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RESEARCH ARTICLE

First Record of *Cunninghamella echinulata Var. Nodosa* as a New Entomopathogenic Fungus Infecting Melon Weevil (*Acytopeus curvirostris persicus,* Curculionidae)

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ABSTRACT

Melon weevil, *Acytopeus curvirostris persicus* Thompson (Col.: Curculionidae), is one of the most important pests of melons that is spread in the Middle East countries. In February and March 2013, during a sampling from different regions in the South Khorasan province, a fungus was isolated from deformed pupae cultures of *A. Curvirostris persicus*. The isolation, purification and identification processes of fungi were carried out, and its pathogenicity was approved through Koch's postulates. To perform pathogenicity test, purified isolates of the mentioned fungus with certain spore concentration were sprayed as suspension on the larvae and adult melon weevil. The fungal isolates were identified as *Cunnighamella echinulata var. nodosa* (Zygomycota: Mucorales) based on morphological characteristics and sequencing of ITS region of ribosomal DNA. This is the first report of the fungus on melon weevil worldwide.

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INTRODUCTION

Melon weevil, *Acytopeus curvirostris persicus* Thompson (Col.: Curculionidae), is one of the most important pests of melons that is spread in the Middle East countries (Mohammadpour, 2013). Its wild host watermelon is *Citrullus colocynthis* (commonly is known as the colocynth, bitter apple, bitter cucumber, desert gourd, egusi, or vine of Sodom), which grows in salt and gypsum lands. This insect attacks a great number of cucurbitaceae. Due to larval feeding and bacterial penetration fruits of host plant would not be useable. This pest is multi-generation and so for its control a large amount of phosphorus pesticides were used by the farmers (Ghavami, 1969).

In recent years, a great number of fungi have been isolated from different orders of insects. The fungi in Entomophtorales order have been reported from different orders of insects. For example, since 2002, different species of Zoophthora fungus have been isolated as entomopathogenic fungi of a variety of Diptera, Coleoptera, Hemiptera, etc. (Keller, 2007).

Genus *Cunnighamella* is the only genus in family cunnighamellaceae, which was identified in 1903 by Matrokot. The genus contains 15 taxa, 12 species and 3 varieties. Recently, classification of this genus is done based on morphological methods regarding zygospore and sporangiole formation stages. The maximum growth temperature and the formation or non- formation of zygospores are characteristics crucial to identify and separate the species from each other (Zheng and Chen, 1992, 1994, 1996, 1998, 2001). Using molecular methods for determination the percentage of C+G and DNA sequencing has recently drawn attention recently in the determining of these species (Su *et al*., 1999).

So far the fungus has been directly isolated from different plant and animal sources such as faded flowers, animal feces, the remainder of flora and fauna, fruits and foods kept in moist places, soil of grains and their seeds (Zheng and Chen, 2001). However, there is no report suggesting its pathogenicity in insects.

The aim of the present study was to isolate indigenous entomopathogenic fungi that naturally occur on *A. curvirostris persicus* in regions of the easte of Iran and, second, to test isolates against larval and adult stages of the pest to assess their entomopathogenic potentiality.

MATERIALS AND METHODS

Field-collected pupae were sampled in clean plastic bags and malformed pupae were surface-sterilised in sodium 1% hypochlorite solution for 3 minutes and in

70% ethanol for 3-4 minutes, rinsed three times with distilled water, cultured onto the potato dextrose agar (PDA) and incubated at 25 C in the dark. Conidia concentrations of 1×10^3, 1×10^4, 1×10^5 and 1×10^6 conidia/ml were applied in 1 ml aliquots to the test adult and larvae. The controls were treated with sterile distilled water. Each experiment was replicated 5 times with 10 number of larva or adult for each replicate. The entire experiment was conducted twice. (Majumbar et al 2008). A moistened filter paper was placed in each container (plastic Petri dish) for maintaining high relative humidity. After 5 days, watermelon weevils and larvae were checked for mortality and the number of dead insects was recorded.

To confirm identity of the fungus, the complete internal transcribed spacer (ITS) rDNA region of the causal fungus was amplified using the primers ITS4 and ITS5. Genomic DNA was extracted using a modified Chelex method with an initial step of grinding the mycelia in liquid nitrogen (Walsh *et al.,* 1991). Growing mycelia were scraped off from the 3-day-old PDA cultures with a sterile scalpel and transferred to a micro centrifuge tube containing 200 µl of 8% w/v Chelex 100 resin suspension. The tubes were vortexed, incubated at 56° C overnight and for 7 min in a boiling water bath. The extracts were thoroughly vortexed, then incubated again for 8 min in a boiling water bath and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to new sterile tube and used directly for ITS amplifications. PCR was performed in a 25 µl volume reaction mixture with 4.4 µl Taq master mix containing dNTPs, MgCl2, reaction buffer,1µl of each primer (10 pmol) and 5 µl DNA template. PCR amplification was carried out under the following conditions: initial denaturation at 94ºC for 3 min; followed by 30 cycles of 94ºC denaturation (45 s), 54ºC annealing for 45 s, and 72ºC extension for 2 min and a final extension at 72ºC for 5 min. The PCR products were purified with the Applied Biosystems 3730/3730xl DNA Analyzers Sequencing PCR Purification Kit (Bioneer, Korea).

