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Research Article

Metarhizium majus and *Metarhizium robertsii* show enhanced activity against the coleopteran pests *Holotricha serrata* and *Oryctes rhinoceros*

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ABSTRACT: Studies were conducted to systematically isolate *Metarhizium* isolates from the insect cadavers and soils of South India. Morphological and PCR amplified sequences of 5.8S ITS regions and RNA polymerase II largest subunit (RPB1) gene regions were used to identify the isolates at species level. Eight *Metarhizium* isolates were isolated and initially identified by morphological and microscopic studies. Further identification was confirmed through 5.8SrRNA ITS and RPB1 analysis. They were identified as three isolates of *M. robertsii* J.F. Bisch., Rehner & Humber sp. nov. (ArMz3R, ArMz3S and ArMz6W), one isolate of *M. majus* (J.R. Johnst.) J.F. Bisch., Rehner & Humber (VjMz1W) and four isolates of *M. anisopliae* (WnMz1S, NlMz2S, BgMz2S and DhMz4R). Topical conidial suspensions (TCS) and powder based formulations (PBF) of the eight indigenous isolates of *Metarhizium* spp. that were isolated from insect cadavers and soils of South India were tested against coleopteran pests *Holotricha serrata* L. and *Oryctes rhinoceros* L. that cause serious damage to sugarcane and palm trees respectively. Against *H. serrata* TCS of *M. robertsii* (ArMz6W) was the most effective with an LC_{50} of 6.893×10^5 cfu/ml and caused 100% mortality against the 3rd instar larvae in 5 days; PBF elicited an LC_{50} of 7.502×10^5 cfu/ml with 96% mortality in 10 days. Against *O. rhinoceros* TCS (LC_{50} of 9.75×10^5 cfu/ml) of *M. majus* (VjMz1W) caused 90% mortality in 7 days and the PBF (LC_{50} of 9.57×10^5 cfu/ml) caused 86% mortality in 14 days. The results establish that *M. robertsii* is highly effective against *H. serrata* and against *O. rhinoceros M. majus* was the most effective. The TCS formulations of these two strains can be readily deployed for field applications.

KEY WORDS: *Holotricha serrata, Metarhizium* spp. *Oryctes rhinoceros,* Powder based formulation (PBF), Topical conidial suspension (TCS)

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INTRODUCTION

Holtrichia serrata (Fabricius) commonly known as white grub is a serious pest of many agricultural crops of South India, it causes serious damage to sugarcane roots. They survive by feeding and mating in sugarcane root system and damage is caused by the larvae (Anitha *et al.,* 2006). Apart from sugarcane they are also serious pests of groundnut and potato. *H. serrata* are widely disturbed around the southern states viz. Tamil Nadu, Karnataka, Kerala, Andhra Pradesh (Veeresh, 1977). Another serious pest is the rhinoceros beetle *Oryctes rhinoceros* L. (Scarabaeidae: Dynastinae) and can be devastating to coconut not only in India but across Southeast Asia leading to severe economic losses (Nair *et al.,* 1997; Norman and Basri, 1997; Bedford, 2014;). As with many beetles, adults and larvae have different feeding preferences. Young adults of *O. rhinoceros* feed on healthy leaves and larvae feed on rotting plant material in soil.

The entomopathogenic fungus, *M. anisopliae,* discovered 100 years ago by Mednichoff has a rather wide host range and is widely used as a microbial

biocontrol agent on various types of pests, which include insects from different orders: Lepidoptera, Coleoptera, Hemiptera, Diptera, Orthoptera, Hymenoptera, as well as non-insect arthropods like elm bark beetle, mosquito larvae, plant hoppers, coconut leaf beetle, rhinoceros beetle, onion thrips, storage pests, white grub and cattle tick. There are 60 commercial mycoinsecticides based on *M. anisopliae* strains (Shanmugam *et al.,* 2014; Chandal *et al.,* 2015). There are several studies conducted to prove the efficacy of *Metarhizium* against many insect pests, they are mostly based on *M. anisopliae*. Against *Holotrichia serrata* strains of *M. anisopliae* have been shown to cause infection and formulations have been used in sugarcane fields to bring down the white grub population (Srikanth *et al.,* 2011; Shanmugam *et al.,* 2014; Sathayaraj and Karthick 2008; Thamarai Chelvi *et al.,* 2011; Pradya and Mohit, 2014). *M. anisopliae* have been tested for control scarab species of several coleopteran beetles (Hurpin and Robert, 1972). *O. rhinoceros* is a serious pest of oil palm in Malaysia (Bedford, 2014; Norman and Basri, 1997), and formulations of *M. anisopliae* were used for biological control (Ramle *et al.,* 2009).

