Efficacy of a Local Isolate of *Metarhizium pinghaense* Against Females of *Margarodes prieskaensis* (Homoptera: Coccoidea) in Field Trials

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The indigenous soil-dwelling scale insect, *Margarodes prieskaensis*, can severely damage and even kill grapevines in the northern grape-growing regions of South Africa. There are no registered means of control, and soil applications of insecticides raise environmental concerns. Using local isolates of entomopathogenic fungi (EPF) that are better adapted to local conditions to target female margarodes could add a valuable biocontrol component to an integrated management strategy. The objective of this research was to evaluate the efficacy of a local isolate of *Metarhizium pinghaense* against *M. prieskaensis* females under field conditions. Dry conidia suspended in water and 0.05% v/v Tween 20, applied as a soil drench, achieved 19.1% and 17.7% infection of margarode females in the Northern Cape and Limpopo, respectively, in 2021. Conidia stored in canola oil, suspended in water and 0.05% v/v Tween 20 and applied as a soil drench achieved infection rates of 38.5% and 62.8%, respectively, at the same sites in 2022. These results confirm the importance of formulating conidia for protection against adverse environmental conditions to improve EPF efficacy in the field. This study is the first to demonstrate the efficacy of *M. pinghaense* against margarode females at the soil surface and confirms the potential of this EPF for the biological control of margarodes.

INTRODUCTION

Margarodes prieskaensis (Jakubski) (Homoptera: Coccoidea) is an indigenous subterranean insect that attacks the roots of grapevines (*Vitis vinifera* L.) along the Lower Orange River and in certain regions in the provinces of North West, Limpopo and Mpumalanga in South Africa (De Klerk & Vermeulen, 2007). It is one of five indigenous *Margarodes* spp. known to feed on grapevine roots in South Africa (De Klerk, 1985). The camel thorn tree, *Vachellia erioloba* (Fabaceae), is the only indigenous host plant identified to date (De Klerk & Vermeulen, 2010). The larvae of *M. prieskaensis* feed on grapevine roots, which weakens the root system and shortens the productive lifespan of the vines. Heavily infested grapevines can die within as little as four years (De Klerk & Vermeulen, 2007).

The life cycle of *M. prieskaensis* was first described by Du Toit (1975). Eggs are laid in the vicinity of host plant roots during summer (October to November). The first instar larvae are mobile, but the second instar larvae settle to feed on the host roots, where they are protected in a cyst formed by waxy secretions and exuviae. The exact number of larval instars is still unknown. Mature larvae in cysts can remain inactive but viable in the soil for years. In autumn and early winter, male pre-pupae emerging from cysts burrow upwards to approximately 15 mm below the soil surface, where they form pupal cells. The pre-pupae moult to form pupae after 50 to 60 days, and winged males appear after about 16 days. Wingless females emerge from the cysts later in winter and burrow to the surface to coincide with the emergence of the winged males (Du Toit, 1975; De Klerk & Vermeulen, 2007). Adult *M. prieskaensis* lack functional mouthparts and do not feed. Consequently, they die soon after mating and egg-laying (Du Toit, 1975).

Currently, no insecticides are registered specifically for the control of margarodes, although field and laboratory

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trials by De Klerk (2010, 2020) showed that soil applications of systemic insecticides could target root-feeding larvae successfully and that adult females and males could be controlled with contact insecticides. Increasing demands from consumers for residue-free produce, as well as the banning or discontinuation of existing insecticides, drive the search for environmentally sustainable pest management options. Burger *et al*. (2017) identified and synthesised the female sex pheromone of *M. prieskaensis*. However, synthesis proved to be very intricate and costly, rendering commercial production of the pheromone for the purposes of mating disruption unprofitable.

