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Article

Efficacy of soil isolates of entomopathogenic fungi against the bulb mite, *Rhizoglyphus robini* (Acari: Acaridae)

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Abstract

The bulb mite, *Rhizoglyphus robini*, is a serious pest of garlic, onion and other crops. The mite is usually found in association with dangerous fungal pathogens such as *Fusarium* spp. Control of this pest has relied upon the use of synthetic acaricides but chemical control of the bulb mite is difficult because it is able to develop resistance quickly. Thus, alternative control methods, e.g. biological control, need to be developed and implemented. The aim of this study was to assess efficacy of selected strains of entomopathogenic fungi (EPF) against adult females of *R. robini* under laboratory conditions. New EPF strains were isolated from soil samples collected in onion and garlic fields in the Czech Republic and Israel using soil elution and cultivation on selective media. Fungal species were determined using macroscopic, microscopic and molecular markers. The efficacy against *R. robini* females was tested in 17 isolated and 3 reference strains of EPF. Results revealed high variability among species and strains. The highest efficacy against *R. robini* mites was found in strains of *Metarhizium anisopliae* isolated from soil samples collected in the Czech Republic which caused mortality up to 99.3%, and a *Metarhizium indigoticum* strain from Israel causing 98.3% mortality after four days of bioassay. *Isaria fumosorosea* strains did not caused mortality higher than 40%. The lowest virulence was found in *Beauveria* spp. strains causing mortality of mites between 5 and 25%. Median lethal time (LT_{50}) and median lethal concentration (LC_{50}) in the three most virulent strains ranged between 2 and 4 days and between 1.01×10^4 and 2.36×10^5 spores/ml, respectively. The concentration-response models indicated that the *M. indigoticum* strain is more lethal than *M. anisopliae* strains. The present study showed that some strains of entomopathogenic fungi, especially from the genus *Metarhizium*, could be perspective biocontrol agents against *R. robini*.

Key words: Alliaceae, soil mites, *Metarhizium*, *Isaria*, *Beauveria*, biological pest control, mycoacaricides, virulence

Introduction

Bulb mites of the genus *Rhizoglyphus* are economically important pests of plants with bulbs, corns, and tubers. Their main hosts are species in the family Liliaceae but they often attack other important crops such as potatoes (*Solanum* sp.) and carrots (*Daucus carota*) (Díaz *et al*. 2000). Fan and Zhang (2004) published a revision of the Australasia and Oceana species of *Rhizoglyphus* and more recently Barbosa and Moraes (2020) reported on the species in Brazil.

Rhizoglyphus robini (Claparède) (Acari: Acaridae) is considered one of the most serious pests of onion, garlic and ornamentals such as lily, tulips and hyacinths in storage, greenhouse and in the field around the world (Díaz *et al*. 2000; Fan & Zhang 2004). This species has a very high reproductive rate. When offered peanuts as a sole food source, the mean total fecundity was 690 eggs

and the intrinsic rate of population increase (r_m) was estimated to be 0.285 (Gerson *et al*. 1983). Besides causing direct feeding damage, this pest also disseminates phytopathogenic bacteria and fungi, e.g. *Fusarium oxysporum*, which infect bulbs, facilitating the pathogens entry into host plants (Poe *et al*. 1979; Okabe & Amano 1991; Díaz *et al*. 2000; Hanuny *et al*. 2008; Zindel *et al*. 2013; Ofek *et al*. 2014).

With the exceptions of solarization of soil (Gerson *et al*. 1981) or hot-water treatment of bulbs (Conijn 1992), the control of this pest is still based almost entirely on broad-spectrum pesticides, even though it has been known for many years that bulb mites quickly develop resistance to pesticides (Poe *et al*. 1979; Kuwahara 1988; Díaz *et al*. 2000). Furthermore the application of these chemicals to soil negatively impacts non-target organisms, e.g. earthworms and soil microorganisms, leaves residues in food crops and contaminates groundwater. For these reasons broad-spectrum pesticides have been targeted by the European community for deregistration (EC 2009). Thus, alternative, environmentally safe control strategies, e.g. biological control, need to be developed and implemented. Efforts to develop biocontrol techniques for bulb mites have been undertaken in many countries and mostly involved the use of soil-dwelling predatory mites, e.g. *Gaeolaelaps aculeifer* (Canestrini) (Acari: Laelapidae) (Lesna *et al*. 1995, 1996). A recent study by Nermuť *et al*. (2019) showed that some, especially small, entomopathogenic nematodes (EPN) are able to invade and kill adult females of *R. robini*. The most promising species were *Steinernema huense* (Nematoda: Steinernematidae), *Heterorhabditis bacteriophora* and *H. amazonensis* (Nematoda: Heterorhabditidae) causing mortality in *R. robini* up to 30%. Mortality of mites treated by culture supernatants of the nematode symbiotic bacteria of the genus *Xenorhabdus* was generally lower but some bacterial strains showed repellent effect to mites. Due to their relatively low efficacy, EPNs and the metabolites of their symbiotic bacteria do not seem to represent a viable option for bulb mite biocontrol as a standalone approach (Nermuť *et al.* 2019).

