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Production of thermotolerant entomopathogenic fungal conidia on millet grain

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Abstract Thermotolerance of entomopathogenic (insect-killing) fungi should be seriously considered before industrialization. This work describes the feasibility of millet grain as a substrate for production of thermotolerant *Beauveria bassiana* (*Bb*) GHA and ERL1170 and *Metarhizium anisopliae* (*Ma*) ERL1171 and ERL1540 conidia. First, conidial suspensions of the *Bb* isolates, produced on millet grain in polyethylene bags, were exposed to five temperatures (43–47°C) at 15-min intervals for up to 120 min (experiment I). Agar-based quarter-strength (¼) Sabouraud dextrose agar supplemented with yeast extract (SDAY) and whey permeate media served as controls. Millet-grain-based culture was superior in producing the most thermotolerant *Bb* conidia, followed by whey permeate agar and ¼SDAY-based cultures. Secondly, to compare the thermotolerance of conidia produced at the same conditions, the *Bb* isolates were then produced on agar-based millet powder medium, with ¼SDAY and whey permeate agar media as controls, and the two *Ma* isolates were added (experiment II). They were then exposed to the same temperatures as above. More thermotolerant *Bb* and *Ma* conidia were produced on millet powder agar than on whey permeate agar and ¼SDAY overall. These results suggest that millet grain can be used as a substrate to produce thermotolerant conidia in a mass production system.

Keywords Thermotolerance · *Beauveria bassiana* · *Metarhizium anisopliae* · Millet · Whey permeate

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Introduction

Entomopathogenic fungi are widely available biological control agents for management of agricultural pests, especially insects with piercing and sucking mouthparts. Several commercial products made of hypocrealean entomopathogenic fungi have been developed and industrialized as follows: *Beauveria bassiana* (BotaniGard®, Mycotrol®, and Beauverin®), *Beauveria brongniartii* (Betel®), *Lecanicillium lecanii* (Vertalec®), *Metarhizium anisopliae* (Bio-Catch-M® and Green Muscle®), *Metarhizium flavoviride* var. *flavoviride* (Biogreen®), and *Isaria fumosorosea* (PreFeRal® and Priority®) [5]. These fungal products, based on conidia, constitute a small percentage of the total insecticide market [47], possibly due to slow progress and high variation in their ability to kill insects, and short shelf-life. In the last decade, efforts have been focused on improving the shelf-life of these products. The minimum shelf-life of biopesticides required for successful development is 12–18 months [3]. Still, most of them have a 6- to 12-month shelf-life at room temperature [5]. One of the major reasons for this short shelf-life is their instability at high temperatures, i.e., low thermotolerance (percentage conidial germination after exposure to high-temperature conditions) [6, 9, 18, 24, 29].

Consideration should therefore be given to storage and application of fungi. Fungal conidia may be exposed to high-temperature conditions during distribution of products

in the market or after application on crops [3]. In the summer, the temperature of a storage room for fungal products can reach >40°C. Packing or stacking may accelerate a high-temperature effect. Transportation of fungal products under high-temperature conditions can be another reason for viability reduction. Ideally storage at <10°C is recommended, but this is not always feasible. Secondly, fungi can lose their viability after application in hot temperatures. The viability of fungal conidia should be guaranteed until their penetration into the insect cuticle.

Physiologically, thermotolerance is closely related to the production of heat shock proteins (HSPs) required for heat tolerance [27, 37, 49] and the endogenous accumulation of polyols and trehalose [13, 14, 17, 32, 39] in fungi, and the alteration of cell-wall lipid composition in yeast and bacteria [10, 11, 34, 45]. Such tolerance can be acquired by careful manipulation of the osmotic pressure of culture media [13, 33, 44], by supplying materials as substrates for induction of thermotolerance [4, 20, 36, 38, 40], or by formulation technology [23].

