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Full Length Research Article

Morphological Characteristics of *Metarhizium anisopliae* and its Bioactive Compounds

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Abstract

Biological control consists of the introduction of beneficial predatory (or) parasitic species into the cultivation system. Integrated pest management (1PM) is an alternative to unilateral intervention strategies using agrochemicals with a wider focus on the ecology of the insect pests as well as the crop plants. *Metarhizium anisopliae* is the most utilised fungal species and the natural genetic variability of entomopathogenic fungi is considered one of the principle advantages of microbial insect control. *Metarhizium anisopliae*, an anamorphic fungus related to the phylum Ascomycota is the most intensively studied genus of *Metarhizium*, considering that the teleomorph is *Cordyceps brittlebank isoides*. The reproductive structure of *Metarhizium anisopliae* (the anamorph, the most commonly encountered form) consists of conidiophores and conidic *Metarhizium anisopliae* produces leveduri-form structures (or) blastospores and appressoria via mycelia differentiation. Blastospores can function in certain cases as reproductive units and are produced in submerged cultures. Entomopathogenic fungi are unique in that they can produce a wide range of bioactive compounds, including helvolic acid (1,2-dihydrohelvolic acid), each with its own molecular formula and molecular weight. *Metarhizium anisopliae* was discovered to be insect material dependent and to produce antibacterial compounds, which were purified from culture to homogeneity. This specific novel bioactive compound indicated that insect-derived material would be useful for enhancing the diversity of compounds produced by entomopathogenic fungi.

***Key words*:** Entomopathogenic fungi, *Metarizhium anisopliae,* Insect medicating bioactive compounds

Tamil Nadu is one among the important agricultural zones in India. The indiscriminate use of pesticides and chemical fertilizers in agriculture has raized a number of ecological problems such as resistance development in plant pathogen and pests. Environmental pollution and negative impact on human health. *Metarhizium anisopliae* with the potential for excreting other roles in the plant. The entomopathogenic fungal isolate as a potential bio-tool in the plant pest management. Soil is the main reservoir of infective propagules of entomopathogenic fungi including *Metarhizium anisopliae* [1-2] many environmental factors like moisture soil type and temperature, aeration, light and natural as well as artificial antagonists, either directly (or) indirectly affect both the persistence and survival of entomopathogenic fungi in terrestrial habitats [3-4]. Compensate for this natural mortality of many species produce copious quantities of infective spores (or) form modified adaptive structure like resistant hyphae, chlamydospores, resting spores microsklerotia and macrocycliccoindia to maximum survival. In developing effective replacement of bioactive compounds of entomopathogenic fungi have been considered as an alternative treatment for the insects [5]. Adoption of a biological control of *M. anisopliae* in integrated pest management system for more suitable methods. Entomopathogenic fungi are unique owing to their versatile ability to produce many bioactive compounds and form the dependence of their morphological differentiation of the presence of insect derived materials [6].

MATERIALS AND METHODS

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Collection of soil sample

The present investigation was studied in the Laboratory of Indian Biotrack Research Institute, Thanjavur, Tamil Nadu, Soil samples were collected from coconut field of Orathanadu, Thanjavur District.

Preparation of potato dextrose agar media (PDA)

Two hundred grams of potatoes were cleaned and peeled off. The peeled potatoes were cut into small thin pieces, weighed and rinsed rapidly under running water. Thereafter the rinsed potatoes were boiled in one litre of distilled water until it was fully boiled. The boiled potatoes were crushed using pestle and mortar and this was filtered through a muslin cloth. Twenty grams of mycological agar and 10 gm of dextrose (Himedia, Mumbai) were added to the filtrate and were stirred until the dextrose dissolved completely. The suspension was diluted with tap water and was made upto onelitre. The final medium was autoclaved at 121ºC / 15 1bs for 15 minutes. After cooling, Streptomycin (20mg/litre) was added in order to inhibit the bacterial growth.

