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ORIGINAL ARTICLE

Viability of *Beauveria bassiana* isolates after storage under several preservation methods

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Abstract *Beauveria bassiana* is a worldwide distributed entomopathogenic fungus, which is used nowadays as an alternative to chemical pesticides in the control of several agriculture pests. Therefore, a preservation method must be established in order to maintain fungal culture and stocks, while fungal characteristics, like morphological features, spore production and viability, are kept well preserved. Although a large number of different storage methods are available and described, each fungus presents different characteristics, and therefore a new challenge for its preservation. In this work, we evaluate the effect of 1-year preservation by lyophilization, glycerol-freeze at −20°C and sub-culturing on growth, production and viability of spores and macro- and micro-morphology of three isolates of *B. bassiana*. Overall, results indicate that sub-culturing is the best method for conservation of this fungal species. However, if long-term storage is required, glycerol-freeze proved to be the most capable method of preservation.

Keywords *Beauveria bassiana* · Preservation · Fungus morphology · Viability

Introduction

Beauveria bassiana is a ubiquitous entomopathogenic fungus, from the class Deuteromycetes, currently under intensive study as a promising biocontrol agent against a large number of agricultural insect pests (Quesada-Moraga et al. 2007; Akello et al. 2009). In addition to being a necrotrophic parasite, it presents the capability to inhibit the growth of several phytopathogenic fungi (Ownley et al. 2008), and no known phytopathogenic activity is associated to this fungus (Reddy et al. 2009). Considering all these facts, *B. bassiana* has become an alternative to the use of chemical pesticides under several conditions (Uribe and Khachatourians 2004).

The establishment of entomopathogenic fungal collections is very important for biotechnology and agricultural research. Without them, there would be no stocks to support research on fungi for potential biocontrol of insects. Thus, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological, and genetic integrity of the fungi cultures over time.

Many methods for the preservation of fungal isolates have been developed. According to Gallo et al. (2008), they can be divided in two groups, according to the continued or suspend metabolism of the fungus. The methods that allow continued metabolism of the fungus include storage in sterile water, sub-cultures in agar, cool storage at temperatures ranging from 5 to 8°C and deep freeze at −20°C. Although storage in sterile water has been proven to be an easy and inexpensive method for preservation of most fungi, it can lead to a loss in the sporulation rates of fungi, as well as of changes in morphological characteristics, and should always be complemented with other methods (Borman et al. 2006). Sub-culturing fungal isolates in agar is a routinely used method to preserve fungi due to its simplicity. When it comes to storage of a large number of specimens, it becomes time-consuming, contaminations are more likely to occur, and genetic and physiological changes are not prevented (Homolka et al. 2007). Cool storage and deep freezing storage have demonstrated success in preserving long-term fungal isolates. However, even these

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methods can prove to be inadequate, due to some of the variabilities, biological or innate, of some organisms (Pasarell and McGinnis 1992).

To suspend the fungal metabolism, preservation through drying, silica gel, lyophilization, liquid nitrogen or cryogenic freezer beads at −70°C can be used. Although lyophilization and cryopreservation are considered to be the storage methods that reduce the possibility of mutation (Borman et al. 2006), both methods require specialized and expensive equipment, not available in many laboratories (Nakasone et al. 2004).

Several methods of preservation of *B. bassiana* have been proposed and studied. Most of them analyzed the effect of storage temperatures on the viability of both formulated and unformulated conidia. The temperatures tested ranged from ambient and refrigerator temperatures (Sandhu et al. 1993; Alves et al. 1996) to freezer temperatures at −10 to −7°C (Alves et al. 1996; Marques et al. 2000). In all these studies, both refrigerating and freezing were proven to be good methods for storing pure conidia for, respectively, 24 (Sandhu et al. 1993) and 80 months (Marques et al. 2000). In formulated conidia, the preservation of viability occurred for longer periods of time, for at least 7 years (Alves et al. 1996). Conidia of *B. bassiana* cryopreserved at low temperatures and in liquid nitrogen have also been subject to viability tests. Cultures have been satisfactorily maintained on sawdust medium containing 10% glycerol at −85°C, for 20 months (Kitamoto et al. 2002), and on potato dextrose agar medium at −70°C, for periods ranging from 6 months to 13 years (Pasarell and McGinnis 1992). Storage in liquid nitrogen, using 10% glycerol as cryprotectant, also seems to be an effective way to preserve *B. bassiana*, for up to 84 months (Faria et al. 1999). Only one study has been performed to evaluate the effect of lyophilization on *B. bassiana* viability (Faria et al. 1999). In the lyophilization of conidia, a mixture of 3% glucose and 3% gelatin was used and a loss of viability was verified, ranging from 41 to 94%, after 49 months of storage at 4°C.

