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Infectivity of Ten *Metarhizium anisopliae* Isolates to the Coffee Berry Borer *Hypothenemus hampei* (Coleoptera: Curculionidae)

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ABSTRACT
Ten isolates of the entomopathogenic fungus *Metarhizium anisopliae* were tested in the laboratory for infectivity to the coffee berry borer *Hypothenemus hampei*. The adult beetles were treated with four spore concentrations (10^5, 10^6,10^7 and 10^8 per ml), incubated and observed for mycosis. Eight isolates caused above 90% infection at the highest spore load whereas only three were on par with this at the standard dose of 10^7 conidia per ml. Based on the bioassay, three highly virulent isolates- BCRL 6911, CCRI *Ma*1 and CCRI *Ma*2 were identified for further evaluation.

Keywords: Coffee berry borer, *Hypothenemus hampei*, *Metarhizium anisopliae*, strains, virulence, bioassay.

1. Introduction
The coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) is a major pest of coffee in all the coffee growing countries except Nepal and Papua New Guinea. Invading India about 25 years back, it is now present in all the coffee growing belts of Karnataka, Kerala and Tamil Nadu. The host range of CBB is restricted to *Coffea* species, with an exceptional record of breeding in stored seeds of the Brazilian para nut *Bertholletia excelsa* belonging to the family Lecythidaceae [1]. As no such breeding hosts are recorded in India, maintenance of coffee plants without any fruits on them after harvest is the most effective method to control the pest. However, large size of robusta bushes in old plantations, and the development of off-season crop due to running blossom, particularly in areas under North East monsoon, render this option impractical. Shortage of workers during harvest season is another constraint for clean harvesting. Consequently, easily adoptable interventions like insecticide application becomes a choice in cases of severe infestation. Concerns over the use of hazardous insecticides like chlorpyriphos and endosulfan for CBB management led to the search for eco-friendly strategies. Results of classical biological control using parasitoids have been disappointing, whereas entomopathogens are considered as promising bioagents [2]. *Beauveria bassiana* and *Metarhizium anisopliae* are two fungal pathogens which could be effectively utilized for CBB management [3]. Though laboratory and field efficacy of the former have been tested in many countries including India and elsewhere [4,5,6], attempts to exploit the potential of the latter are scanty [7,8,9,10,11,12]. The present study was undertaken to identify virulent isolates of *M. anisopliae* which could subsequently be developed as effective myco-insecticide to combat the CBB.

2. Materials and methods
2.1. Maintenance of isolates
Ten isolates of *M. anisopliae* collected from different hosts/sources (Table 1) were maintained on potato dextrose agar medium.

Table1: Isolates of *M. anisopliae* collected and maintained

Sl No	Strain No.	Host	Locality	Source
1	BCRL Ma6	Cockroach	Bangalore	BCRL
2	BCRL 6911	*Plocaederus ferrugineus* (Coleoptera:Cerambycidae)	Pondicherry	,,
3	CCRI *Ma*1	*Pheidologeton diversus* (Hymenoptera:Formicidae)	CCRI*, CRS, Chickmagalur Dt, Karnataka	CCRI
4	CCRI *Ma*2	Dipteran larva	”	”
5	CCRI *Ma* 3	Soil	”	
6	IWST *Ma*11	Soil (termite mount)	Karnataka	IWST
7	IWST *Ma*15	Adult Coleoptera	Kerala	,,
8	MTCC 3210	Cricket	Jorhat,Assam	IMTECH
9	MTCC 6060	Forest soil	Moondumuzhi, Pathanamthitta, Kerala	,,
10	MTCC 6067	Forest soil	Nilambur, Malappuram, Kerala	,,

BCRL=Biocontrol Research Laboratories, Pest Control India Ltd., Bangalore; CCRI=Central Coffee Research Institute; IMTECH=Institute of Microbial Technology, Chandigarh; IWST=Institute of Wood Science and Technology, Bangalore