Since most of the research work is based on *M. anisopliae* strains, reports on the effectiveness of other *Metarhizium* species are very limited. In the present study systematic survey was done to collect forest soil and insect cadaver samples for isolation of *Metarhizium* species and to see if apart from *M. anisopliae* whether other species could be more effective in control of insect pests such as *O. rhinoceros* and *H. serrata*.

MATERIALS AND METHODS

Survey and collection of the *Metarhizium* isolates

The survey was made to various forests of South India (Tamilnadu, Kerala, Karnataka and Andhra Pradesh) dead insects and soil samples were collected from these forests (wet evergreen, moist deciduous, dry deciduous and scrub forest) (Table-1). *Metarhizium* was isolated from insect cadaver and from soil using *Galleria* larvae as bait. Infected cadavers and *Galleria* larvae were surface-sterilized by dipping sequentially in 70% ethyl alcohol, 1% sodium hypochlorite, and sterile distilled water, each for 2-3 minutes. They were dissected and placed on potato dextrose agar plus yeast extract (PDAY medium containing (1% yeast extract 0.6g, 100µg/ml Chloramphenicol, 50 µg/ml Streptomycin, 2 mg crystal violet) and incubated at 28-1°C and 90% RH to facilitate growth and sporulation of the fungus. The purification of the *Metarhizium* spp. was done using Veen's medium (Veen and Ferron, 1966; Hu and St. Leger, 2002). Final purification was done by hyphal tip method. Plates were incubated for 7 to 14 days at 27°C to induce growth and sporulation of the fungus. After 15 days emerged mycelia were harvested by scraping off the content from each Petri plate. Slants of *Metarhizium* fungal cultures were prepared and stored. *Matarhizium* spp. were initially described based on morphological, conidiophores, and phialides mounted microscopes. Further identification was done by sequence analysis of 5.8S rRNA of the internal transcribed spacer (ITS) and RNA polymerase II largest subunit (RPB1) as per protocols by Bischoff *et al.* (2009) and Nishi *et al.* (2011) (Table 1).

Production of *Metarhizium* spp.

Topical conidial suspension (TCS) was prepared as per the procedure described by Fleming (1968) and Tashiro *et al.* (1973). Powder based formulation (PBF) was prepared as per the procedure of Samiyappen *et al.* (2003) and Ramle *et al.* (2009). TCS was prepared by scraping out the mycelia from 10-15 days old culture grown PDA in Petri plates in an aqueous solution of 0.02% Tween 80 and 1% glycerol**,** with continuous stirring in a tube and filtered through a single layer of linen to remove debris and mycelia. The conidial concentration was estimated with haemocytometer under light microscope. Subsequently the spore suspension was diluted to make a final suspension of 1×10^5 spores/ml with 0.02% Tween 80. PBF was prepared by harvesting 15 days old mycelia grown in Potato dextrose broth (PDB). Harvesting was done centrifugation at 10,000 rpm for 15 min. The pellet was mixed @ 100g in mixture containing 200g rice flour, Yeast extract 50g, 10ml glycerol, 10ml honey and

630g of talc.

Collection and rearing of white grubs and *Oryctes rhinoceros*

Adults and larvae of *Holotrichia serrata* and *Oryctes rhinoceros* were collected from agricultural fields (palm, rice and banana and sugarcane) in North-Thanjavur district, Sathanur Village Latitude and Longitude: 10.82035 N: 79.08925 E and surrounding areas in plastic boxes (23x20cm diameter x height) containing the native soil. In the laboratory larvae, pupae and adults were separated into individual plastic boxes and kept at 27°C and 70% RH. The feed consisted of organic matter, decaying wood trash carrot, potato tree yam, ground nuts and other debris. The rearing room was protected from direct sun-light and UV-light by making it dark. The boxes were covered with muslin cloth and tied with rubber bands to ensure good aeration. The moisture level was maintained through wet cloth and soil with gentle moistening when the soil felt dry. While rearing of the grubs, the following biological parameters such as, date of collection, date and causes of grub mortality, date of pupation and adult emergence were recorded. After six week of rearing, healthy grubs of 3rd instar larvae were used in bioassay.