Isolation and enumeration of total fungal population (TFP) of soil and insects

Soil samples were collected from each site and manually grounded using a mortar and a pestle under aseptic conditions. One gram of the ground soil sample was taken in a test tube containing sterile distilled water (10ml). Each test tube was agitated using a vortex mixture for 15 seconds. The suspension was diluted thrice according to tenfold dilution series (10^{-1} to 10^{-6}). From each diluted resultant preparation, 0.1ml was transferred onto separate PDA plates and were spreaded using a sterile L-shaped glass rod. The seeded plates were then incubated at 26ºC ± 2ºC and were observed for fungal growth after 7 days. After eminent growth, the fungi that were suspected entomopathogen [7]. Fungal growth was assessed to quantify the number of colony forming units.

Isolation of Metarhizium anisopliae

Ten gram of soil samples was added to 300ml of sterile distilled water. The contents were evenly mixed for half an hour and later supernatant was serially diluted upto 10^{-6} dilution factor, 0.1ml of sample from 10^{-3},10^{-4} dilution was spread on to the semiselective PDA medium with streptomycin 20mg/l was added before pouring on the petriplates [8]. The petriplates were incubated at 25ºC for 5 days. Colonies morphological identical to be target fungi were sub cultured on the plate and identified morphologically using standard taxonomic identification keys [9].

Sub-culture and identification of M. anisopliae

The selected fungi, which were suspected to be *Metarhizium* spp. were sub-cultured to obtain pure cultures of the entomopathogen. The spores were selected from the initial cultures using a sterile loop and were streaked on PDA plates. The inoculated plates were then incubatedat 26ºC ± 2ºC (Remi BOD incubator) for 7 days. Spores growing on these secondary cultures were again sub-cultured for 7 days, using the same media to obtain pure cultures. The identification of *Metarhizium* was carried out based on their cultural characteristics, colonical morphology, sporulating structures and nutritional requirements [10-12]. The description of pathogenic fungi and bacteria according to CMI (Commonwealth Mycological Institute) was also used to identify the insect pathogenic fungi *M. anisopliae*.

Gas chromatography mass spectrum analysis [13]

Extraction of the bioactive compounds of Metarhizium anisopliae

The fungus which showed promising activity and cultured in a liquid PD broth at 25ºC in darkness for three days. After incubation, the culture was filtered twice with Whatman-1 filter paper and then through scitz filter (G.S) *Metarhizium anisopliae* 100ml culture filtrate and 10ml of ethyl acetate was added in a separating funnel (250ml) shaken well for 3min and the solvent and aqueous layers were separated. The ethyl acetate layer of the culture filtrate was used for further analysis.

Gas chromatography mass spectrum (GC-MS)

The compounds were identified by GC-MS using a column Elite-1 (100% Dimethyl polysiloxane) 30×1µm equipped with GC clasus 500 perkin Elmer maintained initially at 110ºC for 2min (hold) followed by increased upto 200ºC at the rate of 10ºC/min (nohold) upto 250ºC at the rate of 5ºC/min-9min (hold). The electron impact energy was 70eV julet, line temperature was set at 200ºC and the source temperature was set at 200ºC. Electron impact (E1) mass searches on the NIST Ver.2.1.Ms data library and comparing the spectrum obtained through GCMS, the compounds present in the crude samples were identified.

RESULTS AND DISCUSSION

The isolates were identified, based on their colonial morphology and sporulating structures and was confirmed by the description of pathogenic fungi and bacteria according to CMI (Commonwealth Mycological Institute). Identified *M. anisopliae* isolates were named as CPRS series (Crop Protection Research Centre). Standard *Metarhizium anisopliae* was used as reference and also for entomopathogenic fungi.

Metarhizium anisopliae is a ubiquitous component of the mycoflora of the soil [14]. Similarly, [15] obtained 58 isolates of *Metarhizium* from soil samples, either by "plating" or "live baiting" method. But [16] used "*Galleria* bait method" [17] for the isolation of *M. anisopliae*. [18] isolated *M. anisopliae* from 42% of soils in the Darmstadt region of Germany, while [19] isolated 17% of this fungus from agro forestry soils in Finland. The *M. anisopliae* was isolated from the upper 2cm soil layers by [15] the isolated same fungi at a depth of 5cm soil layer. The soil collected from more than 2cm, there would have been a chance of getting more *M. anisopliae* isolates.

