REDIA, XCVIII, 2015: 49-55

ANNARITA CITO (*) - VALERIA FRANCARDI (*) - GIAN PAOLO BARZANTI (*)
AGOSTINO STRANGI (*) - SAURO SIMONI (*) - PIO FEDERICO ROVERSI (*)

EFFECTS OF CRYOPRESERVATION AND FREEZE-DRYING ON PROTEASES ENZYMATIC ACTIVITY OF ENTOMOPATHOGENIC STRAINS OF *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN AND *METARHIZIUM ANISOPLIAE* (METCHNIKOFF) SOROKIN

(*) *Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CRA-ABP), Research Center for Agrobiology and Pedology, Via Lanciola 12/A, 50125, Florence, Italy;* e-mail: annarita.cito@isza.it

Cito A., Francardi V., Barzanti G.P., Strangi A., Simoni S., Roversi P.F. – Effects of cryopreservation and freeze-drying on proteases enzymatic activity of entomopathogenic strains of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin.

Beauveria bassiana and *Metarhizium anisopliae* are fungal species known for their entomopathogenic activity; they represent an important alternative to chemical control of many pest insects and appropriate preservation methods are thus necessary to maintain their properties over time. In the present study the effects of cryopreservation and freeze-drying were investigated on virulence determinants such as proteases and specifically on subtilisin-type Pr 1 and Pr 2 that seem to play a predominant role in insect fungal penetration. Three *Metharizium anisopliae* strains (M 13/ I05, M 13/ I12 and M 13/ I33) and three *Beauveria bassiana* strains (B 13/ I03, B 13/ I57 and B 13/ I63) were tested. Data obtained from our experiments show that the decrease of Pr 1 and Pr 2 enzymatic activity in *B. bassiana* and *M. anisopliae* strains 7 months after storage does not affect their entomopathogenic ability against larvae of *Tenebrio molitor* compared to the fresh cultures before storage. The role of Pr 1 and Pr 2 as predominant factors in the entomopathogenicity of both fungal species is discussed. No significant differences were found in any strain tested for morphological features using both preservation methods.

KEY WORDS: Ascomyceta, entomopathogenic fungi, preservation methods, subtilisin-type Pr 1, trypsin-like protease Pr 2.

INTRODUCTION

Beauveria bassiana (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin are entomopathogenic fungi that have recently received an increasing interest for their promising application as biocontrol agents of a large number of agricultural pest insects and their ecological sustainable impact (VEGA *et al.,* 1995; 2000; EL-SUFTY *et al.,* 2009; FRANCARDI *et al.,* 2012, 2013; PETLAMUL & PRASERTSAN, 2012; MOHAMMADBEIGI & PORT, 2013).

Appropriate methods of cultivation and preservation are thus required to ensure the integrity of morphological, physiological and especially entomopathogenic features of the strains against target insect pests over time. At this regards, several preserving methods were tested in laboratory to maintain fungal cultures for short to long time; sub-culturing of the fungal strains is a common preserving method but it requires consumables and contamination risks are possible. Therefore, freezing, including cryopreservation, with or without cryoprotectants, at low or ultra low temperature, and freeze-drying have been proposed as more preservative methods of fungal strains for long-term storage (NAKASONE *et al.*, 2004; HOMOLKA, 2013).

While viability of *B. bassiana* (FARIA *et al.,* 1999; KITAMOTO *et al.,* 2002; OLIVEIRA *et al.,* 2011) and *M. anisopliae* strains (FARIA *et al.,* 1999; ROBERTS & ST LEGER, 2004) was previously detected after their cryopreservation and freeze-drying, the effects of these two preservation techniques on their entomopathogenic properties were poorly investigated (MARQUES *et al,.* 2000; RYAN *et al.,* 2001; TOEGEL *et al.,* 2010). In particular, the effects of cryopreservation and freeze-drying have been investigated for the first time by our group. Results are reported in the present paper on virulence determinants such as proteases and specifically on subtilisin-type Pr 1 that seems to play a key role in insect penetration (ST. LEGER *et al.,* 1988; SHAH *et al.,* 2005), and trypsin-like protease Pr 2 that could be involved in induction or activation of Pr 1 (ST LEGER *et al.,* 1996; GILLESPIE *et al.,* 1998).

The aim of the present study is to investigate the effects of the cryopreservation and freeze-drying methods adopted on the *M. anisopliae* and *B. bassiana* strains selected in order to assess if their morphological features are stable and well-preserved and overall if possible changes in enzymatic activity of total protease, Pr 1 and Pr 2 may affect their entomopathogenic activity that has been tested against larvae of *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae).

MATERIALS AND METHODS

ISOLATION OF THE FUNGAL STRAINS

Metarhizium anisopliae and *B. bassiana* strains used in the experiments were isolated from dead larvae and adults of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryo-

– Received 23 June 2015 Accepted 28 September 015 Published 10 December 2015

phthoridae) collected from infected palm trees in Tuscany and Sicily (Italy) during 2013. Fragments of infected tissues were placed in Petri dishes (Ø 60 mm) containing Sabouraud Dextrose Agar added with Yeast extract 0.25% (SDAY). The plates were placed at 25°C in darkness for 5-7 days. Fungal strains were subcultured and identified for morphological characters and by sequencing of the internal transcribed spacer region (ITS), using the universal primers ITS1 and ITS4. Morphological, enzymatic and entomopathogenic features were determined on fresh cultures before storage and 7 months after storage; each experiment was carried out in three replicates.

