

## A Quick Method for *Metarhizium anisopliae* Isolation from Cultural Soils

<sup>1</sup>M.A. Tajick Ghanbary, <sup>2</sup>A. Asgharzadeh, <sup>1</sup>A.R. Hadizadeh and <sup>1</sup>M. Mohammadi Sharif

<sup>1</sup>Department of Plant Protection, Faculty of Agronomic Sciences,

Sari Agricultural Sciences and Natural Resources University, Sari, Iran

<sup>2</sup>Department of Soil Biology, Soil and Water Research Institute, Tehran, Iran

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**Abstract: Problem statement:** Fungi are one of the most active members in biological community of cultural soils. Many saprophyte and facultative parasitic fungi live in soil. *Metarhizium anisopliae*, one of the most famous soil inhabitant entomopathogens has a virulence potential on plant and animal pests. **Approach:** Introducing a new method for its isolation from soil was an applied method to find it without any limitation. *Metarhizium anisopliae* shifts to saprophytic phase and remain alive within soil in absence of susceptible host. As a shortcut, we can transfer the fungus from soil to lab by culturing soil suspension. One hundred cultural soil samples from different regions of Iran were tested to finding *Metarhizium* isolates. Culturing 1:5000-1:10000 soil suspension on artificial medium containing necessary macro and micronutrients for fungal growth were resulted in isolation. *Metarhizium anisopliae* isolates were harvested seven days after culturing the suspensions. All isolates were inoculated in 50 mL PDB in destruxin production assay and 7 days later broth medium was filtrated by using filter paper. Culture filtrates were extracted and in bioassays they were sprayed on larva of citrus leaf miner. **Results:** Nine isolates of *Metarhizium anisopliae* were harvested. Microscopic studies showed that morphological features had complete coincidence with valid descriptions of the fungus. Bioassay confirmed that all harvested isolates secrete active and effective destruxin in broth. **Conclusion:** Isolation of *Metarhizium* by culturing the soil suspension, a useful method for more studies of the entomopathogen at different geographical regions. Native populations of this fungus had special importance in local biological control programs. This procedure was a costs- and time-effective method for pathogen isolation.

**Key words:** *Metarhizium anisopliae*, Destruxin, Entomopathogenic fungi, Soil

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### INTRODUCTION

Nowadays biological control as a practical science is very appreciated and as a solvent for long term usage of chemical pesticides problem is completely notified. There has been an increasing interest in employing fungal pathogens to combat insect pests. New application and production combined with a greater understanding of both fungal and insect ecology have shown that biological insecticides can now compete traditional chemical pesticides much faster. *Metarhizium anisopliae* the agent of green muscardin disease of insects is an important fungus in biological control of insect pests<sup>[6]</sup>. It is a Deutromycete belonging to Hyphomycetes. The fungus is a facultative parasite which as an entomopathogen can affect a group of insects. There are many similar entomopathogens in nature that live in different ecological environments.

The exact number of entomopathogenic genera and species is indefinite but in some reviews about 90 genera and 700 belonging species were reported<sup>[17]</sup>. Some are obligate parasites and others facultative. The obligates are living on special insects and their laboratory studies is only possible on naturally infected hosts. But facultative have at least two positive scores: (1) Easy *in vitro* studies and assays, (2) Saprophytic life in absence of suitable host in nature. *Metarhizium* is an interesting organism that further more direct attacking its host, produces a series of biological active metabolites *in vitro* and *in situ*<sup>[18]</sup>. A group of destruxins were identified and purified in most species specially *M. anisopliae*. Other species and varieties have similar biologically active metabolites too. Some important biopesticides are produced as different names in the world from selected *Metarhizium* isolates. In spite of hundreds of reports about *M. anisopliae* and its

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**Corresponding Author:** M.A. Tajick Ghanbary, Department of Plant Protection, Faculty of Agronomic Sciences, Sari Agricultural Sciences and Natural Resources University Sari, Iran  
Tell: +98 911 254 6616 Fax: +98 151 3822578

features, the researches are insufficient and more and more articles are published monthly. In this article a new and quick method for isolating *Metarhizium* is introduced which can serve as a shortcut for quick preparation of local populations of the genus for different studies.

## MATERIALS AND METHODS

**Soil sampling and fungal isolation:** A total of 100 sample each containing 1 kg cultural soil in depth of 0-20 cm from different provinces in Iran were collected. The samples in plastic bags were stored at 4°C until culture. For isolation 10 g of each soil sample was subjected to a 1:5000-1:10000 soil suspension and one ml of final solute was transferred to steril 9 cm petri plates then culture medium containing 0.5 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{K}_2\text{HPO}_4$ , 0.5 g peptone, 0.5 g  $\text{MgSO}_4$ , 10 g dextrose, 0.5 g yeast extract, 0.05 g rosebengal, 0.03 g streptomycin sulphate. The rose-bengal and streptomycin were added to medium after sterilization before transferring to petries. The isolates were purified by single spore method.

**Identification and storage:** All morphological features of isolated *Metarhizia* were compared with valid descriptions of its different species. For long term storage of collected *Metarhizia*, colonies transferred to PDA slants and stored at 4°C.

**Destruxin production tests and bioassay:** For this purpose the fungus was inoculated in 50 mL PDB (potato dextrose broth) in 250 mL erlenmeyers for one week at room temperature. The broth filtrated from pelletes via whattman No. 1 and culture filtrates subjected to secreted products extraction. For destruxins extraction culture filtrates were mixed with 10 mL chloroform and shake vigorously for 10 min. After an hour chloroform was separated from broth and completely evaporated. The residue was resolved in 10 mL distilled water and stored at -20°C until application. Fresh infected different citrus leaves to citrus leaf miner from gardens were prepared for bioassay in 9 cm petri plates. The crude extract and its dilutions were sprayed on leaves then treatments were observed daily on a stereomicroscope.