2.2 Laboratory bioassay

Laboratory bioassay was carried out using all the above isolates during January to May 2012. Conidia from three weeks old cultures on PDA were used. Germination tests were conducted to confirm spore viability before the start of every bioassay. The conidia scraped from the plates were uniformly suspended in sterilized distilled water containing 0.01% Triton X100 and the load adjusted to 10^5, 10^6,10^7 and 10^8 per ml using an improved Neubauer hemocytometer. Female berry borer beetles collected from infested berries were surface-sterilized with 0.5% sodium hypochlorite solution, followed by two washes with distilled water. For each treatment, the beetles were divided into 5 batches of 30 each, immersed in the respective spore suspension (control immersed in water containing 0.01% Triton X100), agitated for 2 minutes and transferred into 60 ml plastic specimen containers (Qualigens®) lined with a piece of sterilized filter paper. Beetles dead within 24 hours were discarded. Twenty each of the healthy insects were transferred into 5 ml homeopathic glass vials (five replications), provided with thin flakes of coffee beans as food, and maintained at 90 $\pm$5% RH in plastic containers having a mixture of 18.80 % (w/v) glycerol in water. The caps were provided with stainless steel wire mesh to allow aeration. The bean slices were renewed as and when required. Mortality was recorded in the morning and evening (at about 12 hours intervals). Dead beetles were removed immediately, maintained individually in micro test plates (Tarsons) at 90 $\pm$5% RH and observed for mycosis using a stereo zoom microscope (Nikon SMZ1000). A randomized block design was followed. The data on per cent infection were subjected to one way ANOVA and DMRT using SPSS 7.5 for windows. The median lethal time (LT_{50}- time taken to kill 50% of the test insects) was also calculated. The data are presented in Table 2.

3. Results and discussion

All the isolates, except BCRL Ma 6, CCRI Ma 3, MTCC 3210 and MTCC 6067 were 100 % infective at 10^8 spores per ml. CCRI Ma 1 and CCRI Ma 2 were superior to all other treatments, followed by BCRL 6911, resulting in above 90% infection at 10^7 conidia per ml. The LT_{50} after treatment with 10^7 spores per ml was as low as 3.0 days for BCRL 6911 and MTCC 6067. Though CCRI Ma1 and Ma2 showed slightly higher LT_{50} at this dosage, the mortality was as high as 100% in both.

Based on these findings, BCRL 6911, CCRI Ma 1 and CCRI Ma 2, which caused the highest mortality at the standard dose of 10^7 conidia per ml, were identified as the most promising isolates to be selected for further studies.

The pioneering work on *M. anisopliae* against CBB carried out in Brazil [10] revealed 79 % and 91% mortality on immersing the beetles for five seconds in suspensions of $1.5x10^7$ and $1.5x10^8$ conidia per ml respectively; exposure to treated leaves at the above dosages resulted in 62 and 79% mortality respectively and that to green berries caused 25 and 60 % death respectively. It is likely that dipping for a longer period as in the present experiment would have resulted in still better mortality. Out of 14 isolates lab tested in Colombia, the strain *Ma* 9101 at 10^7 conidia per ml was highly infective (95%) with a median survival time of 3.4 days [8]. Five strains were tested under laboratory conditions in Mexico [9] and mortality was as high as 94 % at 2.9 x 10^9 spores per ml and less than 35% at 10^7 conidia per ml. Though dipping and agitation method was followed, the actual period of exposure, which possibly determines the number of conidia adhering to the cuticle, was not mentioned in the publication. The Brazilian isolate CG46 was reportedly less infective (46.7 $\pm$ 11.5 %) with an LT_{50} of 9.4 days at 10^7 conidia per ml [11]. The wild and genetically modified (with the scorpion toxin AaIT gene) strains of Ma 549 was highly infective (above 96%) to CBB at spore loads above 10^4 per ml. The average survival time was 3.73$\pm$0.1 days for the wild and 2.98 $\pm$ 0.1 days for the genetically engineered strain [12]. In an earlier experiment, the IWST isolates *Ma* 11 and *Ma* 15 (used in the present study) at 10^7 conidia per ml resulted in 75.0 $\pm$ 5.7 % and 77.5 $\pm$ 9.5% mortality respectively in the arboreal termite *Odontotermes* sp[13] . In our study also these strains were only moderately active against the CBB. The infectivity of a strain isolated from naturally infected CBB was 70 % to the same host species and 80% to the black twig borer (BTB) *Xylosandrus compactus* at 10^7 conidia per ml with one minute dip under laboratory condition; the LT_{50} was 6.2 days and 7 days respectively [7]. CCRI *Ma*1 was highly virulent to the BTB on coffee, resulting in 100% mortality in laboratory in 5 days and 98% in field [14]. The present study also indicated that three isolates from other sources were equally infective to CBB with BCRL6911 showing the lowest LT_{50} value. As immersion and agitation in the spore suspension for 2