The isolation rate of *M. anisopliae* in the present study was considerably more than that of the observations by [17], [19], [2], [14], [18], [20], [15] and [21]. Possible reasons for the great success in isolating the entomopathogenic fungi, during this the study might have been done due to the use of new media and the fact that most samples were collected from wet soils and in irrigated zones. In the soils samples, *M. anisopliae* appeared to dominate the other entomopathogenic mycoflora [15]. It was also reported that *M. anisopliae* was more often isolated from loamy soil, relative to other soil types [1], [2] too. *M. anisopliae* is considered to be thermophilic as it is rare in cold areas [1].

The environment of the target pest for suitable strains of entomopathogenic fungi and simultaneously testing isolates from other hosts [14]. A very good example for the "old association" approach is the *M. anisopliae* var. *acridum* strain that had been isolated from grasshoppers and *M. anisopliae* has utilized against many locust and grasshopper species in sub-Saharan Africa till today. The effects of geographical location, climatic condition, habitat type, soil conditions and pesticide uses have determined the occurrence and distribution of insect pathogenic fungi [1], [22], [15]. Comparisons between the occurrence of insect

pathogenic fungi in organically versus conventionally farmed soil has so far only been undertaken on a minor scale. The strong mosquitocidal activity and the low toxic effect on non-target organisms exhibited by *M. anisopliae* indicate that, besides entomopathogenic fungal conidia, their metabolites may also have a significant role in efficient microbial-derived mosquito control tools that can be used in mosquito control programmes as effective, cheaper, biodegradable, target-specific alternatives to chemical insecticides. Further research into the single crude metabolite chemical constituents under laboratory and semi-field conditions may result in the development of effective *M. anisopliae* derived bio-pesticides [23].

Table 1 Determination of bioactive compounds of *M. anisopliae* by GC-MS Method

RT	Name of the compounds	Molecular formula	MW (KDa)
2.64	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296
3.89	2,6,10-trimethyl-Tetradecane	$C_{17}H_{36}$	240
5.57	Cetene	$C_{16}H_{32}$	224
5.71	Tritetracontane	$C_{43}H_{88}$	604
8.34	Tritetracontane	$C_{43}H_{88}$	604
8.45	Decane, 2-methyl-	$C_{11}H_{24}$	156
10.45	Dodecane, 2-methyl-	$C_{13}H_{28}$	184
27.02	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	322
29.78	5-Octadecene, (E)-	$C_{18}H_{36}$	252
31.46	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296
32.73	Tetratetracontane 1,2-dihydrohelvolic acid	$C_{44}H_{90}$	618
34.89	2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl-	$C_8H_{14}O_4$	174
35.74	Benzamide, 3-amino-N-[4,5-dihydro-5-oxo-1-(2,4,6-trichloropheny	$C_{16}H_{11}Cl_3N_4O_2$	396