Bioassay against *Holotrichia serrata*, *Oryctes rhinoceros* larvae

Third instar larvae of *Holotrichia serrata* and *Oryctes rhinoceros* were transferred aseptically to fresh plastic boxes (4cm diameter and 6cm height). Each box contained moist soil medium with some humus and potato tubers as food. Already prepared TCS/PBF were tested at three concentrations ($1x10^5$, $1x10^4$ and $1x10^3$ spores/ml). Into each box 5ml suspension was added by droplets onto the larvae. Three replications were maintained and total larvae tested for each treatment was fifteen. For control sterile water with 0.02% sterile water was used. Observations on mortality was recorded up to 7 days.

Statistical analysis

Cumulative mortality at the end of the experiment was analysed and LC_{50} were determined using the probit analysis program. SAS 15.0 for windows.

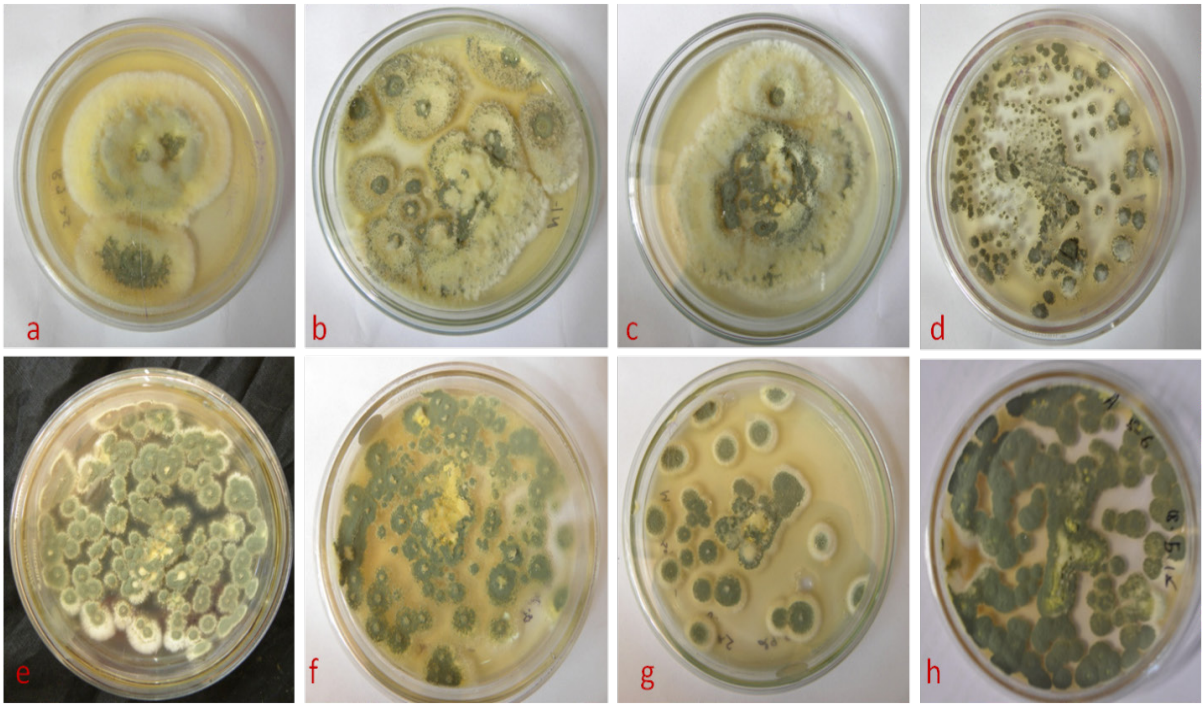

Fig. 1. ***Metarhizium*** **colonies grown on PDAY at room temperature for 15 days (a)** ***M. robertsii*** **(ArMz3R), (b)** ***M. robertsii*** **(ArMz3R), (c).** ***M. robertsii*** **(ArMz6W), (d).** ***M. majus*** **(e).** ***M. anisopliae*** **(f).** ***M. anisopliae*****, (g).** ***M. anisopliae*** **and (h).** ***M. anisopliae*****.**

