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Isolation and characterization of native insect pathogens from soils of North Afghanistan

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Abstract: In a survey of entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) in the north province of Afghanistan, we identified two EPN which belong to genus *Oscheius* and *Diploscapter* and two species of EPF belonging to genus *Metarhiziun*. We used the *Galleria mellonella* baiting method. For morphological identification of EPN and EPF light microscope and scanning electron microscope were used. For molecular identification of EPN three genomic regions including ITS, D2/D3 and 18S were used. The collected nematodes were identified as *Oscheius tipulae* and *Diploscapter coronatus*. The species of EPF were identified as *Metarhizium robertsii* and *Metarhizium anisopliae*. The conidia suspension was applied against subterranean termite, *Anacanthotermes vagans*, at four different concentrations (1×10^5, 1×10^6, 1×10^7, 1×10^8 conidia/ml). The highest mortality rate was obtained from 1×10^8 conidia/ml for both *M. robertsii* and *B. bassiana*. In general, the indigenous isolate *M. robertsii* was more virulent than *B. bassiana* while *B. varroa* does not affect termites.

Key words: insect natural enemy, entomopathogen, biocontrol, entomopathogenic nematodes, entomopathogenic fungi

Introduction

Entomopathogenic nematodes

A huge number of entomogenous nematodes have been reported from insects (Morimoto et al., 2006). These vectors entomopathogenic bacteria inside the hemocoel, the insect is killed, and the nematode propagates feeding on the bacteria. (Lacey, 2017). Rhabditid nematodes are an interesting zoological taxon (Sudhaus and Fitch, 2001). They are very abundant in all types of soil and sediments of fresh water bodies and play important ecological roles mainly and primary consumers (Sudhaus, 2011). Their free-living forms display saprophagous or bacteriophagous feeding habits- but also as animal parasites, in particular, entomopathogenic form (Godjo et al., 2018). *Oscheius* genus presents soil-dwelling Rhabditidae family species with evolutionary differences in mode of reproduction and body sizes. *Oscheius* was divided into two groups: *Insectivora* and *Dolichura*. *Dolichura* group species are smaller in size and have reduced gonads whereas *Insectivora* group species with large and wide body size (Andrássy, 1983; Noujeim et al., 2017).

Entomopathogenic fungi (EPF)

100 genera with approximately 750 species, reported from different insects and living in diverse habitats including fresh water and soil surface, many of them have potential in pest management

(Azmi et al., 2011). Mitosporic fungi the *Metarhizium* and *Beauveria* are widespread in soil and isolates are known with virulence to most arthropods (Perez-Gonzalez et al., 2014). These genera have shown great potential for the management of various insect pests (Singha et al., 2011). *Metarhizium anisopliae* is being developed as insecticide for use against locusts and grasshoppers in Australia and Africa (Dong et al., 2007). Development of similar biopesticides is being undertaken in many countries. In this paper, isolation, and characterization a Metarhizium *robertsii* strain which obtained from the soil of Afghanistan and its efficacy along with some isolate of *Beauveria bassiana* and *Beauveria varroa* on subterranean termite (*Anacanthotermes vagans*) has surveyed.

Material and methods

Collection of soil samples: The soil sample was collected from Badakhshan province of Afghanistan, located in the north part of Afghanistan and was transferred to Ferdowsi University of Mashhad, Iran. Extraction of the entomopathogenic nematodes and entomopathogenic fungi from the soil samples adapted from Lephoto and Gray (2015).

Molecular characterization

For extraction The DNA of EPF DNeasy Blood and Tissue Kits was used and for extraction The DNA of EPN 20 µl Chelex solution and 2 µl proteinase K was utilized. The PCR mixture was carried out in a reaction volume of 25 µl, containing 12.5 µl of master mix, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), and 4 µl of template DNA. For EPN three genomic regions including ITS, D2/D3 and 18S were used. For ENF the ITS gene of ITS4 and ITS5 primers was utilized (White et al., 1990). The PCR products were electrophoresed on 1% agarose gels and subsequently, the gels were stained using Green viewer (SYBR). The PCR products were sequenced by Bioneer Company of South Korea. For phylogenetic analysis of the recovered population, the DNA sequences were compared with those of other available in the Gen-Bank using the BLAST homology search program.

