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ORIGINAL ARTICLE

Comparison of survival and pathogenicity of *Beauveria bassiana* A1-1 spores produced in solid and liquid state fermentation on whitefly nymph, *Trialeurodes vaporariorum*

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Abstract

In order to use entomopathogenic fungi (EPF) as biological control agents, it is necessary to mass produce the EPF in an economical and cost-effective manner. Currently, the mass production of EPF is carried out mainly in two ways: solid-state fermentation in which the aerial conidia are produced, and liquid fermentation in which the blastospores and submerged conidia are produced. This research compares the survival of *Beauveria bassiana* A1-1spores from solid and liquid culture media, after 0, 3, 6 and 9 months of storage at room temperature (25 ± 5°C) and in the refrigerator (4°C). Furthermore, it compares the pathogenicity of spores immediately after production and after 9 months of storage on third nymphs of greenhouse whitefly, *Trialeurodes vaporariorum*. The aerial conidia and blastospores were slightly more virulent than the submerged conidia on whitefly nymphs. In laboratory bioassays, blastospores indicated more pathogenicity on nymphs than submerged conidia, even though there was no significant difference in the pathogenicity of the spores produced in liquid culture media in greenhouse bioassays. Moreover, survival of the aerial conidia at a low temperature (4°C) was higher than that kept at room temperature (25 ± 5°C). This storage temperature comparison revealed a positive effect on the stability and survival of blastospores and submerged conidia as well. Meanwhile, the survival of spores drastically decreased after 3 months of storage at room temperature.

Keywords: *Beauveria bassiana*, entomopathogenic fungi, greenhouse whitefly, pathogenicity, survival rate

Introduction

The mass production of entomopathogenic fungi (EPF) as biological control agents requires an economical and cost-effective production process that provides a large number of viable and virulent propagules. Two approaches are generally utilized to mass produce EPF. The first approach involves solid-state fermentation on a solid substrate to produce hard and healthy aerial conidia, and the second approach involves liquid fermentation in submerged liquid culture to produce hydrophilic blastospores and submerged conidia (Mc--Neil and Harvey 2008). In general, myco-insecticides made from common EPF like *Beauveria bassiana* have used aerial conidia more often than other spore types. The main reasons may be attributed to the longer life span of aerial conidia than of blastospores, their lipophilic nature, and their ability to spread in

oils (oily formulations make the conidia stick more to the hydrophobic cuticle of insects). Meanwhile, the final products of liquid fermentation primarily include blastospores and hyphal bodies. Blastospores germinate much faster than aerial conidia (Hegedus *et al*. 1992). Liquid fermentation, especially if done in a fermenter, provides more controlled nutritional and environmental conditions to produce blastospores, which reduces both production time and the possibility of contamination. However, special methods are required to make blastospores resistant to drying since they quickly lose viability during storage (Lohse *et al*. 2014). In industry, a two-stage fermentation method is more common than other approaches. In this method, mycelium or hyphae bodies are produced in the liquid medium in mobile flasks or fermenters and then transferred to the solid culture medium for the production of aerial conidia. This two-stage fermentation method has the advantages of mass production in liquid fermentation and the production of stable, hydrophobic aerial conidia on a solid substrate at the same time. The disadvantages of this method are the need for more time for reproduction and the high probability of contamination of the culture medium with saprophytic fungi (Jackson 1997; Jackson *et al*. 2010; Mascarin and Jaronski 2016).

The greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) is one of the major pests in vegetable and ornamental plantings in greenhouses. *Trialeurodes vaporariorum* weakens the plants by sucking sap from the host with its piercing-sucking mouthparts. As a by-product of feeding, honeydew is excreted and causes saprophytic fungi to grow on the leached honey, which reduces the quality and marketability as well as the yield. In addition, the direct feeding of this pest transmits several plant viruses (Capinera 2008). Currently, the usual chemical pesticides are mainly being used to control this pest. Due to its relatively short generation time, it quickly becomes resistant to pesticides (Bi and Tosccano 2007; Gorman *et al*. 2007; Li *et al*. 2000). Considering the resistance of *T. vaporariorum* to common pesticides and the environmental hazards chemicals pose, improving alternative environmentally friendly control solutions is urgently necessary. Due to the fact that whiteflies feed on plant sap by dipping their piercing-sucking mouthparts into the vascular tissue of the plant, the use of EPF that act through the insect cuticle is important to control the pest.

Javar *et al*. (2020) confirmed that *B. bassiana* A1-1 is the best EPF isolate to control greenhouse whitefly. Therefore, this study was designed to compare the pathogenicity of spores produced in solid and liquid media immediately after production and after 9 months of storage at room and refrigerator temperatures in nymphs of the greenhouse whitefly, *T. vaporariorum*. Additionally, a comparison of the survival rate and stability of the spores has been made.

