YARA EL KHOURY[a*] - GIUSEPPE BARI[a] - CARLO SALVEMINI[b] - GIAMBATTISTA MARIA ALTIERI[a] - JAVAD KARIMI[c] - MICHELE POLISENO[a] - NIKOLA GRUJIĆ[d] - GIOVANNI BUBICI[e] - EUSTACHIO TARASCO[a]

SUSCEPTIBILITY OF FOUR STORED-PRODUCT INSECT PESTS TO *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE* STRAINS

[a]*Department of Soil Plant & Food Sciences, University of Bari, Bari, Italy*
[b]*Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Naples, Italy*
[c]*Department of Plant Protection, Ferdowsi University of Mashhad, Mashhad, Iran*
[d]*University of Belgrade-Faculty of Agriculture, Department for Entomology and Agricultural Zoology, Belgrade-Zemun, Serbia.*
[e]*Istitute for sustainable plant protection, National Research Council of Italy, Bari, Italy*
***** Corresponding Author: yara.elkhoury@uniba.it

El Khoury Y., Bari G., Salvemini C., Altieri G.M., Karimi J., Poliseno M., Grujić N., Bubici G., Tarasco E. - Susceptibility of four stored-product insect pests to *Beauveria bassiana* and *Metarhizium anisopliae* strains.

Insect infestations are considered one of the major problems causing enormous economic losses in stored grains. Laboratory bioassays were performed to establish the mortality induced by the commercially available *Beauveria bassiana* strain ATCC74040 (formulated product: Naturalis®) and by *Metarhizium anisopliae* strain CIST8 against four stored products pests. Adults of four grain and legume pest species (*Cathartus quadricollis*, *Callosobruchus maculatus*, *Sitophilus granarius*, and *Oryzaephilus surinamensis*) were exposed in laboratory assays to three different concentrations (10^3, 10^5 and 10^7/mL) of each entomopathogenic fungal strain. For each insect species, fungal strain and concentration, the mortality was recorded daily over a period of 7 days. Mean survival time and final cumulative mortality were determined, and 7-day mortality curves were established. A significant effect of insect species, fungal strain, and conidial concentration was observed on the 7-day mortality curves and the final cumulative mortality. Also, the mean survival time varied significantly with the conidial concentration. In addition, a significant interaction between insect species and fungal strain was recorded for all three assessed parameters. In general, at all tested concentrations, *B. bassiana* strain ATCC 74040 caused higher mortality than *M. anispliae* strain CIST8 in all four insect species. Our results suggest that entomopathogenic fungi could be effectively used as part of an integrated pest management program for the control of legumes and grain pests.

Key Words: Entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae,* stored-products pests

INTRODUCTION

In a rapidly growing human population and uneven resource distribution, food security is endangered. According to the FAO, between 720 and 811 million people faced hunger in 2020 (FAO, 2020). This was worsened by the COVID-19 pandemic as between 118 and 161 million people were affected by undernourishment in 2020 more than in 2019 (FAO, 2020). To ensure food security, the agricultural systems are concerned mainly to increase production and productivity of farming systems but less attention is given to ensuring the preservation of the commodities and reduction of losses in the post-harvest phase. However, as much as 30% of the quantity of produced grains and legumes is wasted globally (Sawicka, 2019).

Insect infestations are considered one of the major problems causing enormous economic losses in the stored grains. In addition to the loss of food commodities, thus endangering food security, insect infestation results in the limitation of international trade. Hagstrum *et al.,* (2013) have identified a high number of stored-product insects that have reached 1663 species. For instance, the cowpea weevil or the bean beetle *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae) is a primary pest that infests both the pods in the field and the seeds in the storage facilities (Stoll, 1988; Kéïta *et al.,* 2000; Beck and Blumer, 2014). *Callosobruchus maculatus* is widespread in the subtropical and tropical areas of Asia and Africa (Beck and Blumer, 2014). The cowpea weevil feeds only in the larval stage, and the lifespan of the adults lasts for just two weeks to mate and lay the eggs (Beck and Blumer, 2014). The pupa develops inside the seed then the adult emerges leaving behind a single hole in the seed (Beck and Blumer, 2014). Another main stored-product insect pest is the granary weevil *Sitophilus granarius* L. (Coleoptera: Curculionidae) that can only be found in human-made grain storage (Plarre, 2010). The granary weevil infests the stored grain such as rye, wheat, corn, and rice (Dobie and Kilminster, 1978; Schwartz and Burkholder, 1991; Plarre, 2010). The larval development takes place within the grain seeds. In addition, the adults live from a few months to one year, they feed also on the stored grains, causing damage (Plarre, 2010). The development of one generation at a temperature of 25 degrees Celsius takes about 40 days (Plarre, 2010).