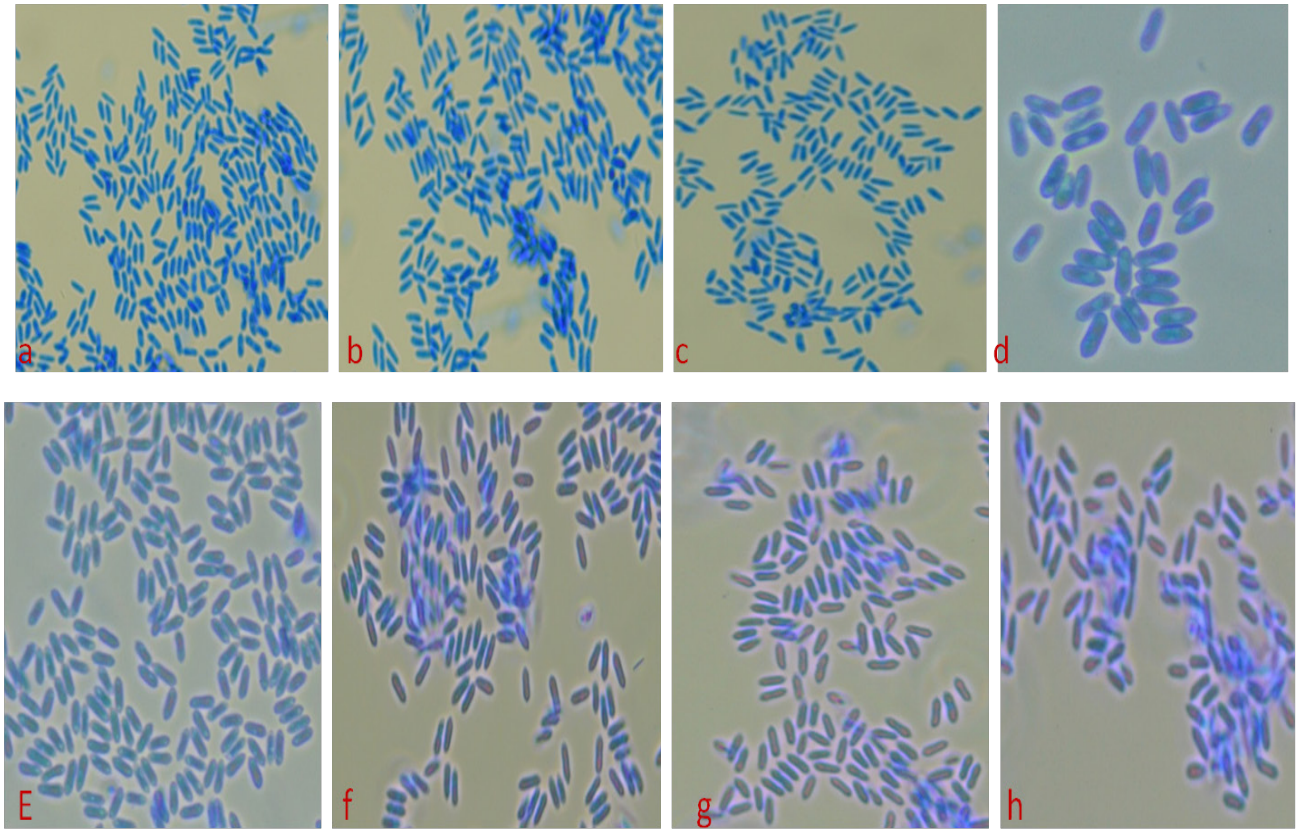

Fig. 2. The morphology of conidia under bright field light microscope (100X); (a-c), *Metarhizium robertsii* conidia small, (d), *M. majus* conidia large, (e-h), *M. anisopliae* conidia medium.

