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METARHIZIUM ANISOPLIAE BIOPESTICIDES AND FUNGUS ISOLATES: CONTROL EFFICACY AGAINST *RHYNCHOPHORUS FERRUGINEUS* (OLIVIER) (COLEOPTERA DRYOPHTHORIDAE) ON DIFFERENT CONTAMINATION SUBSTRATA

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Francardi V., Benvenuti C., Barzanti G.P., Roversi P.F. – *Metarhizium anisopliae* biopesticides and fungus isolates: control efficacy against *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae) on different contamination substrata.

The lack of biological insecticides based on *Metarhizium anisopliae* (Metsch.) Sorokin registered in Italy for the control of the Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier), prompted studies to evaluate the efficacy of two commercial products, Met52® and BioStorm™ and of their fungal isolates, *M. anisopliae* (Man52) and (ManBS) respectively, against the adults. The virulence of the *M. anisopliae* strains (Man52) and (ManBS) was compared with that of an indigenous *M. anisopliae* (Man08/I05) strain obtained from *R. ferrugineus* specimens collected in the wild and that showed to be very virulent against the RPW in previous studies. In both tests the sublethal effects of the treatments on female reproductive potential were examined in relation to the infective substratum. Laboratory results indicated that the commercial formulations mixed directly into the soil were not active in transmitting the infection to RPW adults and in reducing female fecundity and fertility. Diversely the fungal *M. anisopliae* (ManBS), (Man52) and (Man08/I05) strains inoculated on a rice substratum caused over 80% mortality of the phytophagous. In particular, *M. anisopliae* (ManBS) and the *M. anisopliae* (Man08/I05) produced the highest mortality (100%), with LT_{50} e LT_{90} reached in 3 and 6 days respectively. *M. anisopliae* (Met52) strain instead led to 85% mortality of RPW specimens in 28-days but it took longer to reach LT_{50} (6 days) and LT_{90} (12 days). The reproductive potential of females infected with the fungal strains was also significantly reduced with respect to the control. This study indicates the possibility of using also *M. anisopliae* strains in use against other insects of agricultural importance, for the control of *R. ferrugineus* although their effectiveness is conditioned by the formulations and/or methods employed against the RPW.

KEY WORDS: RPW adults, entomopathogenic fungus, virulence, microbiological control.

INTRODUCTION

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier), is the main pest of palm trees in many parts of the world. Various studies have been conducted in newly infested countries for the development of effective control strategies based on chemicals, semiochemicals, the sterile insect technique (SIT) and biological control (FALEIRO, 2006a, b; GINDIN *et al.*, 2006; DEMBILIO *et al.*, 2010a, b; FIABOE *et al.*, 2012; CITO *et al.*, 2014; MAZZA *et al.*, 2014). Although chemical insecticides are important for an efficient recovery of recently infested palm trees, in maintenance "therapy" they must be complementary to the use of strategies that are environmentally friendly and not harmful to the public health. Various Authors have shown that the control of the RPW by means of entomopathogenic fungi can be increased and validated through the use of virulent strains of *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. isolated from different matrices (soil or other insect groups) or directly from dead RPW specimens (GINDIN *et al.*, 2006; EL-SUFTY *et al.*, 2009; SEWIFY *et al.*, 2009; DEMBILIO *et al.*, 2010a; FRANCARDI *et al.*, 2012, 2013; RICAÑO *et al.*, 2013). A strain of *M. anisopliae* (Man08/I05) recently isolated from RPW adult in Italy was found to have higher virulence against RPW adults than *B.bassiana* strains obtained from the same pest or from soil and an infection method was studied for its use in field (FRANCARDI *et al.*, 2012; 2013).

Biopesticides containing *M. anisopliae* were available in the control of different insect pests and inside coleopterans against other curculionids (*Otiorhynchus* spp.) but they were not tested for their employment in the biological control of RPW so far.

The aims of the study were to investigated the efficacy of *M. anisopliae* biopesticides , Met52® EC (Novozymes Biologicals France S.A.) and BioStorm™ (Varsha Bioscience and Technology PVT. LTD., India) against adults of *R. ferrugineus* and to compare the virulence of their *M. anisopliae* strains (Man52 and ManBS, respectively) with that of the indigenous one (Man08/I05). The sublethal effects of the bioinsecticide formulations and of the fungal strains on the reproductive potential of RPW infected females were also investigated.