Materials and Methods

Fungal isolate

An isolate of *B. bassiana* (Code: A1-1) was obtained from the Iranian Research Institute of Plant Protection, Tehran. The fungal isolate was cultured in potato dextrose agar (PDA; Merck, Germany) medium and kept in darkness for 12–14 days in an incubator at 25°C before being used in the experiment.

Insect colony

The initial culture of greenhouse whiteflies, *T. vaporariorum* was collected from tomato plants in a greenhouse. White Burley tobacco was grown and a whitefly colony was established in a greenhouse a 25 ± 5°C, a relative humidity of 60 ± 5%, and a natural lighting period.

Solid-state fermentation

Solid culture substrates, including wheat bran, rice bran, corn bran, and barley residues, were used in the mass rearing of *Sitotroga cerealella* Olivier (hereafter called barley). At first, 100 grams of wheat and rice bran substrates at ratios of 0.5 : 1 (sterilized water: culture substrate) and corn bran and barley substrates at ratios of 1.5 : 1 (sterilized water: culture substrate) were mixed with sterile distilled water and transferred to plastic bags (30 × 40 cm). After closing the bags and placing some cotton for aeration, they were sterilized for 45 minutes in an autoclave at 121°C, and under a pressure of 1.5 atm. The solid substrates inside the bags were inoculated with two discs of 10 mm diameter from the 14-day-old fungal culture and then placed in the incubator (with a photoperiod of 16L/8D at 25 ± 5°C) for 20 days. Every 3 days, the bags were mixed by hand from the outside so that the conidia were completely distributed in the bag. To evaluate the amount of conidia production, 1 g of each treatment was poured into 9 ml of sterilized distilled water containing 0.05% Tween 80. Then, they were completely mixed with a vortex until the conidia on the surface of the substrates were separated and immersed in the water. The obtained suspension was passed through a three-layer muslin cloth, then 1 ml of it was diluted in 9 ml of distilled water containing 0.05% Tween 80. The concentration of conidia per ml was estimated using a hemocytometer slide. Eventually, a fungal suspension with a concentration of 10^7 conidia · ml^{-1} was prepared.

Liquid-state fermentation

The liquid media, including TKI broth culture medium with 5% sugar beet molasses (TKI broth) (Thomas *et al*. 1987; Lohse *et al*. 2014), potato extract medium (PE), and potato extract with 8% whey medium (PE + W) were investigated. The implemented basic salts were dissolved in 1 l of distilled water. The basic salts included a carbon source – 50.0 g; KNO_3 – 10.0 g; KH_2PO_4 – 5.0 g; $MgSO_4$ – 2.0 g; $CaCl_2 \cdot 2H_2O$ – 50.0 mg; $FeC1_3 \cdot 6H_20$ – 12.0 mg; $MnS0_4 \cdot H_2O$ – 2.5 mg; $Co(NO_3)_2 \cdot 6H_2O$ – 0.25 mg; $Na_2MoO_4 \cdot 2H_2O$ – 0.2 mg; $ZnSO_4 \cdot 7H_2O$ – 2.5 mg; and $CuSO_4 \cdot 5H_2O$ – 0.5 mg. The liquid medium was sterilized by autoclaving for 15 minutes at 121°C. Sugar beet molasses was also separately sterilized and added to the medium as a carbon source (Lohse *et al*. 2014). To prepare PE medium, 200 g of peeled potatoes were boiled in 1 l distilled water, and after cooking them, the extract was taken and the volume was increased to 1 l. Subsequently, 8% whey was added to the medium of potato extract to prepare the PE + W medium. All media in the amount of 250 ml were poured into 500 ml Erlenmeyer flasks and were inoculated with two 10-mm-diameter discs from the 14-day fungal culture medium. Then, they were placed in a shaker incubator (HYSC-SL-300RF--South Korea) at 25°C and 150 rpm rotation speed for 5 days. The obtained suspension was passed through a three-layer muslin cloth, while 1 ml of it was diluted in 9 ml of distilled water. Finally, the concentration of blastospores per ml was estimated using a hemocytometer.

Survival of spores produced in solid and liquid media

To see how stable and long-lasting aerial conidia made on different solid substrates were, the substrates with conidia were put in sterile containers and put under a laminar flow hood to dry. The substrates were kept at two different temperatures, room temperature (25 ± 5°C) and in a refrigerator (4°C) for 9 months. Conidia colony forming units (CFUs) were counted every 3 months. At the end of 9 months, a virulence test of the spores was performed on the nymphs according to the method explained in the sections below (laboratory and greenhouse bioassays).