Manipulation of culture media allowed, in particular, entomopathogenic fungi to have enhanced thermotolerance [26]. A medium containing 4% glucose or 1% starch as carbon source greatly enhanced the thermotolerance of *B. bassiana* conidia; however, the thermotolerance decreased remarkably when sucrose was used [48]. Rangel et al. [30] reported that *M. anisopliae* conidia produced under nutritive stress were two times more thermotolerant than conidia produced under nutrient-rich conditions such as potato dextrose agar with yeast extract (PDAY) medium. Recently, Kim et al. [22] reported that a corn grain–corn oil mixture as a source of unsaturated fatty acids improved the thermotolerance of *I. fumosorosea* conidia.

The feasibility of using millet grain as a granular substrate for production of thermotolerant *B. bassiana* and *M. anisopliae* conidia is described herein. A millet-grain-based mass production system has been established at the University of Vermont to produce entomopathogenic fungi for management of western flower thrips and whiteflies in greenhouses. For this research, two *B. bassiana* isolates, GHA and ERL1170, were produced on millet grain in polyethylene bags, similar to a commercial mass production system, and exposed to high temperatures (experiment I). Conidia produced on quarter-strength (¼) Sabouraud dextrose agar supplemented with yeast extract (SDAY) and whey permeate agar were used as controls. Information on the relationship of millet grain to conidial thermotolerance was found. Quarter-strength (¼) SDAY is a standard medium widely used for propagation of entomopathogenic fungi [17]. Whey permeate has industrial potential as a substrate to produce entomopathogenic fungi [21]. To compare the thermotolerance of conidia produced at the same conditions, the *B. bassiana* isolates were then produced in agar-based millet powder medium, with ¼SDAY and whey permeate agar media as controls, and two *M. anisopliae* isolates, ERL1171 and ERL1540, were added (experiment II). They were then exposed to the same temperatures as above.

Materials and methods

Fungal isolates

B. bassiana (*Bb*) GHA and ERL1170 and *M. anisopliae* (*Ma*) ERL1171 and ERL1540 were obtained from the Entomology Research Laboratory (ERL) Worldwide Collection of Entomopathogenic Fungi at University of Vermont. ERL1170 is included, as a backup culture, in the USDA ARS collection at Cornell University as ARSEF 2060. All isolates, stored in 10% (v/v) glycerol at −80°C, were propagated initially on ¼SDAY [/l distilled water (DW), pH 6: dextrose 10 g, yeast extract 2.5 g, Bacto peptone 2.5 g, and agar 15 g] in darkness at 25 ± 1°C for 14 days [17].

Solid cultures

Millet grain and powder were used as culture substrates in experiment I and II, respectively. Two agar-based media, ¼SDAY and whey permeate agar media, served as control treatments in each experiment. In experiment I, millet grain and the two agar-based media as controls were used for observation of the potential of millet grain to increase the thermotolerance of the two *Bb* isolates. To provide additional details, experiment II was designed to investigate the thermotolerance of conidia produced at the same culture conditions (all in agar media). The two *Ma* isolates were added to experiment II.

In experiment I, *Bb* GHA and ERL1170 conidia were produced on millet grain in polyethylene bags [16, 19]. First, 500 g millet (*Panicum miliaceum* L.) grain was placed in a polyethylene bag (60 × 30 cm_2), and then 250 ml distilled water containing citric acid at 0.4 ml [50% (w/v) stock solution]/l was added to the bag. The bag was placed at 90 ± 2°C in a water bath for 1 h, followed by autoclaving at 121°C for 30 min. Each isolate treatment had four bags within an experimental replicate. After cooling to ambient temperature, each bag was inoculated with a 5-ml aliquot from a liquid culture of each isolate. The inoculum was produced in 75 ml potato dextrose broth (PDB) (Difco, MD) in a 250-ml baffled flask which was held on a rotary shaker (180 rpm) at 25 ± 1°C for 3 days. All inoculated bags were shaken for ca. 1 min to ensure complete distribution of the inocula throughout the millet grain. Bags were held at 25 ± 1°C and a 16:8 (L/D)

