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Orijinal araştırma (Original article)

Molecular characterization and virulence of the entomopathogenic fungus *Beauveria bassiana* from *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae)[1]

Ostrinia nubilalis (Hubner) (Lepidoptera: Pyralidae)'den elde edilen entomopatojenik fungus *Beauveria bassiana*'nın moleküler karakterizasyonu ve virulansı

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Summary

The European corn borer [*Ostrinia nubilalis*, (Hubner) Lepidoptera: Pyralidae] is one of the most important pest of maize and other crops, such as potato, green pepper, and winter wheat almost all over the world. In this study, we collected larvae from corn fields infested with *O. nubilalis* during 2008-2010 in the Eastern Black Sea Region of Turkey and investigated fungal pathogens of this pest. One fungal species was isolated from *O. nubilalis* and it was characterized according to morphological and molecular characteristics. Based on morphology, ITS sequence and partial sequencing of the EF1-α gene, the isolate was identified as *Beauveria bassiana*. Additionally, virulence of the isolate was tested against *O. nubilalis* larvae by testing different concentrations of conidia (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}). The LC_{50} value was calculated as 4.8×10^5 conidia ml^{-1}. These results indicate that *Beauveria bassiana* Ost3 has a potential in control of *O. nubilalis*.

Key words: *Beauveria bassiana*, european corn borer, corn, LC_{50}

Özet

Mısır kurdu [*Ostrinia nubilalis*, (Hubner) Lepidoptera: Pyralidae] hemen hemen bütün dünyada mısır, patates, yeşil biber ve kışlık buğday gibi pek çok ürünün en önemli zararlılarından birisidir. Bu çalışmada, 2008-2010 yılları arasında Doğu Karadeniz Bölgesi'nde *O. nubilalis* tarafından istila edilmiş mısır tarlalarından böcek larvaları toplanmış ve entomopatojenik fungus yönünden araştırılmıştır. *O. nubilalis*'ten bir fungal örnek izole edilmiş, morfolojik ve moleküler verilere göre karakterizasyonu yapılmıştır. Morfolojik, ITS ve kısmi EF1-α gen dizin analizine göre, izolat *Beauveria bassiana* olarak tanımlanmıştır. İlave olarak, bu izolatın *O. nubilalis* larvaları üzerindeki virülensliği farklı konsantrasyonlarda spor süspansiyonları (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 spor/ml) kullanılarak test edilmiştir ve LC_{50} değeri 4.8×10^5 spor/ml olarak hesaplanmıştır. Bu sonuçlar, *Beauveria bassiana* Ost3'ün *O. nubilalis*'in mücadelesinde bir potansiyele sahip olduğunu göstermektedir.

Anahtar sözcükler: *Beauveria bassiana*, mısır kurdu, mısır, LC_{50}

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Introduction

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner) (Lepidoptera: Pyralidae) is a serious pest in many regions of the world and it causes worldwide economic damage on maize plants (*Zea mays*) (Gaspers, 2009). Apart from maize, there are more than 200 plants such as hemp, hop, peppers, sorghum, cowpea, soybean, beans and cotton (Lewis, 1975; Gasper, 2009). Conventional control of *O. nubilalis* relies on a large extent on the use of foliar application of insecticides such as carbaryl, profenofos ,thiodicarb, methomyl and chlorpyrifos-ethyl (Hoffmann et al., 2002; Anonymous, 2008). Another effective method to prevent an infestation and damage to maize is the cultivation of Bt-maize. Maize plants carrying Bt toxins of *Bacillus thuringiensis* make possible the expression of these toxins which is specific to Lepidopteran pests. Therefore, *O. nubilalis* larvae feeding on these plants die during the whole cultivation period (Gasper, 2009).

Entomopathogenic fungi are effective and environmentally safe biological control agents that can be used against many important pest species in both agriculture and forestry because they are safe for animals, plants and environment (Chandler et al., 2000; Shah & Pell, 2003; Goettel et al., 2005; Gökçe & Er, 2005). Entomopathogenic fungi differ from other insect pathogens since they are able to infect through the host's integument, therefore ingestion is unnecessary and infection is not limited to chewing insects (Fuxa, 1987). Therefore, they are unique to control insect pests which feed by sucking plant or animal fluid (St Leger & Roberts, 1997).