The method explained by Lohse *et al*. (2014) was applied to the drying and storage of blastospores. Accordingly, the liquid medium containing blastospores of the fungus was passed through the three-layer muslin cloth and centrifuged for 10 minutes then washed twice with sterile distilled water and centrifuged. The samples were placed in sterile Petri dishes under a laminar flow hood to dry in the air with <3% humidity. This fungal mass was kept at room temperature (25 ± 5°C) and in the refrigerator (4°C) for 9 months and the CFUs and their survival was checked every 3 months. After 9 months of storage, a bioassay test of blastospores was performed on whitefly nymphs according to the method explained below in the bioassay tests section under laboratory and greenhouse conditions.

Laboratory and greenhouse bioassays

The laboratory bioassay was conducted according to the method of Oreste *et al*. (2016). In order to provide a population of the same age whiteflies and to perform bioassays, four-leaf plants of green bean were placed in the whitefly colony for 24 h. After the egg incubation period and the appearance of most third-instar nymphs, leaves were sprayed with a fungal suspension at a concentration of 10^7 spores per ml using a hand sprayer. Consequently, the leaves were placed on filter paper for 5 min. to absorb excess moisture, and then transferred to Petri dishes containing 2% agar medium. Finally, the treatments were transferred to an incubator (at 26 ± 5°C, 60 ± 5% RH and a photoperiod of 16L/8D). The mortality rate was checked daily and recorded for 8 days. The experiments were set up in a completely randomized design with five replications. For the control treatment, sterilized distilled water containing 0.05% Tween 80 was sprayed on the leaves containing nymphs.

In the greenhouse bioassay, bean seeds were planted in 30 cm diameter pots, and after the plants reached the multi-leaf stage, the pots were kept inside the whitefly colony for 2 days (25 ± 5°C, 60 ± 5% RH, 16 hours of light) to allow eggs to be oviposited on the underside of the leaves. Following successful oviposition, the pots were removed from the colony and placed in the greenhouse inside a cage free from any contamination. Following the egg incubation period and the appearance of mostly third-instar nymphs, the plant leaves were sprayed with a fungal suspension of 10^7 spores per ml in five replications (each replication included one bean leaf). The number of nymphs was counted using a magnifier before conducting the experiment on the specified leaves. Seven days after fungal application, the number of dead and alive whiteflies was counted under a stereomicroscope. Dry and discolored nymphs or nymphs covered with white fungal mycelium were considered dead nymphs.

Statistical analysis

Bioassay experiments were performed in a completely randomized design in five replications for both the laboratory and greenhouse bioassays. One-way analysis of variance (ANOVA) was used in the bioassay experiments. The mortality percentage of whitefly

nymphs was corrected based on Abbott's formula. The average percentage of pest deaths was compared using Tukey's test at a 5% probability level using SPSS software. Graphs were drawn with Excel software.

Results

Survival of spores produced in solid and liquid media

The survival percentages of aerial conidia grown on solid substrates including wheat bran, rice bran, corn bran, and barley for 9 months at room and refrigerator temperatures were checked every 3 months (Fig. 1 and Fig. 2, respectively). The mean survival percent of conidia kept at room temperature in all solid substrates was more than 80% after 6 months (Fig. 1). After 9 months, the highest survival percentage of conidia was recorded in the corn bran substrate (97.33%), but the lowest percentage was in the barley substrate (79%). On the other hand, at refrigerator temperature, the survival percentage of conidia in all substrates (Fig. 2) was higher than that at room temperature (>80%) after 9 months. The highest percentage was observed in corn bran and rice bran substrates with 100% and 97%, respectively, whereas the lowest one belonged to barley substrate with 80%.

In the TKI broth, the observed spores were in the form of submerged conidia, while in PE and PE + W