The selected strains were compared to the cultures of the entomopathogenic microorganism collection of the Council for Agricultural Research and Economics, Florence and then they were deposited in the collection for further investigations.

Cryopreservation and freeze-drying treatments

Fungal strains were subcultured on SDAY and incubated at 25±1°C with a relative humidity (RH) of 80% for 15 days. Half of the agar plugs (Ø 7 mm) obtained from each fungal culture were single placed in sterile cryotubes (1.5 ml) containing 10% glycerol in water and stored at -80°C , the rest were single placed in tubes (1.5 ml) frozen at -20°C for 24 h, freeze-dried by a vacuum pump system (Edwards Modulyo Freeze-Dryer, West Sussex U.K.) overnight and then stored at -20°C.

Assessment of the radial growth of the colonies, conidia production and germination rate of the fungal strains tested

An agar plug of each strain obtained from fresh cultures was placed in Petri dishes containing SDAY and incubated at 25±1°C for 15 days. Radial growth of the colonies (mm/day^{-1}), conidia production (number of $conidia/mL^{-1}$) and germination rate (%) were detected according to Petlamul & Prasertsan (2012). For comparative purposes, the same features were determined using an agar plug from each freeze-dried strain and an agar plug from those cryopreserved 7 months . Both were compared to fresh cultures. The agar plugs were incubated at 25±1°C in darkness and after 15 days radial growth of the colonies, conidia production and germination rate were determined.

Enzymatic activity assays

Culture conditions

A basal salts medium (1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.5 g/l NaCl, Carlo Erba Reagents, Italy), containing 1 g/L casein (Sigma-Aldrich, Missouri U.S.A.) was inoculated with a concentration of $1x10^7$ conidia/ml and then incubated at 25±1°C for 72 hours. Conidial suspensions were collected from the cultures obtained from the three tested treatments : fresh, freeze-dried and cryopreserved. To determine the activity of total proteases, Pr 1 and Pr 2, for the fresh and the stored cultures, mycelia were harvested by centrifugation and the supernatants were used to determine enzymatic activity by UV-VIS spectrophotometer assays (SmartSpec™ Plus, Bio-Rad, California, U.S.A.).

Total protease enzymatic activity

Total protease activity assay was performed according to Lakshmi *et al.* (2010). Briefly, 0.4 ml of casein substrate (1 g casein/ 10 ml of 0.01 M Tris-Buffer at pH 8.0), 0.2 ml of supernatant and 0.2 ml of 0.01 M Tris Buffer at pH 8.0 were incubated for 10 min at 37°C. The reaction was stopped by adding 0.7 ml of 1.2 M trichloroacetic acid (Sigma-Aldrich, Missouri U.S.A.). The samples were centrifuged at 1000 rpm for 5 min and the resulting supernatants were used for absorbance determination at 280 nm. One unit of total protease activity was defined as the amount of enzyme that produced 1.0 mM of tyrosine per minute under the above conditions.

Pr 1 and Pr 2 enzymatic activity

Pr 1 and Pr 2 enzymatic activity was assayed by a modified method described by St. Leger *et al.* (1987). N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich, Missouri U.S.A.) was used as the specific synthetic substrate for Pr 1, while N-benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma-Aldrich, Missouri U.S.A.) was used as the specific substrate for Pr 2. In detail, 0.05 ml of both substrates were mixed with 0.85 ml of Tris buffer 0.05 M pH 8.0 and 0.025 ml of culture supernatant. The reaction mixture was incubated for 45 min at 30°C and the reaction was terminated using 0.25 ml of 30% acetic acid. Samples were centrifuged at 4000 rpm for 10 min and placed in ice for 15 min before spectrophotometer determination.

Absorbance of para-nitro aniline produced by the reaction was observed at 410 nm; one unit of Pr 1 and Pr 2 enzymatic activity was defined as the amount of enzyme that produced 0.001 mM of para-nitro aniline per minute under the above conditions. One unit of Pr 1 and Pr 2 enzymatic activity was defined as the amount of enzyme that produced 0.001 mM of para-nitro aniline per minute.

Bioassays against larvae of T. molitor

Entomopathogenic activity of the fungal strains was tested against larvae of *T. molitor*, that are known to be susceptible to these two entomopathogenic fungal species and often used as laboratory insect test for bioassays (Bharadwaj & Stafford, 2011; Oreste *et al.*, 2012).

Mature larvae of *T. molitor* were obtained from laboratory insect breeding, maintained in plastic boxes at 23±2°C and fed with bread and bran. Twenty larvae were dipped for 30" in 10 ml of conidial suspension (1×10^7 conidia/ml) of each fungal strain; three replicates were made for each fungal strain. The concentration of the conidial suspension adopted for the bioassays was determined on the basis of the previous entomopathogenicity tests performed in laboratory (Francardi *et al.,* 2012). Conidia used for bioassays were obtained from 15 days old colonies from SDAY Petri dishes used for the morphological and enzymatic determination for both fresh and stored cultures. Larvae immersed in water solution 0.02% Tween 80 were used as control. The excess moisture was removed from the larvae with a filter paper.