The lack of information regarding the storage of *B. bassiana* as well as the complexity of some of the conservation methods described until now suggest that a specific protocol must be selected having regard to the characteristics of the fungus. Thus, the objective of the present work was to estimate the viability and macro and micro-morphology changes of three autochthonous *B. bassiana* isolates after being stored for 1 year at −20°C in glycerol (30%, v/v), freeze-dried (lyophilized) and continually sub-cultured in potato-dextrose agar (PDA) medium. This work will allow a direct comparison of storage methods that may be applied to unformulated conidia of *B. bassiana*, and introduces, for the first time, the use of lyophilization without the use of cryoprotective and lyoprotective agents.

Materials and methods

Fungal isolates

Autochthonous isolates of *Beauveria bassiana* were obtained during 2007 from field-collected pupae of *Prays oleae* from several localities in the Trás-os-Montes region (northeast Portugal). Fungal isolation was performed on potato-dextrose agar (PDA) medium containing 0.01% (w/v) chloramphenicol (Oxoid). Pure cultures were obtained by sub-culture in the same medium, and the different isolates were identified by amplification and sequence of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White et al. 1990). The isolates obtained were deposited in the culture collection of Escola Superior Agrária of Instituto Politécnico de Bragança (Portugal), with the designations Bb 1T/07, Bb 2T/07 and Bb 3T/07. Colonies produced from single spores were used as pure isolates.

Storage methods

The three *B. bassiana* isolates were produced in 9-cm Petri dishes containing PDA medium, for 15 days at 25±1°C in the dark, in order to provide mycelium and conidia for the different storage methods. After that, fungal isolates were stored using three different methods: (1) freeze at -20°C in an aqueous glycerol solution (30%, v/v); (2) freeze-dried (lyophilized) and maintained at ambient temperature; and (3) by continual sub-culture in PDA medium. In the first two methods, mycelium and conidia of each isolate were removed from the agar surface by scraping with a sterile scalpel and placing in sterile 2-ml cryovials tubes containing 1 mL of 30% (v/v) glycerol (freeze method) or in sterile 15-mL Falcon tubes (freeze-dried method). In the first method, the tubes were stored at −20°C, and in the second method, the fungal cultures are frozen at −20°C for 24 h and subsequently dried under vacuum in a freeze-dryer model Ly-8-FM-ULE (Snijders) for 24 h. In the continuous sub-culture method, Petri dishes containing PDA medium were centrally inoculated with a single circular 5-mm mycelial plug removed from the edge of an actively growing colony. After sealed with Parafilm, the Petri dishes were stored in the dark at 25±1°C and sub-culture every month.

Viability assessment

The viability of each isolate was assessed after 1 year of storage. Growth assessment was achieved by inoculating the centre of a 9-cm Petri dishes containing PDA medium with 2 μL of stored spores' suspension containing 2×10^6 spores/mL. Five replicate of each isolate per storage

method were performed. The plates were incubated at 25± 1°C in the dark, and the radial growth of fungal cultures was measured during 18 days. Mycelium growth indicated that the culture was viable.

Conidia production

The conidia produced by each fungal strain were evaluated after 18 days of incubation. For this, a conidia suspension was retrieved from PDA cultures, obtained in the viability assessment, to 500 μL of an aqueous solution of Tween 80 (0.02%, v/v). The number of conidia was counted in a Thoma counting chamber. Results were expressed in conidia per mL.

Spores' viability

The spores' viability was measured by quantifying the percentage of germinated conidia. Therefore, 1 mL of stored spore suspension, containing approximately 10^7 spores/mL, was spread in 9-cm Petri dishes containing agar medium (15 g/L agar-agar). Five replicates of each isolate per storage method were performed. After incubation, at 25±1°C in the dark for 24 h, the percentage of germination was evaluated by counting the number of germinated and non-germinated spores, from a total of 300 spores per Petri dish.