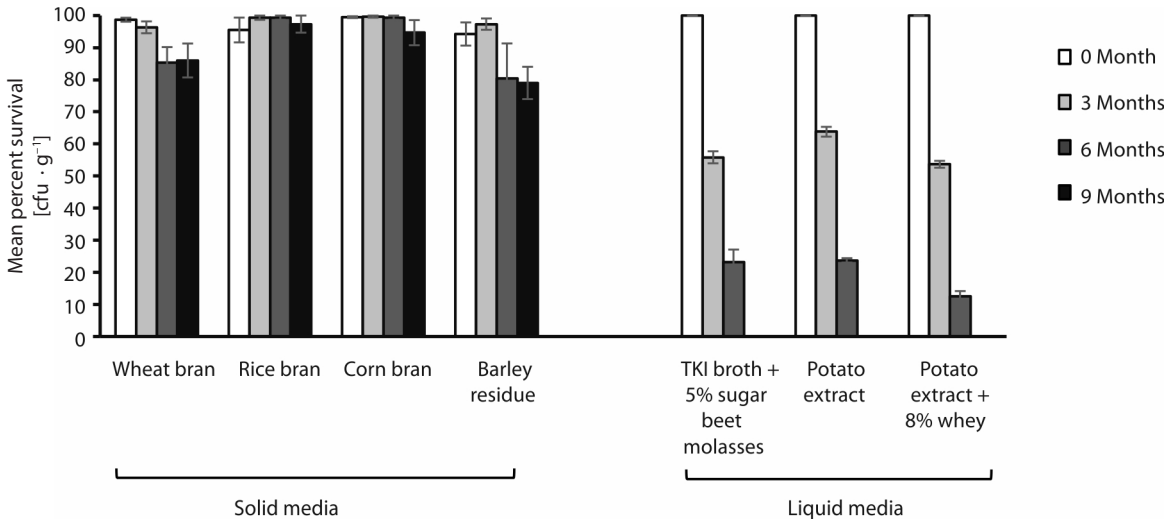

Fig. 1. Comparison of the mean survival percent (±SE) of all spore types produced on/in solid and liquid media after 0, 3, 6, and 9 months of storage at room temperature (25 ± 5°C)

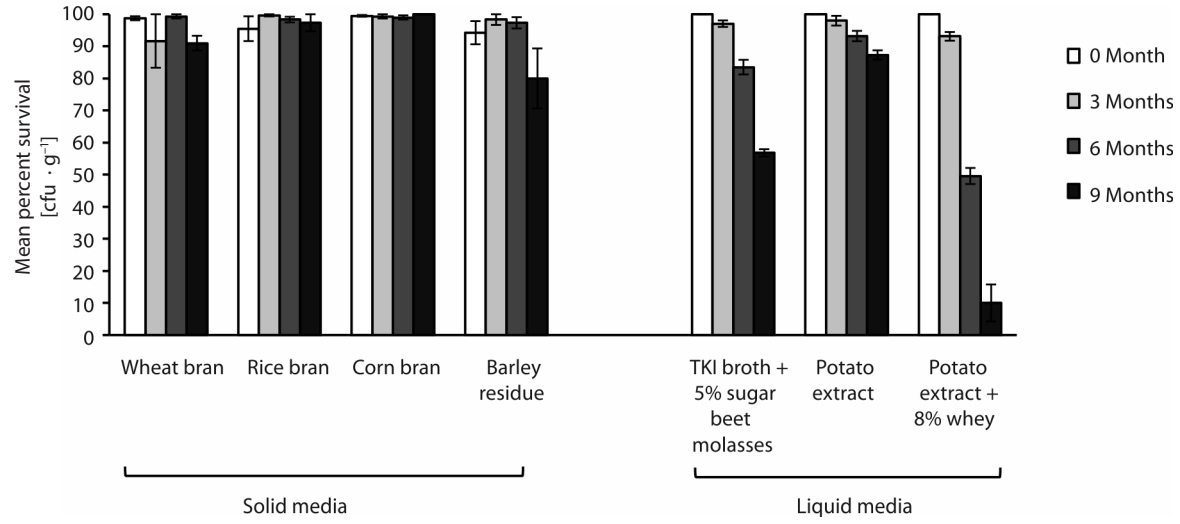

Fig. 2. Comparison of the mean survival percent (±SE) of all spore types produced on/in solid and liquid media after 0, 3, 6, and 9 months of refrigeration (4°C)

medium, spores were produced as blastospores. Figures 1 and 2 show the survival (cfu · g^{-1}) of immersed conidia produced in TKI broth as well as blastospores in PE and PE+W after harvesting from the medium and storage at 3, 6, and 9 months at room temperature (Fig. 1) and refrigerator temperature (Fig. 2). When the obtained spores were stored at room temperature (Fig. 1), the slope of changes in the survival was downward in all media. After 3 months of storage at room temperature, the viability of blastospores obtained from PE medium decreased to 63.8%, while that of the other spores decreased to about 50%. After 9 months of storage at room temperature, none of the spores were alive.

When spores were stored in the refrigerator, more than 80% of survival was observed after 3 months (Fig. 2). Afterwards, the survival of spores presented a downward trend. The greatest decline in survival was observed in the blastospores obtained from the PE + W media. Therefore, about 10% of the blastospores were alive after 9 months of storage. The blastospores obtained from PE showed the highest stability in terms of survival, with more than 80% of the spores still alive after 9 months. At the same time, the survival of submerged conidia after 9 months was decreased to about 50%.

Laboratory and greenhouse bioassay

Laboratory bioassay

The laboratory bioassay showed that storing the aerial conidia in the refrigerator for 9 months affected the virulence of conidia harvested from various substrates on the whitefly nymphs. There was a significant difference in the mean mortality rate of whitefly nymphs after infection with conidia from different solid substrates 9 months post-production and storage ($df = 3$, $F = 68.700$, $p < 0.05$). Conidia kept on corn and rice bran substrates caused the highest rate of mortality of about 89.4 ± 1.69% and 79 ± 1.18%, respectively. However, the conidia grown on barley caused the lowest mortality rate with an average of 34 ± 4.5% (Fig. 3). Comparing the mean mortality rate of conidia after 9 months of storage in the refrigerator to the newly produced one revealed that the virulence of conidia grown on rice and corn bran substrates did not change significantly, causing the highest mortality in whitefly nymphs, while on barley and wheat bran substrates decreased significantly after 9 months (Fig. 3).