Treated and control larvae were then placed in Petri dishes (Ø 120 mm) with a filter paper on the bottom, incubated in a climatic chamber at 25±1 °C and a RH 80%. Mortality was recorded daily for twenty days. Mycosis was confirmed by the external growth of the mycelia and by sporulation of the fungus on the dead larvae bodies.

Statistical Analysis

To evaluate the effect of both the preservation methods used, data were analyzed by paired Student's *t* test, after checking normality and homogeneity of variance by Shapiro-Wilk and Levene tests, respectively. All tests and analyses were performed with SPSS 15.0 software (SPSS, 2006).

RESULTS

Identification of the fungal strains

The colonies obtained from cultures of the three *B. bassiana* strains showed a whitish and flocculent mycelium and subglobose conidia (2-4 mm) (Fig. I, 7-12). The cultures of the three *M. anisopliae* strains showed olivaceous-green colour due to the abundant presence of long chains of cylindrical conidia (5-8 x 2.5-3.5 mm) (Fig. I, 1-6). *B. bassiana* strains were defined by the following codes: B 13/ I03, B 13/ I57 and B 13/ I63 while *M. anisopliae* strains by the following codes: M 13/ I05, M 13/ I12 and M 13/ I33.

The ITS sequences belonging to *B. bassiana* B 13/ I03, B 13/ I57 and B 13/ I63 and *M. anisopliae* M 13/ I05, M 13/ I12 and M 13/ I33 were found equal to their corresponding type strains *B. bassiana* ARSEF 1564 (accession numbers NR_111594) and *M. anisopliae* ARSEF 7487 (GeneBank accession number NR_132017).

Radial growth of the colonies, conidia production and germination rate of the fungal strains tested

Radial growth of the colonies, conidia production and germination rate of the *B. bassiana* and *M. anisopliae* strains in fresh cultures and 7 months after both storage treatments are reported in Table 1. No significant differences were found in all the strains tested for morphological features after both preservation methods.

Enzymatic activity assay

Total protease enzymatic activity

Metarhizium anisopliae strains M 13/ I05, M 13/ I12 and M 13/ I33 and *B. bassiana* strain B 13/ I03 showed a total protease enzymatic activity after cryopreservation and freeze-drying not significantly different from that observed before storage (Fig. II,1).

A significant decrease of total protease enzymatic activity was instead found in *B. bassiana* strains B 13/ I57