photoperiod for 3 weeks. Conidia were separated from mycotized millet grain by sieving (75 mesh), dried in a dessicator for 2 days, and considered ready for use when the moisture content of the conidial powder was <5% (measured with a moisture analyzer, Sartorius-Omnimark, CO). Simultaneously, GHA and ERL1170 conidia were produced in ¼SDAY and whey permeate agar media [/l DW: whey permeate powder (Agri-Mark Inc., VT) 75 g and agar 15 g] in Petri dishes. Each had pH 6, adjusted using 1 M HCl and 1 M NaOH. A conidial suspension (1×10^6 conidia/ml) from each isolate was inoculated onto these media (100 µl/plate) by spreading with a sterile iron spreader with four replicates (four Petri dishes). Petri dishes were then sealed with Parafilm and held in darkness in an incubator at 25 ± 1°C for 14 days [17]. After incubation, dishes were left open in a drying room with a dehumidifier for 24 h. Conidia were aseptically removed from the agar media using a sterile spatula (when <5% moisture was observed). The entire experiment was repeated once.

In experiment II, the two *Bb* isolates and two *Ma* isolates were produced on millet powder agar, ¼SDAY, and whey permeate agar. The millet powder agar medium consisted of 75 g ground millet powder (≈300 mesh) (local market, VT) and 15 g agar per liter of DW (pH 6). The same amount of inocula (100 µl/plate) as above ≈ was introduced onto these agar media by spreading with three replicates (three Petri dishes) per treatment. The inocula were produced according to the same procedures described above. Petri dishes (without sealing) were placed in covered transparent plastic boxes ($45 \times 25 \times 10$ cm^3), and the boxes were held in darkness at 25 ± 1°C in the incubator for 14 days. After incubation, no further drying was conducted. The entire experiment was repeated once.

Thermotolerance assay

In experiment I, ca. 0.01 g dried conidia powder from each culture bag or Petri dish was suspended in 10 ml sterile 0.02% (v/v) polysiloxane polyether copolymer (Silwet L-77®; Loveland Industries, Greeley, CO) solution [21, 46] in a test-tube (four tubes/treatment). All test-tubes had glass beads (2 mm diameter, 10 beads/tube) to aid displacement of conidia. Tubes containing the fungal materials were shaken on a vortex mixer (Vortex-Genie 2™; VWR Scientific, NY) for 30 s. Following vortexing, suspensions were filtered through a layer of sterilized mesh (150×150 µm^2 pore size) placed on top of a sterile test-tube and adjusted to 1×10^7 conidia/ml. Each conidial suspension was distributed into five Eppendorf tubes (1 ml/tube), then individually placed in water baths (PreciTemp CW12, Julabo Inc., PA, USA) at 43°C, 44°C, 45°C, 46°C or 47°C [1]. When the Eppendorf tubes were placed in the water baths, temperatures of the conidial suspensions equalized to the set temperatures within 2 min. They were held at these conditions for 120 min. During the experiment, the equalized temperatures were kept constant. The temperature range was selected because, within the exposure time, comparison among the treatments was feasible. For exposure temperatures <43°C or >47°C, overall conidial germination was too high or low to compare treatments. The test temperatures were tightly spaced within the range to investigate the thermotolerance of conidia per change of 1°C at high temperatures. Prior to and after exposure, at intervals of 15 min up to 120 min, 100 µl conidial suspension from each tube was removed and pipetted onto ¼SDAY in Petri dishes. Every conidial suspension was taken from the tube after 4-5 times of sucking and pumping using a pipette to keep the remaining suspension with the same conidial concentration. Conidia were suspended in all tubes without settling. Following pipetting, Petri dishes were held at 20 ± 1°C in darkness for 24 h. Percentage germination was assessed by randomly counting 100 conidia using a microscope (400×). A conidium was considered to be germinated when a germ tube was visible. The entire experiment was repeated once using a different batch of conidia on a different day.