The obtained results of the laboratory bioassay after the production of spores in the liquid medium showed that all treatments caused a high percentage (over 70%) of mortality of the whitefly nymphs (Fig. 3). No significant difference between the virulence of spores harvested from the different substrates on whitefly nymphs was found ($df = 2$, $F = 3.70$, $p \geq 0.05$). The blastospores obtained from PE + W medium recorded the highest rate of mortality (86.40%). However, the submerged conidia from TKI broth showed the lowest mortality rate (74.03%).

After 9 months of storage, only the spores at refrigerator temperature were used in bioassays, because none of the spores were alive after being kept at room temperature. In the laboratory bioassay, a significant difference was observed between the virulence of spores from different liquid media on whitefly nymphs. After 9 months of storage in the refrigerator, submerged conidia from TKI broth and blastospores from PE caused 25% to 30% losses in nymphs, respectively (Fig. 3). On the other hand, the blastospores obtained

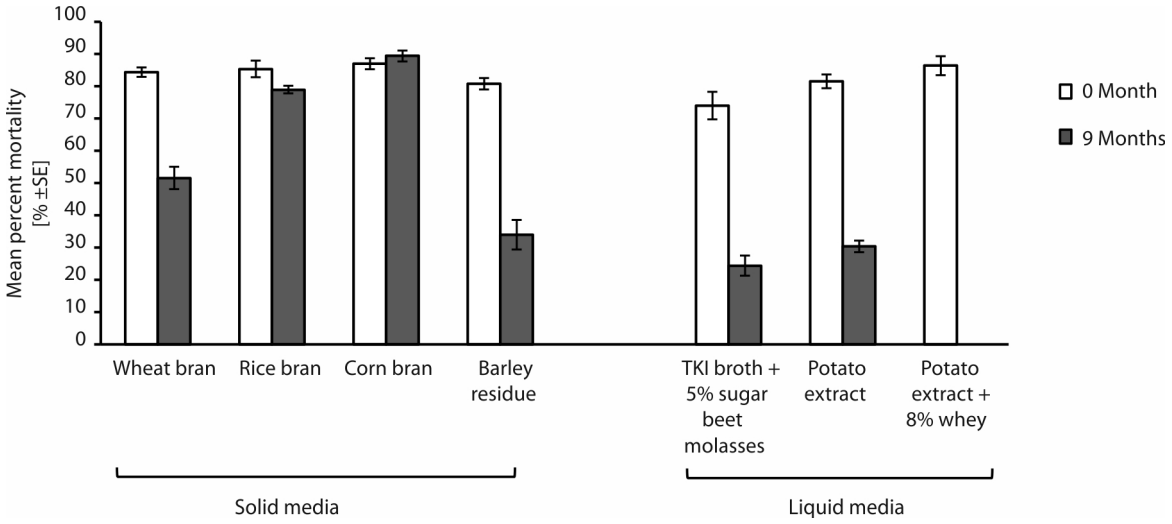

Fig. 3. Comparison of the virulence of all spore types produced on/in different solid and liquid media immediately and 9 months after production under the laboratory bioassay conditions. Third instar nymphs of *Trialeurodes vaporariorum* were sprayed with a fungal suspension (107 spores · ml^{-1}). The daily mortality rate was checked and recorded for a period of 8 days. Bars are the standard error of the mean

from the PE+W were not pathogenic for nymphs. Comparing the mortality percentage of different spores at the beginning of production and after 9 months revealed that their mortality rate decreased significantly. In the greenhouse bioassay, no deaths were observed in the tested nymphs.

Greenhouse bioassay

There was a significant difference in the virulence of conidia harvested from different solid substrates after storage for 9 months in the refrigerator on the whitefly nymphs ($df = 3$, $F = 92.334$, $p < 0.05$). The conidia on rice bran and corn bran substrates registered the highest mortality rate in nymphs with an average of 52.15 ± 2.64 and 51.16 ± 2.51%, respectively. Meanwhile, the conidia produced on barley had the lowest rate (8.69 ± 1.63%) (Fig. 4). The mean mortality percent of third instar nymphs of the greenhouse whitefly was caused by conidia grown on different solid substrates after 9 months of refrigeration which differed from newly produced conidia. The laboratory bioassay results indicated that the rate of mortality in conidia grown on rice bran and corn bran substrates did not change considerably after 9 months of storage. At the same time, the virulence of conidia produced on barley and wheat bran decreased significantly after 9 months. The virulence results of aerial conidia in the greenhouse bioassay were similar to those recorded in the laboratory after 9 months (Figs. 3 and 4). Despite this, the rate of mortality in nymphs in the laboratory bioassay was higher than that in the greenhouse for all substrates (Figs. 3 and 4).