Entomopathogenic fungi (EPFs) represent another promising group of biocontrol agents. Their advantages are that they do not need to be ingested as they are able to penetrate the host cuticle and can be relatively easily produced (Shahid *et al*. 2012). Many EPFs species attack Acari and can be used for biological control of mite pests. Besides Acari-specific pathogens such as *Hirsutella thompsonii* (Fisher) and *Neozygites* spp. (Entomophthorales), 'nonspecialist' mitosporic fungi (Hyphomycetes) like *Beauveria bassiana* (Bals.-Criv.) Vuill., *Metarhizium anisopliae* (Metsch.) Sorokin, *Isaria fumosorosea* (Wize), *I. farinosa* (Holmsk.), and *Lecanicillium lecanii* (Zimm.) Zare & W. Gams have potential to control some mite species (Chandler *et al*. 2000). Most studies on efficacy of EPFs against mites have targeted ticks (Kaaya *et al*. 1996; Kaaya & Hassan 2000; Fernandes & Bittencourt 2008), spider mites (Chandler *et al*. 2005; Wekesa *et al*. 2005; Shi & Feng 2009; Ullah & Lim 2017; Shang *et al*. 2018; Khoury *et al*. 2020) and eriophyoid mites (Latge *et al*. 1988; McCoy 1996; Van der Geest *et al*. 2000). To our knowledge, only three EPFs species have been tested against *R. robini* under laboratory or greenhouse conditions: *Hirsutella kirchneri* (Rostrup) Minter, Brady and Hall (Sztejnberg *et al*. 1997), *I. fumosorosea* (Zemek *et al*. 2018) and *Metarhizium brunneum* Petch (Ment *et al*. 2020). While the first two species were not able to control *R. robini*, *M. brunneum* was found to be a promising biocontrol agent against this pest.

The aim of our study was to assess the possibility of fungal biocontrol of *R. robini* by new EPF strains isolated from soil samples collected in onion and garlic fields in the Czech Republic and Israel. The efficacy of these strains was compared with *I. fumosorosea* strain CCM 8367 and two commercially used strains, *B. bassiana* strain GHA and *M. brunneum* Petch strain F52.

Material and methods

Rhizoglyphus robini

The laboratory culture of *R. robini* was established from mites originating from rotting onion plants collected in Israel. The mites were maintained in large Petri dishes lined with wet filter paper using crushed raw peanuts as a food source. The dishes with mites were kept in darkness at 20 °C.

Entomopathogenic fungi

In total, 17 EPF strains were isolated from soil of several, mostly pesticide free onion and garlic fields in Pilsen and South Bohemian regions in the Czech Republic and in the Beit She'an Valley, Jezreel Valley and Lower Galilee in Israel. Soil sampling was performed during vegetation season in 2017. EPF isolates were obtained by water elution of soil samples and cultivation using selective medium containing dodine (Chase *et al*. 1986). Strains of EPF were identified on the basis of macroscopic, microscopic and genetic characteristics.

DNA for genetic analysis was extracted from fresh mycelium grown at 25±1 °C for 7 days on Petri dishes with PDA (Sigma-Aldrich, Darmstadt, Germany) medium. Each mycelium was collected to a sterile 1.5 mL microtube. The extraction method used was based on CTAB-PVP (Doyle 1991) with modification for fungi. Genomic DNA was amplified by PCR with universal primers NL1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ (forward) and NL4 5′-GGTCCGTGTTTCAAGACGG-3′ (reverse) (O'Donnell 1992; 1993). PCR reactions were carried out in a volume 25 µL containing in 1X reaction buffer (75 mM Tris–HCl, pH=8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween® 20 (Sigma-Aldrich, Darmstadt, Germany), 2.5 mM $MgCl_2$, 200 µM dNTPs), 1.25 U Taq Purple DNA polymerase (PPP Master Mix, Top-Bio, CZ), 10 pmol of both forward and reverse primer and 50 ng template DNA. Microtubes were placed in a thermal cycler (TProfessional Basic Gradient, Biometra) with the following program: 1 cycle of 94 °C for 5 min, 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and 15 s, and final elongation at 72 °C for 5 min. The part of amplified PCR products was visualized on 2% agarose gel. The PCR products were sequenced by SEQme (Czech Republic). The sequences obtained were edited, compiled and aligned using Geneious (New Zealand) software. Sequence similarity searches were performed using NCBI GenBank BLASTn.

Cultures have been deposited at the Biology Centre CAS, České Budějovice. GenBank accession numbers for all 17 strains are listed in Table 1. In addition, three reference strains were used in efficacy bioassays. Two reference strains were re-isolated from commercial mycopesticides: *B. bassiana* strain GHA (BotaniGard® WP, Certis USA, Llc., Butte, MT, USA) and *M. brunneum* strain F52 (Met52® EC, Novozymes Biological, Franklinton, NC, USA). The species *M. brunneum* was previously classified as *M. anisopliae* (Bischoff *et al*. 2009). The third strain was CCM 8367 strain of *I. fumosorosea*, which originates from the horse chestnut leaf miner, *Cameraria ohridella*, Deschka & Dimić (Lepidoptera: Gracillariidae) collected in the Czech Republic (Zemek *et al*. 2007). The strain is patented (Prenerová *et al*. 2013, 2015) and deposited in the Czech Collection of Microorganisms in Brno.

All strains were cultivated on PDA medium at 25±1 °C and 16L:8D photoperiod. After 10 days of incubation, the spore suspensions were prepared from each strain by scraping off conidiospores into a sterile solution of 0.05% (v/v) Tween® 80 (Sigma-Aldrich, Darmstadt, Germany). Suspensions were filtered through sterile gauze to separate the mycelium and clusters of spores. In uniform suspension, the number of spores was counted with a Neubauer improved counting chamber (Sigma-Aldrich, Darmstadt, Germany) and subsequently the suspension was adjusted to the required concentration. The suspension was left for approximately 12 hours at temperature 23±1 °C to accelerate and synchronize germination of conidia (Dillon & Charnley 1985, 1990) before its

application. Viability of spores was verified using a standard germination test (Skalický *et al*. 2014). Ten drops from suspension were applied using a 1 µl inoculation loop on the surface of 2% water agar, which was poured in a thin layer onto the surface of a sterile slide. After the drops had dried, the slides were moved into a wet chamber and incubated at 25±1 °C for 24 h. Percentage of germinating spores was determined using an Olympus CH20 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan); bright field, 400× magnification. The spore germination of all strains was >95%.