Other grains pests like *Cathartus quadricollis* (Guerin-Meneville) (Coleoptera: Silvanidae) and *Oryzaephi-*

Received 8 September 2022 Accepted 11 November 2022

lus surinamensis Linneaus (Coleoptera: Silvanidae) are considered secondary pests (CABI, 2022). The square-necked grain beetle, *C. quadricollis* can coexist with *C. maculatus* (Fabricius), *Sitophilus oryzae* (Linnaeus), and other grain pests (Allotey and Morris, 1993; Sousa *et al.,* 2009). Although the sawtoothed grain beetle is a secondary pest of stored grain (Champ and Dyte, 1976; Beckel *et al.,* 2007), *O. surinamensis* Linneaus (Coleoptera: Silvanidae) is a very important storage pest throughout the world (Champ and Dyte, 1976; Beckel *et al.,* 2007). It attacks broken grain seeds or cereals damaged by the primary storage pests (Mathlein, 1971; Howe, 1973; Pricket *et al.,* 1990; Beckel *et al.,* 2007), and processed products such as flour, nuts, and dried fruits (Highland, 1991; Mowery *et al.,* 2002; Beckel *et al.,* 2007).

To manage post-harvest insect infestations, air recirculation, grain protectants, fumigation, and storage insecticides are used (Coceral, 2018). However, operators are struggling to comply with the zero tolerance for live insects as the most effective pesticides are gradually phased out of the market; high resistance to the approved storage insecticides (organophosphates and pyrethroids in the EU) and fumigation techniques, although safe and practical, do not kill premature insect stages (Coceral, 2018).

Beauveria bassiana (Bals.-Criv.) Vuill. (Hypocreales, Cordycipitaceae) and *Metarhizium anisopliae* (Hypocreales, Clavicipitaceae) are two entomopathogenic EPF ascomycetes. Both EPF inhabit various soils around the world (Quesada-Moraga *et al.,* 2007). These EPF are used as biological insecticides to control numerous pests such as beetles, aphids, whiteflies, thrips, grasshoppers, and termites (Roberts and Hajek, 1992; Vega *et al.,* 2012; El Khoury *et al.,* 2020; Quesada-Moraga *et al.,* 2020; Keszthelyi *et al.*, 2021). Both species require arthropod hosts to develop the mitosporic conidia; they quiesce in a conidial stage in the soil before infecting a host. *Beauveria bassiana* and *M. anisopliae* attack insect hosts via attachment to cuticular substrates and the production of enzymes for insect cuticle degradation and penetration (Rustiguel, 2012; Pedrini *et al.,* 2013).

According to several studies, EPF could be a potential alternative to chemical insecticides against various numbers of insects infesting stored grains and legumes (Flinn and Schöller, 2012; Rumbos and Athanassiou, 2017; Batta and Kavallieratos, 2018; Wakil, 2021). The application of different strains of EPF, *M. anisopliae* and *B. bassiana*, on grains has increased the mortality of adult stages of the lasser grain borer, *Rhyzopertha dominica*, the granary weevil, *Sitophilus granarius*, the red flour beetle, *Tribolium castaneum*, and Khapra beetle, *Trogoderma granarium*, etc. (Riasat *et al.,* 2011; Kavallieratos *et al.,* 2014; Wakil *et al.,* 2015; Wakefield, 2018; Saeed *et al.,* 2020, Wakil *et al*., 2021). However, further studies are needed to test the efficacy of commercial EPF on a wider range of cereal pests. Therefore, our study aims to conduct a preliminary evaluation of the pathogenicity of EPF *B. bassiana* ATCC74040 and *M. anisopliae* CIST8, by conducting bioassays against four legume Coleoptera species under laboratory conditions.

MATERIALS & METHODS

Stored products pests: rearing and collecting

Colonies of *Callosobruchus maculatus* were reared in the Entomology Laboratory of the Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy. Colonies were kept in flasks containing chickpea seeds (*Cicer arietinum*) and covered with a fine mesh for ventilation. Flasks were incubated in a climate chamber at 28±2°C and 70±10% relative humidity. Bioassays were performed with 24-42 h old adults. The remaining pests were collected from various sources such as infested chickpeas and infested pea bags in grain and legume stores of farms in the Apulia region of Italy. *Cathartus quadricollis*, *Sitophilus granarius*, *and Oryzaephilus surinamensis* were identified and used in the bioassays within one to two weeks.