RESULTS AND DISCUSSION

Experiments were conducted to collect forest soil and insect cadaver samples for isolation of *Metarhizium* species and to see if apart from *M. anisopliae* whether other species could be more effective in control of insect pests such as *O. rhinoceros* and *H. serrata*. Eight *Metarhizium* isolates were isolated and initially identified by morphological and microscopic studies (Table 1 and Fig. 1-2) as per conidial shape and size (Driver *et al.,* 2000; Bischoff *et al.,* 2009). Further identification was confirmed through 5.8SrRNA ITS and RPB1 analysis. They were identified as three isolates of *M. robertsii* (ArMz3R, ArMz3S and ArMz6W), one isolate of *M. majus* (VjMz1W) and four isolates of *M. anisopliae* (WnMz1S, NlMz2S, BgMz2S and DhMz4R). The partial sequences for 5.8SrRNA ITS and RPB1 were submitted to NCBI (Table 1). Bischoff *et al.* (2009) employed a multigene phylogenetic approach from nuclear encoded EF-1, RPB1, RPB2 and - tubulin gene regions and proposed nine terminal taxa in the *M. anisopliae* complex which included *M. majus* and *M. robertsii*. Similarly Nishi *et al.* (2011) analyzed the internal transcribed spacer (ITS) and 50 end of the EF-1a sequence of 145 isolates of *Metarhizium* spp. isolated from soil and e identified six species: *M. anisopliae, M. brunneum, M. guizhouense, M. majus, M. pingshaense* and *M. robertisii*. In our studies morphological, 5.8SrRNA ITS and RPB1 analysis was done to confirm the species.

Bioassay against *Oryctes rhinoceros*

Bioassay was conducted against 3rd instar larvae of *Oryctes rhinoceros* with TCS formulation containing the eight *Metarhizium* isolates. It was observed that at 7 days after treatment the highest mortality (90%) and infection (86%) was with *Metarhizium majus* treated

Table 1. List of *Metarhizium* spp. isolates obtained and sequenced for phylogenetic analysis. All isolates obtained for this study (GC) were from locations within south Indian

Strain	Fungi	Type of forest	Conidia and Phialides (µm)	Colony colour	Region	Geographical location	Accession Number	
							ITS	RPB1
ArMz3R	*M. robertsii*	WEF	3.84-0.14 x 3.84-0.14	Olive green	Aralam, Kerala	11´99°N 75´76°E	KU983799	KU680339
ArMz3S	*M. robertsii*	WEF	3.78-0.19 x 3.28-0.12	Olive green	Aralam, Kerala	11´99°N 75´76°E	KU983775	KU680335
ArMz6W	*M. robertsii*	WEF	2.18-0.04 x 2.13-0.04	Olive green	Aralam Kerala	11´99°N 75´76°E	KU983797	KU680341
VjMz1W	*M. majus*	WEF	11.17-13.1x 4.42-3.21	Dark green	Coorg, Karnataka	12´20°N 75´80°E	KU983771	KU680342
WnMz1S	*M. anisopliae*	WEF	7.69-0.22 x 3.23-0.12	Light green	Aralam, Kerala	11´60°N 76´08°E	KU983788	KU680323
NlMz2S	*M. anisopliae*	MDF	7.12-14.5 x 2.31-3.0	Light green	Bengaluru	12´97°N 77´59°E	KU983785	KU680325
BgMz2S	*M. anisopliae*	DDF	8.44-0.13 x 3.48-0.18	Light green	Bengaluru	12´80°N 77´57°E	KU983780	KU680320
DhMz4R	*M. anisopliae*	MDF	7.30-0.26 x 3.20-0.24	Light green	Palakkad Kerala	10´77°N 76´65°E	KU983784	KU680328

Wet Evergreen Forest (WEF), Moist Deciduous (MDF) and Dry Deciduous Forest (DDF); 5.8SrRNA Internal Transcribed Sequence (ITS), RNA polymerase II largest subunit (RPB1)

Table 2. Probit analysis of mortality response in field-collected third instar larvae of *Oryctes rhinoceros* following treatment with TCS of *Metarhizium*