Bioassays

Efficacy of EPF strains was assessed in single-dose bioassays using 24-well polystyrene tissue culture plates (Orange Scientific, Braine-L'Alleud, Belgium). Plate dimensions were 128×86 mm and bottom area of single well was 193 mm^2. Filter paper discs of 14 mm in diameter were placed into each well and moistened with 100 µL of sterile distilled water. Water did not only provided moisture, but also created a surface tension that prevented the mites from escaping from the experimental arena (Chen 1990). Four *R. robini* females were placed into each well and the plate was sprayed with 2 mL of fungus suspension with concentration 1×10^7 spores per 1 mL using a Potter spray tower (inner diameter of cylinder 29 cm, spray pressure 50 kPa). Density of conidia was thus approx. 3×10^4 spores per cm^2, i.e. 5.8×10^4 spores per a single plate well. Control variant was treated with a sterile solution of 0.05% Tween 80®. After treatment, plates were covered with lids and incubated at 25±1°C and constant darkness for four days. After this period mortality of mites was recorded using a dissection microscope Technival 2 (Carl Zeiss, Jena, Germany) at a magnification 25× and 40×. Mycosis on cadavers was documented by Olympus SZX12 equipped with an Olympus E-3 digital camera (Olympus Optical Co., Ltd., Tokyo, Japan) at 50× magnification. Each strain bioassay was conducted in 3 replicates, i.e. 3 times one plate with 96 mites tested.

Dose-response of *R. robini* to EPFs was assessed in three selected strains of *Metarhizium* spp. which showed high efficacy in previous single-dose experiments. The mean and the median time to death (LT_{50}, the number of days until 50% of mites were dead) and lethal concentrations (LC_{50} and LC_{90}) of conidia were estimated from cumulative mortality of mites at five concentrations ranging from 1×10^3 to 1×10^7 spores/ml of suspension. The bioassays were performed as described above except that mortality was checked daily. The control was treated with 0.05% Tween 80® solution. Each concentration test was repeated twice; 96 mites were used per replication.

Scanning electron microscopy of pathogenesis

Pathogenesis was studied in two selected EPF strains, BEA 02 and MET 08. Samples of *R. robini* females treated by either strain were collected at 24, 48, 72 and 96 hours after the application of fungal suspension. Mites were fixed and dehydrated in vapors from crystals of osmium tetroxide in Petri dishes properly sealed with Parafilm® at -20 °C in a freezer. After three weeks the Petri dishes with mites were placed in the fume hood and kept open for 24 hours to evaporate remaining osmium tetroxide. The following day the mites were mounted on aluminium stubs using a double-sided carbon tape and coated with gold using a Sputter Coater (Baltec-SCD 050). The mites were examined in the scanning electron microscope JEOL 7401-FE (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 4 kV.

Statistical analysis

Mortality in *R. robini* females was expressed as mean percentage ± standard error of the mean. A generalized linear model with a binomial distribution and logit link was used to analyse data. Treatment and replication were set as fixed effects. The analysis was performed in SAS® Studio for Linux (SAS Institute Inc. 2018) using the GLM procedure (PROC GENMOD) of SAS/STAT

module (SAS Institute Inc. 2017). Means were separated by the least-square means (LSMEANS) statement of SAS with Tukey-Kramer adjustment for multiple comparisons. Dose-response experiment data were first subjected to survival analysis. The Kaplan–Meier product limit estimate calculated in the LIFETEST procedure in SAS/STAT module was used to determine both the mean and the median time to death (LT_{50}) for each selected strain. Wilcoxon and log-rank test statistics (PROC LIFETEST) were used to test the global hypothesis that mortality (time to death) differed between strains. Data were further analysed using Probit analysis (PROC PROBIT) to estimate lethal concentrations (LC_{50} and LC_{90}). In all tests P values <0.05 were considered statistically significant.

Results

In total, 17 strains of EPFs were isolated and identified as species *B. bassiana*, *Beauveria brongniartii* (Sacc.) Petch, *I. fumosorosea, M. anisopliae* and *Metarhizium indigoticum* (Kobayasi & Shimizu) Kepler, S.A. Rehner & Humber (Table 1). The results of bioassays revealed that while in the control treatment mortality of *R. robini* females was only 2.8% without any evidence of fungal infection, some strains belonging to genus *Metarhizium* were able to kill almost all treated mites within four days (Fig. 1). Mycosis on the cadavers followed by sporulation was observed in few mites treated by *Beauveria* spp. strains (Fig. 2 A) and almost on all cadavers of mites treated by *Metarhizium* spp. strains (Fig. 2 B, C). No obvious symptoms of mycosis were found four days after the treatment by strains of other EPF species. Observation of pathogenesis revealed that conidia germinated in 24 hours and in 48 hours were able to form appressoria (Fig. 3 A1-2). Strains of *Metarhizium* genera were the fastest in development of conidiophores and sporulation was observed as early as 72 hours after fungus application (Fig. 3 B3).

TABLE 1. Strains of entomopatogenic fungi isolated within this study and used in bioassays.

Species	Strain	Country of origin	Genbank accession number
Beauveria bassiana	BEA 01	Israel	MN960362
	BEA 02	Israel	MN960361
	BEA 03	Czech Republic	MN960359
	BEA 04	Czech Republic	MN960363
Beauveria brongniartii	BEA 05	Czech Republic	MN960372
Isaria fumosorosea	ISA 01	Israel	MN960358
	ISA 02	Czech Republic	MN960357
Metarhizium indigoticum	MET 01	Israel	MN960355
Metarhizium anisopliae	MET 02	Israel	MN960371
	MET 03	Israel	MN960367
	MET 04	Israel	MN960373
	MET 05	Czech Republic	MN960356
	MET 06	Czech Republic	MN960369
	MET 07	Czech Republic	MN960366
	MET 08	Czech Republic	MN960370
	MET 09	Czech Republic	MN960374
	MET 10	Czech Republic	MN960365