Preparation of formulated conidial suspensions

To prepare the fungal suspensions, fresh green colonies of *M. anisopliae* developed for 15 days on potato dextrose agar (PDA) culture medium at a temperature of 22°C and a ratio of 16:8 (L:D) were scrubbed with a sterile Pasteur pipette and 15 to 20 ml of sterile distilled water was added. The Petri dish was then sealed and stirred for 1 minute to ensure maximum dissociation of conidia from the colonies into the solution. This conidial suspension was filtered through 6 layers of gauze cloth to remove mycelial fragments. The filtrate was collected in Falcon tubes and used as stock solution. Conidia were counted using a Malassez chamber hemacytometer under an optical microscope prior to bioassays. To prepare the bioassay solutions, the starting solutions were decimally diluted several times to obtain suspensions containing 10^3, 10^5, and 10^7 conidia/mL for both strains. Tween 80 (0.02%) was added as a surfactant. The procedure was performed near a flame and under a laminar flow hood to ensure high standards of hygiene.

Conidial suspensions of *B. bassiana* strain ATCC 74040 were obtained by diluting appropriate amounts of the formulated product Naturalis® (an oil dispersion (OD) containing 2.3 x 10^7 viable conidiospores/mL) from CBC (Europe) S.r.l. - BIOGARD Division (Grassobbio, Italy) in sterile distilled water.

Laboratory mortality bioassays

The experiment was carried out under laboratory conditions in sterile Petri dishes (diameter of 15 cm). Sterile filter papers were placed in the Petri dishes and 20 adults of one pest species were introduced. Then the pests were sprayed with 0.5 mL of the conidial suspension at a known concentration using a micropipette. For each fungal strain, *B. bassiana* ATCC74040 and *M. anisopliae* CIST8, three concentrations were tested: 10^3, 10^5 and 10^7 conidia/mL. For each pest species, the experiment was repeated five times with each fungal concentration.

Table 1 - Results of ANOVA analysing 7-day mortality curves, final mortality mean value and mean survival time as a function of insect species (IS), Fungal strain (FS) and Conidial concentration (C) (general analysis and analysis by insect species).

Source of variation	7-day mortality curves[a,b]	Final cumulative mortality mean value[b,c]	Mean survival time of individuals who died from treatment[c]
Phase 1: general analysis			
Insect species (IS)	**<0.0001**	**<0.0001**	0.2158
Fungal strain (FS)	**0.0029**	**0.0012**	0.8457
Conidial concentration (C)	**<0.0001**	**<0.0001**	**0.0008**
IS×FS	**<0.0001**	**0.0182**	**0.0025**
IS×C	0.8888	0.3606	0.2363
FS×C	0.9722	0.4749	0.3685
Phase 2: analysis by insect species (IS)			
Catartus quadricollis			
Fungal strain (FS)	**<0.0001**	**0.0001**	**0.0062**
Conidial concentration (C)	**0.0005**	**0.0010**	0.4197
Callosobruchus maculatus			
Fungal strain (FS)	**<0.0001**	**0.0001**	**0.0085**
Conidial concentration (C)	**<0.0001**	**<0.0001**	0.0730
Sitophilius granarius			
Fungal strain (FS)	**<0.0001**	**<0.0001**	0.1394
Conidial concentration (C)	**<0.0001**	**0.0061**	**0.0337**
Oryzaephylus surinamensis			
Fungal strain (FS)	**0.0076**	**0.0134**	1.0000
Conidial concentration (C)	**0.0013**	**0.0017**	1.0000

Bold values denote statistical significance at the p < 0.05 level
[a] Repeated measures analysis of variance (ANOVA)
[b] Data subjected to angular transformation before analysis
[c] Analysis of variance (ANOVA)

An untreated control was also replicated five times by spraying the adults with 1mL of sterile distilled water containing 0.02% Tween 80. The treated pests in the plates were then incubated for 7 days in a dark climate chamber at 25 °C and 75% relative humidity. Mortality was recorded daily for each treatment. Mortality caused by fungi was considered and confirmed by re-isolating the pathogen on a PDA medium.

Statistical data analysis

Mortality curves data were analyzed by repeated measures analysis of variance ANOVA. The final cumulative mortality data (on the 7th day after treatment) and the mean survival time MST, (MST for each strain and each concentration, were obtained by applying the Kaplan - Meier analysis) were analyzed by ANOVA. The data of the mortality curves and those of final cumulative mortality were subjected to angular transformation before analysis. Means were compared using the LSD test ($p < 0.05$).