Macroscopic and microscopic characterization of the colonies

Macroscopic characteristics of the colonies were registered during 18 days of fungal culture and include mycelium texture, color and border appearance of the colony, medium coloration and exudates production. Microscopic characterization was evaluated under bright-field conditions after 18 days of culture with a Leica model CTR 5000 microscope, using lactophenol cotton blue staining.

Data analysis

Data from radial growth of colonies, spore germination (%) and number of spores (conidia/mL) were presented as the mean of five independent experiments displaying the respective standard error (SE) bars or standard deviation (SD) values. Differences among means were done by analysis of variance (ANOVA), using SPSS v.17 software, and averages were compared using Tukey test ($p<0.05$).

Results and discussion

The preservation of fungal strains as reference stocks for ongoing research requires that the stored cultures remain

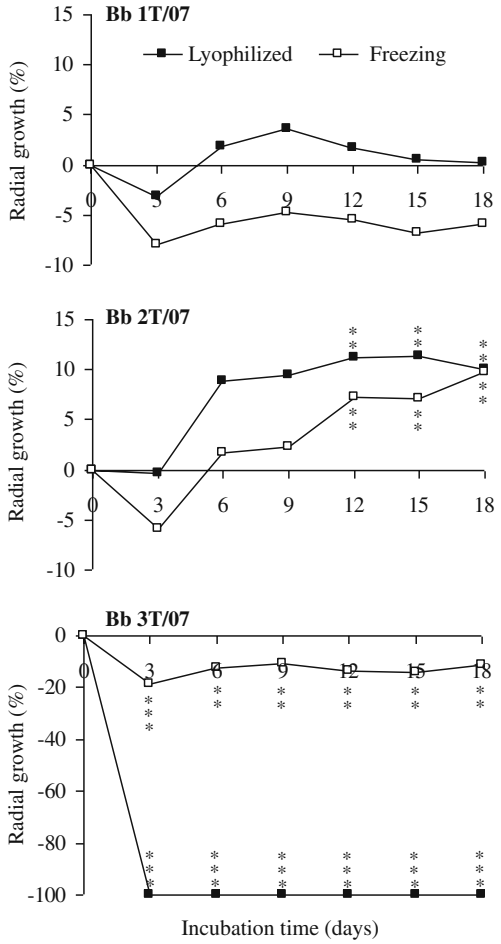

Fig. 1 Differences on radial growth (%) of *Beauveria bassiana* strains (Bb 1T/07, Bb 2T/07 and Bb 3T/07) maintained by sub-culture and preserved by two methods (lyophilization and freezing on 30% glycerol), for 18 days. Statistical significance: $^{**}p<0.01$, $^{***}p<0.001$

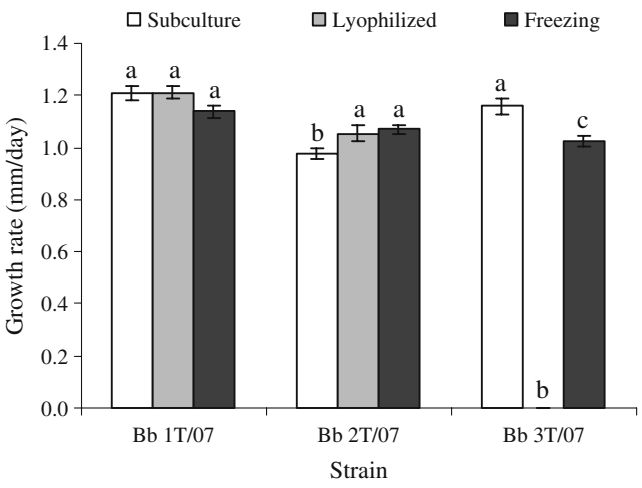

Fig. 2 Growth rates (mean ± SE, $n=5$) of *Beauveria bassiana* strains (Bb 1T/07, Bb 2T/07 and Bb 3T/07) preserved by sub-culture, lyophilized and freezing (30% glycerol) methods, on PDA medium, at day 18. *Bars* with different *letters* indicate values with significant differences ($p<0.05$), within each strain

Table 1 Spore germination and spore production (mean ± SD, n=5) of *Beauveria bassiana* strains after 1-year preservation by sub-culture (*Sb*), lyophilization (*Ly*) and freeze in aqueous solution of glycerol (30%, v/v) (*Fr*)