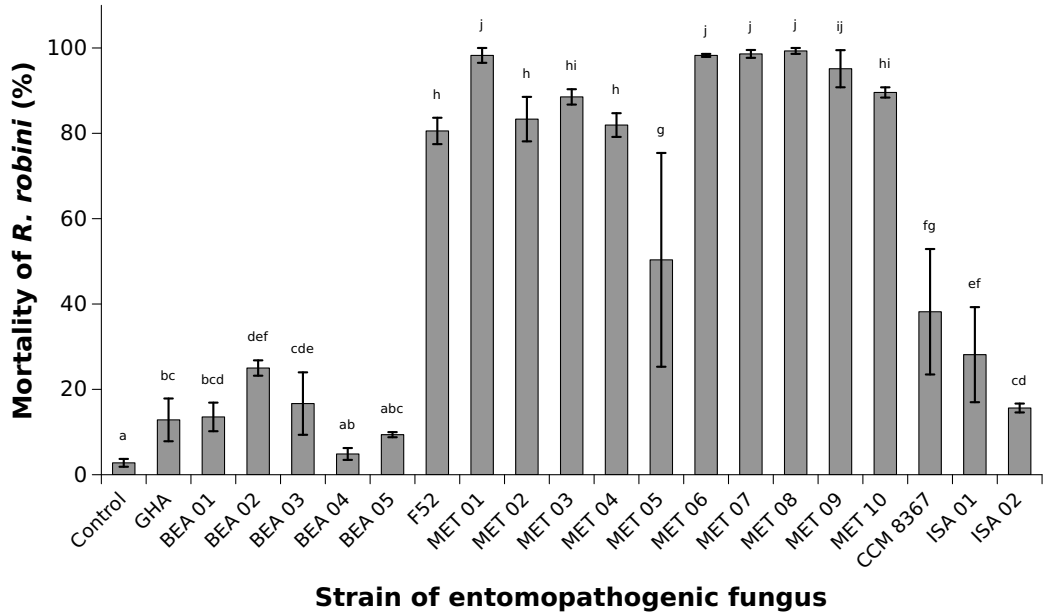

FIGURE 1. Mortality of *Rhizoglyphus robini* adult females treated with various strains of entomopathogenic fungi. See Table 1 for the key to EPF strains. Data presented are means (± SE), with three replicates of 96 mites for each strain. A generalized linear model was fitted and pairwise between treatment differences were tested using the least-square means. Different letters indicate significant differences between columns (P<0.05).

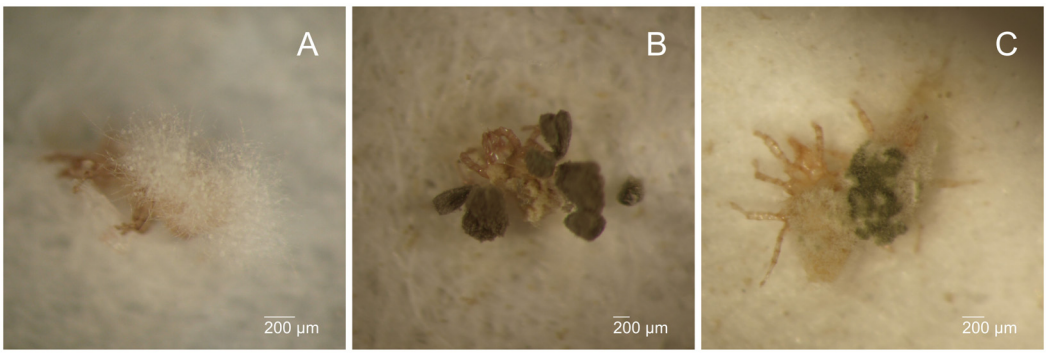

FIGURE 2. Photographs (dissection microscope, 50×) of sporulated entomopathogenic fungi on *Rhizoglyphus robini* cadavers. A: *Beauveria bassiana* strain BEA 02, B: *Metarhizium indigoticum* strain MET 01, C: *Metarhizium anisopliae* strain MET 08.

The most virulent were particularly *M. anisopliae* strains MET 08 and MET 07 from the Czech Republic and *M. indigoticum* strain MET 01 from Israel causing mean mortality 99.3, 98.6 and 98.3%, respectively. The strain F52 caused also high mortality of *R. robini* females (80.6%). Other species of EPF turned out to be much less virulent against *R. robini*. The reference strain of *I. fumosorosea* CCM 8367 caused mortality less than 40%. Strains of the genus *Beauveria* showed very low acaropathogenic effect against *R. robini* females. Mortality ranged from 4.9% (BEA 04) to 25.0% (BEA 02). The effect of strain on virulence against *R. robini* was highly significant (χ^2=4086.32, df=20, P<0.001). No significant differences were found among replications (χ^2=1.41, df=2, P=0.493).

