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Fusarium cf nygamai as a new entomopathogenic fungus infecting melon weevil (Acytopeus curvirostris persicus)

N.Sepasi, M.Jahania, M.R.Mirzaee, K.Mohammadpour

Abstract

Melon weevil is one of the important pests which attack many plants of cucurbit family. A species of fungus belonging to Fusarium cf nygamai was isolated during sampling of dead larvae of melon weevil from different districts of South Khorasan province during February and March 2013. Stages of isolation, culture, purification, identification was carried out. Pathogenesis of the weevil was established based on Koch's postulates. Based on valid stages of identification of Fusarium species,and formation of binding and parabolic hyphae and microconidia long chains distinctions were made between this fungus and Fusarium cf nygamai species. Other characteristics of fungus such as formation of chlamydospore and polyphialides were also studied. Results of molecular data based on ribosome DNA nucleotide sequence analysis indicated homology of 99% with Fusarium cf nygamai. Study of different concentrations of spore on melon and adult insect showed that this fungus had high potential to control this pest as the mortality rate of the larvae at the concentration of 106 spore/ml after lapse of 10 days was measured to be 83% ±5 and 62 %±5 on the Weevil larvae and mature insects, respectively. Since inoculation of fungus on the host plants is asymptomatic, this strain can be used as a promising biocontrol agent for management of melon weevil.

Key words: entomopathogenic fungus, melon weevil, fusarium cf nygamai

Introduction

The genus *Fusarium* comprises a large group of species of filamentous fungi widely distributed in soil usually in association with plants. Most species are saprotrophic and relatively abundant members of the soil microbiota (Leslie and Summerell., 2006). Although many *Fusarium* species are well-known as pathogens of plants, insects, and humans (Majumbar et al. 2008), there are some *Fusarium* species that are insect pathogens but are not pathogenic to the plants (Kuruvilla and Jacob 1979a, 1979b, 1980). Toxins have been reported from *Fusarium* species, some of which, or their derivatives, have insecticidal properties (Robert., 1981).A large number of *Fusarium* spp. are entomopathogenic; some are weak, facultative pathogens, especially of the lepidopteron and coleopteran orders, and some colonize their dead hosts as saprophytes. Strong pathogens were reported primarily from homopterans and dipterans from field observations of natural mortalities as well as from pathogenicity tests. Potential *Fusarium* isolates which cause high insect mortalities also show high host specificity and no damage to crop plants. Mycotoxins, such as trichothecenes (T-2) and other secondary metabolites, contributed to mortalities of termites, mealworms, flour beetles, maize borers and blow flies, while zearalenone(F-2) exhibited a beneficial effect on egg production in flour beetles and a detrimental effect on fecundity in mammals (Teetor-Barsch and Roberts., 1983).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 known as the colocynth, bitter apple, bitter cucumber, desert gourd, egusi, or vine of Sodom), which grows in salt and gypsum lands. This pest is multi-generation and so for its control a large amount of phosphorus pesticides were used by the farmers (Ghavami., 1969).Through sampling from different parts of South Khorasan Province during 2013 a fungus with the features of *Fusarium* was isolated from dead larvae on PDA.The aim of the present study was to isolate indigenous entomopathogenic fungi that naturally occur on *A.curvirostris persicus* in regions of the eastern Iran and, second, to test isolates against larval and adult stages of the pest to assess their entomopathogenic potentiality.

Materials & Methods

Fungal isolates and culture conditions: In August 2012, during sample collection from melon farms in Mohammedieh area, Birjand, the dead larvae of melon weevil were collected in clean plastic bags and transported to the laboratory for culturing. The larvae were surface sterilized with 1% sodium hypochlorite for 3 min, 70% ethanol for 3 min and then were rinsed with sterile water three times (Lacey and Brock., 1997). After sterilization, the larvae were cultured in broth PDA medium and kept in the dark at 25°C. The morphological identification stages of fungus were performed in different PDA (Potato Dexterose Agar), CLA (Carnation Leaf Agar) and SNA (Synthetic Nutrient Agar) media, and it was kept on barley stubble medium and sterile sand.

Pathogencity test: To perform pathogenicity tests, a suspension with spore concentrations of 10^2, 10^3, 10^4, 10^5 and 10^6 of conidia per milliliter were sprayed on the larvae and adult weevils. The experiment negative control was performed with sterile distilled water. Each experiment was done with five replicates and 15 larvae or adult insects at each replicate. In every plastic Petri dish containing larvae or adult, wet filter papers were placed to maintain relative the humidity; after 10 days, the percentage of mortality was recorded.