In experiment II, six mycotized agar discs (10 mm diameter) were randomly cut from a Petri dish using a sterile cork borer. They were placed in a test-tube containing 6 ml 0.02% (v/v) Silwet L-77® and 10 glass beads (2 mm diameter) (three tubes/treatment). All tubes were shaken, and the suspensions were filtered using the same methods described above. Most fungal materials (conidia and hyphae) were detached from the surface of mycotized agar discs after vortexing. Each conidial suspension was pipetted into five Eppendorf tubes (1 ml/tube), then individually placed in water baths at 43°C, 44°C, 45°C, 46°C or 47°C. The same procedure described above was used to examine conidial thermotolerance. The entire experiment was repeated once using a different batch of conidia on a different day.

Data analysis

Data on percentage conidial germination were analyzed by the general linear model (GLM), considering a possible block effect caused by experimental repetitions using SPSS version 17.0 (SPSS Inc., 2009) software. This was followed by Tukey's highly significant difference (HSD) test for multiple comparisons. In the analysis of experiment I, due to the variability in culturing and harvesting procedures, each entire production process was considered a treatment: treatment 1 (millet grain in polyethylene bags), 2 (¼SDAY in Petri dishes), and 3 (whey permeate agar in Petri dishes). The medium was not considered a factor to be analyzed.

However, in experiment II, isolate and medium were used as factors to be analyzed. Median survival time (LT_{50}; time for conidia to lose half of their initial viability, min) of conidia for each temperature was estimated by probit analysis using SAS version 9.1 (SAS Institute Inc., 2003) software. To provide an overall outlook of the influences of the two factors (isolate and medium) on conidial germination, main-effect analyses were conducted using Minitab version 15.1 (Minitab Inc., 2007) software. For analyses, a 0.05 (α) confidence level was considered significant.

Results

Thermotolerance of *Bb* conidia produced on millet grain

More thermotolerant *Bb* conidia were produced on millet grain in polyethylene bags than on whey permeate agar and ¼SDAY media in Petri dishes (Table 1). The germination rates of unexposed conidia (initial viability) were >95%. The LT_{50} values of conidia from the millet grain treatment were greater than those of the whey permeate treatment, followed by the ¼SDAY treatment. Overall the conidia from millet grain treatments showed higher percentage germination than the other two treatments ($F_{2,1620} = 3650.7$, $P < 0.001$), and *Bb* ERL1170 conidia had higher percentage germination than GHA ($F_{1,1620} = 4200.7$, $P < 0.001$). Germination was affected by not only isolate and production method but also exposure temperature ($F_{4,1620} = 14304.4$, $P < 0.001$) and exposure time ($F_{8,1620} = 6422.4$, $P < 0.001$). All two- and three-way interactions were significant ($P < 0.001$).

In each *Bb* isolate, the conidia from millet grain treatment showed the highest percentage germination, followed by the whey permeate and the ¼SDAY treatments (GHA: $F_{2,810} = 269.9$, $P < 0.001$; ERL1170: $F_{2,810} = 5309.4$, $P < 0.001$). Within the millet grain treatment, *Bb* ERL1170 had greater germination than GHA ($F_{1,540} = 12593.1$, $P < 0.001$) but vice versa in the ¼SDAY treatment ($F_{1,540} = 28.8$, $P < 0.001$).

Thermotolerance of *Bb* and *Ma* conidia produced on millet powder agar

Overall, millet powder agar was superior in producing the most thermotolerant conidia, as compared with the whey permeate agar and the ¼SDAY (Table 2). The germination rates of unexposed conidia were >95%. The millet powder agar treatment showed higher percentage conidial germination than the whey permeate agar treatment, followed by the ¼SDAY treatment ($F_{2,2160} = 590.9$, $P < 0.001$), for all four isolates. *Ma* ERL1540 isolate showed the highest percentage conidial germination ($F_{3,2160} = 208.1$, $P < 0.001$) (Fig. 1). All factors, not only isolate and medium but also exposure temperature ($F_{4,2160} = 1791.9$, $P < 0.001$) and exposure time ($F_{8,2160} = 160.2$, $P < 0.001$), affected germination. All two- and three-way interactions were significant ($P < 0.001$).