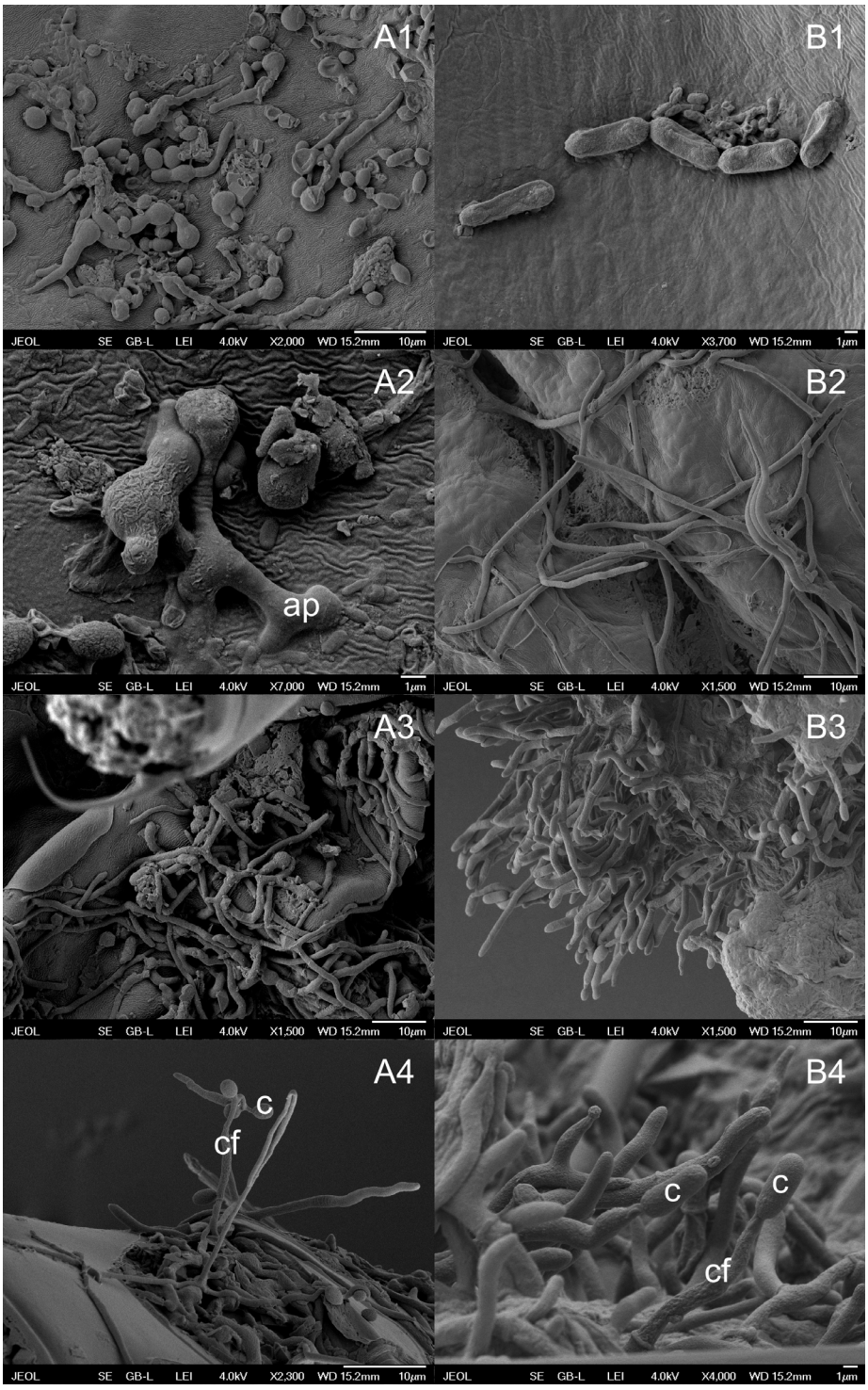

FIGURE 3. Scanning electron micrographs showing pathogenesis of *Beauveria bassiana* strain BEA 02 (A) and *Metarhizium anisopliae* strain MET 08 (B) on *Rhizoglyphus robini* females at 24 (1), 48 (2), 72 (3) and 96 (4) hours after fungus application. A1: germinating conidia; B1: attachment of conidia to mite cuticule; A2: appressorium (ap) formation; B2 and A3: mycelium on the edge of the anal opening; B3: the beginning of sporulation; A4 and B4: details of conidiophores (cf) with conidia (c).

Cumulative mortality at the end of dose-response experiments reached 97.9, 99.0 and 99.5% in mites treated with the highest concentration of MET 01, MET 07 and MET 08 strain, respectively (Fig. 4). First mycosis was observed on the 3rd day after fungus application at the highest concentration when 22.3%, 12.0% and 14.0% of cadavers had symptoms of mycosis in MET 01, MET 07 and MET 08 strain, respectively. Four days after the treatment, mycosed cadavers were found also at concentrations 1×10^5 and 1×10^6 spores/ml (Fig. 5). Survival analysis of obtained data revealed no statistically significant effect of strain at the lowest concentration, i.e. 1×10^3 spores/ml (Wilcoxon test, χ^2=1.705, P=0.426; log-rank test, χ^2 = 1.654, P = 0.437) but highly significant differences were found at all higher concentrations (Wilcoxon test and log-rank test, P<0.001). The shortest median survival time (LT_{50}=2.0 days) was estimated in strain MET 07 applied at concentration 1×10^7 spores/ml (Table 2). The log-probit regression lines describing relationship between concentration of MET01, MET07 and MET08 strains and mortality of *R. robini* (Fig. 6) have a form $y = -3.830 + 0.957x$, $y = -3.315 + 0.617x$ and $y = -3.324 + 0.711x$, respectively. The estimated values of LC_{50} and LC_{90} were lowest in strain MET 01 (Table 3).

TABLE 2. Corrected mortality, mean survival time (± SE) and median lethal time (LT_{50}) of *Rhizoglyphus robini* adult females treated by suspensions of selected strains of *Metarhizium* spp.

Species	Strain	Concentration(spores/ml)	Mortality[a] (%)	Survival time[b] (days)	LT_{50} (95% CI) (days)
M. indigoticum	MET 01	1×10^3	11.96	3.81±0.04	NA
		1×10^4	40.76	3.64±0.06	NA
		1×10^5	95.11	3.09±0.07	3.0 (3.0–4.0)
		1×10^6	95.65	2.84±0.07	3.0 (NA–NA)
		1×10^7	97.83	2.55±0.07	3.0 (2.0–3.0)
M. anisopliae	MET 07	1×10^3	4.14	3.74±0.05	NA
		1×10^4	10.06	3.72±0.06	NA
		1×10^5	12.42	3.67±0.06	NA
		1×10^6	48.52	3.45±0.07	4.0 (4.0–NA)
		1×10^7	98.82	2.29±0.07	2.0 (NA–NA)
	MET 08	1×10^3	7.78	3.69±0.06	NA
		1×10^4	19.16	3.67±0.06	NA
		1×10^5	31.14	3.54±0.07	NA
		1×10^6	85.63	3.12±0.07	3.0 (3.0–4.0)
		1×10^7	99.40	2.64±0.06	3.0 (NA–NA)

[a] Percent of dead individuals at the end of experiment corrected for mortality in control using the Abbott equation (Abbott 1925).
[b] The mean survival time and its standard error were underestimated because the largest observation was censored and the estimation was restricted to the largest event time.