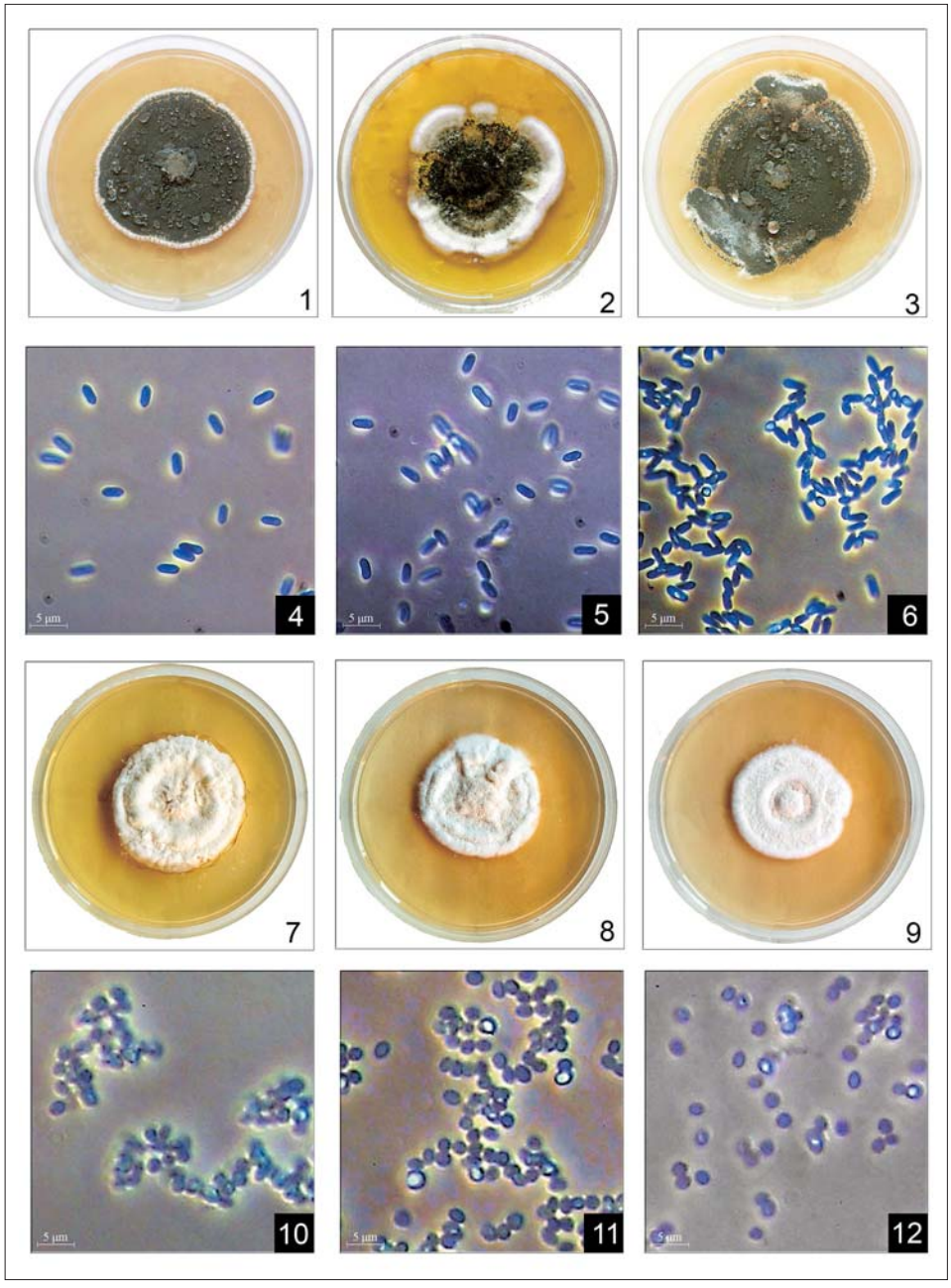

Figure I – Colonies of *M. anisopliae* strains M 13/ I05 (1), M 13/ I12 (2), M 13/ I33 (3) and *B. bassiana* B 13/ I03 (7) B 13/ I57 (8) and B 13/ I63 (9) incubated at 25±1°C for 15 days on SDAY Petri dishes Ø 90 mm and their conidia at a phase contrast light microscope, 400 × (4-6 for *M. anisopliae* strains; 10-12 for *B. bassiana* strains).

and B 13/ I63 after cryopreservation ($t = 5.341$, $df = 4$, $p < 0.05$) *vs* data obtained before storage (Fig. II,1)

Pr 1 enzymatic activity

Pr 1 enzymatic activity decreased in *M. anisopliae* strain M 13/ I05 after cryopreservation ($t = 2.900$, $df = 4$, $p < 0.05$) but not after freeze-drying. Pr 1 enzymatic activity significantly decreased 7 months after cryopreservation in *M. anisopliae* strains M 13/ I12 ($t = 10.629$, $df = 4$, $p < 0.01$), M 13/ I33 ($t = 10.229$, $df = 4$, $p < 0.01$) and in the same strains after freeze-drying: M 13/ I12 ($t = 9.202$, $df = 4$, $p < 0.01$) and M 13/ I33 ($t = 8.883$, $df = 4$, $p < 0.01$) (Fig. II, 2).

A significant decrease of Pr 1 enzymatic activity was also detected 7 months after cryopreservation in *B. bassiana* strain B 13/ I03 ($t = 4.192$, $df = 4$, $p < 0.05$), B 13/ I57 ($t = 21.427$, $df = 4$, $p < 0.01$) and B 13/ I63 ($t = 10.938$, $df = 4$, $p < 0.01$). Pr 1 enzymatic activity also decreased after freeze-drying in B 13/ I03 ($t = 4.806$, $df = 4$, $p < 0.05$) B 13/ I57 ($t = 10.938$, $df = 4$, $p < 0.01$) and B 13/ I63 ($t = 10.938$, $df = 4$ $p < 0.01$) (Fig. II, 2).

Pr 2 enzymatic activity

Pr 2 enzymatic activity observed in *M. anisopliae* strain M 13/ I05 significantly decreased after cryopreservation ($t = 3.313$, $df = 4$, $p < 0.05$) but after freeze-drying it was similar to that observed before storage. M 13/ I12 and M 13/ I33 strains showed a significant decrease of Pr 2 after cryopreservation ($t = 8.453$, $df = 4$, $p < 0.01$ and $t = 14.080$, $df = 4$, $p < 0.01$ respectively) and similarly after freeze-drying ($t = 14.476$, $df = 4$, $p < 0.01$ and $t = 10.968$, $df = 4$, $p < 0.01$ respectively) (Fig. II, 3).

Pr 2 enzymatic activity decreased after cryopreservation in *B. bassiana* strains B 13/ I03 ($t = 25.663$, $df = 4$, $p < 0.01$), B 13/ I57 ($t = 13.953$, $df = 4$, $p < 0.01$) and B 13/ I63 ($t = 13.953$, $df = 4$, $p < 0.01$) and in the same fungal strains after freeze-drying ($t = 9.004$, $df = 4$, $p < 0.01$, $t = 10.370$, $df = 4$, $p < 0.01$ and $t = 10.370$, $df = 4$, $p < 0.01$ respectively) (Fig. II, 3).

BIOASSAYS AGAINST LARVAE OF T. MOLITOR

Data obtained from entomopathogenic bioassay experiments are reported in Table 2.

All of the three *M. anisopliae* strains tested showed a cumulative mortality of *T. molitor* larvae > 90 % before storage and 7 months after cryopreservation while after freeze-drying, cumulative mortality varied from 88% to 99%.

Tenebrio molitor larvae cumulative mortality due to *B. bassiana* strains B 13/ I03 and B 13/ I57 recorded before storage (77% and 80% respectively) is similar to that observed after cryopreservation (70% and 75% respectively) and freeze-drying (68% and 70%, respectively).