Entomopathogenic fungi (EPF) are specialised insect pathogens that occur naturally in soil, where they attack live insects and serve as important natural regulators of insect populations (Meyling & Eilenberg, 2007). Most EPF are non-pathogenic and non-toxic to plants and other non-insect fauna, including humans, making them ideal biocontrol agents for insect pests (Meyling & Eilenberg, 2007; Hatting *et al*., 2019; Sharma & Sharma, 2021). The optimum temperature for EPF lies between 25ºC and 35ºC, but they are resilient and able to tolerate harsh environmental conditions (Alves *et al*., 1998; Burges, 2012). Various EPF species, particularly *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metchn.) Sorokin (Ascomycota: Hypocreales), have been developed commercially as biocontrol agents for a variety of agricultural insect pests worldwide (De Faria & Wraight, 2007; Sharma & Sharma, 2021; Sharma *et al*., 2023), and in South Africa (Hatting *et al*., 2019).

In South Africa, the potential of local EPF isolates and commercially available EPF as biocontrol agents has been demonstrated against a range of fruit pests that include the soil-dwelling life stages of false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Coombes, 2012), banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Dlamini *et al*., 2020), adult stages of obscure mealybug, *Pseudococcus viburni* (Signoret) (Mathulwe *et al*., 2022a), soil-dwelling stages of the Mediterranean and Natal fruit flies, *Ceratitis capitata* (Wiedemann) and *C. rosa* Karsch (Goble, 2009), and eggs and larvae of the codling moth, *Cydia pomonella* L. (Abaajeh & Nchu, 2015), and the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Stokwe, 2016; Mathulwe *et al*., 2023).

The bright yellow females of *M. prieskaensis* are highly conspicuous when waiting at the soil surface to mate, and present an ideal target for control. When the adult margarodes emerge during winter, daytime temperatures in the Northern Cape and the northern provinces where this insect occurs are considerably lower than during summer (ARC-Natural Resources & Engineering, vgenti@arc.agric.za) and closer to the optimum temperature range of 25ºC to 35ºC for EPF (Alves *et al*., 1998; Burges, 2012).

The use of local isolates of EPF that are better adapted to local conditions to target female margarodes could add a valuable biocontrol component to an integrated pest management (IPM) strategy. The pathogenicity of five South African *Metarhizium* (Hypocreales: Clavicipitaceae) species complex isolates and one *Beauveria* (Hypocreales: Cordycipitaceae) isolate, obtained from the Stellenbosch University EPF collection, was tested against females of *M. prieskaensis* under laboratory conditions (Erasmus, 2023). *Metarhizium pinghaense* isolate 5HEID emerged as the best candidate for field trials.

This article reports the results of field trials conducted with a local mass-cultured isolate of *M. pinghaense* to evaluate its efficacy against *M. prieskaensis* females under field conditions.

MATERIALS AND METHODS

Mass culturing of EPF conidia for field trials with *M. prieskaensis*

Conidia of *M. pinghaense* isolate 5HEID (GenBank number MT367414), obtained from the Stellenbosch University EPF collection, were mass cultured according to the protocol developed by Mathulwe *et al*. (2022b). Sabouraud dextrose agar plates were used to maintain stable stock cultures of the EPF isolate. Liquid blastospore inoculum was prepared by the fermentation of fungal conidia in a broth consisting of sterile distilled water, glucose, yeast extract, K_2HPO_4 and corn steep liquor. White parboiled rice was used as the solid substrate to produce conidia over a period of four to five weeks. The rice cultures were then dried for approximately 10 days. To harvest the conidia, sieves mounted on a collection pan were used along with a vibratory shaker to separate and filter them. Harvested conidia were weighed and aliquots of 5 g were stored in airtight and waterproof resealable bags. Spore counts were used to calculate that a dose of 5 g conidia suspended in 5 L water would equate to a spore concentration of 10^7 conidia/mL. Bags with conidia were stored at ± 15°C until used in the field trials.

Field trial sites

Field trials were conducted in table grape vineyards over two seasons, with trial site 1 (-28.5000, 20.1575) located in the Blouputs Valley, Northern Cape province and site 2 (-24.3170, 29.1803) near Mokopane in Limpopo province. At each trial site, 10 plots measuring 1 m^2 each were demarcated with 1 m × 11 cm plastic strips in areas within the vineyard where *M. prieskaensis* females were present at the soil surface. Five replicates of the EPF and control treatments were randomly assigned to the demarcated plots in a randomised block design.