Discussion

Fungal insect pathogens are important natural control agents for many insect and other arthropod pests. These pathogens have a potential to significantly reduce host insect populations (Burges 1981; Carruthers & Soper 1987; McCoy *et al*. 1988). Many EPF can be not only entomopathogenic but also acaropathogenic. The study by Chandler *et al*. (2000) describes many EPF species capable of attacking mites. In theory, Acari make good hosts for fungal pathogens because they are generally soft bodied and many inhabit environments with humid microclimates (Ferro & Southwick 1984) which favour infection and disease transmission (Hajek & St Leger 1994).

There are many studies that have focused on the use of the EPF *H. thompsonii* against mites. In laboratory experiments, Gerson *et al*. (1979) observed 94% mycosis of the carmine spider mite, *Tetranychus cinnabarinus* Boisduval (Acari: Tetranychidae), within four days of inoculation at a constant 100% relative humidity (RH), 68% mycosis at 100% RH for 18 h per day, and 23% mycosis at 100% RH for 6 h per day. *Hirsutella thompsonii* was also shown to be effective against the two-spotted spider mite, *Tetranychus urticae* Koch in laboratory bioassays, causing 96.5% mortality with unformulated conidia and up to 99% mortality with the formulated Mycar® (Gardner *et al*. 1982). McCoy *et al*. (1971) applied the mycelia of *H. thompsonii* against the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead) (Acari: Eriophyiidae) infestation on citrus trees, observing sporulation after about 48 h, followed by a decline in mite infestation, which only began to recover 10–14 weeks later. In contrast, Sztejnberg *et al*. (1997) reported a failure of an isolate of *H. kirchneri*, obtained from the cereal rust mite (original accession number CMI 257456) to infect *R. robini*. Another EPF that has an acaropathogenic effect is *Neozygites floridana* (Weiser & Muma) Remaud. & S. Keller. It was first described as the cause of population declines in the Texas citrus mite, *Eutetranychus banksi* (McGregor) in Florida (Weiser & Muma 1966). *Neozygites floridana* is highly effective against the two-spotted spider mite, *T. urticae* and the tobacco spider mite, *Tetranychus evansi* Baker and Pritchard in several major crops where it causes natural epizootics (Humber *et al*. 1981; Klubertanz *et al*. 1991; Nordengen & Klingen 2006; Duarte *et al*. 2009).

TABLE 3. Lethal concentrations of selected strains of *Metarhizium* spp. against *Rhizoglyphus robini* adult females.

Species	Strain	LC_{50}	LC_{90}	χ^2	P
M. indigoticum	MET 01	1.01×10^4	2.20×10^5	306.79	<0.001
M. anisopliae	MET 07	2.36×10^5	2.81×10^7	286.42	<0.001
	MET 08	4.74×10^4	3.01×10^6	319.56	<0.001

In the present study, new strains of "nonspecialist" mitosporic fungi *Beauveria* spp., *Isaria* sp. and *Metarhizium* spp. were tested against the bulb mite *R. robini* under laboratory conditions. The most effective strains were those belonging to the genus *Metarhizium*. This genus is one of the most widely used fungus in mycoinsecticides throughout the world, mainly as an inundative control agent (Zimmermann 2007). According to Goettel *et al*. (1990) the host range of *M. anisopliae* includes Symphyla, Orthoptera, Dermaptera, Isoptera, Homoptera, Heteroptera, Diptera, Coleoptera, Hymenoptera, Siphonaptera, Lepidoptera and Acari, as well as some nontarget hosts, e.g. those belonging to Malacostrata (Amphipoda) and Ephemeroptera. A summary of the safety of *M. anisopliae* has been reviewed by Zimmermann (2007).

Metarhizium genus shows very high virulence to many mite species, as evidenced by several studies (Kaaya *et al*. 1996; Smith *et al*. 2000; Zimmermann 2007; Tomer *et al*. 2018). The results of the present study revealed that Czech strains of *M. anisopliae* MET 07 and MET 08 caused almost 100% mortality in *R. robini* females in four days of incubation. The highest virulence was found in *M. indigoticum* strain MET 01 from Israel in which the lowest median lethal concentration ($LC_{50}=1.01\times10^4$) was estimated compared to the above mentioned *M. anisopliae* strains. Their LC_{50} values were still much lower (4.74×10^4 and 2.36×10^5) than that reported by Wekesa *et al.* (2005) for *M. anisopliae* strain in which the lowest LC_{50} was 0.7×10^7 conidia/ml 7 days after application on *T. evansi* under laboratory conditions. The differences in efficacy may be due to different EPF strain or host species or due to the fact that in the present study conidia in suspensions were activated for 12 hours prior to their application which is known to synchronize germination of spores (Dillon & Charnley 1985, 1990). Recent study by Ment *et al*. (2020) demonstrated high efficacy against *R.*

robini in *M. brunneum* isolate Mb7. Conidia of this fungus applied *in-vitro* at concetration 1×10^7 caused mortality of mites 43% and 100% at three and seven days post inoculation, respectively and the estimated LT_{50} value was 4.3 days. Drench application in potted onion experiments also significanly reduced bulb mite population compared to untreated control (Ment *et al*. 2020). Reference strain F52 of *M. brunneum*, the active ingredient of mycopesticide Met52® EC, is effective against many arthropod pests including the larvae of the black vine weevil *Otiorhynchus sulcatus* (Moorhouse *et al*. 1993; Bruck & Donahue 2007; Ansari & Butt 2013), chilli thrips *Scirtothrips dorsalis* (Arthurs *et al*. 2013), Japanese beetle larvae *Popillia japonica* (Behle *et al*. 2015; Krueger *et al*. 1992), the Asian longhorned beetle *Anoplophora glabripennis* (Gardescu *et al*. 2017; Clifton *et al*. 2020) and the tick *Ixodes scapularis* (Bharadwaj & Stafford 2010; Stafford & Allan 2010). In the present study F52 strain also caused high mortality of *R. robini* (80%) but lower than other *Metarhizium* spp. strains tested.