RESULTS

The entomopathogenic fungal strains used in the experiment have different efficacy on the insects tested and the interactions between the insect species and the fungal strain were significant $p < 0.05$ for all three parameters assessed (Table 1). However, the interactions between the insect species and the conidial concentrations ($p=0.88$; $p=0.36$; $p=0.23$) and those between the fungal isolate and the conidial concentration ($p=0.97$; $p=0.47$; $p=0.36$) were not significant (Table 1). The effect of the conidial concentration is therefore neither dependent on the insect species nor on the fungal strain. Furthermore, both strains caused mortality rates directly proportional to exposure time and conidial concentration (Fig. I).

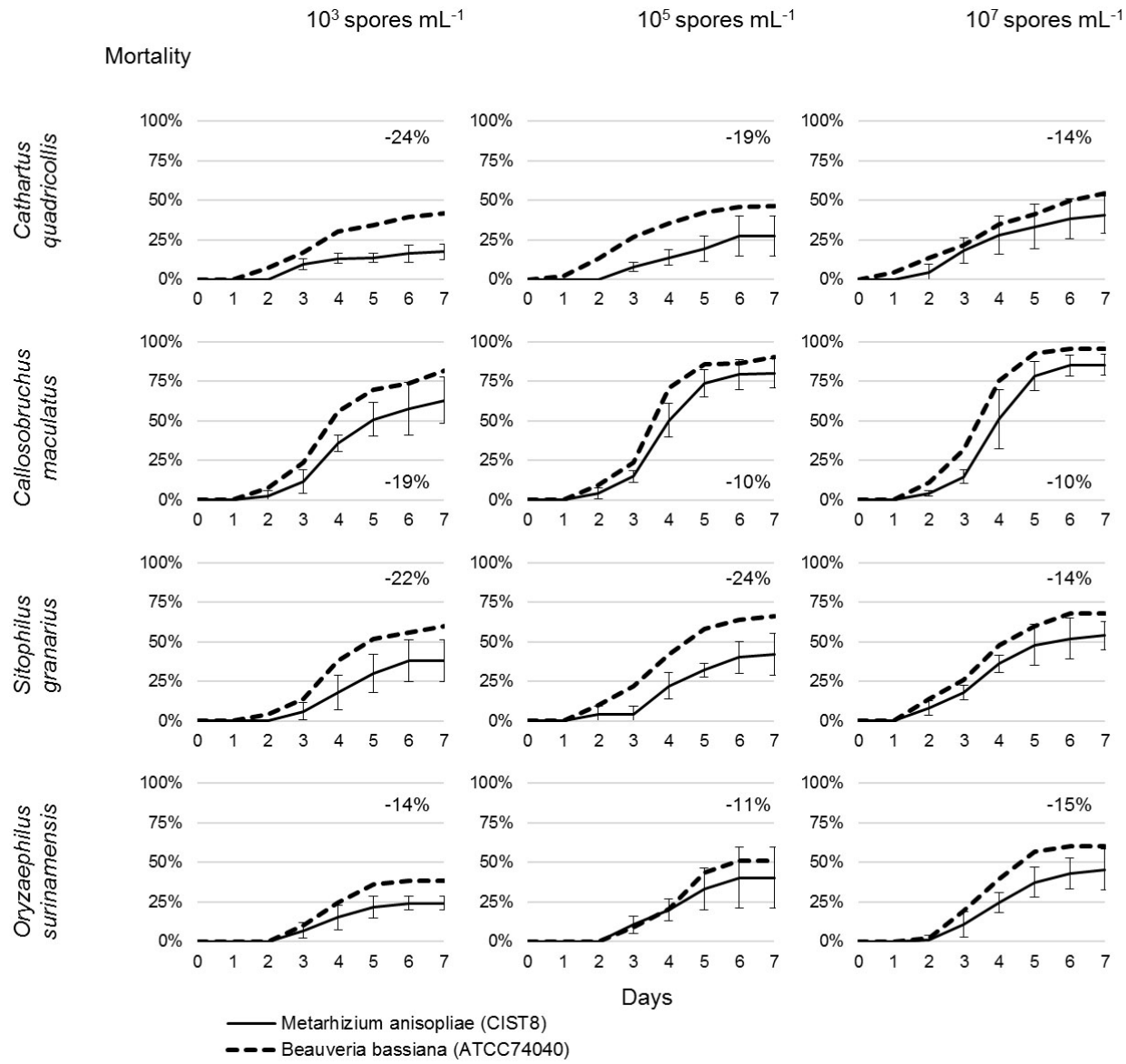

Fig. I - 7-Day mortality curves (± SE) of *C. quadricollis*, *C. maculatus*, *S. granarius*, and *O. surinamensis* adults treated with three concentrations of *Beauveria bassiana* ATCC 74040 and *Metarizium anisopliae* CIST8.