MATERIALS AND METHODS

M. ANISOPLIAE COMMERCIAL PRODUCTS

The *M. anisopliae*-based commercial products used in this study were: Met52® EC in a granular formulation

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containing 2% of the strain *Metarhizium anisopliae* var. *anisopliae* F52, made by Novozymes Biologicals France S.A. (100 g of the product contain 2 g [9x10^8 cfu/g] of the fungal strain plus inert compounds and sterile rice grains up to 100 g); BioStorm™ in a powder formulation containing 1.15% of *M. anisopliae*, made by Varsha Bioscience and Technology PVT. LTD., India.

M. anisopliae rearing strains

The fungal strains (Man52) and (ManBS) were obtained from larvae of *Galleria mellonella* L. according to the method proposed by ZIMMERMANN (1986). The dead larvae were collected and placed in humid chambers until full development of the mycelium and conidial production. Conidia of the two strains were then collected and grown separately in Petri dishes on SDAY1/4 (16.25 g/l Sabouraud Dextrose Agar, Fluka, Ch, 2.5 g/l yeast extract) in a climatic chamber at 25°C until complete development and sporulation of the mycelium. The indigenous *M.anisopliae* (Man08/I05) strain was obtained from infected Red Palm Weevil adults collected on attacked palms in Sicily, and it was grown on the same *substratum* under the same temperature conditions of *M.anisoliae* (Man52) and (ManBS) strains.

M. anisopliae (Man08/I05) is stored in culture tubes on SDAY1/4 in the entomopathogenic fungi collection of CREA-APB – Research Centre for Agrobiology and Pedology, Florence (Italy).

R. ferrugineus adults

RPW adults, emerged from cocoons sent to CREA-ABP Centre from Sustainable Management of Agro-ecosystems Laboratory, UTAGRI ECO ENEA CR, Casaccia, (Rome), were placed separately in plastic basins with ventilation holes and fed on apple slices for 7 days in a climate-controlled room at 24±2°C and 70±5% RH to ensure their good health conditions prior to be employed in the bioassays.

Bioassays with the commercial formulations in the soil

RPW cocoons were frequently observed on the soil around the trunk of infested *Phoenix canariensis* Chabaud palms in different urban environments in Italy as also reported in the soil for the adults in date palm plantations in Arabian Gulf region and in Egypt by EL-SUFTY *et al.* (2011). The temporary permanence of *R. ferrugineus* adults on the soil was the basis to verify the effect of the two biopesticides BioStorm™ and Met52® EC against the insect pest. To simulate field conditions 50g of potting compost moistened with 150 ml of sterile water was put in three plastic basins (20x18 cm, 4L capacity, GIO'Style); in one basin the soil was mixed with 25 g of BioStorm™, in the other one with 25 g of Met52® while in the control basin the soil was only moistened with the same amount of sterile water. For each basin we used 40 *R. ferrugineus* adults (20♂ + 20♀) kept in contact with the treated or control soil for 30 minutes, after which they were separated and reared individually on apple slices renewed weekly. Adult mortality and female fecundity and fertility were checked daily. The bioassay took place in a controlled climatic chamber (24± 2°C and 70±5% RH) and lasted 28 days. In this test was not employed *M.anisopliae* (Man08/I05) for the difficulty to set up an appropriate formulation for the fungal strain.

Bioassays with *M. anisopliae* strains grown on rice substratum

Rice is a substratum proved to ensure a good growth of entomopathogenic fungal mycelium and conidia production, to be effective in inoculum transmission, suitable to be employed in autocontamination traps and to mantain fungal inoculum stability in field (PRIOR & ARURA, 1985; GINDIN *et al.*, 2006; FRANCARDI *et al.*, 2012; 2013) so it represented a good infective substratum to be employed in virulence bioassays.

Three tests were set up with *M. anisopliae* strains: one with *M. anisopliae* (ManBS), another with *M. anisopliae* (Man52), and still another with *M. anisopliae* (Man08/I05). A last test was the control. For each test we used a 500 ml flask containing 200 g of rice following the method of GINDIN *et al.* (2006). After sterilization in an autoclave the rice contained in the three flasks was inoculated with an agar plug (ca. 4x4x4 mm) of sporulated colonies of *M. anisopliae* (ManBS), (Man52) and (Man08/I05) strains respectively. In the control the rice was inoculated with a sterile agar plug. All flasks were maintained at 27±2°C in a climatic chamber. After 20 days the rice inoculated with the fungal strains was completely colonized by the mycelium, with abundant conidial production. For each test we used a plastic basin (20x18 cm, 4L capacity, GIO'Style) with a perforated cover closed by wire mesh containing the contaminated or control rice on which were mantained *R. ferrugineus* adults (20♂ + 20♀ per test) for 30 min. The males were then separated from the females and each specimen was reared individually on apple slices in plastic containers with ventilation holes. Adult mortality and female fecundity (number of eggs/female) and fertility (number of larvae/female) were checked daily for 28 days. Dead adults were placed in humid chambers to verify the presence of *M. anisopliae.* Each test was replicated two times.