DNA extraction: To identify the fungus, the regions of ITS (ITS4 & ITS5) and tubulin (T1 & T222) from fungal DNA were used. A value of 100 mg of 3-day fungal mycelium was transferred to a sterile microtube for DNA extraction. DNA extraction was done using the CHELEX 100 substance by Walsh et al (1991) method. The polymerase chain reaction was done using the Thermocycler, (BIO-RAD Model, made in America) with the final reaction volume of 25 µL, containing a 4.4 micro-liters mixture of *taq* DNA polymerase enzyme, $MgCl_2$, PCR buffer, dNTps, 1 µL of each forward-reverse primer (10 pmol), and 5 µL of template DNA. 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 reaction products were stored at -20°C.A volume of 25 microliter of each PCR product with each of the forward-reverse primers at a concentration of 10 pm (picomole) was sent to Bioneer Company, South Korea for sequencing. The samples were sequenced by automated Sanger method using the PCR purification kit. To enhance the accuracy of the sequencing, each sample was sequenced with both forward and reverse primers.

Results

During sample collection from different melon farms of South Khorasan province (Table 1), from the dead larvae of *Acytopeus curvirostris* cultures with general characteristics of the genus *Fusarium* was isolated with a frequency of 71%. Based on the morphological characteristics of the fungus on PDA, SNA and CLA media, the fungus was identified as *Fusarium cf nygamai*. On PDA culturing medium, after ten days, the average colony diameter reached to 90 mm in the dark at 25°C.; The fungal aerial mycelia were white cottony, and the purple pigments were visible below the Petri dish. On the PDA, CLA and SNA media terminal, intercalary and chain chlamydospores were formed. Abundant spiral hyphae and aerial mycelia conidiophores in single, branching and terminating to 1-3 phialides, were observed. On carnation leaf- agar (CLA) medium, mono- phialides and poly-phialides with 2-3 pores were seen; the microconidia were formed as chain and over the false heads. The number of conidia in each chain was more than 30; sporodochia were sporadic and light brown in color, and the phialides were ampule-shaped. Microconidia were transparent and very abundant in various clubbing, cylindrical and parabolic forms. Parabolic microconidia were also formed on the phialides. The microconidia average diameter was equal to 2.5-12.5 micrometers. The macroconidia were transparent, with 2-5 lateral walls, thin, right or sickle shape and 22.5-45 micrometers in size. The number of microconidia in each chain varies between 12-30 microconidia. Formation of parabolic and spherical microconidia, spiral hyphae and long chains distinguishes this fungus from Burgess &Trimboli *Fusarium nygamai* (Klason and Nilsson, 1998). Parabolic microconidia were also formed on the phialides.The results of nucleotide sequence analysis of ITS region of ribosomal DNA in Gene Bank represents 99% homology with *Fusarium nygamai* fungus with accession number of U34568 and X94174. BLAST results of sequences obtained from the amplification of β tubuline gene of two isolates of the studied fungus (598 and 469 bp) represents 99% homology with several species of *Fusarium* fungus such as *F. proliferatum* with access codes of KF466444 and KC964151 and *F. solani* with access code of KC964151.

The results of fungal pathogenicity test on weevil larvae at various spore concentrations showed that the concentration of 10^6 spores has had the highest mortality rate on larvae and adult insects, 83 ± 5% larval mortality and about 62 ± 5% of deaths in adult insects. After 40 days of fungi inoculation on plants, the results of both concentrations on plants lacked any symptoms. Thus, it can be concluded that the studied fungal isolates can be commercially used as promising biocontrol isolate for biological pest control.

Discussion

So far, more than 13 species of *Fusarium* genus have been reported as pathogens of insects in orders of Diptera, Homoptera and Coleoptera. Some reports have been also provided at Curculionidae family level as *Fusarium* fungus as insect pathogen. But this is the first report of *Fusarium* pathogenicity on adult melon weevil.*F. avanaceum* has been reported as a pathogen of *Sitophilus oryzae(Betta.,*2012). This report was the first report of a pathogenic fungus on Curculionidae family members.His research results showed that this species of the genus *Fusarium* at different levels is capable of causing mortality on adult insect of *Sitophilus oryzae*, and direct spray of fungi on rice weevil caused 94% mortality.In other studies on order coleopteran, several species of the genus *Fusarium* such as *F. solani, F. monoliform, F. roseum* have been introduced as pathogens of insects of this order (Loke and Norris., 1970). The first species of *Fusarium* isolated from Coleopteran insects order was *F. solani* fungus, which was isolated in 1970 from the insect of *Xyleborus ferrugineus* (Coleoptera: Scolytidae).Based on the analysis of morphological characteristics and molecular data from the sequencing of the studied fungal isolates, comparing with different identification keys and different species of *Fusarium*, the studied fungus can be introduced as a new species of the *Fusarium* genus. The pear-shaped and spherical conidia, spiral hyphae and long microconidial chains are distinguishing aspects of this fungus from *Fusarium nygamai*.The results of molecular data analyses indicate that the use of two loci is not sufficient to distinguish the species, and amplification of other genes fragments such as translation elongation factor 1-a (TEF) is required. This gene encodes an important part of proteins in the translation process, and contains high levels of information regarding the genus *Fusarium* at species level (Gisser*et al.*, 2004).Fungal isolates are quite close together with *F. proliferatum* in terms of DNA ribosomal sequence similarity; however, they have important differences in the morphological characteristics. The studied fungus forms single and chain chlamydospores on the PDA medium after two weeks. Spiral hyphae were also observed abundantly in the PDA and CLA media. *F. proliferatum* lacks chlamydospores, spiral hyphae and spherical conidia. The studied fungal isolate, considering the mortality rate on pest biological processes, can be considered as a promising pathogen. The results obtained in this study are consistent with the results obtained by Peliza *et al*. (2010) and Betta (2012). It seems that depending on the concentrations of isolates and hosts of other