Field trials 2021

Treatments were applied on 2021-07-13 and 2021-07-31 when the margarodes female emergence peaked at trial sites 1 and 2, respectively. The bags containing 5 g of mass-cultured conidia were transported to the field in a cooler box. The conidia in each bag were mixed with 5 L water and 1 mL 0.05% v/v Tween 20 and applied to each marked plot as a soil drench using a watering can with a sprinkler head.

Three days after application, the top 10 cm of the soil in each plot was watered lightly, removed with a shovel and placed into individually marked 50 kg polypropylene bags. The bags were folded to prevent desiccation and left in the vineyard for four more days to increase the exposure time of the margarodes to the EPF. Seven days after EPF application, the soil was sieved and all margarodes females were collected. The collected females for each replicate were sealed in a separate container with moist soil and transported

to the laboratory in a cooler box for evaluation of insect infection by the EPF.

In the laboratory, the containers with soil and margarodes females were further incubated in a growth chamber at ± 25°C. The insects in the containers were checked every second day for signs of mortality or overt mycosis. Margarodes female mortality was recorded after seven days. Females that did not respond to prodding with fine forceps were considered dead. To determine whether mortality was due to EPF infection, the margarodes cadavers were surface-sterilised and incubated on nutrient-free water agar plates at ± 25°C. Mycosis was recorded after incubation for four days. Females that exhibited mycosis, with the grey-green spores typical of *M. pinghaense* (Fig. 1), were deemed to have died of EPF infection.

Field trials 2022

Trial layout was the same as in 2021. Treatments were applied on 2022-06-04 and 2022-08-12, when the margarodes female emergence peaked at trial sites 1 and 2, respectively. The postgraduate student who produced the dry conidia for the laboratory trials had concluded her studies, therefore dried conidia of the EPF were not available and conidia stored in canola oil were used. The contents of each storage tube, containing 5 g conidia, were mixed vigorously with 2 mL 0.05% v/v Tween 20 and 5 L water and applied to a treatment plot as a soil drench using a watering can with a sprinkler head. The control plots were treated with 5 L water.

The exposure of females to the EPF in bags of soil in the vineyard and subsequent assessment of EPF efficacy in the laboratory were as described for 2021, with one notable change: the retrieval of margarodes females from the soil seven days after EPF application was done by removing individual females by hand rather than by sieving so as to reduce mortality due to injury.

Data analysis

Data for each site were analysed separately. The homogeneity of year*treatment variances was verified by Bartlett's test (Bartlett, 1937), before submitting the data to a combined analysis of variance (ANOVA) using the general linear models procedure (PROC GLM) of the SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Normality of the standardised residuals was confirmed by the Shapiro-Wilk test (Shapiro & Wilk, 1965). Fisher's least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott & Longnecker, 2010). A probability level of 5% was considered significant for all significance tests.

RESULTS

The results of the field trials conducted over two seasons with *M. pinghaense* at site 1 in the Northern Cape are presented in Table 1. In 2021, mortality in the control treatments was high, with a mean of 60.5%, but there was zero infection by the EPF. Mean mortality did not differ significantly between the EPF treatment (82.0% ± 8.8%) and the control (60.5% ± 2.9%). However, the mean percentage *M. pinghaense* infection in the EPF treatment (19.1% ± 6.1%) did differ significantly from the control (0%). In 2022, mean mortality in the control treatments was significantly lower than in 2021 (10.7% ± 6.4% vs 60.5% ± 2.9%). Mean mortality in the EPF treatments (47.5% ± 8.8%) was significantly higher than in the control (10.7% ± 6.4%). While mean mortality in the EPF treatments was significantly lower than in 2021 (82% ± 8.8%), the percentage *M. pinghaense* infection (38.5% ± 7.5%) was significantly higher than in 2021 (19.1% ± 6.1%).