Metarhizium Species	Mortality %	Infection %	LC_{50}	95% Fiducial limits		Slop± SE	X2	P
M. robertsii (ArMz3R)	84	56	14.78	10.8037	21.5689	1.106±0.634	6.42	0.0113
M. robertsii (ArMz3S)	80	46	14.86	6.9628	74.6436	1.092±0.069	4.09	0.0430
M. robertsii (ArMz6W)	76	53	17.69	14.4800	31.9684	1.143±0.857	7.34	0.0067
M. majus (VjMz1W)	90	86	9.75	4.2387	12.0897	1.168±1.047	8.16	0.0043
M. anisopliae (WnMz1S)	66	43	21.10	16.9789	56.0096	1.195±0.876	7.25	0.0071
M. anisopliae (NlMz2S)	66	40	25.68	19.3167	24.9661	1.291±0.592	5.85	0.0156
M. anisopliae (BgMz1S)	67	43	18.62	15.4102	33.2553	1.162±1.059	8.24	0.0041
M. anisopliae (DhMz4R)	70	43	18.55	16.1216	24.7683	1.298±2.178	13.24	0.0003

*LC_{50} values are expressed as $x10^5$ spores/ml; TCS = topical conidial suspension

and the LC_{50} was calculated as $9.75x10^5$ conidia/ml. Significant mortality was also observed with *M. robertsii* (ArMz3R) treated wherein 84% mortality was seen with a LC_{50} value of $14.78x10^5$ condia/ml. Surprisingly *M. anisopliae* isolates were less virulent (Table 2 and Fig. 3). When tested with PBF again *M. majus* (VjMz1W) was the most virulent with highest mortality of 90% in 10 days and the LC_{50} was $9.57x10^5$ spores/ml. similar mortality (90%) was observed with *M. robertsii* (ArMz3S) treated and the LC_{50} was $11.98x10^5$ spores/ml. Two *M. anisopliae* isolates (WnMz1S and DhMz4R) exhibited 86% mortality with LC_{50} ranging from 14.18 to 19.12 x 10^5 spores/ml (Table 3 and Fig. 3). The results establish that *M. majus* (VjMz1W) was an ideal candidate to combat *O. rhinoceros*. This is the first report to indicate the usefulness of *M. majus* and *M. robertsii* in biological control. Reports are available in using *M. anisopliae* against *O. rhinoceros*, *M. anisopliae* formulated in powder form caused 90% mortality and 73% infection and the fungus could infect all stages of *O. rhinoceros* larvae (Ramle *et al.*, 2007). Powder formulations of *M. anisopliae* based on kaolin and rice were tested under laboratory conditions and 93% mortality was

Table 3. Probit analysis of mortality response in field-collected third instar larvae of *Oryctes rhinoceros* following treatment with PBF of *Metarhizium*

Metarhizium species	Mortality %	Infection %	LC_{50}	95% Fiducial Limits		Slop± SE	X^2	P
M. robertsii (ArMz3R)	83	53	17.78	14.0107	57.2927	1.118±0.414	5.43	0.0198
M. robertsii (ArMz3S)	90	60	11.98	3.9459	15.3456	1.104±0.396	5.38	0.0204
M. robertsii (ArMz6W)	86	56	13.62	11.5536	15.5961	1.180±2.620	17.47	0.0001
M. majus (VjMz1W)	90	70	9.57	0.6618	12.5190	1.126±0.327	5.07	0.0244
M. anisopliae (WnMz1S)	86	52	14.18	11.2460	17.4497	1.128±1.376	10.11	0.0015
M. anisopliae (NlMz2S)	85	55	16.47	12.5825	40.5832	1.107±0.384	5.32	0.0210
M. anisopliae (BgMz1S)	80	50	19.97	16.9391	31.0107	1.280±1.745	11.04	0.0009
M. anisopliae (DhMz4R)	86	46	19.12	16.1844	29.4487	1.224±1.571	10.52	0.0012

*LC_{50} values are expressed as $x10^5$ spores/ml; PBF = powder based formulation