minutes do not asphyxiate the beetles, this exposure period, as followed for screening of *B. bassiana* isolates against CBB [15] appears to be ideal for acquisition of conidia in optimum quantity. Furthermore, a uniform accepted protocol will also help to compare and interpret the results of different experiments.

Table 2: Coffee berry borer mortality caused by *M. anisopliae* isolates from various sources/ host insects

Sl No	Strain	Spore concentration/ml	% mortality (infection)* (mean ± SD)	LT_{50} in days (mean ± SD)
1	BCRL Ma6	10^8 10^7 10^6 10^5	96.0 ± 1.00j 42.0 ± 6.04g 12.0 ± 2.55cd 5.0 ± 1.58abc	3.0 ± 0.0
2	BCRL 6911	10^8 10^7 10^6 10^5	100.0 ± 0.0 0j 91.0 ± 1.87j 27.0 ± 3.39f 3.0 ± 2.00abc	3.0 ± 0.0 3.0 ± 0.0
3	CCRI *Ma*1	10^8 10^7 10^6 10^5	100.0 ± 0.00 j 100.0 ± 0.00 j 21.0 ± 3.67ef 2.0 ± 2.00abc	3.5 ± 0.0 4.0 ± 0.0
4	CCRI *Ma*2	10^8 10^7 10^6 10^5	100.0 ± 0.0 j 100.0 ± 0.0 j 26.0 ± 3.31f 0.0 ± 0.00a	4.0± 0.0 5.4 ± 0.5
5	CCRI *Ma* 3	10^8 10^7 10^6 10^5	84.0 ± 4.58i 26.0 ± 1.87f 0.0 ± 0.00a 0.0 ± 0.00 a	7.0 ± 1.7
6	IWST*Ma*11	10^8 10^7 10^6 10^5	100.0 ± 0.00 j 74.0 ± 7.81i 29.0 ± 2.91f 8.0 ± 1.22abc	3.0 ± 0.0 3.8 ± 0.3
7	IWST*Ma*15	10^8 10^7 10^6 10^5	100.0 ± 0.00 j 61.0 ± 6.00h 16.0 ± 2.91de 0.0 ± 0.00a	3.0 ± 0.0 3.6 ± 0.2
8	MTCC3210	10^8 10^7 10^6 10^5	91.0 ± 4.58j 38.0 ± 4.06g 5.0 ± 2.23abc 1.1 ± 1.00a	4.0 ± 0.0
9	MTCC6060	10^8 10^7 10^6 10^5	100.0 ± 0.00j 11.0 ± 1.00bcd 0.0 ± 0.00a 0.0 ± 0.00a	3.0 ± 0.0
10	MTCC6067	10^8 10^7 10^6 10^5	78.0 ± 2.55i 58.0 ± 5.61h 8.1 ±3.74abc 1.1 ± 1.00a	3.0 ± 0.0 3.0 ± 0.0

Values followed by the same alphabet(s) do not differ significantly by DMRT (P=0.05)

4. Conclusion

The present investigation indicated that different strains of *M.anisopliae* significantly differed in their infectivity to coffee berry borer. Highly virulent strains like BCRL6911, CCRI *Ma* 1 and CCRI *Ma*2, which caused above 90% infection, should be qualified for further evaluation of conidial productivity on cost-effective substrates, field efficacy, safety to non target organisms and compatibility with other plant health management practices.

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