The results of the field trials conducted over two seasons with *M. pinghaense* at site 2 in Limpopo are presented in Table 2. In 2021, mean mortality in the control treatments (44.3% ± 7.4%) was high and did not differ significantly

FIGURE 1

Margarodes prieskaensis female showing mycosis and grey-green sporulation typical of infection by *Metarhizium pinghaense*.

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from that in the EPF treatment (59.5% ± 9.6%). However, the percentage infection did differ significantly between the EPF treatments (17.7% ± 4.2%) and the controls (0%). In 2022, mean mortality in the control treatments was significantly lower than in 2021 (17.2% ± 3.4% vs 44.3% ± 7.4%) and differed significantly from mortality in the EPF treatments (94.1% ± 3.6%). The mean percentage infection in the EPF treatments (62.8% ± 9.0%) was significantly higher than in the controls (0%). Moreover, mean mortality in the EPF treatments (94.1% ± 3.6%) and mean percentage infection

TABLE 1
Mortality (%) of *Margarodes prieskaensis* females and percentage of females infected by *Metarhizium pinghaense* in field trials conducted in the Blouputs Valley, Northern Cape in 2021 and 2022 (site 1).

Blouputs		**2021**			**2022**		
Treatment	**Replicate**	**No. females (*n*)**	**% Mortality***	**% EPF infection****	**No. females (*n*)**	**% Mortality***	**% EPF infection****
M. pinghaense	1	52	90.4	0.0	110	50.0	44.6
	2	212	67.0	12.3	46	80.4	65.2
	3	34	100.0	32.4	54	37.0	27.8
	4	46	91.3	19.6	85	30.6	25.9
	5	83	61.5	31.3	48	39.6	29.2
Mean			82.0 ± 8.8^{a}	19.1 ± 6.1^{b}		47.5 ± 8.8^{b}	38.5 ± 7.5^{a}
Control	1	448	66.3	0.0	27	3.7	0.0
	2	86	55.8	0.0	85	2.4	0.0
	3	395	60.3	0.0	230	35.2	0.0
	4	325	67.4	0.0	44	11.4	0.0
	5	1418	52.8	0.0	128	0.8	0.0
Mean			60.5 ± 2.9^{ab}	0.0^{c}		10.7 ± 6.4^{c}	0.0^{c}

Notes: * least $LSD_{(0.05)}$ for percentage mortality = 22.887; ** $LSD_{(0.05)}$ for percentage infection = 15.669 [LSD = least significant difference]
Different letters in a column and between columns for the same parameter indicate significant differences between treatments.

TABLE 2
Mortality (%) of *Margarodes prieskaensis* females and percentage of females infected by *Metarhizium pinghaense* in field trials conducted in 2021 and 2022 near Mokopane in Limpopo (site 2).

Limpopo		**2021**			**2022**		
Treatment	**Replicate**	**No. females (*n*)**	**% Mortality***	**% EPF infection****	**No. females (*n*)**	**% Mortality***	**% EPF infection****
M. pinghaense	1	318	36.8	7.2	19	84.2	52.6
	2	14	85.7	28.6	34	100.0	79.4
	3	92	60.9	26.1	18	100.0	88.9
	4	71	39.4	11.3	7	100.0	42.9
	5	111	74.8	15.3	22	86.4	50.0
Mean			59.5 ± 9.6^{b}	17.7 ± 4.2^{b}		94.1 ± 3.6^{a}	62.8 ± 9.0^{a}
Control	1	113	38.9	0.0	23	21.7	0.0
	2	680	31.8	0.0	21	14.3	0.0
	3	128	57.0	0.0	15	13.3	0.0
	4	334	27.8	0.0	34	8.8	0.0
	5	357	65.8	0.0	18	27.8	0.0
Mean			44.3 ± 7.4^{b}	0.0^{c}		17.2 ± 3.4^{c}	0.0^{c}

Notes: * $LSD_{(0.05)}$ for percentage mortality = 19.622; ** $LSD_{(0.05)}$ for percentage infection = 16.171 [LSD = least significant difference]
Different letters in a column and between columns for the same parameter indicate significant differences between treatments.