orders of insects, the percentage of mortality can be different. Weather conditions can also affect the virulence of the strains of this genus. Some research suggests that in conditions of high relative humidity and lower temperatures, the *Fusarium* can be more virulent on the host. With examining the rate of humidity, maximum and minimum daily temperatures in isolation areas of fungal strains and proper temperature conditions for the onset of disease in the host, one can recommend that each fungus has the ability to control pests in different areas. The studied areas in this research, with a maximum temperature of 27 degrees Celsius in winter and 45 degrees Celsius in summer and the relative low humidity in these regions suggest that the strains isolated are effective on their host in hot and dry areas. However, they need to be also studied more in areas with high relative humidity and low temperatures average.Producing specific metabolites and toxic toxins by the genus *Fusarium* has been proven. According to Tomanov studies in 1965, the production of toxin is the main cause of death in adults insects tested with genus *Fusarium*. Due to risks of this toxin in humans, animals and insects, examining the fungus effects on natural enemies of pests is of great importance.The multi-gene analysis, phylogenic studies and drawing the phylogenetic trees are being used in identifying and describing fungal arrays, including members of the genus *Fusarium*. Using the EF-1α, along with β-tubulin is essential for molecular identification of *Fusarium* species. Having a high number of phylogenic isolates, the relationship between the isolates and with other close species can be examined. In this study, we only performed morphological and molecular identification with a low number of isolates. Thus, it is recommended in later stages, the experiments will be repeated with more number of isolates and using other specific primers to determine the limits of the species (Scoflyer, 2010).

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: 33.629907/ E: 58.3778153
Mohamadiyeh	1352	N: 32°52.521/ E: 58°59.875

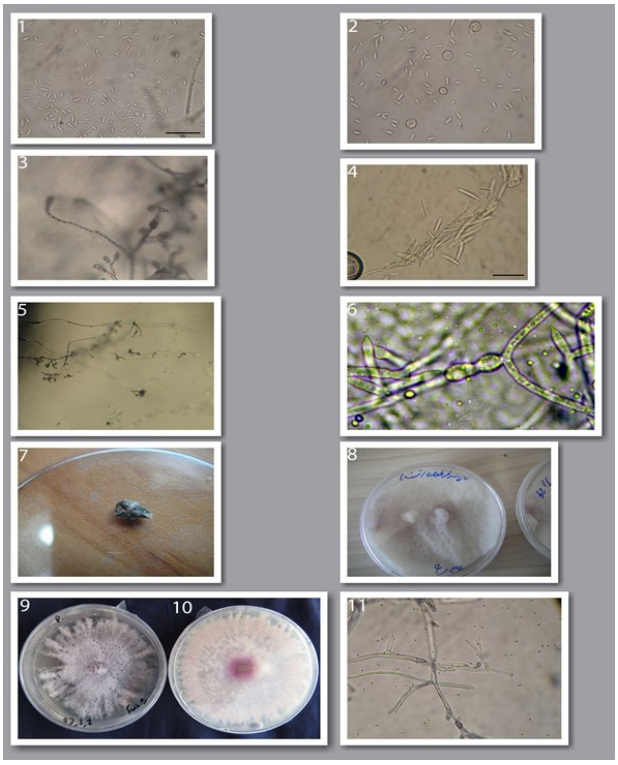

Fig. 1.*Fusarium nygamai*. 1. Napiforme micro conidia (Bar: 20µm). 2. Globose micro conidia. 3. Microconidia in chain. 4. Macro conidia(Bar:25µm). 5. Coiled. 6. Chlamydospore . 7. Dead adult insect. 8. Dead larva. 9-10. Colony of fungus. 11. Poly phialid and mono phialid.

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N.Sepasi, Department of plant protection, College of Agriculture, University of Birjand, Birjand, Iran
M.Jahania, Department of plant protection, College of Agriculture, University of Birjand, Birjand, Iran
Corresponding author: mjahani@birjand.ac.ir
M.R.Mirzaee, K.Mohammadpour, Agriculture and Natural Resources Research Center of South Khorasan, P. O. BOX:413, Birjand,Iran