Beauveria bassiana strain B 13/ I63 caused 98% cumulative mortality of *T. molitor* larvae before storage, that significantly decreased to 65% after cryopreservation ($t = 41.131$, $df = 4$, $p < 0.01$) and to 55% after freeze-drying ($t = 6.507$, $df = 4$, $p < 0.01$).

DISCUSSION

In the present study the effects of cryopreservation and freeze-drying on total proteases, Pr 1 and Pr 2 enzymatic activity were investigated in *B. bassiana* and *M. anisopliae* strains. These fungi penetrate the host cuticle by mechanical force and cuticle-degrading enzymes such as chitinases, lipases and proteases (KHACHATOURIANS & QAZI, 2008). Penetration of insect cuticle, mainly composed up of chitin and protein, is mediated by proteases and specifically by Pr 1 that seems to play a key role in entomopathogenic fungi infection process (ST. LEGER *et al.,* 1988; SHAH *et al.,* 2005) and by Pr 2 that could induce/and or activate Pr 1 during the early stages of cuticle colonization (ST LEGER *et al.,* 1996; GILLESPIE *et al.,* 1998). Pr 1 and Pr 2 are the most studied serine proteases involved in the mechanism of fungal penetration of *B. bassiana* and *M. anisopliae* (CASTELLANOS-MOGUEL *et al.,* 2007; DHAR & KAUR, 2010; PERINOTTO *et al.,* 2014; SÁNCHEZ-PÉREZ *et al.,* 2014).

Data obtained in the present study showed that although total protease enzymatic activity resulted unchanged in almost all fungal strains, Pr 1 and Pr 2 enzymatic activity decreased in fungal strains similarly after cryopreservation and freeze-drying with the exception of M 13/ I05 strain. The decrease of Pr 1 and Pr 2 enzymatic activity seems to be a consequence of the low temperatures adopted rather than of the preservation methods applied. Previously, RYAN *et al.* (2001) observed that different preservation methods can affect fungal secondary metabolite profiles and extracellular enzyme production of *M. anisopliae* and that response to preservation may be strain specific; similarly, KIRSOP & DOYLE (1991) proposed that metabolism alteration is a consequence of the different

Table 1 – Radial growth of colonies, conidia production and germination rate of the *B. bassiana* and *M. anisopliae* strains assayed in fresh cultures and 7 months after both storage treatments.

Fungal strains	Daily Radial Growth (mm/day^{-1})			Conidia Production (number of conidia/mL^{-1})			Germination rate (%)[a]		
	Before storage	Cryo.	Freeze-drying	Before storage	Cryo.	Freeze-drying	Before storage	Cryo.	Freeze-drying
M 13/I05	1.8 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.7 ± 1.4	2.4 ± 1	2.6 ± 1.3	96 ± 2.2	93 ± 5.2	98 ± 1.1
M 13/I12	2.0 ± 0.6	2.1 ± 0.1	2.2 ± 0.1	1.6 ± 0.2	1.3 ± 0.4	1.5 ± 0.4	98 ± 5.9	99 ± 0.8	99 ± 0.1
M 13/I33	1.8 ± 0.7	2.1 ± 0.1	2.2 ± 0.1	2.7 ± 0.4	2.6 ± 1.4	2.6 ± 1.6	98 ± 3.1	99 ± 0.4	98 ± 2.2
B 13/I03	2.0 ± 0.2	1.9 ± 0.3	1.8 ± 0.5	1.7 ± 0.7	1.6 ± 0.8	1.4 ± 0.3	97 ± 1.1	98 ± 0.9	97 ± 1.1
B 13/I57	1.9 ± 0.4	1.8 ± 0.2	2.2 ± 0.2	2.4 ± 0.5	2.3 ± 0.9	2.6 ± 0.2	98 ± 1.9	99 ± 0.4	99 ± 0.3
B 13/I63	1.8 ± 0.1	2.1 ± 0.3	1.9 ± 0.4	3.0 ± 0.7	2.5 ± 0.5	2.4 ± 1.2	99 ± 1.1	99 ± 0.5	99 ± 0.8