In the greenhouse bioassay, the mortality rate of spores obtained from the liquid media on whitefly nymphs significantly decreased compared to the laboratory bioassay in all treatments (Figs. 3 and 4). No significant difference between the virulence of spores harvested from the different liquid media on whitefly nymphs was found ($df = 2$, $F = 1.83$, $p > 0.05$), with the mortality ranging between 43 and 50% (Fig. 4) immediately after production. After 9 months, no mortality was observed in the tested nymphs.

Discussion

For the use of microbial agents in a biological pest control program, the viability and stability of these agents are important for their pathogenicity (Glare *et al.* 2012). There are different factors affecting the survival of insect pathogenic fungi such as the type of fungal isolate, production conditions, moisture content of conidia (spores), humidity level, and storage temperature (Faria *et al.* 2009; Blanford *et al.* 2012). In this regard, Blanford *et al.* (2012) revealed that aerial conidia of *B. bassiana* maintains its stability with the least decrease in survival rate under low humidity (less than 5%) and storage temperature (less than 5°C) conditions for 2 years. Based on the findings reported previously by Kim *et al.* (2019) that *B. bassiana* conidia grown on millet and rice seeds when stored at low temperatures (4°C) and medium temperatures (25 and 30°C) more than 80% survived up to 24 and 18 months, respectively. The present results confirmed that more than 85% of conidia cultured on solid substrates of wheat

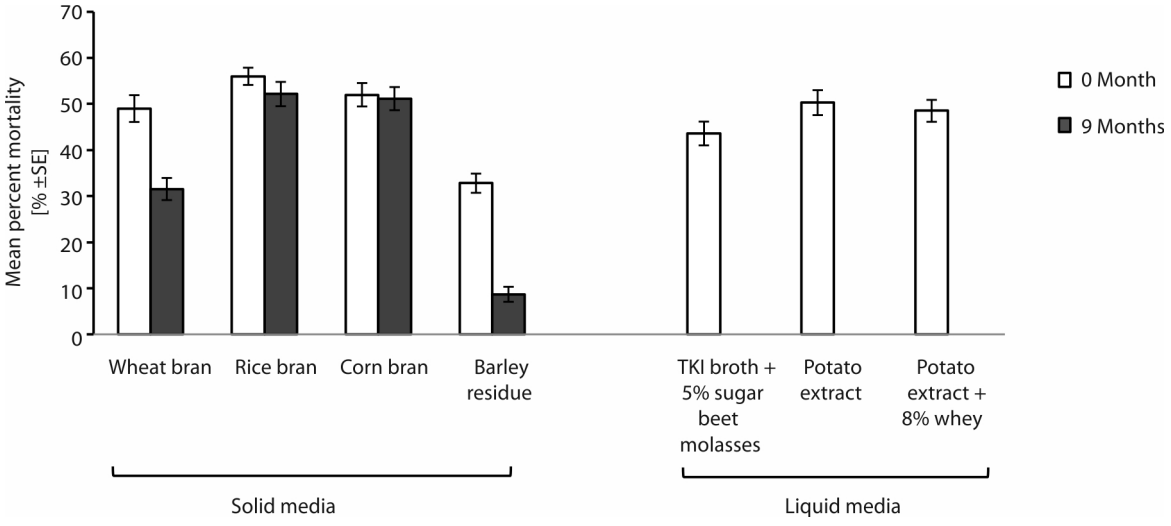

Fig. 4. Comparison of the virulence of all spore types produced on/in different solid and liquid media immediately and 9 months after production under the greenhouse bioassay conditions. Third instar nymphs of *Trialeurodes vaporariorum* were sprayed with a fungal suspension (107 spores · ml^{-1}). Seven days after fungal application, the number of dead and living whiteflies was counted under a stereomicroscope. Bars are the standard error of the mean