(62.8% ± 9.0%) were significantly higher in 2022 than in 2021.

DISCUSSION

Although restricted in distribution, *M. prieskaensis* can cause serious economic damage in vineyards in which it is endemic, including the death of grapevines (De Klerk & Vermeulen, 2007). In some locations in the northern regions, table grape production has been deemed no longer economically viable, partly due to the lack of any registered control options for margarodes (A. Bredell, personal communication). Systemic insecticides applied as soil drenches that target margarodes larvae feeding on grapevine roots were shown to be effective against *M. prieskaensis* in South Africa (De Klerk, 2010) and against the margarodes *Eurhizococcus brasiliensis* Wille, which attacks grapevine roots in Brazil (Botton *et al*., 2010). However, the use of these chemicals is problematic due to environmental concerns, particularly in export markets. The application of contact insecticides to target female and male margarodes at the soil surface, shown to be effective by De Klerk (2010, 2020), also raises environmental concerns.

EPF present an insect-specific, residue-free and environmentally sustainable option for pest control (Meyling & Eilenberg, 2007; Hatting *et al*., 2019; Sharma *et al*., 2023). Lopes *et al*. (2012) showed that cysts of *E. brasiliensis* are susceptible to local isolates of *Isaria fumosorosea* Wize (formerly known as *Paecilomyces fumosoroseus*) and that females are susceptible to infection by naturally occurring *M. brunneum* in the soil. Since females make up only a small proportion of the *E. brasiliensis* population, the potential of *M. brunneum* for margarodes control is limited. To our knowledge, this study is the first to demonstrate the efficacy of mass-produced EPF for the control of margarodes females at the soil surface.

Field trials showed that *M. pinghaense* was able to successfully infect and kill margarodes females when applied as a soil drench. In 2021, mortality in the control treatments was high, but in 2022, when females were retrieved by hand to eliminate physical injuries sustained during the sieving of soil, mortality was reduced significantly.

Fluctuations in temperature and humidity, as well as solar radiation experienced under field conditions, are often not ideal for EPF and could affect their efficacy in the field (Alves *et al*., 1998; Burges, 2012). According to Grant *et al*. (2001), the development rate of *Metarhizium* species, and therefore their effectiveness to control pests, is directly influenced by temperature. Data obtained from weather stations on both trial farms showed that the minimum and maximum temperatures during the trial periods were higher and more favourable for EPF development in 2022 than in 2021 (data not shown) and could have contributed to the improved performance of the EPF in 2022.

In 2022, conidia of *M. pinghaense* preserved in canola oil were used, rather than dry conidia as in 2021, which resulted in a significant increase in mean percentage infection at both sites – from 19.1% to 38.5% in the Northern Cape and from 17.7% to 62.8% in Limpopo. Improved activity of *Metarhizium* conidia under sub-optimal conditions when formulated in oil is well documented (Moore *et al*., 1993; Rodrigues *et al*., 2019). This is attributed to the fact that oil-formulated conidia adhere better and more homogeneously to insect cuticles and are protected against abiotic stresses. Rodrigues *et al*. (2019) also found that the addition of diatomaceous earth to the formulation increased conidial activity because it causes abrasive damage to the target insect cuticles, which facilitates EPF penetration. The results reported here confirm the importance of correct formulation, combined with optimum temperature conditions, to achieve effective EPF performance in the field.

CONCLUSIONS

The infection of female *M. prieskaensis* by a local isolate of *M. pinghaense* applied as a soil drench in field trials confirmed the potential of this EPF for the biological control of margarodes during the winter, when the females appear at the soil surface to mate. The importance of formulating conidia for protection from adverse environmental conditions to improve EPF efficacy in the field was also demonstrated. Further field trials with *M. pinghaense* formulated in oil and/or diatomaceous earth to target margarodes females as well as male pre-pupae are recommended.

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