Entomopathogenic fungal species belong to *Beauveria* genus attack many insect pests worldwide and species within the genus range from the ubiquitous insect pathogen such as *B. bassiana* to rare species. However, the entomopathogenic life-style is dominant (Glare, 2004; Glare et al., 2008; Sevim et al., 2010a). A total of six species were described within this genus and they were designated as *B. bassiana*, *B. bassiana* cf. Clade C, *B. brongniartii*, *B. caledonica*, *B. vermiconia* and *B. amorpha* (Glare & Inwood 1998, 2008; Glare, 2004; Rehner & Buckley, 2005; Sevim et al., 2010b). Among these species, *B. bassiana* is the most studied one and remarkable effort were spent to develop microbial control agent using this species. Moreover, the most widely used species available commercially is *B. bassiana*. (Goettel et al., 2005; Meyling & Eilenberg, 2007). The entomopathogenic fungus *B. bassiana* is extensively used for the control of many important pests of various crops around the world and it was tested on different target insects (Campbell et al., 1985; Leathers & Gupta, 1993; Padmaja & Kaur, 2001; Todorova et al., 2002; Tafoya et al., 2004; Sevim et al., 2010a; Sevim et al., 2010b).

In this study, we isolated and characterized *B. bassiana* isolate Ost3 and tested its effectiveness on *O. nubilalis* larvae as a possible biocontrol agent. This isolate appears to be a promising candidate in the biological control of *O. nubilalis*.

Material and Methods

Collection of larvae

Larvae of *O. nubilalis* were collected from the Eastern Black Sea Region of Turkey between 2008 and 2010. Larvae were removed from inside of corn stems by cutting with a scalpel and collected larvae were placed individually into plastic boxes (20 mm). A small corn stem was provided as food. The collected larvae were immediately taken to the laboratory and were regularly checked for possible fungal infection. Fungus isolation was done from the mycosed larvae.

Isolation of fungi

The fungus was isolated from dead larvae showing external mycelia growth at outside of cadaver. A small part of mycelia was taken from cadaver using an inoculation loop and the mycelium was placed on Sabouraud dextrose agar medium with 1% yeast extract (SDAY medium, Difco Laboratories) including 50 µg/ml ampicillin (AppliChem) and 50 µg/ml tetracycline (AppliChem) to prevent bacterial growth. After obtaining pure culture, it was kept in a refrigerator at 4°C and was subcultured monthly. Glycerol stock was prepared by adding spores into microcentrifuge tubes including 10% glycerol and was stored at -80°C.

Morphological identification

Morphological identification of the isolate was done according to identification key prepared by Dr. Richard Humber (Humber, 1997). The appearance of dead larvae, the shape of colony and spores and the size of spores were used for initial identification process.

DNA extraction and gene sequencing

The fungus was derived from single conidial spores grown on SDAY plates. To do this, conidia from culture on SDAY plate were suspended in 0.1% Tween 80 up to 10^5 conidia ml^{-1} and were plated on Potato Dextrose Agar (PDA) (Merck, Germany) and incubated for 2 days at 25°C under 12:12 photoperiod. At the end of the incubation period, single colony was used to inoculate into 250 ml flask containing 100 ml Potato Dextrose Broth (PDB) (Merck, Germany). Liquid culture was shaken at 250 rpm at 28ºC for 1-2 weeks. After the incubation period, mycelia were collected by filtering, frozen into liquid nitrogen, crushed with mortar and 50 mg fungal mycelia were used for DNA extraction. Total genomic DNA was extracted using Nucleospin Plant kit (50 preps). Isolated DNAs were stored at -20ºC until use.