Strain	Spore germination (%)			No. spores (conidia/mL)		
	Sb	Ly	Fr	Sb	Ly	Fr
Bb 1T/07	100±0 a	100±0 a	100±0 a	20×10^6±9.7 a	25×10^6±10.7 a	149×10^6±76.3 b
Bb 2T/07	66.7±57.7 a	66.7±57.7 a	100±0 a	53×10^6±18.0 a	36×10^6±20.7 a	530×10^6±307.6 b
Bb 3T/07	100±0 a	n.d.	100±0 a	26×10^6±10.3 a	n.d.	28×10^6±4.3 a

Means within a row with different letters differ significantly (p<0.05.) If there was a significant difference between strains in the spore germination and number, then means were compared by Tukey's test, since equal variances could be assumed (p>0.05 by means of Levene test).
n.d. Not determined

viable for long time periods without any morphological or physiological alterations. The entomopathogenic fungal (EF) collection, of the School of Agriculture–Polytechnique Institute of Bragança, has been established by us since 2007, especially with the aim to support biocontrol research involving these fungi. Considering the lack of available information regarding the storage of entomopathogenic fungi, we have decided to study the effect of several preservation methods on the viability of autochthones *B. bassiana* isolates. Therefore, in a first attempt, we were interested in studying the viability over short periods of time (up to 1 year) in order to: (1) prevent loss of autochthones *B. bassiana* isolates, since this is the only EF collection in Portugal; and (2) provide fungal material, to perform laboratory bioassays, with the aim to selected the most virulent strains. We also intended, in a future work, to proceed to the evaluation of the viability of *B. bassiana* storage over long periods. Taking this into consideration, we decided to include the lyophilization method in the present study.

The results obtained from the radial growth shown considerable differences in the fungal isolate response to storage method (Fig. 1). Isolate Bb 1T/07, although presenting smaller radial growth when frozen in glycerol and slightly higher growth when lyophilized, did not present significant differences to the sub-culture method (considered as control assay). Isolate Bb 2 T/07, when glycerol frozen and lyophilized, presented, in the first 3 days of culture, minor growth when compared with sub-culture in PDA medium. After that, the radial growth of fungi frozen and lyophilized increased as compared to control, leading to significantly statistical differences (p< 0.01) after 12 days of culture. By contrast, isolate Bb 3T/07 showed a significantly decrease on radial growth in the freeze and freeze-dried methods when compared to control culture. It was inclusively verified, for this isolate, that lyophilized conidia were not able to grow.

Similar results were obtained for fungal growth rates (Fig. 2). In the strain Bb 1T/07, similar growth rates were observed, with no differences between control assay (PDA sub-cultures) and lyophilization or glycerol freeze storage. For strain Bb 2T/07, higher growth rates were observed for lyophilization and glycerol freeze storage, with no significant differences between both methods, while sub-cultures presented a slower rate of growth, with significant differences (p<0.05). Results for strain Bb 3T/07 showed differences between all preservation methods. For this strain, PDA sub-cultures present higher growth rates, while glycerol freeze led to a decrease on this parameter.

The number of spores produced by the different isolates showed differences related to the storage method (Table 1). While sub-cultures and lyophilization presented a similar result for two of the three isolates, the colonies obtained after the glycerol freeze storage produced a much higher

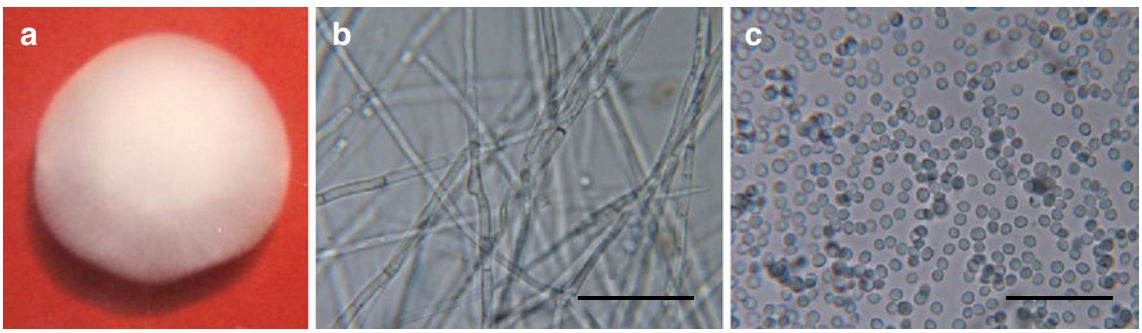

Fig. 3 Macroscopic (**a**) and microscopic (**b, c**) morphology of *Beauveria bassiana* isolate Bb 3T/07, after 1-year preservation by glycerol-freeze at −20°C. Colony (**a**), hyphae (**b**) and conidia (**c**) of *B. bassiana* grown in PDA medium. *Bar* 10 μm