[a] data were processed after Arcsin transformation

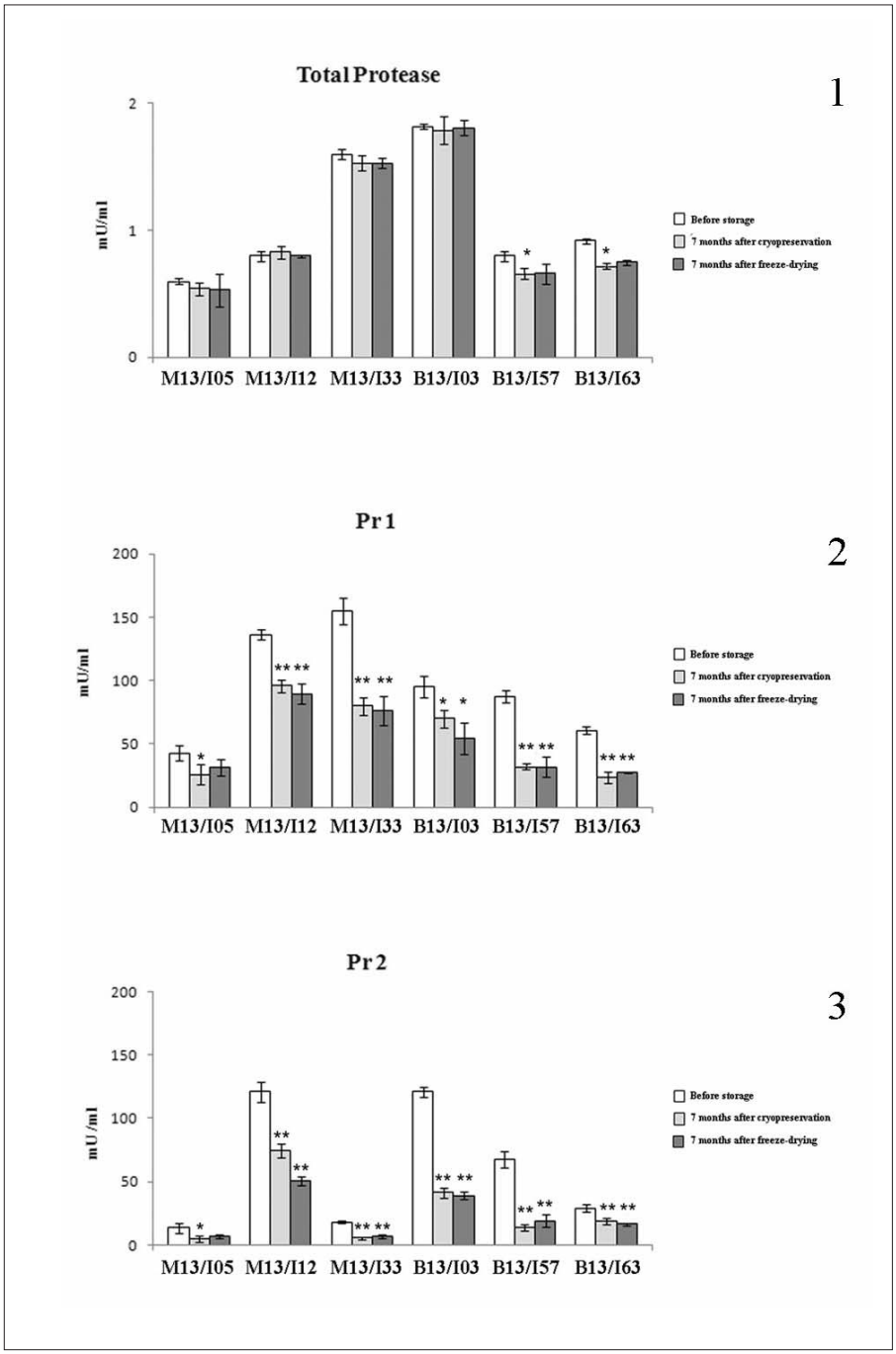

Figure II – Enzymatic activity of total protease (1), Pr 1 (2) and Pr 2 (3). Each data represents the average (Mean ± Standard Deviation) of three replicates. Statistical significance * $p < 0.05$; ** $p < 0.01$.

Table 2 – Bioassays against *Tenebrio molitor* larvae.

Fungal strains	Mortality 20 days after inoculation (%)[a]		
	Before storage	7 months after cryopreservation	7 months after freeze-drying
M 13/I05	95	95	99
M 13/I12	98	93	85
M 13/I33	98	90	88
B 13/I03	77	70	68
B 13/I57	80	75	70
B 13/I63	98	65**	55**

[a] data were processed after Arcsin transformation. Statistical significance * $p < 0.05$; ** $p < 0.01$.

response of fungal strains to stressful conditions imposed by the storage procedure.

In the present paper, in spite of the decrease of Pr 1 and Pr 2 enzymatic activity after cryopreservation and freeze-drying, the entomopathogenicity of *M. anisopliae* and *B. bassiana* strains tested against *T. molitor* larvae after both the adopted storage methods was similar to that observed before the storage, with the exception of B 13/ I63 strain. These results support hypothesis that entomopathogenicity of *B. bassiana* and *M. anisopliae* is complex and involves several mechanisms and that although cuticle-degrading proteases, Pr 1 and Pr 2 especially, are important virulence determinants, they are not predominant factors (WANG *et al.,* 2003; CITO *et al.,* 2014). On the contrary, the total protease activity is well-

preserved independently from the strains and the storage method tested; as a consequence the total protease activity could be considered a reliable virulence factor for the fungal strains tested. The hypothesis that other Pr isoforms could play a crucial role in the entomopathogenicity and virulence of the strains is likely (St. Leger *et al.,* 1994). The present study also showed that both the adopted cryopreservation and freeze-drying methods do not affect the growth and the biology of *B. bassiana* and *M. anisopliae* strains 7 months after storage in agreement with Pasarell & McGinnis (1992), Marques *et al.* (2000), Kitamoto *et al.* (2002) and Oliveira *et al.* (2011). On the contrary, Faria *et al*. (1999) observed a loss of total viability (from 40.9% to 80.7%,) in *B. bassiana* strains 48-49 months and in *M. anisopliae* strains (from 89.5% to 96.9%), 47-48 months after freeze-drying. Oliveira *et al*. (2011) pointed out that the adopted freeze storage method at -20°C was the best promising preservation method for spore viability (100%) and spore production in all the *B. bassiana* strains tested compared to freeze-drying procedure which caused a loss of viability in some of the fungal strains 1 year after the storage. The successful set up in freeze-drying protocol could be due to keeping the same temperature (-20 °C) before and after the freeze-drying process to maintain fungal strains. The absence of cryoprotectants did not influence viability of *M. anisopliae* strains despite data previously reported by Toegel *et al*. (2010).