Evaluation of fungal pathogenicity against termites at laboratory assay

To determine the insecticidal activity of three entomopathogenic fungi, three strains including *Metarhizium robertsii* (Afghan isolate), *Beauveria bassiana (FUM102)* and *Beauveria varroa (FUM121)* were selected to use for assay against *Anacanthotermes vagans.* The fungal strains were tested at a series concentration of 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml. 600 µm suspension of each fungal concentration were poured by a pipit into sterile 9 cm Petri dish and allowed to dry partially. Termites were allowed to walk on the partially dried fungal suspension for 1 min. One milliliter 0.05 Tweem-80 solution was added on the filter paper in Petri dishes as the control. Three replicates were performed for each concentration of conidial suspensions with ten individual termites which maintained. Mortality was observed at different day's intervals (every 24 h) for 10 days.

Evaluation of pathogenicity against termites at semi-field assay

For semi-field experimental setup, we prepared plastic dishes at 14 cm on the level of the balcony. The soil with saw dust and added moisture was placed on container. The open shape plastic dishes were laid out on the soil and saw dust to prevent termite's scope and ant attack and also protect the termites from direct sunlight. In this test, two strains including *Metarhizium robertsii* and *Beauveria bassiana* were selected. The fungal concentrations were 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml. 1 ml suspension of *Metarhizium robertsii* and *Beauveria*

bassiana was sprayed by small sprayer into each plastic container and put 10 termites in each plastic container. Three replicates were performed on each concentration of conidial suspensions with ten individual termites which maintained and mortality was recorded at different day's intervals (every 24 h) for 12 days.

Data analysis

Mortality data were corrected according to Abbott's formula (Abbott 1925). Differences between the fungal isolates and control group, with respect to mortality, was determined by analysis of variance (ANOVA). And subsequently by LSD multiple comparison test. All analyses were performed using SAS (SAS Institute, 2002).

Results

Molecular analysis of O. tipulae

The ITS amplification resulted in single fragments in rDNA sequence of 850 base pairs (bp), and the size of 28S rDNA D2/D3 fragments was 580 bp. Blast search using newly obtained ITS and 28S rDNA sequence of the *O. tipulae* isolate revealed that the ITS sequence has 99-100% identity with the available ITS sequence of the species in the database (indicate what sequence with the accession number and the locality). Blast search using 28S rDNA D2/D3 fragment yielded the same result.

Molecular analysis of Diploscapter coronatus

From the results of the BLAST search, *D. coronatus* Afghan isolate was close to with available sequences, with 100% of bases identical for the 18S sequences. Unfortunately, no *Diploscapter coronatus* D2/D3 region sequence is available in the GenBank, thus phylogenetic analysis for D2/D3 gene did not perform.

Molecular analysis of EPF

For identity of the *Metarhizium* species the partial sequence (550 fragments) of the ITS4-ITS5 gene was sequenced and it identify compared with representative valid sequences of *Metarhizium* isolates. According to phylogenetic analysis, the new *Metarhizium* isolates made a single clade along with other isolates of *M. robertsii* and *M. anisopliae* with high bootstrap. The Afghan strain of *Metarhizium* had the lowest genetic distance with those of other isolates of *M. robertsii* and *M. anisopliae.* The partial DNA sequence was deposited in the GenBank with accession numbers of MN128533 and MN078271, respectively.

Insecticidal activity of Metarhizium and Beauveria under the laboratory condition

Four different conidial concentrations (1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia/ml) of *M. robertsii*, *B. bassiana*, and *B. varroa* were administrated for insecticidal activity against *A. vagans* in the laboratory condition. After application of each conidial concentration the cause of mortality so few during the 24 h, although termites were very weak and hardly moving. It is certain that *M. robertsii* was virulence and pathogenic to the termite *A. vagans* the termite mortality was increased along with the indigenous of a newly identified Afghani species of *M. robertsii*. In the laboratory, all isolates caused significant different mortality rate ($F = 104.08$, $df = (2, 30)$, $p < 0.0001$) in comparison to control groups. All concentration caused significant different mortality rate in comparison to each other's ($F = 28.98$, $df = (4, 30)$, $p < 0.0001$). The interaction between fungal species and concentrations were significant difference ($F = 7.10$, $df = (8, 30)$, $p < 0.0001$). Conidia from the *M. robertsii* were highly

virulent for *A. vagans* causing approximately 100% mortality 4 days after inoculation in the concentration of 1×10^8 conidia/ml. other concentration respectively caused 100% mortality approximately 5-6 days post inoculation.

The significant effect of fungal filtrates, observation time intervals were recorded in laboratory assay for concentration of 1×10^8 6 days of treatment of *B. bassiana*. Other concentration respectively caused 100% mortality approximately 7-10 days post inoculation. On the other hand, minimum mortality (so less than others) was recorded on 10^{th} days by filtrate application of *B. varroa* (table 2). The results showed that indigenous *M. robertsii* was very virulent due to it has significant highly mortality 4 day post inoculation ($F = 33.34$, $df = (4, 30)$, $p < 0.0001$), *B. bassiana* also significant effect 4 days post inoculation ($F = 9.47$, $df = (4, 30)$, $p < 0.0001$) and *B. varroa* was not significant effected 4 days post inoculation ($F = .0.38$, $df = (4, 30)$, $p < 0.8246$). The *M. robertsii* and *B. bassiana* were selected for semi-field bioassay due to the high pathogenicity.