Within the two *Bb* isolates, the millet powder agar treatment showed higher percentage germination than the ¼SDAY treatment but lower than the whey permeate agar treatment ($F_{2,1080} = 410.6$, $P < 0.001$). No significant difference in germination between the two *Bb* isolates was observed ($F_{1,1080} = 3.1$, $P = 0.083$). In each *Bb* isolate, the whey permeate agar treatment showed the highest percentage germination (GHA: $F_{2,540} = 19578.6$, $P < 0.001$; ERL1170: $F_{2,540} = 12486.2$, $P < 0.001$). The overall mean difference in conidial germination between the whey permeate agar and the millet powder agar treatments was 7.3%.

For the two *Ma* isolates, the millet powder agar treatment showed higher percentage conidial germination

Table 1 Median survival time (LT_{50}) (min) of *B. bassiana* conidia produced on millet grain in polyethylene bags and two agar-based media (¼SDAY and whey permeate) as controls after exposure to 43–47°C (experiment I)

Isolate	Temp. (°C)	LT_{50} (min) (95% confidence level)		
		Treatment 1 (millet grain in bags)	Treatment 2 (¼SDAY agar in Petri dishes)	Treatment 3 (whey permeate agar in Petri dishes)
Bb GHA	43	156 (138–186)	104 (93–119)	168 (145–210)
	44	152 (133–183)	62 (55–68)	78 (73–84)
	45	73 (70–77)	39 (36–42)	49 (45–52)
	46	46 (44–49)	15 (14–17)	24 (21–26)
	47	33 (30–35)	9 (8–11)	20 (17–22)
Bb ERL1170	43	177 (151–226)	93 (87–100)	148 (131–175)
	44	134 (122–153)	45 (42–49)	57 (53–61)
	45	88 (84–93)	25 (23–28)	54 (50–57)
	46	49 (46–52)	13 (11–15)	30 (27–32)
	47	38 (35–40)	10 (7–12)	23 (19–27)

Table 2 Median survival time (LT_{50}) (min) of *B. bassiana* and *M. anisopliae* conidia produced on three agar-based media (millet powder, ¼SDAY, and whey permeate) in Petri dishes after exposure to 43–47°C (experiment II)

Isolate	Temp. (°C)	LT_{50} (min) (95% confidence level)		
		Millet powder agar	¼SDAY agar	Whey permeate agar
Bb GHA	43	235 (183–377)	69 (62–78)	208 (169–297)
	44	131 (120–147)	55 (49–61)	160 (141–192)
	45	72 (68–76)	22 (19–25)	109 (102–118)
	46	37 (35–42)	12 (9–15)	51 (49–54)
	47	34 (32–36)	9 (7–11)	45 (43–48)
Bb ERL1170	43	150 (133–176)	70 (65–75)	179 (152–229)
	44	169 (146–208)	73 (68–78)	169 (146–210)
	45	76 (73–80)	37 (34–39)	86 (82–91)
	46	46 (44–49)	17 (14–19)	52 (49–54)
	47	39 (37–42)	10 (9–12)	45 (42–47)
Ma ERL1171	43	124 (115–138)	94 (88–101)	83 (78–88)
	44	134 (123–151)	82 (77–86)	79 (75–84)
	45	65 (61–68)	42 (40–45)	42 (40–45)
	46	46 (44–49)	33 (31–35)	33 (31–35)
	47	39 (36–42)	25 (23–27)	32 (29–34)
Ma ERL1540	43	201 (166–278)	148 (134–170)	148 (133–171)
	44	187 (159–247)	138 (128–155)	129 (119–143)
	45	126 (117–137)	92 (88–97)	77 (74–80)
	46	56 (53–59)	46 (43–49)	37 (34–39)
	47	51 (49–54)	37 (34–39)	31 (28–33)