bran, rice bran and corn bran survived after 9 months of storage at both refrigerator and room temperatures. Padmanaban *et al.* (2009) indicated that the shelf life of *B. bassiana* conidia grown on wheat bran decreased from 4×10^8 CFU · g^{-1} to 9×10^7 CFU · g^{-1} after 90 days of storage. Meanwhile Abas *et al.* (2014) found that up to 55.7% of *B. bassiana* conidia grown on the mixture of wheat bran and corn cob powder (WC) was viable after 3 months of storage. In addition, the viability of conidia was sharply reduced after 3 months storage. Aregger (1992) also showed that the viability of different strains of *B. brongniartii* grown on barley decreased from 1.8% (strain 166) up to 100% (strain 169) within 2 years when stored at 2°C. Some previous studies have shown that adding whey as a source of carbon and nitrogen (the presence of lactose and protein) can have a positive effect on the survival and stability of the fungus as well as its pathogenicity (Kassa *et al*. 2008). The positive effect of adding whey on the amount of blastospore production has also been seen by Rashid *et al*. (2019). These findings were in agreement with our results when 8% of whey was added to the solid substrate. The germination rate of conidia from barley residue was acceptable (80%), while the conidia showed lower virulence on whiteflies in the laboratory bioassay. The conidial germination was only checked on agar plates. Ultimate infection occurs on an insect cuticle where texture, exudates, and microflora have a role in the pre-infection stages. Since barley residues used in the mass rearing of *S. cerealella* were used in our research instead of barley, it seems that the nutrient content of barley residue affects the virulence factors of obtained conidia. According to Blanford *et al*. (2012), the dry conidia of *B. bassiana* obtained from barley showed no loss in viability over the monitoring period at 7°C with a spore germination rate of 98.1 ± 0.55% after 750 days. Moreover, a clear temperature dependent decline was observed in viability when conidia were transferred to different temperatures (32, 26 and 22°C).

The storage temperature influenced the stability of conidia. Our results clearly showed that the survival rate of conidia at a lower temperature (4°C) was better than that kept at a higher temperature (25 ± 5°C). This finding is in line with previous studies reported by Sandhu *et al*. (1993), Roshandel *et al*. (2016), and Kim *et al*. (2019). Kim *et al.* (2019) reported that the viability of *B. bassiana* ERL836 conidia from mycotized grains (millet and rice) stored at a high temperature (37°C) showed a lower germination rate of about 80% for only 5 months. In contrast, the samples stored at low (4°C) and moderate (25 and 30°C) temperatures showed a germination rate of more than 85% for 24 and 18 months, respectively, along with improved insecticidal activity. Moreover, the germination rate of an emulsifiable concentrate formulation of *B. bassiana* strain CG425 decreased when both storage time and temperature were increased. The time for initial conidial germination to drop 50% (GT_{50}) varied from over 180 to less than 90 days at 4 and 35°C, respectively (Lopes and Faria 2019). The highest viability loss of conidia at high storage temperatures can be related to greater active metabolism at these temperatures (Grijalba *et al.* 2018). With an increase in environmental temperature, a higher metabolic rate is generally anticipated in cells. Thus, heat response pathways will be initiated to utilize the extra energy and the physiological changes taking place. Production of toxic metabolites affects conidial germination and causes irreversible damage to the fungus, and leads to loss of viability (Bakar *et al.* 2020). The total duration of heat exposure is another important parameter. Shorter exposures to heat can lead to different proteomic changes compared to longer exposures. This is because native proteins take time to be denatured or unfolded, and finally to be degraded under high-temperature conditions (Albrecht *et al.* 2010).

Beauveria bassiana can produce three types of conidia in addition to hyphae (or mycelium) in culture media such as aerial conidia, blastospores, and submerged conidia (Hegedus *et al*. 1992). The aerial conidia are produced from mycelia grown on solid or semi-solid materials (MacLeod 1954), while blastospores (Bidochka 1987) and submerged conidia (Thomas *et al*. 1987; Rombach 1988) are produced in liquid culture media. Blastospores are unicellular hyphal bodies with thick walls larger than aerial conidia and they are produced in vitro using artificial nutrient-rich broth. Submerged conidia are similar in shape and size to aerial conidia, but different in surface morphology and are seen in food-rich environments (Thomas *et al*. 1987; Holder and Keyhani 2005). Production of submerged conidia in TKI broth medium has been reported by Thomas *et al*. (1987) and Lohse *et al*. (2014). Obtained results revealed that the submerged conidia were only observed in the TKI medium with 5% sugar beet molasses while blastospores were observed in other tested liquid media. The blastospores and submerged conidia produced in liquid medium show pathogenicity on whitefly nymphs as well. Lacey *et al*. (1999) reported that blastospores of *Paecilomyces fumosoroseus* produced in a liquid culture medium were more effective than aerial conidia in terms of pathogenicity and mortality of *Bemisia argentifolii*. According to our results, the virulence of aerial conidia produced in solid media was almost the same as spores produced in liquid media on greenhouse whitefly nymphs. The blastospore yield, drought tolerance, viability, and pathogenicity of two fungal species of *B. bassiana* and *Isaria fumosorosea* were investigated by Mascarin *et al*. (2015). They used two liquid fermentation environments containing casein and cottonseed flour as nitrogen sources

and they reported that both fungal species had higher blastospore yields within 3 days in the medium containing cottonseed flour. Moreover, dried blastospores had more pathogenicity at low concentrations than aerial conidia on whitefly nymphs. In our laboratory bioassays, blastospores showed more pathogenicity on whitefly nymphs than submerged conidia, even though in greenhouse bioassays, there was no significant difference between the pathogenicity of spores produced in liquid culture media. Hegedus *et al*. (1992) showed that the outer surface of the blastospore is less hydrophobic than the submerged conidia, helping the spores stick to the cuticle of the insect. Therefore, their pathogenicity rate is slightly higher than the submerged conidia.