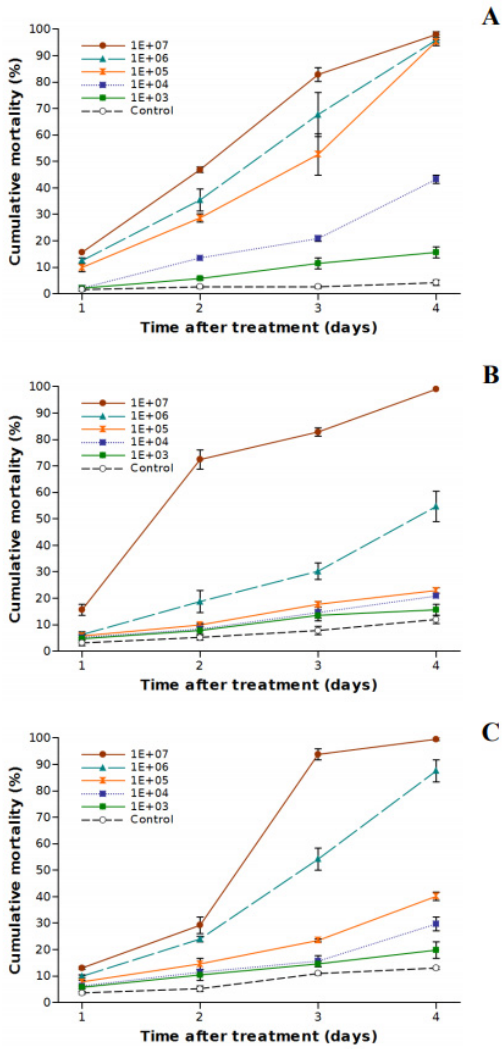

FIGURE 4. Cumulative mortality of *Rhizoglyphus robini* treated by *Metarhizium indigoticum* strain MET 01 (A) and *Metarhizium anisopliae* strains MET 07 (B) and MET 08 (C). Data presented are means (± SE), with two replicates of 96 mites for each strain and concentration.

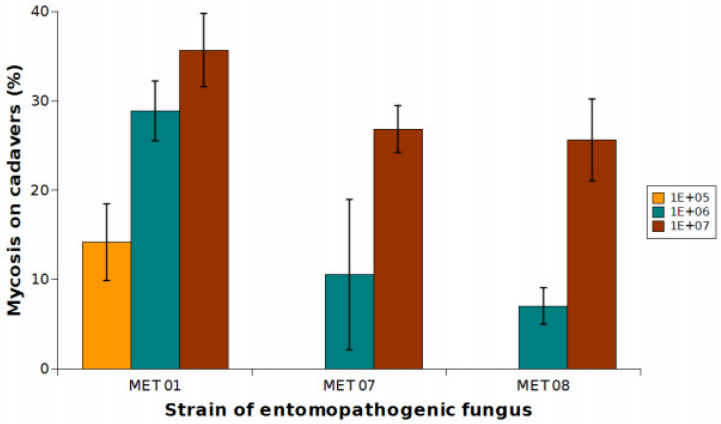

FIGURE 5. Percentage of mycosed cadavers of *Rhizoglyphus robini* adult females treated by *Metarhizium indigoticum* strain MET 01 and *Metarhizium anisopliae* strains MET 07 and MET 08. Data presented are means (± SE), with two replicates of 96 mites for each strain and concentration.

The strains of other EPF species, *Isaria* sp. and *Beauveria* spp. tested in the present study showed much lower efficacy against *R. robini* than strains of *Metathizium* spp. Reference strain CCM 8367 of *I. fumosorosea* caused 40% mortality which was slightly higher compared to the two other *I. fumosorosea* strains tested. This strain was reported to be a promising biocontrol agent against several insect pests (Hussein *et al*. 2013, 2016; Prenerová *et al*. 2013) although recent findings demonstrated that some pests might be resistant to infection (Zemek *et al*. 2020). The strain also turned out to be virulent against *T. urticae* (Zemek *et al*. 2016). High virulence of *I. fumosorosea* against *T. urticae* was found earlier by Kim *et al*. (2008). This EPF species was found to be effective also against the broad mite, *Polyphagotarsonemus latus* (Bank) (Acari: Tarsonemidae) (Pena *et al*. 1996), *T. cinnabarinus* (Shi & Feng 2004a,b), *Eutetranychus orientalis* (Klein) (El-Sharabasy 2015) and the European red mite, *Panonychus ulmi* (Koch) (Graeff *et al*. 2017) whereas it provided only moderate effect against *P. oleivora* compared to *B. bassiana* and *M. anisopliae* (Robles-Acosta *et al*. 2019). When *I. fumosorosea* is applied together with other biological control agents, its side effects against non-target species like predatory mites or parasitoids needs to be assessed (Zemek *et al*. 2017). For example, in a recent study by Chen *et al.* (2020), the fungal entomopathogen *I. fumosorosea* exhibited low toxicity to the predatory mite *Neoseiulus cucumeris* (Oudemans).

