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Storage of *Beauveria bassiana* Conidia Suspension: A Study Exploring the Potential Effects on Conidial Viability and Virulence against *Dermanyssus gallinae* De Geer, 1778 Acari: Dermanyssidae

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ABSTRACT

Beauveria bassiana are usually formulated as conidia infection suspensions CIS for field and laboratory applications as biocontrol agent, but the factors affecting CIS shelf-life and virulence, are unexplored. With the aim of investigating the best storage conditions for extending the CIS shelf-life, the potential effects of storage at room 22 ± 1°C and refrigerated temperatures 4 ± 1°C, with and without light and agitation, on viability and virulence of B. bassiana CISs, was assessed.

The viability of conidia was evaluated monthly, over a one year period, and the B. bassiana virulence against D. gallinae, five times in one year. B. bassiana had a significantly higher rate of survival at 4 ± 1°C compared to 22 ± 1°C, regardless of the other conditions of storage. The highest mortality rate of D. gallinae was registered for CIS stored at 4 ± 1°C, in dark and without agitation $p<0.05$.

These results indicate that refrigerating CIS in the dark and without agitation seems to guarantee the best viability and performance of B. bassiana against D. gallinae. Data obtained in this study confirm B. bassiana as potential promising candidate for controlling red mites and provide evidences that appropriate storage condition extend CIS shelf-life for up to one year.

Keywords: *Beauveria bassiana*; Bioassay study; Conidia viability; *Dermanyssus gallinae*

INTRODUCTION

The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* are the most promising bio controllers against both agricultural [1,2] and medical/veterinary pests, including some important arthropods ticks and mites, which parasitize humans and/or animals, i.e., *Rhipicephalus sanguineus*, *Rhipicephalus* (Boophilus) *microplus, Psoroptes* spp., *Dermanyssus gallinae* [3-7].

Although the advantages of using fungi as bio pesticides are well known, mainly for reducing the hazards related to the use of chemical products, their large-scale application is limited. Such limitations are largely due to the inconsistency of their performance because abiotic factors i.e., temperature, humidity, UV-radiation, habitat type can significantly reduce conidial viability, speed of germination, hyphal growth rate and spore production [8-10]. Nonetheless, the above abiotic factors are not restrictive when fungal strains are first isolated from the environment or from naturally infected hosts also known as native strains and used as myco-insecticides/acaricides [6,7,11].

To date, studies on entomopathogenic fungi have focused on direct measures of efficacy on the target arthropod species and/or to on the persistence of spores' post-treatment [6,7,12]. Data on the storage potential of spores and/or conidia, however, are limited to dry-or unformulated conidia [12-15]. Factors affecting the shelf-life, as well as the virulence, of conidia infected suspensions CIS, which are commonly employed in field and in vitro studies, are unknown. Recently, a native strain of *B. bassiana* has been shown to be highly virulent, particularly against the poultry red mite PRM *Dermanyssus gallinae*, the most economically important pest of the poultry industry [6,7]. Despite these encouraging results, it would be desirable that the performance of *B. bassiana* conidia, as well as other fungi, could be ensured overtime, particularly in large-scale studies.

To investigate the best storage conditions for extending the CIS shelf-life a study on the viability and virulence against PRM of a native *B. bassiana* CIS stored at different temperatures, and with and without light and agitation was carried out.

MATERIALS AND METHODS

Beauveria bassiana identification and conidial infection suspension preparation

A native strain of *B. bassiana* CD1123 was obtained from naturally infected ticks collected in a private dog shelter in Putignano 40°50'N, 17°07'E, 372 m a.s.l., Bari, Italy and morphologically and molecularly identified as previously described [6]. The strain was maintained on potato dextrose agar PDA and kept at 4 ± 1°C. The conidial infection suspension CIS of *B. bassiana* was obtained by growing the strain on PDA for 3 weeks at 25 ± 1°C. Conidia were harvested by washing the surface of these cultures with sterile distilled water and by transferring them to an assay tube containing sterile distilled water plus 0.1% Tween 80 v/v. Five dozen glass beads 6 mm in diameter were used to ensure complete homogenization. Turbidity was adjusted spectrophotometrically Biosan DEN 1 to an optical density of 7.5 McFarland, corresponding to 1-5 × 10^7 conidia/ml [6,7,16]. CIS was divided into 50 mL tubes Falcon® Centrifuge Tubes, (Aptaca, Asti, Italy) and the concentration of conidia was calculated as reported before and adjusted in order to obtain the same concentration in each replicate at the start of the experiments T_0.