In conclusion the results obtained show that the adopted cryopreservation and freeze-drying procedures ensure *B. bassiana* and *M. anisopliae* strains preservation for 7 months. Both methods are rapid, with no need of expensive equipment and do not affect viability and entomopathogenicity of the fungi strains tested, essential factor for their use in biological control.

Further studies based on the evaluation of the factors involved in the virulence expression have to be performed to confirm our preliminary data.

ACKNOWLEDGMENTS

The research was supported by a grant from the Italian Ministry of Agriculture, Food and Forestry national project "PROPALMA" (D.M. 25618/7301/11, 2012/12/01).

We are grateful to Dr. Tullio Turchetti, National Research Council, for his precious advises.

REFERENCES

Bharadwaj A., Stafford K.C., 2011 – *Potential of* Tenebrio molitor *(Coleoptera: Tenebrionidae) as a Bioassay Probe for* Metarhizium brunneum *(Hypocreales: Clavicipitaceae) Activity Against* Ixodes scapularis *(Acari: Ixodidae).* - J. Econ. Entomol., 104**:** 2095-2098.

Castellanos-Moguel J., Gonzalez-Barajas M., Mier T., Reyes-Montes Mdel R., Aranda E., Toriello C., 2007 – *Virulence testing and extracellular subtilisin-like (Pr1) and trypsin-like (Pr2) activity during propagule production of* Paecilomyces fumosoroseus *isolates from whiteflies (Homoptera: Aleyrodidae).* - Rev. Iberoam. Micol., 24: 62-68.

Cito A., Mazza G., Strangi A., Benvenuti C., Barzanti G.P., Dreassi E., Turchetti T., Francardi V., Roversi P.F., 2014 – *Characterization and comparison of* Metarhizium *strains isolated from* Rhynchophorus ferrugineus. - FEMS Microbiol. Lett., 355: 108-115.

Dhar P., Kaur G., 2010 – *Cuticle-Degrading Proteases Produced by* Metarhizium anisopliae *and Their Induction in Different Media.* - Indian J. Microbiol., 50: 449-455.

El-Sufty R., Awash S.A., Al-Bgham S., Shahdad A.S., Al-Bathra A.H., 2009 – *Pathogenicity of the Fungs* Beauveria bassiana *(Bals.) Vuill to the Red Palm Weevil,* Rhynchophorus ferrugineus *(Oliv.) (Col.: Curculionidae) under Laboratory and Field Conditions.* - Egyptian J. Biol. Pest Control, 19: 81-85.

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

Francardi V., Benvenuti C., Roversi P.F., Rumine P., Barzanti G.P., 2012 – *Entomopathogenicity of* Beauveria bassiana *(Bals.) Vuill. and* Metarhizium anisopliae *(Metsch.) Sorokin isolated from different sources in the control of* Rhynchophorus ferrugineus *(Olivier)(Coleoptera Curculionidae).* - Redia, 95: 49-55.

Francardi V., Benvenuti C., Barzanti G.P., Roversi P.F., 2013 - *Autocontamination trap with entomopathogenic fungi: a possible strategy in the control of* Rhynchophorus ferrugineus *(Olivier) (Coleoptera Curculionidae).* - Redia, 96: 57-67.

Gillespie J.P., Bateman R., Charnley A.K., 1998 – *Role of Cuticle-Degrading Proteases in the Virulence of* Metarhizium *spp. for the Desert Locust,* Schistocerca gregaria. - J. Invertebr. Pathol., 71: 128-137.

Homolka L., 2013 - *Methods of Cryopreservation in Fungi.* - In: Gupta VK, Tuohy MG, Ayyachamy M, Turner KM,O'donovan A (Eds.), Laboratory Protocols in Fungal Biology. Springer, New York, pp. 9-16.

Khachatourians G., Qazi S., 2008 - *Entomopathogenic Fungi: Biochemistry and Molecular Biology.* - In: Brakhage, A., Zipfel, P. (Eds.), Human and Animal Relationships. Springer Berlin Heidelberg, pp. 33-61.

Kirsop B., Doyle A., 1991 - *Maintenance of microorganisms and cultured cells*.- Academic Press London, 308 pp.

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.

Lakshmi S.B., Kuar S.G., Palem P.P., 2010 – *Isolation and purification of cuticle degrading extracellular proteases from entomopathogenic fungal species of* Beauveria bassiana *and* Metarhizium anisopliae. - Int. J. Appl. Biol. Pharmaceut. Technol., 1: 1150-1156.

Marques E.J., Alves S.B., Marques I.M., 2000 – *Virulence of* Beauveria bassiana *(Bals.) Vuill. to* Diatraea saccharalis *(F.)(Lepidoptera: Crambidae) after conidia storage at low temperature.* - An. Soc. Entomol. Bras., 29: 303-307.