In the second phase of analysis, the efficacy of the treatments on the different species of arthropods was evaluated separately. The results showed that the efficacy of the strains of *B. bassiana* and *M. anisopliae* varied for all pests tested (Table 1). In terms of final mortality mean, the *M. anisopliae* strain is on average 7-14% less effective than *B. bassiana* (Table 2), with differences ranging from 10% (*C. maculatus* at concentrations 10^5 and 10^7) to 24% (*C. quadricollis* at concentration 10^3; *S. granarius* at concentration 10^5) (Fig. I). Thus, the *M. anisopliae* CIST8 strain showed low efficacy against *O. surinamensis* (24-46%) compared to the other strains (38-60%).

Looking at the mean survival time, this is on average higher for the CIST8 strain than for ATCC74040 for *C. quadricollis*, *C. maculatus* and *S. granaries*, but the same for the experiment against *O. surinamensis* (Table 3).

The mortality rates of the four beetles differed in all experiments. In particular, *C. maculatus* recorded the highest mortality (82-95%) when treated with *B. bassiana*, regardless of concentration. Similarly, *M. anisopliae* at the highest concentration of 10^7 spores/ml caused the highest mortality in *C. maculatus* (85%) compared to *S. granarius* (54%), *O. surinamensis* (46%) and *C. quadricollis* (41%).

DISCUSSION

EPF, especially *B. bassiana* and *M. anisopliae*, parasitize on a wide range of insect pests (Quesada-Moraga *et al.,* 2007). Recently, several studies have been conducted to test the pathogenicity of entomopathogenic fungi against pests of stored products, especially beetles (Rumbos and Athanassiou, 2017; Batta and Kavallieratos, 2018). However, none of these strains are approved and registered for use against insects in stored products (Rumbos and Athanassiou, 2017).

Table 2 - Final cumulative mortality rates (% mortality ± S.D.) of 7-days exposed *C. quadricollis*, *C. maculatus*, *S. granarius*, and *O. surinamensis* adults to three doses of *M. anisopliae* CIST8 and *B. bassiana* ATCC74040. Mean values of five replicates.

	Treatments							
	Metarhizium anisopliae CIST8 spore/mL			Control	*Beauveria bassiana* ATCC74040 spore/mL			Control
Insect species	10^3	10^5	10^7	1mL 0.02% Tween80	10^3	10^5	10^7	1mL 0.02% Tween80
Cathartus quadricollis	17±4.93	27±12.7	41±11.65	14±3.4	42±11.97	46±11.54	55±14.48	15±3.4
Callosobruchus maculatus	63±14.66	80±8.96	85±6.8	25±4.4	82±7.83	91±4.16	95±2.77	29±4.6
Sitophilius granarius	38±13.04	42±13.04	54±8.94	12±3.2	60±7.07	66±5.48	68±4.47	12±3.2
Oryzaephilus surinamensis	24±4.32	40±19.68	46±13.32	5±2.1	38±7.4	51±17.51	60±18.38	8±2
Averages	**35.5%**	**47.25%**	**56.5%**	**14%**	**48%**	**56%**	**63%**	**16%**
	46.4%				**55.6%**			

Table 3 - Mean survival time (MST) (days ± S.D.) of *C. quadricollis*, *C. maculatus*, *S. granarius*, and *O. surinamensis* adults exposed to three doses of *M. anisopliae* CIST8 and *B. bassiana* ATCC74040.