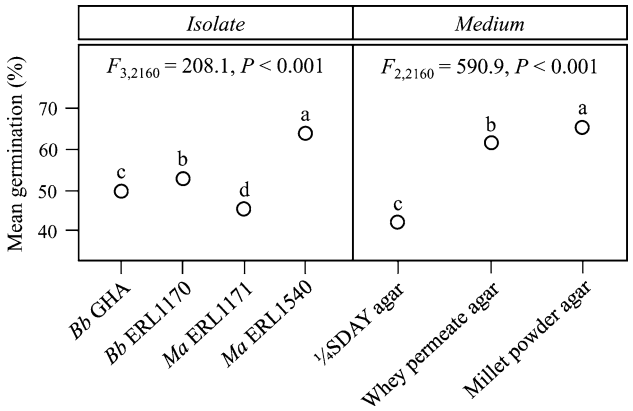

Fig. 1 Effects of isolates and media on germination of conidia in experiment II, analyzed by general linear model (GLM) analysis. *Circles* followed by the *same lower case letter* in each factor (isolate and medium) do not significantly differ by Tukey's HSD ($P = 0.05$). The GLM analysis considered all factors, isolate and medium, and also exposed temperature and exposed time

than the other two media treatments ($F_{2,1080} = 89.3$, $P < 0.001$). *Ma* ERL1540 conidia had higher percentage germination than *Ma* ERL1171 ($F_{1,1080} = 314.3$, $P < 0.001$). In the *Ma* ERL1171 isolate, the millet powder agar treatment showed the highest percentage germination ($F_{2,540} = 3050.9$, $P < 0.001$), and the other two treatments showed no difference in germination ($P = 0.887$). Similarly for *Ma* ERL1540, the millet powder agar treatment showed the highest percentage germination, but the whey permeate treatment showed lower percentage germination than the ¼SDAY treatment ($F_{2,540} = 4194.8$, $P < 0.001$).

Within the millet powder agar treatment, *Ma* ERL1540 conidia had the highest percentage germination, followed by *Bb* ERL1170, *Bb* GHA, and *Ma* ERL1171 ($F_{3,720} = 2366.6$, $P < 0.001$). However in the whey permeate agar treatment, *Bb* GHA conidia showed the highest germination, followed by *Bb* ERL1170, *Ma* ERL1540, and *Ma* ERL1171 ($F_{3,720} = 5494.7, P < 0.001$). In the ¼SDAY treatment, *Ma* ERL1540 showed the highest percentage germination, followed by *Ma* ERL1171, *Bb* ERL1170, and *Bb* GHA ($F_{3,720} = 12812.9, P < 0.001$).

Bb conidia produced on millet grain (experiment I) and millet powder agar (experiment II) had similar levels of thermotolerance (Tables 1, 2). At each temperature, LT_{50} values between experiment I and II were not significantly different, except at 46°C for *Bb* GHA and 45°C for *Bb* ERL1170. However, though the *Bb* conidia were produced on the same ¼SDAY medium in experiment I and II, the suspensions made of conidia from experiment II (naturally dried conidia during culture) had better conidial thermotolerance than the suspensions made of conidia from experiment I (compulsorily dried conidia). This difference was also found in the whey permeate agar treatment ($F_{1,900} = 1115.4$, $P < 0.001$).

Discussion

The present work explored whether millet grain could be used to produce thermotolerant conidia in a mass production system. Initially the potential of millet grain as a substrate for producing thermotolerant conidia was compared with ¼SDAY and whey permeate agar media (experiment I). Then, their effect was investigated using conidia produced on agar-based media (experiment II).