Beauveria bassiana conidia viability

A total of eight 50 mL tubes Falcon® Centrifuge Tubes, Aptaca, Asti, Italy, containing 40 mL each of CIS, were used in the test. Each tube containing CIS was stored at ambient or refrigerated temperatures (i.e., about 22 ± 1°C and 4 ± 1°C), respectively, with/without light and agitation, according to the scheme reported in Table 1. A light-tight box was used for incubation in the dark. Agitation was achieved using a rotatory oscillating plate with and without the thermostatic cupola (ASAL s.r.l. Milan), Italy at 150 rpm. The viability of conidia in CIS in each tube was evaluated at T_0, (i.e., immediately after CIS distribution into the tubes) and monthly for one year using a quantitative plate count of colony forming units CFU/ml on PDA after incubation at 25°C for 4 days. Two replicates were prepared for each time point and the results reported as mean values of Log_{10} CFU/ml.

Table 1: Storage condition of *Beauveria bassiana* conidia infection solution (CIS) at two different temperatures (22 ± 1°C and 4 ± 1°C) in presence and absence of light and agitation

Storage tubes	Experimental conditions
A	22 ± 1°C; light, with agitation
B	22 ± 1°C; light, without agitation
C	22 ± 1°C; dark, with agitation
D	22 ± 1°C; dark, without agitation
E	4 ± 1°C; light, with agitation
F	4 ± 1°C; light, without agitation
G	4 ± 1°C; dark, with agitation

H	4 ± 1°C; dark, without agitation

Beauveria bassiana virulence

The virulence of *B. bassiana* against *D. gallinae* was tested five times in one year, i.e., at T_0 and every 3 months of storage and performed as previously reported [7]. Mites were collected from an egg-laying hen farm in Bitritto 41°03′00″N 16°50′00″E, 102 m a.s.l., Bari, Italy. The farm was naturally infested by the PRM, and no treatments were conducted one month before the collection. Mites were put in sealed plastic bags and delivered to the Department of Veterinary Medicine, Unit of Parasitology and Mycology, University of Bari. After morphological identification as *D. gallinae* [17,18], adult mites were stored at 22 ± 1°C to be used for the experiment within 24 h of collection.

A total of 3,600 adult specimens were tested. All bioassays consisted of two groups of mites, one control group CG and eight treated groups TGs, i.e., one for each CIS stored under different condition. Each group consisted of two subgroups of twenty adult mites. Mites were put into Petri dishes 60 mm diameter containing filter paper Whatman N. 1, 10 × 10 mm Labor, 67 g/m^2, Tecnochimica Moderna, Italy of the same diameter. The filter paper was soaked with 0.2 ml of each CIS (i.e., CISs from tubes A to H) for the TGs and with 0.2 mL of sterile distilled water plus 0.1% tween 80 for the CG. The mites were placed on paper soaked with either CIS or control solution. The petri dishes were covered with a lid, sealed with ParafilmTM and stored at 22 ± 1°C RH 80 ± 5%. Mortality was evaluated at 5 and 10 days' post infection, (i.e., T+5 and T+10 DPI), respectively. Mites were considered dead if they exhibited no movement after repeated mechanical stimulation with an entomological pin by three different examiners. One dead mite for each group was cultured on PDA to verify the presence of viable fungus. Dead mites were not removed from the bioassay room. Death caused by fungal infection was confirmed according to Koch's postulate. All experiments were repeated in duplicate. The mortality rate was expressed as the corrected mortality rate using Schneider-Orelli's formula: Corrected mortality % = (Mortality % in treated plot- Mortality in control plot)/(100 - Mortality % in control plot) x 100 [19].

Statistical analysis

All experiments were performed in duplicate and repeated two times on different days. The *B. bassiana* conidia viability and the mortality rates of PRM at each time point of two independent experiments were compared using Chi-square tests, with 5% significance $p<0.05$. Subsequently, the data was averaged and the viability of *B. bassiana* under different conditions over time was compared using the T test for paired samples. Statistical significance was set at $p<0.01$. The rates of mortality of *D. gallinae* after contact with *B. bassiana* stored under different conditions were compared using the Fisher's exact test. The software used was SPSS for Windows, version 13.0. Statistical significance was set at $p \leq 0.05$.