		Mean survival Time (MST) ± St Dev. (days)		
Insect	Treatment	10^3 spore/mL	10^5 spore/mL	10^7 spore/mL
Cathartus quadricollis	*Metarhizium anisopliae* CIST8	3.8±0.6	4.3±0.5	3.8±0.3
	Beauveria bassiana ATCC74040	3.8±0.1	3.2±0.2	3.5±0.3
Callosobruchus maculatus	*Metarhizium anisopliae* CIST8	4.5±0.4	4.2±0.1	4.3±0.3
	Beauveria bassiana ATCC74040	4.2±0.4	3.9±0.1	3.8±0.6
Sitophilus granarius	*Metarhizium anisopliae* CIST8	4.6±0.6	4.4±0.9	4.0±0.3
	Beauveria bassiana ATCC74040	4.3±0.4	4.0±0.2	3.8±0.2
Oryzaephilus surinamensis	*Metarhizium anisopliae* CIST8	4.2±0.6	4.3±0.4	4.3±0.6
	Beauveria bassiana ATCC74040	4.2±0.3	4.6±0.1	4.1±0.2

In our study, all treatments were effective against the stored-product pests tested, albeit with varying results. This is consistent with previous studies that have shown that the application of *M. anisopliae* and *B. bassiana* on grains increased the mortality of adult stages of different grain and legume pests. The bean beetle *C. maculatus*, the lasser grain borer *R. dominica*, the granary weevil *S. granarius*, the rice weevil *Sitophilus oryzae*, the red flour beetle *T. castaneum* and Khapra beetle *T. granarium* were all susceptible to entomopathogenic fungi (Rice and Cogburn, 1999; Cherry *et al.*, 2005; Kavallieratos *et al.,* 2014; Wakefield, 2018; Saeed *et al.,* 2020, Wakil *et al.,* 2021). On average, *B. bassiana* (55.6%) achieved higher mortality rates than *M anisopliae* (46.4%) for all insect pests tested. Regardless of the fungal species, the commercial formulation of *B. bassiana* (Naturalis®), which is an oil dispersion (OD), may have improved the performance and viability of the strain's conidia compared to the unformulated local strain *M. anisopliae* (CIST8) with tween80. For example, the formulation containing the oil suspension of *B. bassiana* was the most virulent among all formulations and provided the best protection for maize grains against *S. zeamais* (Hidalgo *et al.,* 1998). Similarly, treatments with formulated *M. anisopliae* and *B. bassiana* were significantly more effective than their non-formulated counterparts against *R. dominica* and *T. molitor*, respectively (Batta, 2005; Batta *et al.,* 2010).

Another enormously important factor in the treatment of pests in stored products is the pace of treatment, especially when treating insects with a short life cycle. The faster the pest respond to the treatment, the more effective the treatment is considered. *Callosobruchus maculatus*, for example, can complete a life cycle in two to three weeks, which explains the relatively higher mortality in the control compared to the other pests. In our study, the highest mortality rates were recorded from the fifth day onwards. As reported by Ashraf *et al.,* 2017, higher mortality rates were positively associated with longer exposure time (7, 14, 21 days). This suggests that increasing the duration of the trial would have resulted in higher mortality.

One of the main limitations of EPF application is the particular need for humidity for the conidia to adhere and germinate. The latter contradicts the basic condition of dry food storage; therefore, the fungal strains should work efficiently under lower humidity conditions (Zeni *et al.,* 2021). In this regard, diatomaceous earth DE has shown promising results in combination with EPF under dry conditions (Lord, 2001; Akbar *et al.,* 2004; Riasat *et al.,* 2011). In his study, *B. bassiana* and DE had synergistic effects on adult *R. dominica* and *O. surinamensis* at all concentrations (Lord, 2001). The general results in the literature show that most combinations of EPF and DE improve the efficacy of control of various stored-product pests through synergistic effects compared to the effect of EPF alone or DE alone (Lord, 2001; Athanassiou and Steenberg, 2007; Dal Bello *et al.,* 2006; Shafighi *et al.,* 2014; Storm *et al.,* 2016; Pourian and Alizadeh, 2021). The best examples of EPF that can be successfully and efficiently coupled with DEs to produce the synergistic effect mentioned above were *B. bassiana* and *M. anisopliae*.

In summary, our results suggest that EPF can be used as part of an integrated pest management programme to control legume and cereal pests and was most effective against *C. maculatus* and *S. granarius*. Knowing the insect's biological cycle to choose the right time for application and providing adequate moisture levels without compromising grain quality are both critical and challenging criteria for treatment success. Although EPF have high potential for use against stored-product pests, isolation of new strains (Al Khoury *et al.,* 2021) and testing of better dry formulations and combinations with other products are crucial to further pursue the most efficient biological control.

ACKNOWLEDGEMENT

This study was funded by the project: PSR_LEG: P.S.R. Puglia2014/2020 - Misura 16 Cooperazione - SM 16.2 Progetto "Innovazione per potenziare la produttivita', sostenibilita' della filiera dei legumi tipici pugliesi - Acronimo: "PSR_LEG" - DDS N. 94250033001.

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