## RESULTS

After 3-5 days different fungal colonies grew and sporulation completed about 7-10 days. In primary investigations on different fungal colonies via a stereomicroscope and morphological comparisons

suspected *Metarhizium* colonies were selected and transferred to PDA slants. Culture of soil samples lead to nine *Metarhizium* isolates. Species identification process showed that all belong to *M. anisopliae* with small differences. Most of morphological features had high coincidence with species description. Fungal storage at 4°C remained them alive at least for 6 months. All isolates produced total destruxins without shaking at room temperatures in PDB medium after one week. The isolates sporulated easily on PDA and PDB. There was no difference between isolates for destruxin production, in a test some two weeks old cultures proceeded for toxins extraction, total destruxins remained stable at least for two weeks at room temperature. The crude extracts of total destruxins and its serial diluted fractions up to seventh dilution had total mortality on different larval stages on all citrus varieties. Larval mortality started on second day after treatments regardless to their stages. In another test no difference was observed in effects of -20°C stored and fresh extracts on larvae of citrus leaf miner.

## DISCUSSION

In modern and sustainable agriculture chemical pesticides are exchanged for alternative strategies of pests control. Entomopathogenic fungi showed high performance in integrated pest management programs that resulted meaningful decrease in using insecticides. Under natural conditions entomopathogenic fungi are the most important mortality factor of natural insect populations and is safe for non target organisms<sup>[22]</sup>. Potentially all insect groups may be affected by more than 700 species of entomopathogenic fungi<sup>[15]</sup>. Some are facultative parasites (*Fusarium* or *Aspergillus*) and others obligate parasites (*Entomophthora*), some are completely host specific (*Cordyceps*). The Entomophthorales are so effective on aphids and flies in humid and warm regions, but attempts for preparing commercial mycoinsecticides of them failed, because their mass production is only possible on live hosts, furthermore their favorite conditions is not always present in the fields. For this reason most attempts are focused on mitosporic easy amplifiable fungi like *Metarhizium*, *Beauveria*, *Verticillium* and *Paecilomyces*. Most have a wide host range, some very high intraspecific genetic variation, many have exact host specificity. In contrast to other biological control agents fungi can penetrate directly from host cuticle and have no necessity for entrance in its digestive system, then can affect sucker insects. *Metarhizium anisopliae* is a worldwide and interesting soil inhabiting entomopathogen. Its first description was written by

Russian mycologist Metschnikoff in 1879 as an *Entomophthora* species *E. anisopliae*, then was corrected by Sorokin in 1883 to *M. anisopliae*. During the last 50 years *Metarhizium* and allied species specially *M. anisopliae* absorbed the attention of many scientists as mycologists, entomologists, biologists and so on. The most investigated aspect of *Metarhizium* is its application as biological control agent of plants and domestic animals pests. Some examples of successful applications are control of termites<sup>[24]</sup>, onion thrips<sup>[13]</sup>, tobacco whitefly, red spider mite<sup>[4]</sup>, eggs of mites<sup>[21,25]</sup>, fruit flies, mosquitoes<sup>[1]</sup>, green leafhopper<sup>[23]</sup>, rice weevil<sup>[5]</sup>, flour beetle<sup>[23]</sup>, some animal arthropods<sup>[7]</sup>, vein weevil<sup>[20]</sup>, ectoparasitic animal mites<sup>[8]</sup> and more other examples were listed by Bruck<sup>[9]</sup>. Furthermore above research reports, various bioinsecticides against a range of insect pests were released as commercial products<sup>[3,10,14]</sup>. In Bruck's belief<sup>[9]</sup> success in application of biological control agents in nature has close relationship with their related biological systems specially their ecological niche. The subject is exactly true for entomopathogenic fungi. Our information about entomopathogens biology far from their host body is insufficient. A special pathogen may be present and active in an environment then infect insect pest after its entrance to that region. An example is the control of black vine weevil by *Metarhizium anisopliae*<sup>[9]</sup>. Pathogenicity and virulence variation between different species, isolates and situations were discussed by<sup>[19]</sup>. Based on another report produced inocula on naturally infected insects is more virulent and effective than harvested inoculum from axenic culture media on sensitive insect hosts<sup>[2]</sup>. This is important specially for mass production of *Metarhizium* and other entomogens which are using as mycoinsecticides. A suspected reason for decrease in pathogen virulence is ingredients of culture media like carbohydrates<sup>[12,19]</sup>. *Metarhizium* is one of the most promising biological controlling agents against insect pests specially some soil inhabitants like scarab grubs<sup>[11,16]</sup>. There are many attempts for testing some *Metarhizium* formulations and application methods on soil insects. *Metarhizium anisopliae* has been isolated from infected hosts in nature. Collecting native isolates of each geographical region is possible via field search and finding naturally infected dead insects. This method is very time consuming and season restricted, because the environmental factors for infection of live hosts aren't present during the year. The introduced method by this research without no restrictions is executable for ever and cause saving time and costs for all local studies on *Metarhizium anisopliae* biology, populations biology, pathogenicity, viability in soil, variations in total

destruxins production, ecology and many other purposes.

## CONCLUSION

Culture of soil suspension in artificial media has a good chance to find *Metarhizium* in soil. The entomopathogen finding in local regions without susceptible bait is nearly impossible and this method can serve as a shortcut without any host, time and season limitations. Complementary approaches after taxonomic identification like bioassay confirms the isolation results. Local populations of *Metarhizium anisopliae* have special role in integrated pest management programs at different regions.

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