RESULTS

At T_0 the colony counts of CIS was around 1.2 × 10^7 CFU/ml, i.e., Log10 CFU/ml=7.1 (Table 2 and Figure 1). *B. bassiana* had a significantly higher rate of survival at 4 ± 1°C compared to $p<0.01$, regardless of the other storage conditions, i.e., with/without light or agitation.

Table 2: Viability of *Beauveria bassiana* conidia infection suspension (CIS) expressed as mean value of Log10 of CFU/mL (colony forming unit/mL) at different storage conditions (i.e., A, B, C, D, E, F, G and H). No statistically significant differences were marked with the same superscript letter (Student's t-test, $p<0.01$) (A: light, with agitation; B: light, without agitation; C: dark, with agitation, D: dark, without agitation; E: light, with agitation; F: light, without agitation; G: dark, with agitation; H: dark, without agitation SD: Standard deviation value)

Storage conditions								
	22 ± 1°C				4 ± 1°C			
Time+Months	A (SD)	B (SD)	C (SD)	D (SD)	E (SD)	F (SD)	G (SD)	H (SD)
T0	7.1 (0.02)	7.1 (0.02)	7.1 (0.02)	7.10 (0.02)	7.1 (0.02)^a	7.1 (0.02)	7.1 (0.02)^b	7.1 (0.02)^{c,e,f,g,h}
T+1	6.1 (0.10)	5.8 (0.02)	6.1 (0.09)	6.1 (0.10)	6.9 (0.02)^a	6.8 (0.01)	7.0 (0.06)^b	7.0 (0.01)^c

T+2	5.8 (0.02)	5.8 (0.01)	5.8 (0.01)	5.9 (0.01)	6.8 (0.01)	6.8 (0.01)	6.8 (0.01)	7.0 (0.02)[e]
T+3	5.7 (0.03)	5.8 (0.01)	5.7 (0.02)	5.8 (0.01)	6.8 (0.02)	6.8 (0.01)	6.7 (0.03)	6.9 (0.01)[f]
T+4	5.7 (0.02)	5.7 (0.02)	5.7 (0.01)	5.7 (0.02)	6.7 (0.03)	6.8 (0.02)	6.7 (0.01)	6.9 (0.02)[g]
T+5	5.6 (0.04)	5.6 (0.02)	5.6 (0.04)	5.7 (0.03)	6.7 (0.01)	6.7 (0.02)	6.6 (0.01)	6.9 (0.01)[h]
T+6	5.7 (0.02)	5.6 (0.01)	5.6 (0.02)	5.7 (0.02)	6.5 (0.04)	6.5 (0.05)	6.6 (0.02)	6.6 (0.03)
T+7	5.6 (0.02)	5.6 (0.01)	5.7 (0.02)	5.7 (0.02)	6.5 (0.05)	6.5 (0.03)	6.5 (0.01)	6.6 (0.02)
T+8	5.6 (0.01)	5.6 (0.02)	5.6 (0.02)	5.6 (0.02)	6.5 (0.02)	6.4 (0.05)	6.5 (0.03)	6.5 (0.04)
T+9	5.6 (0.02)	5.5 (0.03)	5.6 (0.01)	5.6 (0.02)	6.4 (0.05)	6.4 (0.03)	6.5 (0.01)	6.3 (0.12)
T+10	5.5 (0.04)	5.5 (0.03)	5.5 (0.04)	5.5 (0.03)	6. 4 (0.08)	6.4 (0.03)	6.3 (0.03)	6.2 (0.05)
T+11	5.5 (0.009)	5.5 (0.02)	5.5 (0.03)	5.5 (0.04)	6.44 (0.03)	6.3 (0.05)	6.2 (0.03)	6.1 (0.07)
T+12	5. 5 (0.01)	5.4 (0.03)	5.5 (0.02)	5.3 (0.03)	6.4 (0.01)	6.2 (0.05)	6.0 (0.06)	6.1 (0.06)

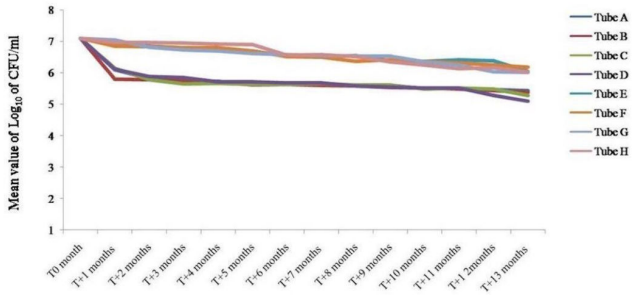

Figure 1: Graphic representation of the viability of *Beauveria bassiana* conidia infection suspension (CIS) expressed as mean value of Log^{10} of CFU/mL (colony forming unit/mL) at different storage conditions (i.e., A, B, C, D, E, F, G and H)