number of spores, with significant statistical differences. Isolate Bb 3T/07 was the exception, presenting analogous results between subcultures and glycerol freeze, but nevertheless a higher production of spores was observed for the glycerol-conserved isolate. In the lyophilized isolate, due to the lack of germinated spores, it was not possible to evaluate the number of spores produced by the colonies. It is known that repeated sub-cultures can lead to changes in some of the characteristics of fungi, such as a decrease on the ability to sporulate (Nakasone et al. 2004), and some fungal species have their sporulation ability decreased when storage with glycerol freeze, when compared to storage in medium at 4°C (Mota et al. 2003).

Regarding spore viability, there are no significant differences ($p<0.05$) between the tested storage methods for *B. bassiana* (Table 1). Although some studies showed that lyophilization can lead to a decrease on spore viability, either by structural damage in spores, due to the formation of ice crystals on the freezing process prior to lyophilization (Nakasone et al. 2004), or in the drying process (Horaczek and Viernstein 2004), two of our *B. bassiana* isolates seemed to tolerate this kind of preservation. Isolate Bb 3T/07 proved to be an exception, presenting 0% of germination of spores. The obtained results showed that none of the preservation methods induced morphological alterations, either macroscopic or microscopic, in the different tested isolates of *B. bassiana*. In the macroscopic observation, all the colonies presented a whitish-yellow mycelium, with aerial growth and cotton-like aspect, and regular borders (Fig. 3a). The back of the colony also presented with a white color, although slightly more yellow in the center. Those characteristics are in accordance with those described for *B. bassiana* (Varela and Morales 1996; Fernandes et al. 2006). Microscopic characteristics of *B. bassiana* are similar to those previously described for this fungus, either for hyphae or conidia (Liu et al. 2003). The hyphae were septated, with hyaline aspect and thin walls (Fig. 3b), and conidia showed a rounded or ovoid shape, also with a hyaline aspect, and about 4 μm in diameter (Fig. 3c).

Although no differences were observed in the spore viability and fungal macro- and microscopic characteristics, some differences were found in the growth and production of spores of the tested isolates. It is known that response to stressful conditions, either induced on the conservation process or in the recovery process, may differ considerably among different taxonomic groups, or even between strains of some species (Kirsop and Doyle 1991). Some factors may account for the differences observed between the three tested *B. bassiana* strains, such as the rate of cooling, size of fungal propagules or thickness of the cell wall (Faria et al. 1999). Furthermore, studies prove that glycerol is the cryoprotectant most suitable for almost all strains (Homolka et al. 2003), which may account for the promising results achieved with this method of conservation. Overall, results in our work indicate that continual sub-culturing in PDA medium remains the preservation method in which fungal isolates can be stored, while maintain high viability and production of spores, as well as growth rate. Nevertheless, and due to the characteristics of this method, referred to before, as being time consuming, prone to contaminations and not preventing genetic and physiological changes in the long term, there is always the need to maintain isolates by other methods of preservation.

Freeze-drying and especially glycerol freezing could be good methods for permanent preservation of *B. bassiana*. The possibility of storing lyophilized *B. bassiana* cultures at room temperature (20–25°C), without addition of protective agents, was verified for the first time. Although the results demonstrated intra-specific sensitivity to the lyophilization process, it could be a promising method for long-term conservation of this fungus. This hypothesis must be confirmed through the evaluation of the viability of *B. bassiana* storage over long periods. Glycerol freeze seems to be the best preservation method. Higher spore production and 100% viability of spores were achieved by this method, and although presenting some significant differences in growth rate and radial growth when compared to sub-culturing, results were more promising when compared to lyophilization. Furthermore, glycerol-freeze is a rapid method for preserving cultures, with no need of expensive equipment, and which allows a quick retrieval of conidia for use in laboratory assays.