In experiment I, the millet grain treatment had the highest conidial thermotolerance, but further verification was required. Significant differences in conidial production methods were found between the polyethylene-bag-based culture (millet grain) and the agar-based cultures (¼SDAY and whey permeate agar). During culture, the production methods had different culture containers (polyethylene bag versus Petri dish), physical forms of substrates (grain versus agar), sealing of containers (paper towel versus Parafilm), and culture time (3 weeks versus 2 weeks). After culturing, the conidia from mycotized millet grain were dried in a dessicator for 2 days, but those from agar-based media were dried in a room using a dehumidifier for 1 days. The type of container and sealing methods can influence aeration and relative humidity. They are significantly related to mycelial growth and conidiation [19]. Different physical form of substrates can be associated with the usability of nutrition. Culture time is also significantly involved in maturation of conidia, which is responsible for conidial thermotolerance [3]. Though all conidia had <5% moisture content after drying, the two drying conditions may be a reason for different conidial storage stability.

In experiment II, designed for more precise determination of conidial thermotolerance, conidia from millet powder agar had the highest thermotolerance, followed by the whey permeate and ¼SDAY media treatments. In terms of nutritional composition, millet generally has protein (4.0%), fat (1.5%), ash (2.6%), carbohydrate (72.6% as starch), etc. [7], whereas whey permeate, a byproduct from the cheese industry, is composed of protein (3.5%), fat (0.3%), ash (9.0%), carbohydrate (82.0% as lactose), etc. [43]. Relying on the carbon-to-nitrogen (C/N) ratios of these agar-based media (calculated based on the nutrient compositions described above), the millet powder agar has ca. 5.4% starch and 0.30% nitrogen (C/N: 18/1) [7, 43] and the whey permeate agar has ca. 6.2% lactose and 0.26% nitrogen (C/N: 23/1) (Agri Mark Inc., VT), whereas the ¼SDAY has ca. 1.0% glucose and 0.5% nitrogen as peptone and yeast extract (C/N: 4/1). The millet powder and whey permeate agar media have higher C/N ratios (relatively lower nitrogen level) than the ¼SDAY.

Nitrogen starvation, a better inducer of conidiogenesis than carbon starvation [25], can achieve faster conidiogenesis, followed by providing enough time for conidial maturation. Maturation of conidia can be significantly involved in thermotolerance, which is supported by the fact that old, matured conidia have more rodlet proteins which are responsible for thermotolerance in the rodlet layer [2, 15]. This explanation is based on the blastic (chronological) conidiogenesis of *Bb* and *Ma* [17]. The narrow apex of the conidiogenous cells of *Bb* extends sympodially: each new apex becomes converted into a blastic conidium, and then the next apex grows out from behind and to one side of it. In this way, a succession of conidia is produced, with the youngest conidium at the tip of the structure. Similarly, *Ma* produces conidia from a conidiophore in chains by apical and blastic budding, with the oldest conidium at the end of the chain. The influence of these agar-based media on conidial thermotolerance may be further clarified by the positive and negative correlations of thermotolerance with carbohydrates [4, 48] and nitrogen [30], respectively.

In another aspect, nutritional starvation induces production of HSPs, which is mediated by a heat shock factor (HSF) binding to a site in the HSP gene promoter [49]. The induction of HSP gene expression also occurs in response to other stresses, such as temperature, oxidative, or osmotic stress, exposure to weak organic acids, ethanol, or low pH. HSPs, involved in thermotolerance, are divided into several families by their molecular mass: 100-, 90-, 70-, and 60-kDa HSPs [27, 28, 37]. HSPs 100 are proteases with ATPase activity and are thought be required for induction of heat tolerance under lethal stress and for the general stability of aerobically growing cells. In *Saccharomyces cerevisiae*, the *hsp* 104 gene (HSPs 100 family) occurs in the idiophase and at the initial stage of sporulation [35]. HSPs 90 are conservative chaperone (a special class of proteins involved in folding of polypeptide chain in protein molecules)-like proteins with weak ATPase activity. A HSPs 90 family gene, *hsp* 83, is induced by heat shock or upon transition to the stationary growth phase and sporulation [28]. HSPs 70 proteins have ATPase activity and interact with aggregates of denatured proteins, contributing to their deaggregation and further proper chain folding de novo [28]. In addition, small HSPs with a chaperone function are synthesized. They have molecular mass of 12–43 kDa and contain the so-called α-crystalline domain, a small (80–100 amino acids) conservative site at the C-terminus [12]. A 30-kDa HSP is a stress-induced highly hydrophobic integral membrane protein. There is another low-molecular-weight HSP, ubiquitin (8 kDa), characteristic of eukaryotic organisms [37]. Ubiquitin is not required for vegetative growth at optimal temperatures, but it is essential for resistance to heat shock or starvation. It is also synthesized during sporulation for maintenance of conidial viability.