In particular, all CIS stored at showed a reduction of about two logarithmic units of CFU/mL, starting from the 2nd month, persisting until the 12th incubation month. CIS stored at showed a reduction of about one logarithmic unit of CFU/mL starting from the 5th month, remaining unchanged until the end of the monitoring time. Light or agitation did not affect *B. bassiana* survival in the samples kept at the same temperature $p>0.05$. At all storage times, the number of conidia in CIS stored in H was higher or, at least, equal to that under other storage conditions. The in vitro effect of *B. bassiana* CIS stored under different conditions against adults of *D. gallinae* at T+5 and T+10 DPI are reported in Tables 3 and 4 and Figures 2 and 3.

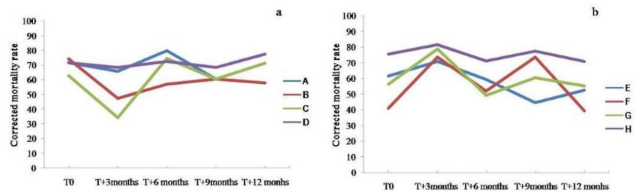

Figure 2: Graphic representation of the corrected mortality rate at 5 days post-infection of *Dermanyssus galllinae* with *Beauveria bassiana* stored at 22 ± 1°C (a) and 4 ± 1°C (b) with and without light and agitation (i.e., A, B C, D, E, F, G and H) during 1 year of observation time

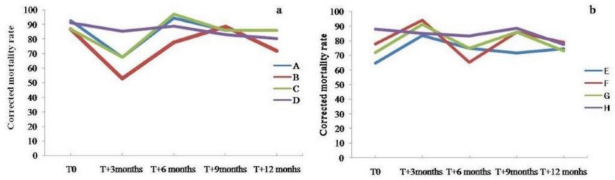

Figure 3: Graphic representation of the corrected mortality rate at 10 days post infection of *Dermanyssus galllinae* with *Beauveria bassiana* stored at 22 ± 1°C (a) and 4 ± 1°C (b) with and without light and agitation (i.e., A, B C, D, E, F, G and H) during 1 year of observation time

No statistical differences between replicates were recorded ($p>0.05$). The mortality rate of *D. gallinae* increased significantly $p<0.01$ according to the time of exposure in all TGs. The corrected mortality rate at T+5 and T+10 DPI varied accordingly to the storage method. A different trend of virulence at 5 and 10 DPI, during the storage time, (i.e., T_0 and every 3 months of storage for one year), was registered according to storage temperature. Overall, at T+5 DPI, solutions A, D and H showed the highest efficacy and stability overtime, with no significant loss of efficacy during the storage time (Table 3). At T+10 DPI, solutions D, G and H showed the best efficacy and stability overtime (Table 4).

Table 3: Corrected mortality rate at T_0 and every 3 months in one year of *Dermanyssus gallinae* with *Beauveria bassiana* stored at different conditions (i.e., A, B, C, D, E, F,G and H) after 5 days post-infection (A: light, with agitation; B: light, without agitation; C: dark, with agitation, D: dark, without agitation; E: light, with agitation; F: light, without agitation; G: dark, with agitation; H: dark, without agitation; NA: Not available; SD: Standard deviation values; *values significantly lower than the others (Fisher exact test; $p<0.05$)

Storage conditions								
Time+Months	22 ± 1°C				4 ± 1°C			
	A (SD)	B (SD)	C (SD)	D (SD)	E (SD)	F (SD)	G (SD)	H (SD)
T0	71.8 (1.4)	74.4 (2.8)	62.9 (2.8)	71.8 (4.2)	61.5 (7.1)	41 (4.2)*	56.4 (4.2)	75.6 (0.7)
T+3	65.8 (4.2)	47.4 (5.7)*	34.2 (1.4)*	68.4 (5.7)	71 (1.4)	73.7 (2.8)	78.9 (2.8)	81.6 (0.7)
T+6	79.7 (2.8)	57 (1.4)	74.6 (0.0)	72.5 (1.4)	59.5 (0.0)	51.9 (4.2)*	49.4 (0.0)*	71.2 (0.7)
T+9	60.5 (7.1)	60.5 (7.1)	60.5 (1.4)	68.4 (0.0)	44.7 (1.4)*	73.7 (0.0)	60.5 (7.1)	77.6 (3.5)
T+12	NA	57.9 (5.7)	71.5 (1.4)	77.6 (0.0)	77.6 (2.8)	39.5 (4.2)*	55.3 (4.2)	71 (4.2)