High efficacy of *B. bassiana* against mite pests has been documented by several authors. Khoury *et al.* (2020) compared the virulence of blastospores and aerial conidia of different strains of *B. bassiana* against different life stages of *T. urticae* under laboratory and greenhouse conditions with high mortality after exposure mainly to blastospores. Under laboratory conditions, the LT_{50} values of conidia of the Lebanese strain of *B. bassiana* (concentration 1×10^7) was estimated to be 5.6 and 7.5 days for adults and for motile juveniles, respectively. Shi *et al*. (2008) demonstrated that *B. bassiana* had a significant ovicidal effect on the two-spotted spider mite, with up to 87.5% egg mortality in laboratory bioassays while a significant negative effect on reproductive potential of *T. urticae* females was reported by Shi and Feng (2009). Chandler *et al*. (2005), in a greenhouse experiment, demonstrated up to 97% reduction in *T. urticae* abundance when the commercial biopreparate Naturalis-L based on *B. bassiana* was applied. Wekesa *et al.* (2005) studied the pathogenicity of *B. bassiana* against *T. evansi* and determined the LC_{50} of 1.1×10^7 conidia/ml. Studies with other mite species reported promising results for *B. bassiana* against *P. latus* in laboratory bioassays and greenhouse trials (Peña *et al*. 1996) and with various fungal isolates against

the false spider mite, *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae) under laboratory conditions (Rossi-Zalaf & Alves 2006). The strain GHA has been reported to be highly efficient in control of many insect pest species (Liu & Bauer 2008; Mukawa *et al*. 2011; Clavet *et al*. 2013; Parker *et al*. 2015) and was found to be virulent also against *T. urticae* (Ullah & Lim 2015). In the present study, however, the efficacies of all tested *Beauveria* spp. strains against *R. robini* were rather low confirming the fact that the virulence of infective propagules of *B. bassiana* may vary with respect to the arthropod host or their developmental stage (Khoury *et al.* 2020).

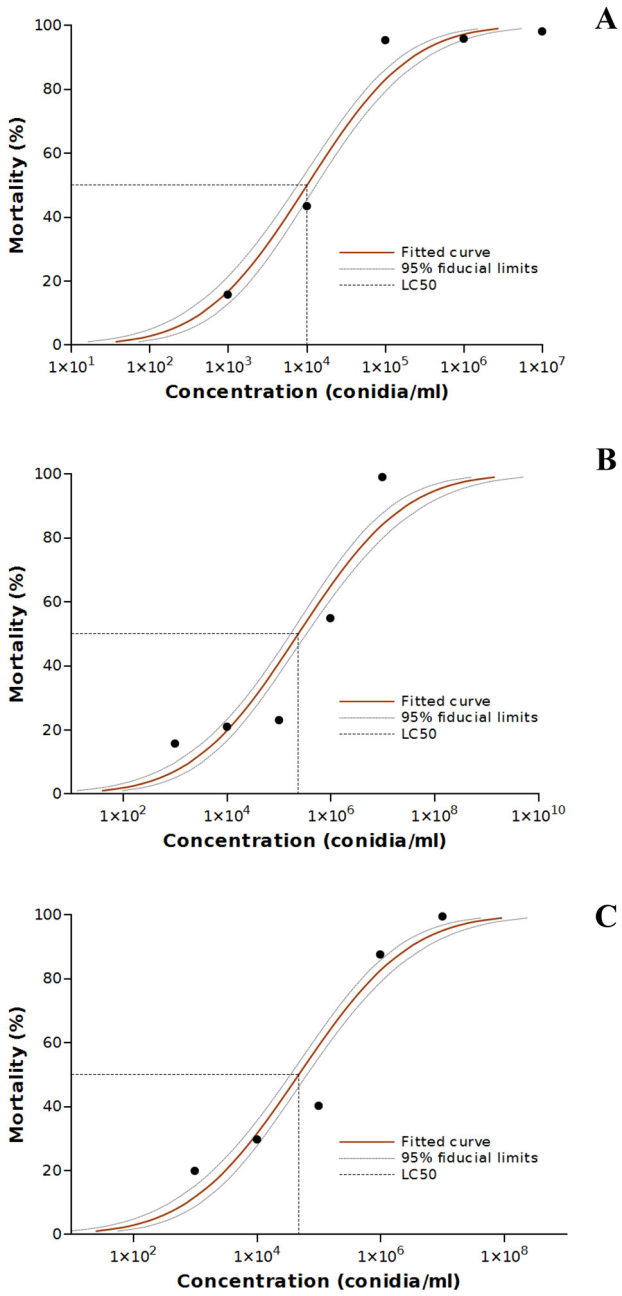

FIGURE 6. Log-probit regression lines of concentration-mortality response of *Rhizoglyphus robini* to *Metarhizium indigoticum* strain MET 01 (A) and *Metarhizium anisopliae* strains MET 07 (B) and MET 08 (C).

The above results indicate that *R. robini* is more resistant to some EPF species than to others. This might be linked to some compounds found in *R. robini* which have been shown to possess antifungal activity. Leal *et al*. (1990a) described hexyl rhizoglyphinate and showed that it inhibited mycelial growth of several species of fungi. Further studies are needed to elucidate if this inhibitory effect is species-specific, e.g. if it negatively affects *Beauveria* spp. or *Isaria* sp. more than *Metarhizium* spp. The role of other compounds, such as the monoterpenoids robinal (Leal *et al*. 1990b) and isorobinal (Sakata *et al*. 1996) in adaptation of bulb mites to live next to some acaro/entomopathogenic fungi in soil environments also remains to be explored.

Conclusions

Acaropathogenic status was demonstrated in most strains of EPF isolated from soil of onion and garlic fields. The most virulent strains of *M. anisopliae* (MET 08) from the Czech Republic and *M. indigoticum* (MET 01) from Israel caused almost 100% mortality in *R. robini* females. Results of the present study thus indicate that particularly that species of EPF belonging to the genus *Metarhizium* can be promising new biocontrol agents against *R. robini.* Further research needs to be carried out to verify if application of EPF is viable as an alternative method to chemical control of bulb mites under greenhouse and field conditions.

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