Nutritional starvation also induces expression of trehalose synthase genes for synthesis of trehalose, which is relevant to fungal thermotolerance [42]. Similar to nutritional starvation, in the process of fungal cell differentiation, trehalose is accumulated in idiophase, when inhibition of growth processes is observed [41]. This disaccharide is referred to as the dormancy sugar, since its active synthesis occurs during sporulation and attains the highest level in resting forms. The expression of the trehalose synthase genes is regulated not only by starvation but also by heat shock and osmotic stress [31]. Trehalose synthase carries out synthesis of trehalose-6-phosphate from uridine diphosphate (UDP)-glucose and glucose-6-phosphate. The reaction requires the presence of magnesium ions and is irreversible. Trehalose is synthesized in the form of trehalose-6-phosphate but stored in the form of a dephosphorylated carbohydrate. The production of trehalose occurs in parallel with the HSPs [12].

This work provides additional information on the evaluation of conidial thermotolerance. First, agar-based millet powder medium can be alternatively used to estimate the thermotolerance of conidia produced on millet grain in polyethylene bags. Many efforts required in polyethylene-bag-based culture can thus be reduced as follows: (1) preparing millet grain medium, (2) handling mycotized grain during culture, and (3) harvesting conidia from grain after culture. Secondly, forced drying of conidia can reduce conidial thermotolerance when they are suspended in a solution and exposed to high temperature (wet-heat exposure), depending on the species isolates and culture methods. Drying may be another abiotic stress to conidia, resulting in imbibitional damage to conidia [8]. In particular, when conidia which are not fully maturated during blastic conidiogenesis are exposed to excessive drying conditions, their viability may be significantly reduced due to the lack of enough surface materials such as rodlet-layer proteins responsible for environmental resistance [13, 14].

Millet grain has further advantages in the production of entomopathogenic fungal conidia. In solid cultures, the small size of millet grain (approx. 2 mm diameter) can provide much larger surface area where fungi can propagate and produce conidia. The size of millet grain is smaller than that of other cereal grains including rice (2.5 mm), wheat (3 mm), and legumes (yellow beans or red kidney beans, 8–13 mm) [19, 22]. If aggregated millet grains caused by mycelial net are broken into pieces appropriately one or two times during culture, the substrate will be properly aerated. Generally, aeration is significantly affected by the levels of mycelial growth and the physical properties of the grain. Millet grain is not as sticky as rice and not as breakable as legumes when cooked at 80–90°C for several hours. Such favorable physical properties may enable millet grain to produce high conidia yield. Secondly, in terms of application, mycotized millet grain can be used directly as a granular product to be applied to soil. The size of mycotized millet grain is small and it can be incorporated into the upper layers of soil, in contrast with other granular products. No further processing to make the grain smaller is necessary. Millet grain can provide nutrition to fungi, allowing them to colonize soil.

These findings suggest that millet grain can be used as a substrate in fungal mass production of thermotolerant *B. bassiana* and *M. anisopliae* conidia. Nutritional starvation, in particular of a nitrogen source, may be related to the enhanced conidial thermotolerance. This needs further clarification. Use of millet grain provides not only conidial thermotolerance but also many benefits in conidial production and field application. Whey permeate can also be used as an additive for grains or legumes for such purpose, particularly to produce thermotolerant *B. bassiana* rather than *M. anisopliae*.

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