Pathogenicity of M. robertsii and B. bassiana under the semi-field condition

M. robertsii and *B.bassiana* were tested an insecticidal activity against *A. vagans* in the semi-fields bioassays. There were significant mortality differences between both isolates ($F = 64.22$, $df = (1, 20)$, $p < 0.0001$) in comparison to control groups. Mortality of *A. vagans* at different doses of the selected *M. robertsii* and *B. bassiana* isolates was significantly different ($F = 65.22$, $df = (4, 20)$, $p < 0.0001$) and the interaction between fungal species and concentration were not significant difference ($F = 7.\ 2.56$, $df = (4, 20)$, $p < 0.0706$).The results showed that in indigenous *M. robertsii* was much virulent due to it has significant highly mortality 5-day post inoculation ($F = 45.72$, $df = (4,30)$, $p < 0.0001$), *B. bassiana* also significant effect 5 days post inoculation ($F = 22.06$, $df = (4,30)$, $p < 0.0001$). In both genera the higher concentration (1×10^8 conidia/ ml) had achieved maximum mortality compared to 1×10^5, 1×10^6 and 1×10^7 conidia/ml. Dead termite infected by entomopathogenic fungi would develop mycosis in five to six days after they were placed in the wet condition. For the dead termite infected by *M. robertsii* they were covered by green conidia, while for *B. bassiana* the white conidia were fully grown on termite cadavers. In pathogenicity test, melanization spots were observed around the thoracic and abdominal segments after inoculation of conidial suspension. The fungal infection changed the color of the insect body with the progressive symptom of sluggishness (slow movement) when compared to control. The highest concentration (1×10^8 conidia/ ml), caused quick sporulation when exposed to individual termites.

Discussion

The genus *Oscheius* has large number of species which are morphologically very close to each other Sudhaus (2011) divided the species into two groups' *Dolichura* group and *Insectivora* group. This genus comprises 42 valid species, which 14 species belong to *Dolichura group* and 28 species belong to the *insectivore* group (Tabassum et al., 2016). Species under *Dolichura* group are represented by species with slender body, peloderan bursa, inconspicuous posterior phasmids and spicules with straight tip without a distal hook, whereas species under *Insectivora* group are characterized by large and wide body with 4 to 6 incisures each in the lateral fields with 3 to 5 ridges, pseudopeloderan/ leptoderan bursa, phasmids posterior to the last genital papillae and crochet needle-like spicules with a distal hook. Variations have been observed in the arrangement of papillae within the same species (Kumar et al., 2019).

Diploscapter coronatus

This species described for the first time by Cobb in 1893 from the rhizosphere of banana growing in Fuji as *Rhabditis coronatus* (Morimoto et al., 2006). In 1913, Cobb erected the genus *Diploscapter* Cobb, 1913 to accommodate the species which he originally described and suggested that this species could be representative of a species complex. Zimmerman (1898) described *Rhabditis bicornis* found associated with the roots of coffee plants, a species later synonymized with *Diploscapter bicornis (*Abolafia and Santiago, 2007*).* Kreis (1929) reported for the first time *Acrobeles armatus* Kreis, 1929 from Peking, China, a species later synonymized with *Diploscapter coronatus* (Van Rensburg, 2010). Here we report and provide additional habitat information on *Diploscapter coronatus* from northern Afghanistan. BLAST search using 18S and D2/D3 sequences showed that the nematodes collected in Afghanistan, having close molecular sequence similarity to the *Diploscapter coronatus.*

EPF

In recent years, some studies have focused on developing fungal insecticides to control insect pest. Four to five hundred species of fungi have pathogenic effects on insects. For the basal fungi, EPF are found in three main groups: the Entomophthoromycota, the Blastocladiomycota, and the Microsporidia. The largest numbers of EPF species in the basal fungi occurring in the phylum Entomophthoromycota (Chandler, 2017). This fungal virulence mostly has been associated with intra and extracellular synthesis of different substances including cuticle-degrading enzyme, and low molecular weight toxic compounds (Mishra et al., 2015; Luangsa-Ard et al., 2017). However, most of the literature has focused on low molecular Wight fungal metabolites (secondary metabolites), whereas the high- molecular Wight compounds, such as proteins, remain poorly studied (Keppanan et al., 2018).

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