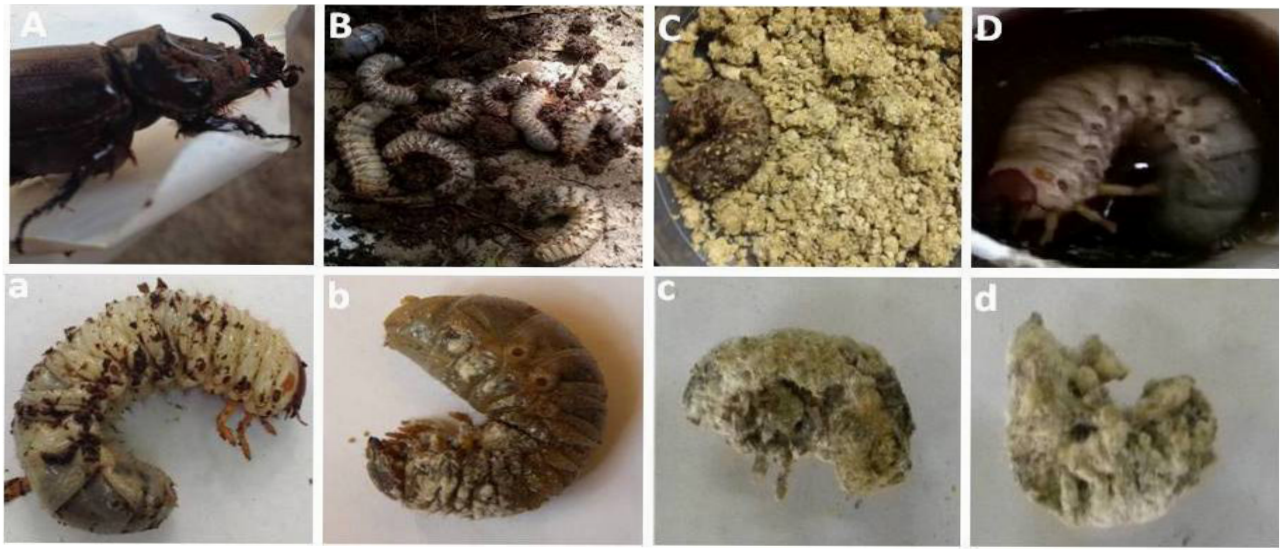

Fig. 3. Bioassy against *Oryctes rhinoceros*: (A). *O. rhinoceros* adult beetle, (B). Breeding site in decaying vermin composting, (C). Healthy third-instar larvae, (D), Treatment with Conidial suspesnion, (a). 3rd and 4th instar larvae, (b-c). Dead infected larvae, (d). Larvae mummified with mycelia and conidia of *Metarhizium majus*.

observed in 18 days (Ramle *et al.,* 2009).Liquid based conidial suspension @ 300ml/L of water was effective in reducing *O. rhinoceros* (Verma, 2013)

Bioassay against *Holotrichia serrata*

The eight *Metarhizium* isolates were also evaluated against 3rd instar larvae of *Holotrichia serrata* with TCS and EBF formulations. With TCS it was observed that at 5 days after treatment the highest mortality (100%) and infection (95-90%) was observed with all the three *Metarhizium robertsii* treated and the LC_{50} was calculated as 6.89 to 9.75x10^5 conidia/ml (Table 4 and Fig. 4). Significant mortality (94-96%) was also observed with *M. anisopliae* (WnMz1S and DhMz4R) and *M. majus* (VjMz1W) treated larvae of *H. serrata*. However the *M. robertsii* strains displayed better virulence and strain ArMz6W was the most toxic. The bioassay with PBF formulation also gave similar results and 93-96% mortality was observed with all the three *M. robertsii* strains and the LC_{50} ranged as 7.50 to 9.50 x10^5 spores/ml. High mortality (90%) was also observed with remaining *Metarhizium* isolates with LC_{50} ranging from 17.10 to 23.60 x 10^5 spores/ml (Table 5 and Fig. 4). The results establish the fact that *M. robertsii* strains will be better choice for combating *H. serrata*.