RESULTS AND DISCUSSION

During a survey conducted in February and March 2013 in watermelon growing areas in Birjand (south Khorasan province) Iran, malformed pupae and dead larvae of melon weevil (*Acytopeus curvirostris persicus*) were observed in the fruit debris (Table 1).

A fungus with the features of *Cunninghamella echinulata var. nodosa* was isolated from the deformed pupae on PDA. Colonies floccose, at first white, then cream to yellowish, reverse light yellow; hyphae branching, at first non-septate later septate; stolons and rhizoids presents; sporophores erect, straight or recumbent, arising from stolons, sometimes opposite rhizoids, or directly from aerial hyphae and stolon-like, hyaline, colorless, of 2 intergrading kinds (typical longer type and shorter type). Septa in sporophores usually absent, vesicles of the longer type sporophores globose, oval to broadly clavate, terminal vesicles (15) 19.23-31.77 (37.5) µm diam., lateral vesicles 9-25 µm diam.; sporangiola hyaline to pale brownish, globose and (7.5) 9-13.9(13) µm diam (figure 1). Pedicels slender, 4.5-7 µm in diam., bearing globose sporangiola 24 µm in diam. Clamydospores absent, maximum growth temperature 42ºC.

Table 1: Geographical details of the sampling regions in south Khorasan, Iran

Region	Altitude (m)	Co-ordinates
Taghab	1356	N:32°50.771/ E:58°55.467
Hajiabad	1420	N:32°52.570/ E: 59°09.131
Bojd	1536	N: 32°51.575/ E: 59°22.615
Ghaen	1436	N: 40°07.037/ E:37°36.638
Sarayan	1199	N: 33°62.9907/ E: 58°37.78153
Mohamadiyeh	1352	N: 32°52.521/ E: 58°59.875

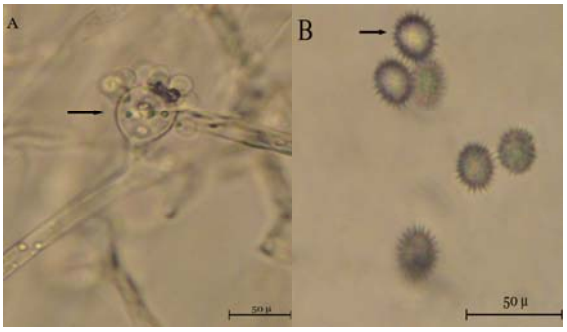

Fig. 1: *Cunnighamella echinulata var. nodosa*: A, Terminal vesicle (arrow) with sporangiolum attached by pedicels; B, Sporangiola with spines on its surface

Fig. 2: Mortality of larval and adult *A. curvirostris persicus* within 10 days after inoculation of the fungal isolates

According to morphological and molecular data, the isolates were identified as *Cunninghamella echinulata var. nodosa*.

A blast search in the NCBI database (http://www.ncbi.nlm.nih.gov/) showed that the pathogen had 99% homology with *Cunninghamella echinulata var. nodosa* isolate Cu-34 Accession no. AF346407.

Two fungal isolates caused high mortality ranging from 30% to 50% on watermelon weevil by 10 days after inoculation. Fungal structures were observed growing within the tissues of dead hosts (Figure 2).

The taxonomic characteristics of the fungus described in this study were consistent with those reported by Zheng and Chen (2001) and Zanganeh *et al.* (2007). *Cunninghamella echinulata* has been reported as an entomogenous fungus on *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Rishi and Balu 2010). To our knowledge, this is the first record of *Cunninghamella echinulata* var. *nodosa* infecting a member of Coleoptera.

Entomopathogenic fungi produce various enzymes which are accepted as key factors in insect mycosis (Włóka 2011). Further investigations are needed to determine the relation between this fungal pathogen and mechanism of its host paralysis. Our results indicate that the new isolates are highly promising biological control agent against *A. curvirostris*, one of the most serious cucurbitaceous pests worldwide.

According to preliminary investigations, the fungus is not pathogenic for cucumber fruit as one of the weevil host; however, concluding in this regard needs further experiments.

Due to using different concentrations of fungi and positive pathogenicity tests on larvae and adult weevils, it seems promising for the future to use the fungi as biocontrol agents of weevil pests. However, the effects of fungi have not been studied on other insects. Also, the interaction between the fungi and other organisms, pests, either beneficial insects or the host itself, need further studies.

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