The conidial concentration was determined in 0.2 g (equal to six grains) of infected rice sampled randomly from each test. Each grain was placed in a 15 ml falcon tube with a drop of polysorbate detergent TWEEN 80 (0.1%) and 1 ml of distilled water and the tube was put in the centrifuge for 1 min. The conidial concentration was estimated with a hemocytometer (Thoma-Zeiss counting chamber) and expressed as the mean value of six counts per grain. The mean conidial concentration/ml of the three *M. anisopliae* strains in two replicates was: 8x10^5 conidia for (Man BS), 1.2x10^6 conidia for (Man52) and 1.4x10^6 conidia for (Man08/I05).

Statistical Analysis

Survival analyses were performed with the Wilcoxon-Gehan test using SPSS 15.0. The significance of the analysis of variance (ANOVA) was assessed with the post-hoc Tukey HSD test. All analyses were conducted with a significance level of P=0.05.

RESULTS

Bioassay with the commercial formulations in the soil

BioStorm™ and Met52® EC were not effective in controlling RPW adults: the mortality levels for both products were not significantly higher than the control level (Table1). The fecundity and fertility of females treated with Met52® EC were significantly higher than the control levels, while those of females treated with BioStorm™ did not differ from the control and Met52® EC levels (Table 1).

Table 1 – *R. ferrugineus* adults mortality and female fecundity and fertility after treatment with the commercial formulations BioStorm™ and Met52® EC.

		Fecundity			Fertility	
Biopesticides	Mortality % *	Mean±SE **	Eggs/♀	% larvae	Mean±SE ***	Eggs/♀
Control	20A	7.38±1.14 A	28.8	40	2.92±0.42 A	11.4
BioStorm™	20A	11.68±1.17 AB	45.85	30	3.53±0.36 AB	13.9
Met52® EC	12.5A	13.94±1.68 B	54.35	34	4.78±0.54 B	18.65

* Test Wilcoxon-Gehan 1.270 df 2 Sig. 0.530
** ANOVA F(2,233) = 6.039 df 2 Sig. 0.003 Post-hoc Tukey HSD test
*** ANOVA F(2,233) = 4.46 df 2 Sig. 0.013 Post-hoc Tukey HSD test

BIOASSAYS WITH THE *M. ANISOPLIAE* ISOLATES GROWN ON RICE MEDIUM

The adult mortality in the three tests with *M. anisopliae* (Man52), (ManBS) and (Man08/I05) strains was significantly higher than the control level (Fig. I). Both *M. anisopliae* strains isolated from the commercial products, *M. anisopliae* (Man52) and *M. anisopliae* (ManBS), showed high virulence against *R. ferrugineus* adults, with mortality of 85% and 100% respectively in 28-days. In particular, *M. anisopliae* (ManBS) caused mortality levels identical to those recorded for the indigenous strain *M. anisopliae* (Man08/I05) (100%) and with similar LT_{50} (2.9 days and 3 days respectively) and LT_{90} (5.4 and 6.2 days respectively). *M. anisopliae* (Man52) took longer to act, reaching LT_{50} in 6 days and LT_{90} in 12 days (Table 2). The fecundity and fertility of *R. ferrugineus* females infected with the three *M. anisopliae* isolates were significantly lower than the control levels; moreover, females contaminated with *M. anisopliae* (Man08/I05) and *M. anisopliae* (ManBS) showed significantly lower fertility than those infected with *M. anisopliae* (Man52) (Tables 3 and 4).

DISCUSSION

In the biological control the use of biopesticides based on entomopathogenic fungi may represent a valid alternative/integrative support to chemical control, particularly due to the identification of fungal strains higly virulent against *R. ferrugineus* and to the possible spread of the inoculum within the weevil population by contact between healthy and infected individuals and/or infected cadavers on which new conidial generations may occur on the palm canopy (LACEY *et al.*, 1999; QUESADA-MORAGA *et al.*, 2004; DEMBILIO *et al.*, 2010a). The present study highlights the possibility of using *M. anisopliae* strains, already employed in commercial biopesticides against