Table 4: Corrected mortality rate at T_0 and every 3 months in one year of *Dermanyssus galllinae* with *Beauveria bassiana* stored at different conditions (i.e., A, B, C, D, E, F, G and H) after 10 days post-infection (A: light, with agitation; B: light, without agitation; C: dark, with agitation, D: dark, without agitation; E: light, with agitation; F: light, without agitation; G: dark, with agitation; H: dark, without agitation; NA: Not available; SD: Standard deviation value; *value significantly lower than the others (Fisher exact test; $p<0.05$)

Storage conditions								
22 ± 1°C					4 ± 1°C			
Time+Months	A (SD)	B (SD)	C (SD)	D (SD)	E (SD)	F (SD)	G (SD)	H (SD)
T0	92.65 (2.1)	86.76 (0.7)	86.76 (0.7)	91.18 (1.4)	64.71 (4.2)*	77.94 (3.5)	72.06 (0.7)	88.24 (2.8)
T+3	67.65 (4.2)*	52.94 (5.7)*	67.65 (1.4)*	85.29 (1.4)	83.82 (4.9)	94.12 (0.0)	91.18 (4.2)	85.29 (0.0)
T+6	94.44 (0.0)	77.78 (5.7)	97.22 (1.4)	88.89 (1.4)	75 (1.4)	65.28 (10.6)*	75 (1.4)	83.33 (2.8)
T+9	85.92 (1.4)	88.73 (2.8)	85.92 (1.4)	83.1 (0.0)	71.83 (0.0)	85.92 (1.4)	85.92 (1.4)	88.73 (0.0)
T+12	NA	71.83 (2.8)	85.92 (1.4)	80.28 (1.4)	74.65 (1.4)	78.87 (3.5)	73.24 (0.7)	77.46 (5.7)

DISCUSSION

The vitality and the virulence of the native strain of *B. bassiana* CIS seems to be affected by the temperature, whilst virulence against PRM by temperature, light and agitation. These findings overlap the available data on *B. bassiana* conidia formulated in dry powder form. Previous studies suggest that temperature, humidity and spore moisture content can all influence the viability of entomopathogenic fungi during long-term storage [12-15,20-23]. Low temperature, i.e., <5°C, as well as low spore moisture content <5%, ensure fungal viability [12,15,20,24], and promote faster development of the fungus, due to higher production of ATP [15,25]. In this study, storage at 4 ± 1°C provided the best vitality of *B. bassiana* CIS, with no significant reduction in CFU detected over five months. It has been shown that the maximum yield of conidia is usually achieved under conditions of hypoxia in the absence of light [26,27]. Conversely, in our study, conidial viability appears unrelated to agitation of CIS and/or the presence/ absence of light. Such contrasting results might be due to the experiment herein designed; in fact, the hypoxic conditions PO_2<16% and the agitation speed >250 rpm, which reduces the fungus viability [27], may have not been reached in our experiment. However, if the vitality of conidia is not affected by the presence or absence of light and agitation, the virulence of *B. bassiana* is linked to the above factors, with CIS stored in the dark and without agitation, (i.e., D and H tubes) being highly virulent and stable overtime against *D. gallinae* at 5 and 10 DPI.

These findings confirm that light may negatively affect the virulence of the fungus as suggested by the effect of the solar radiation, particularly UV-B [26]. Additionally, although the agitation speed might enhance enzyme production by filamentous fungi, it might also damage the cells, interfering with fungal virulence, thus confirming our results [28].

CONCLUSION

The results of this study confirm the high virulence of *B. bassiana* CIS against the PRM [7] and suggest that storage methods affect the vitality of *B. bassiana* and its virulence against the PRM. Under refrigerated conditions, i.e., 4 ± 1°C, RH 80 ± 5% in dark and without agitation, *B. bassiana* CIS can be stored with minimal loss of viability or virulence for up to one year. The suggested conditions of storage, coupled with *B. bassiana* virulence against *D. gallinae*, indicates that this fungus is a potential candidate to be employed as an alternative to the chemical compounds currently used for controlling red mites.

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