The present findings also support the hypothesis that storage at low temperature (4°C) positively affects the stability and survival of the blastospores and submerged conidia more than the higher temperatures. The air-dried blastospores (with <3% humidity) obtained from PE showed the highest stability in terms of survival, with more than 80% of the spores still alive after 9 months. Whilst, after 3 months of storage at room temperature, the viability of blastospores obtained from PE medium decreased to 63.8%. Additionally, the survival of spores drastically reduced after 3 months of storage at room temperature (25 ± 5°C). According to Hegedus *et al.* (1992), all types of spores (aerial conidia, blastospores and submerged conidia) quickly lose their viability at 37°C, which was much higher than ours (25 ± 5°C). Later findings showed that the blastospores, dried and formulated as granules with 1% water content, were relatively viable during the first 5 months of storage at <0°C but their viability decreased thereafter. The average survival rate was 50%, 40% and only 3–10% after 8, 10–12, 12–23 months, respectively (Chen *et al.* 1990). Based on the findings reported by Mascarin *et al*. (2016), the survival of air-dried blastospores with <5% water content after 9 months at 4°C was over 80%. In the current study, the survival of blastospores decreased to 50% after 12 weeks; then it reached <10% after 40 weeks at 28°C. In other research, the survival of blastospores, dried in the air with <3% humidity, was 61% to 86% (Mascarin *et al*. 2015). After 9 months storage at 4°C, 32–56% of the *B. bassiana* blastospores obtained from liquid medium containing complex organic sources of nitrogen such as soy flour, autolyzed yeast and cottonseed, survived (Mascarin *et al.* 2018). No mortality was recorded in nymphs after 9 months storage of TKI and PE produced spores in the greenhouse bioassay, even though 25% to 30% losses were observed in nymphs under the laboratory bioassay conditions. Since the viability of spores produced in liquid medium was reduced with time, the number of insects that are exposed to viable conidia are most likely reduced. Consequently, the mortality rate of nymphs is expected to become reduced. Further studies are required to prolong the shelf life of the spores obtained from the liquid media. Over the last decades, several studies on prolonging blastospore shelf life have been published. Dietsch *et al.* (2021) showed that fermentation media specially sugar concentrations and complex nitrogen sources, drying process, the type of formulation, and the storage conditions highly influence the shelf life of spores.

Selection of the best mass production technique for entomopathogenic fungi depends on different factors such as the cost of mass production and formulation, propagule yield, virulence, survivability, persistence in the field, tolerance to environmental abiotic and biotic factors, pest target, delivery system, and market size (Feng *et al.* 1994; Jackson *et al*. 2010). The solid-state fermentation method contains a simple formulation and production process yielding aerial conidia and is very well suited for low-technology artisanal production. Meanwhile, its time-consuming production process (20 days in this research), the possibility of contamination with saprophytic fungi and high labor costs in industrial production procedure indicate the approach's disadvantages. On the other hand, the liquid fermentation method mainly produces blastospores, which are as pathogenic as the aerial conidia on whitefly nymphs. However, their stability and survival rate are lower, especially under dry and hot conditions. Therefore, special techniques are needed to make it more resistant to drying. Moreover, the advantages of production in liquid fermentation are less production time (5 days in this research), better control of saprophytic contaminations, and low cost of industrial production. A fermenter or stirred bioreactor also provides an optimal homogenous nutritional environment for the rapid growth of a large amount of active and stable blastospores for commercial uses. It is capable of measuring and controlling all the important parameters such as temperature, dissolved oxygen, and pH in order to increase blastospore yield and reduce contamination (Mascarin and Jaronski 2016).

Based on the findings of this research, the survival of aerial conidia obtained from solid substrates, especially rice and corn bran substrates, was almost constant during the study period under two reported temperatures. Moreover, the conidia showed a significant mortality effect on whitefly nymphs. So, it is recommended to use these substrates for the mass production of *B. bassiana* to control greenhouse whiteflies. However, blastospores produced on liquid media containing potatoes indicated a high mortality effect on whitefly nymphs, while the survival of these spores especially at room temperature is low. There are several approaches to increasing the shelf life of blastospores (Dietsch *et al.* 2021). Nitrogen sources may affect the

productivity, desiccation tolerance, and storage stability of *B. bassiana* blastospores (Mascarin *et al.* 2018). Since blastospores infect the host faster than aerial conidia and need less time to produce (Hegedus *et al.* 1992), it is more suitable to use them against small insect pests such as whiteflies living in humid environments of the phylloplane. The results clearly show that if blastospores are to be used, storage of them in the refrigerator is essential. Further studies are essential to improve their stability.

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