Mohammadbeigi A., Port G., 2013 – *Efficacy of* Beauveria bassiana *and* Metarhizium anisopliae *against* Uvarovistia zebra *(Orthoptera: Tettigoniidae) via contact and ingestion.* - Int. J. Agric. Crop Sci., 5: 138-146.

Nakasone K.K., Peterson S.W., Jong S.C., 2004 - *Preservation and distribution of fungal cultures.* - In: Mueller GM, Bills GF,Foster MS (Eds.), Biodiversity of Fungi. Academic Press, Washington DC, pp. 37-47.

Oliveira I., Pereira J.A., Bento A., Baptista P., 2011 – *Viability of* Beauveria bassiana *isolates after storage under several preservation methods.* - Ann. Microbiol., 61: 339-344.

Oreste M., Bubici G., Poliseno M., Triggiani O.,

TARASCO E., 2012 - *Pathogenicity of* Beauveria bassiana *(Bals.-Criv.) Vuill.and* Metarhizium anisopliae *(Metschn.) Sorokin against* Galleria mellonella *L. and* Tenebrio molitor *L. in laboratory assays.* – Redia, 95: 43-48.

PASARELL L., MCGINNIS M. R., 1992 - *Viability of fungal cultures maintained at –70°C.* - J. Clin. Microbiol., 30**:** 1000-1004.

PERINOTTO W., ANGELO I., GOLO P., CAMARGO M., QUINELATO S., SANTI L., VAINSTEIN M., BEYS DA SILVA W., SALLES C., BITTENCOURT V., 2014 – Metarhizium anisopliae *(Deuteromycetes: Moniliaceae) Pr1 activity: biochemical marker of fungal virulence in* Rhipicephalus microplus *(Acari: Ixodidae).* - Biocontrol Sci. Technol., 24: 123-132.

PETLAMUL W., PRASERTSAN P., 2012 – *Evaluation of strains of* Metarhizium anisopliae *and* Beauveria bassiana *against* Spodoptera litura *on the basis of their virulence, germination rate, conidia production, radial growth and enzyme activity*. - Mycobiology, 40: 111-116.

ROBERTS D.W., ST. LEGER R.J., 2004 - Metarhizium *spp., Cosmopolitan Insect-Pathogenic Fungi: Mycological Aspects*. - In: Gadd G,Sariaslani S (Eds.), Adv. Appl. Microbiol. Academic Press, pp. 1-70.

RYAN M., JEFFRIES P., BRIDGE P., SMITH D., 2001 – *Developing cryopreservation protocols to secure fungal gene function*. - CryoLetters, 22: 115-124.

SÁNCHEZ-PÉREZ L., BARRANCO-FLORIDO J.E., RODRÍGUEZ-NAVARRO S., CERVANTES-MAYAGOITIA J.F., 2014 – *Enzymes of Entomopathogenic Fungi, Advances and Insights*. - Adv. Enzyme Res., 2: 65-67.

SHAH F.A., WANG C.S., BUTT T.M., 2005 – *Nutrition influences growth and virulence of the insect pathogenic fungus* Metarhizium anisopliae. - FEMS Microbiol. Lett., 251: 259-266.

SPSS, 2006 SPSS for Windows v. 15.0, pp., SPSS Inc. Chicago (IL) USA.

ST. LEGER R.J., CHARNLEY A.K., COOPER R.M., 1987 – *Characterization of cuticle-degrading proteases produced by the entomopathogen* Metarhizium anisopliae. - Arch. Biochem. Biophys., 253: 221-232.

ST. LEGER R.J., DURRANDS P.K., COOPER R.M., CHARNLEY A.K., 1988 – *Regulation of production of proteolytic enzymes by the entomopathogenic fungus* Metarhizium anisopliae. - Arch. Microbiol., 150: 413-416.

ST. LEGER R.J., BIDOCHKA M.J., ROBERTS D.W., 1994 – *Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by* Metarhizium anisopliae. - Arch Biochem Biophys, 313: 1-7.

ST LEGER R.J., JOSHI L., BIDOCHKA M.J., RIZZO N.W., ROBERTS D.W., 1996 – *Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by* Metarhizium anisopliae *in infected insect cuticles*. – App. and Environ. Microbiol., 62: 1257-1264.

TOEGEL S., SALAR-BEHZADI S., HORACZEK-CLAUSEN A., VIERNSTEIN H., 2010 – *Preservation of aerial conidia and biomasses from entomopathogenic fungi* Beauveria brongniartii *and* Metarhizium anisopliae *during lyophilization*. - J. Invertebr. Pathol., 105: 16-23.

VEGA F.E., DOWD P.F., BARTELT R.J., 1995 – *Dissemination of Microbial Agents Using an Autoinoculating Device and Several Insect Species as Vectors*. - Biol. Control, 5: 545-552.

VEGA F.E., DOWD P.F., LACEY L.A., PELL J.K., JACKSON D.M., KLEIN M.G., 2000 - *Dissemination of Beneficial Microbial Agents by Insects*. - In: Lacey L,Kaya H (Eds.), Field Manual of Techniques in Invertebrate Pathology. Springer Netherlands, pp. 153-177.

WANG C., SKROBEK A., BUTT T.M., 2003 – *Concurrence of losing a chromosome and the ability to produce destruxins in a mutant of* Metarhizium anisopliae. - FEMS Microbiol. Lett., 226: 373-378.