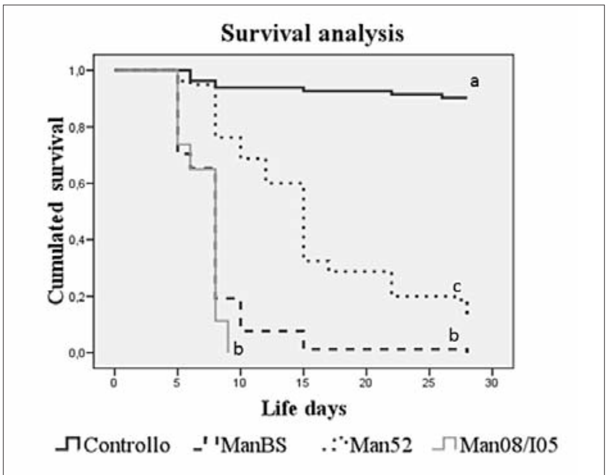

Fig. I – Survival curves of *R. ferrugineus* adults contaminated with the *M. anisopliae* (Man52), (ManBS) and (Man08/I05) strains (Wilcoxon-Gehan test 203.46 df 3 Sig. 0.000). Lines with the same letter are not significantly different.

Table 2 – Mortality of *R. ferrugineus* adults contaminated with the *M. anisopliae* isolates (Man52), (ManBS) and (Man08/I05). Each experiment was replicated two times.

Test	Mortality	LT_{50} days	LT_{90} days
Control	7.5%	/	/
ManBS	100%	2.9	5.4
Man52	85%	6.1	12.4
Man08/I05	100%	3.0	6.2

Table 3 – *R. ferrugineus*: female fecundity (mean number of eggs/female) after contamination with the *M. anisopliae* isolates (Man52), (ManBS) and (Man08/I05). Numbers with the same letter are not significantly different. Each testt was replicated two times.

Test	Eggs laid (mean±SE)	ANOVA
Control	14.50±1.347 A	F= 13.599 df 3 Sig. 0.000
ManBS	4.09±1.002 B	
Man52	8.03±1.490 B	
Man08/I05	5.06±1.204 B	

Table 4 – *R. ferrugineus:* fertility (mean number of larvae/female) after treatment with the *M. anisopliae* isolates (Man52), (ManBS) and (Man08/I05). Numbers with the same letter are not significantly different. Each test was replicated two times.

Test	Larvae (mean±SE)	ANOVA
Control	2.84±0.485 A	F= 19.551 df 3 Sig. 0.000
ManBS	0.21±0.086 B	
Man52	1.28±0.289 C	
Man08/I05	0.13±0.046 B	

insects of agricultural-ornamental importance, for the biological control of the RPW but focused on the importance of the infective method for an effective contamination of the insect pest. In this regard the commercial bioinsecticides in granular Met52® EC and powder formulations BioStorm™ at the dose used and mixed with soil, resulted not effective in the control of the weevil in terms of either adult mortality or a reduction of female reproductive potential. In contrast the *M. anisopliae* strains isolated from the biopesticides, (Man52) (ManBS) and grown on rice grains used as contamination substratum, showed high virulence against *R. ferrugineus* adults as well as negative sublethal effects on female fecundity and fertility. In particular (ManBS), even at a low conidial concentration ($8x10^5$ conidia), caused the same mortality (100%) of the indigenous isolate (Man08/I05) ($1.4x10^6$ conidia). *M. anisopliae* (Met52) strain with a concentration of $1.2x10^6$ conidia, caused 85% mortality in the 28-days of the bioassay, but it took longer to reach LT_{50} (6 days) and LT_{90} (12 days) than (ManBS) and (Man08/I05) (Lt_{50} 3 days and LT_{90} 5-6 days, respectively). In these bioassays all the *M. anisopliae* strains at the tested concentrations resulted suitable for their employment in the biological control of *R.ferrugineus*.

Results also put in light that *M.anisopliae* (Man08/I05) strain is effective against RPW at lower conidia concentration than that ($7.1x10^6$ conidia) tested in a previous study (FRANCARDI *et al.*, 2013).

Reduction in reproductive fitness by *M. anisopliae* strains was also observed in another coleopteran species, *Anoplophora glabripennis*, by HAJEK *et al.* (2008) who observed 60% of unhatched eggs of treated females with signs of fungal infection.