Table 4. Probit analysis of mortality response in field-collected third instar larvae of Holotrichia serrata following treatment with TCS of different *Metarhizium* spp

Metarhizium species	Mortality %	Infection %	LC_{50}	95% Fiducial Limits		Slop± SE	X2	P
M. robertsii (ArMz3R)	100	90	9.753	4.2387	12.089	1.1687±1.0473	8.16	0.0043
M. robertsii (ArMz3S)	100	93	8.172	4.3859	9.9131	1.6899±2.2494	10.83	0.0010
M. robertsii (ArMz6W)	100	95	6.893	0.4011	9.6811	1.3499±0.6167	5.84	0.0157
M. majus (VjMz1W)	95	85	11.63	9.90417	12.974	1.3290±3.8535	23.61	0.0001
M. anisopliae (WnMz1S)	92	74	14.20	11.7666	16.897	1.1482±1.8791	12.93	0.0003
M. anisopliae (NlMz2S)	94	76	13.82	10.1142	17.523	1.1142±0.9702	8.01	0.0046
M. anisopliae (BgMz1S)	96	78	11.10	2.87657	14.063	1.1135±0.4321	5.51	0.0189
M. anisopliae (DhMz4R)	94	76	12.84	9.4172	15.365	1.1274±1.3667	10.06	0.0015

*LC_{50} values are expressed as x10^5 spores/ml; TCS = topical conidial suspension

Table 5. Probit analysis of mortality response in field-collected third instar larvae of *Holotrichia serrata* following treatment with PBF of different *Metarhizium* spp.

	Mortality %	Infection %	LC_{50}	95% Fiducial Limits		Slop± SE	X2	P
M.robertsii (ArMz3R)	93	73	9.478	6.2739	11.1821	1.3471±2.3362	13.65	0.0002
M.robertsii (ArMz3S)	95	75	8.134	3.0253	10.3511	1.3433±1.3380	8.74	0.0031
M.robertsii (ArMz6W)	96	76	7.502	1.3249	10.0135	1.3696±0.8926	6.82	0.0090
M.majus (VjMz1W)	90	73	17.118	13.893	41.7293	1.5405±3.1498	6.80	0.0091
M.anisopliae (WnMz1S)	90	70	17.102	14.139	31.4180	1.5096±3.5736	8.75	0.0030
M.anisopliae (NlMz2S)	90	70	17.148	14.640	24.9644	1.5089±2.8179	10.20	0.0010
M.anisopliae (BgMz1S)	90	63	18.375	16.093	23.7808	1.5022±3.8275	21.00	0.0001
M.anisopliae (DhMz4R)	90	53	23.610	18.061	80.2086	1.3402±1.0473	8.16	0.0040

*LC_{50} values are expressed as $x10^5$ spores/ml; PBF = powder based formulation

Fig. 4. Bioassay against *Holotrichia serrata* (A-B). Adult *H. serrata* (C). Healthy uninfected larvae in control, (D-F). Insect cadavers mummified with mycelia and conidia of *Metarhizium robertsii*.

Reports are available in using *M. anisopliae* against *Holotrichia serrata.* Thamari Chelvi *et al.* (2011) *M. anisopliae* against *H. serrata* and obtained a mean mortality of 81% with liquid formulation containing $1x10^8$ conidia/ml and 76-78% mortality was obtained with powder formulations containing $4x10^8$ spores/g in 15 days. Srikanth *et al.* (2011) also used conidial suspension @ and obtained LC_{50} of $9.28x10^7$ conidia/ml in 7 days. Powder formulations of *M. anisopliae* species were used against lepidopteran or coleopteran larvae primarily employed the method of surface contamination with conidial suspension followed by provisioning of food such as roots or shoots in containers (Easwaramoorthy *et al.* 2004; Srikanth *et al.* 2006; Manisegaran *et al.,* 2011) and application of *M. anisopliae* @ 4×10^9 conida/ml gaveg 92% control of white grub population at 30 days. Talc based on powder formulation of *M. anisopliae* @ 1×10^5 spores/ml gave highest virulence and mortality against *H. serrata* (Thamari Chelvi *et al.,* 2011).

The present study indicate that *M. majus* (VjMz1W) was highly virulent against *O. rhinoceros* and all the isolates of *M. robertsii* proved to be more effective against *H. serrata*. They performed better than the *M. anisopliae* strains as seen in the LC_{50} values. There is no literature available on the use of *M. majus* or *M. robertsii* in biological control. This is the first study to show that these could be exploited along with *M. anisopliae* for the management of *O. rhinoceros* and *H. serrata*. Further studies like lytic enzyme profiling and toxin nature of *M. majus* and *M. robertsii* will throw a better light on their virulence.

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