rice in maintaining the shelf life of fungal biopesticides, given the improved conidial thermotolerance. Long-term exposure to high temperatures may maximize the difference in shelf life between thermotolerant conidia and less thermotolerant conidia. Development of media that produce more

Table 3 Number of conidia (mean ± SE) produced in 100 g of ground corn amended with 1, 2, 4, or 8 g of an unsaturated fatty acid as an additive and germination rate (mean ± SE) after exposure of conidia to 50°C for 2 h

Additive		No. of conidia/g substrate (mean ± SE)	Germination rate (%) (mean ± SE)	
Content	Dosage (g)		Non-exposed	Exposed
Corn oil	1	$1.6 \times 10^9 \pm 1.9 \times 10^8$ a	97.3 ± 1.3 aA	89.3 ± 3.4 aB
	2	$8.2 \times 10^8 \pm 1.5 \times 10^8$ c	97.0 ± 2.5 aA	90.0 ± 3.8 aB
	4	$3.8 \times 10^8 \pm 1.0 \times 10^8$ d	97.8 ± 1.3 aA	87.8 ± 1.0 aB
	8	$1.6 \times 10^8 \pm 4.6 \times 10^7$ e	97.8 ± 1.0 aA	91.8 ± 2.9 aB
Linoleic acid	1	$1.3 \times 10^9 \pm 1.9 \times 10^8$ b	95.8 ± 3.1 aA	79.8 ± 2.6 bB
	2	$7.7 \times 10^8 \pm 1.7 \times 10^8$ c	96.5 ± 2.3 aA	79.5 ± 2.5 bB
	4	$3.9 \times 10^8 \pm 8.9 \times 10^7$ d	95.0 ± 2.5 aA	89.0 ± 2.1 aB
	8	$1.8 \times 10^8 \pm 8.3 \times 10^7$ e	95.8 ± 3.3 aA	89.8 ± 2.3 aB
Oleic acid	1	$1.1 \times 10^9 \pm 1.7 \times 10^8$ b	97.0 ± 1.7 aA	64.0 ± 5.5 dB
	2	$3.0 \times 10^8 \pm 1.1 \times 10^8$ d	96.5 ± 1.8 aA	72.5 ± 3.4 cB
	4	$9.0 \times 10^7 \pm 2.0 \times 10^7$ e	95.5 ± 3.7 aA	73.5 ± 5.4 cB
	8	$1.1 \times 10^8 \pm 6.0 \times 10^7$ e	95.0 ± 2.3 aA	84.0 ± 2.7 bB
Control				
No additive	–	$1.8 \times 10^9 \pm 1.9 \times 10^8$ a	96.8 ± 2.9 aA	36.8 ± 3.5 eB

Means followed by the same lower case letter in columns or by the same upper case letters in rows do not significantly differ by the Duncan's MRT test ($P = 0.05$). No significant differences were not observed over the experimental repetitions (no. of conidia: $P = 0.025$, germination rates: $P < 0.001$)

thermotolerant conidia can also achieve higher cost effectiveness. Cost for managing technical materials or final products at low temperatures can be reduced, and furthermore inventory can be more easily controlled thanks to the improved shelf life. Lastly, this approach—development of media—can allow commercial productions of fungal biopesticides to be more competitive than other approaches that mainly focus on the formulation of conidia to improve fungal thermotolerance. Such formulation needs additional processes in productions, probably accompanying unexpected damages to fungal growth by adding adjuvants.

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