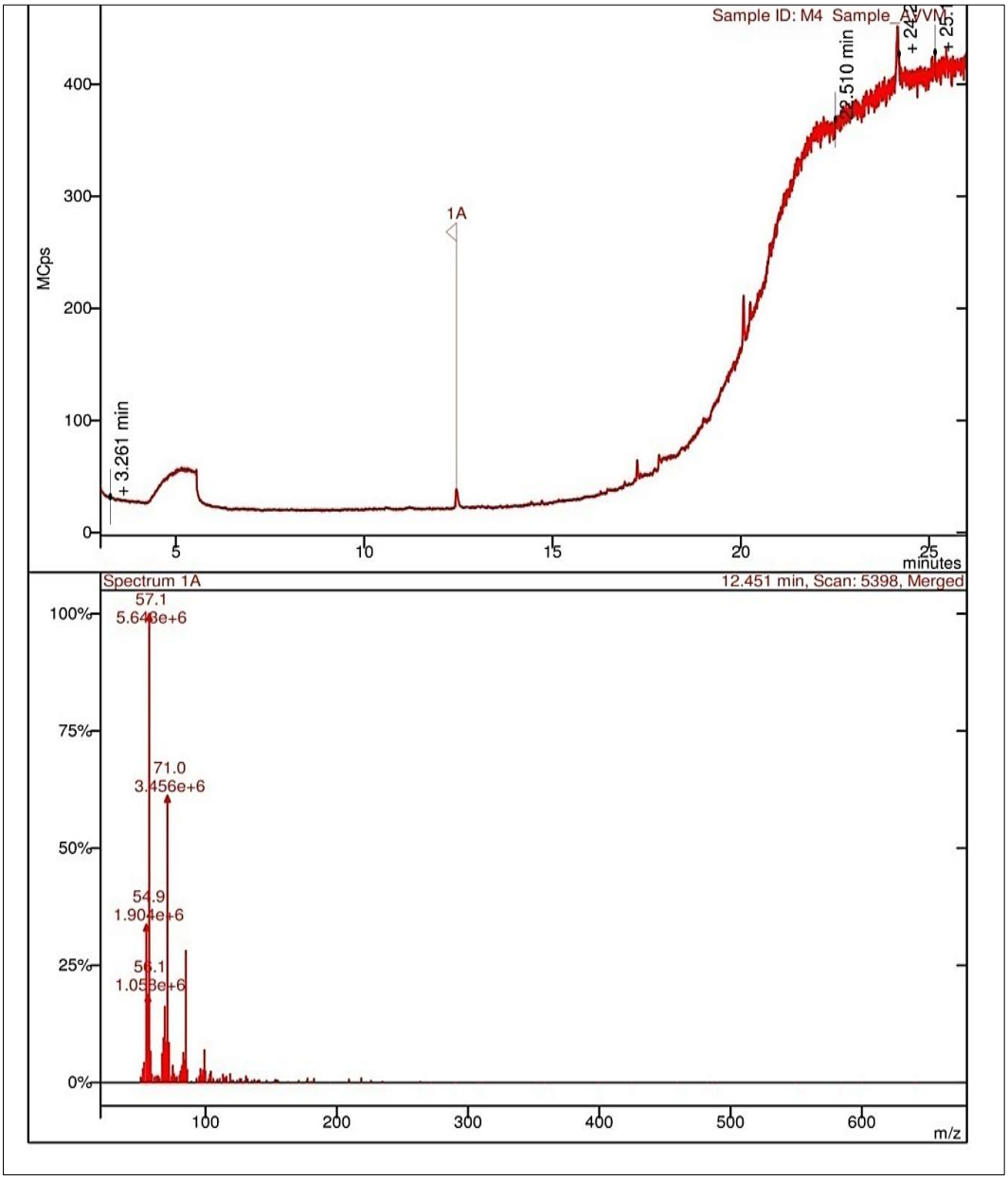

Fig 1 Bioactive compounds of *M. anisopliae* by GC-MS method

In the current investigation suggested that the biological efficiency index model of identification of entomopathogenic fungi to variety based on morphological characteristics alone is extremely difficult because of insufficient morphology based identification key which may results in the misidentification characters, most of the entomopathogenic fungi are mesphilic, with growth between 25ºC and the maximum germination temperature of *Metarhiziumanisopliae*was about 37ºC thermotolerance, saprophytes and in parasitized hosts.

In the recent study stated that the entomopathogenic fungi *Metarhiziumanisopliae*bioactive compounds such as 1-Iodo-2-methylundecane, ,2,6,10-trimethyl-tetradecane, Cetene, Tritetracontane, Decane,2-methyl-, Dodecane,2-methyl-, [1,1′-Bucyclopropyl]-2-octanoic acid,2′-hexyl-,methyl ester, 5-Octadecene, (E)-, 1-Iodo-2-methylundecane, Tetratetracontane 1,2-dihydrohelvolic acid,2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl-, Benzamide, 3-amino-N-[4,5-dihydro-5-oxo-1-(2,4,6-trichloropheny)] were identified. Totally 13 bioactive compounds which is responsible for entomopathogenic activities in the respective insects.

The entomopathogenic properties were high reliability index because of bioactive molecules as well as enzyme activation. The enzymes Acetyl cholinesterase (AChE) is an proteinases enzyme. These enzymes are important neurotranswitters such as acetylcholine and dopamine are released at synapses of nervous system to control the insect's metabolism.

CONCLUSION

Metarhizium, an entomopathogenic fungus, appears to be a promising candidate for controlling the larval and pupal stages of *L. botrana*. It can be concluded that the *Metarhizium anisopliae* was most effective entomopathogenic fungi with respective potential bioactive measures of insecticidal properties in the environment.

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