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References

Akello J, Dubois T, Coyne D, Kyamanywa S (2009) The effects of *Beauveria bassiana* dose and exposure duration on colonization and growth of tissue cultured banana (*Musa* sp.) plants. Biol Control 49:6–10

Alves S, Pereira R, Stimac J, Vieira A (1996) Delayed germination of *Beauveria bassiana* conidia after prolonged storage at low, above-freezing temperatures. Biocontrol Sci Technol 6:575–581

Borman A, Szekely A, Campbell C, Johnson E (2006) Evaluation of the viability of pathogenic filamentous fungi after prolonged storage in sterile water and review of recent published studies on storage methods. Mycopathologia 161:361–368

Faria M, Martins I, Mello R, Tigano M (1999) Entomopathogenic fungal (Hyphomycetes) collection: assessment of conidial viability. Pesqui Agropecu Bras 34:1497–1503

Fernandes E, Costa G, Moraes Á, Zahner V, Bittencourt V (2006) Study on morphology, pathogenicity, and genetic variability of *Beauveria bassiana* isolates obtained from *Boophilus microplus* tick. Parasitol Res 98:324–332

Gallo M, Guimarães D, Momesso L, Pupo M (2008) Natural products from endophytic fungi. In: Jain S (ed) Microbial Biotechnology, Volume 4. Publishing Agency, New Delhi, pp 139–147

Homolka L, Lisá L, Nerud F (2003) Viability of basidiomycete strains after cryopreservation: comparison of two different freezing protocols. Folia Microbiol 48:219–226

Homolka L, Lisá L, Kubátová A, Váňova M, Janderová B, Nerud F (2007) Cryopreservation of filamentous micromycetes and yeasts using perlite. Folia Microbiol 52:153–157

Horaczek A, Viernstein H (2004) Comparison of three commonly used drying technologies with respect to activity and longevity of aerial conidia of *Beauveria brongniartii* and *Metarhizium anisopliae*. Biol Control 31:65–71

Kirsop B, Doyle A (1991) Maintenance of microoganisms and cultured cells – a manual of laboratory methods. Academic, London

Kitamoto Y, Suzuki A, Shimada S, Yamanaka K (2002) A new method for the preservation of fungus stock cultures by deep-freezing. Mycoscience 43:143–149

Liu H, Skinner M, Brownbridge M, Parker BL (2003) Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae). J Invertebr Pathol 82:139–147

Marques E, Alves S, Marques I (2000) Virulência de *Beauveria bassiana* (Bals.) Vuill. a *Diatraea saccharalis* (F.) (Lepidoptera: Crambidae) após Armazenamento de Conídios em Baixa Temperatura. An Soc Entomol Bras 29:303–307

Mota M, Campos A, Araújo J (2003) Sporulation, radial growth and biomass production of *A. robusta* and *M. thaumasium* submitted to different methods of preservation. Braz J Microbiol 34:157–160

Nakasone KK, Peterson SW, Jong S-C (2004) Preservation and distribution of fungal cultures. In: Mueller GM, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier, Amsterdam, pp 37–47

Ownley B, Griffin M, Klingeman W, Gwinn K, Moulton J, Pereira R (2008) *Beauveria bassiana*: Endophytic colonization and plant disease control. J Invertebr Pathol 98:267–270

Pasarell L, McGinnis M (1992) Viability of fungal cultures maintained at -70°C. J Clin Microbiol 30:1000–1004

Quesada-Moraga E, Maranhao E, Valverde-García P, Santiago-Álvarez C (2007) Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity. Biol Control 36:274–287

Reddy NP, Ali Khan AP, Devi UK, Sharma HC, Reineke A (2009) Treatment of millet crop plant (*Sorghum bicolor*) with the entomopathogenic fungus (*Beauveria bassiana*) to combat infestation by the stem borer, *Chilo partellus* Swinhoe. J Asia Pac Entomol 12:221–226

Sandhu S, Rajak R, Agarwal G (1993) Studies on prolonged storage of *Beauveria bassiana* conidia: effects of temperature and relative humidity on conidial viability and virulence against Chickpea Borer, *Helicoverpa armigera*. Biocontrol Sci Technol 3:47–53

Uribe D, Khachatourians G (2004) Restriction fragment length polymorphism of mitochondrial genome of the entomopathogenic fungus *Beauveria bassiana* reveals high intraspecific variation. Mycol Res 108:1070–1078

Varela A, Morales E (1996) Characterization of some *Beauveria bassiana* isolates and their virulence toward the Coffee Berry Borer *Hypothenemus hampei*. J Invertebr Pathol 67:147–152

White Tj, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Shinsky J, White Tj (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322