The rice substratum is an excellent medium for fungal growth favouring abundant conidial production but is also an efficient mean of RPW adults contamination and infectivity on account of their tunneling activity into the substratum mass, which results in a greater transfer of conidia onto the pest's body (LEGER *et al.*, 1991; IBRAHIM & LOW, 1993; GINDIN *et al.*, 2006; SOUNDARAPANDIAN & CHANDRA, 2007; SAHAYARAJ & NAMASIVAYAM, 2008; FRANCARDI *et al.*, 2013). For this last reason in particular it is likely that the infectivity of the (ManBS) and (Man52) strains was enhanced by the rice substratum that likely increase the contact with the conidial mass with respect to the commercial products that mixed with the soil, may not have found favorable conditions for an effective contact of the fungal inoculum with the weevil. For an efficient infection of RPW in field, applications of the contaminant substratum should be used in the most localized way possible on host trees at the sites of greatest aggregation and refuge of adults in the canopy, where they feed, copulate and ovideposit (PRIOR & ARURA, 1985) or outside the host tree in delivery systems that attract insects to entomopathogen infection sites in response to environmental, semiochemical or food stimuli (VEGA *et al.*, 2007). In this regard, EL-SUFTY *et al.* (2011) used autodissemination traps partially buried in the ground provided with a Petri dish containing a powder formulation of a local strain of *B. bassiana* as inoculum in three palm cultivations during two successive seasons (from April 2006 to May 2007); in the last two months they reported a field RPW mortality that ranged 41.2%-51.3% compared with the control 4.8%-4.9%. More recently FRANCARDI *et al.* (2013), in laboratory tests conducted in conditions of semi-freedom of the RPW, demonstrated the effectiveness of an experimental "infect, attract and release trap" containing a rice-based substratum inoculated with *M. anisolpliae* (Man08/I05) in the RPW control (95% mortality of individuals in 28 days). Moreover field tests revealed a good preservation of the infectivity of the fungus (conidial concentration and viability) in rice substratum within these traps in the seasons of highest abundance of the weevil, spring, summer and autumn, with temperatures between 21 and 25°C (FRANCARDI *et al.*, 2013).

Rice is one of the components of some entomopathogenic fungi-based biopesticides but the formulation and/or method of administration must be "fit for purpose" and at this regard the use of an autocontamination trap may be a valid biological control technique in the integrated pest management of *R. ferrugineus* (GINDIN *et al.,* 2006, EL-SUFTY, 2007; FRANCARDI *et al.*, 2013).

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RIASSUNTO

BIOINSETTICIDI A BASE DI *METARHIZIUM ANISOPLIAE* E ISOLATI FUNGINI: EFFICACIA NEL CONTROLLO DI *RHYNCHOPHORUS FERRUGINEUS* (OLIVIER) (COLEOPTERA DRYOPHTHORIDAE) SU DIVERSI SUBSTRATI DI CONTAMINAZIONE

La mancanza di bioinsetticidi a base di *Metarhizium anisopliae* (Metsch.) Sorokin registrati in Italia per il controllo del Punteruolo rosso delle palme, *Rhynchophorus ferrugineus* (Olivier) (RPW) ha promosso studi volti a valutare l'efficacia di due prodotti commerciali a base di *M. anisopliae* attivi nei confronti di altri curculionidi appartenenti al genere *Otiorhynchus*, Met52® e BioStormTM e dei loro isolati fungini, *M. anisopliae*(Man52) e (ManBS), contro adulti di RPW. La virulenza degli isolati di *M. anisopliae* (ManBS) e (Man52) è stata confrontata con quella di un ceppo indigeno di *M.anisopliae* (Man08 / I05) ottenuto da adulti di *R. ferrugineus* raccolti in natura e che, in studi precedenti, si è dimostrato molto virulento contro il fitofago. Nello studio sono stati inoltre indagati gli effetti subletali dei trattamenti sul potenziale riproduttivo delle femmine. I risultati ottenuti indicano che, mentre le formulazioni commerciali usate direttamente nel terreno non sono risultate efficaci nel trasmettere l'infezione ad adulti di *R. ferrugineus,* gli isolati fungini *M. anisopliae* (ManBS), (Man52), (Man08/I05) inoculati su un substrato di riso hanno causato una mortalità del fitofago superiore all' 80%. In particolare, *M. anisopliae* (ManBS) e l'isolato indigeno *M. anisopliae* (Man08/I05) hanno registrato la più alta mortalità del fitofago (100%), con LT_{50} e LT_{90} raggiunti in 3 e 6 giorni rispettivamente. L'isolato *M. anisopliae* (Met52) pur avendo causato l'85% di mortalità degli esemplari del curculionide in 28 giorni, ha impiegato più tempo per raggiungere LT_{50} (6 giorni) e LT_{90} (12 giorni). Il potenziale riproduttivo delle femmine infettate con tutti gli

isolati fungini è risultato significativamente ridotto rispetto al controllo. Questo studio indica la possibilità di utilizzare ceppi di *M. anisopliae* per il controllo biologico di *R. ferrugineus*, già utilizzati nel controllo di altri insetti di interesse agrario ma evidenzia anche che la loro efficacia è condizionata dalla formulazione e/o dalla metodologia impiegata nei trattamenti contro il fitofago.

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