The 570 bp fragment of ITS1-5.8S-ITS2 region of the nuclear rRNA-complex was examined for the isolate to confirm strain identification. Oligonucleotide primers ITS4 (5'– CTCCGCTTATTGATATGC–3') as reverse primer and ITS5 (5'– GGAAGTAAAAGTCGTAACAAGG–3') as forward primer were used for ITS PCR-amplification (White et al., 1990). PCR amplifications were performed in a total volume of 50 µl, including 5 µl 10X *Taq* DNA polymerase reaction buffer, 200 µM of each dNTPs, 50 pmol each amplification primers, 2,5 unit *Taq*-DNA polymerase (Fermentas), and 50 ng genomic DNA. The PCR reaction was carried out in Eppendorf thermocycler, with an initial denaturation at 95ºC for 5 min, followed by 35 cycles at 95ºC 1 min, 55ºC for 55 sec, 72ºC for 2 min and a final extension at 72ºC for 10 min.

Approximately 1200 bp fragment of EF1-α was also amplified by using primers EF1T (5'-ATGGGTAAGGARGACAAGAC-3') and 1567R (5'-ACHGTRCCRATACCACCSATCTT-3') (Rehner & Buckley, 2005). PCR conditions were adapted essentially as described by Rehner & Buckley (2005). PCR products were separated on %1 agarose gel and visualized under UV light. Amplification products were extracted from agarose gels with the QIAquick Gel Extraction Kit (50) and sent to MACROGEN (Korea) for sequencing. Obtained sequences were used to carry out BLAST searches by using the NCBI GenBank database to confirm isolate identification. Additionally, the sequences were used to compare the representative sequences from the study that were included for comparison of *Beauveria* strains of Rehner & Buckley (2005) (Table 1).

Table 1. Genbank sequences data of fungal isolates used in this study (Rehner & Buckley, 2005)

Species	Isolate	Location	Host	Clade[a]	Accession number	
					ITS	EF1-α
B. bassiana	ARSEF937	Brazil	Coleoptera: Chrysomelidae	A	AY532056	AY531965
B. bassiana	ARSEF296	USA	NA	A	AY532013	AY531922
B. bassiana	ARSEF714	People's Republic of China	Homoptera: Delphacidae	A	AY532042	AY531951
B. brongniartii	ARSEF1431	Philippines	Coleoptera: Cerambycidae	B	AY531980	AY531889
B. brongniartii	ARSEF1848	Belgium	Coleoptera: Rhizophagidae	B	AY531995	AY531904
B. brongniartii	ARSEF4850	Korea	Coleoptera: Cerambycidae	B	AY532028	AY531937
B. cf. *bassiana*	ARSEF812	France	Hemiptera: Tingidae	C	AY532051	AY531960
B. cf. *bassiana*	ARSEF156	Poland	Hymenoptera: Ichneumonidae	C	AY531985	AY531895
B. celedonica	ARSEF2567	Scotland	Soil	D	AY532006	AY531915
B. vermiconia	ARSEF2922	Chile	Soil	D	AY532012	AY531920
B. amorpha	ARSEF2641	Brazil	Hymenoptera: Formicidae	F	AY532008	AY531917
Cordyceps cf. *scarabaeicola*	EFCC252	S. Korea	NA	E	AY532057	AY531966

[a] Clades names were given according to Rehner & Buckley (2005)

Bioassay

A hundred micro liter spore suspension of the fungal isolate (1×10^6 conidia ml^{-1}) was plated on SDAY medium and incubated at 25°C for 3-4 weeks under 16-h photoperiod. After growth period, conidia were harvested from 3-week-old cultures by adding 10 ml of sterile distilled water supplemented with 0.1% Tween 80. The conidial suspension was filtered through two layers of sterile muslin into 50 ml falcon tube and then shaken for 5 min using a vortex. The concentrations of conidial suspension were adjusted to desired concentration using a Neubauer haemocytometer. The viability of conidia of the isolate was determined by inoculating them onto PDAY and assessed the germination after 24 h of incubation at 25°C and under 12:12 photoperiod. Conidia were considered to have germinated if the germ tube was longer than the diameter of the conidium. Isolates with a viability of above 95% were used for bioassay experiments.

Larvae of *O. nubilalis* were collected from naturally infested corn field in the Eastern Black Sea Region of Turkey and were used for the pathogenicity test. They were randomly selected and treated with six different doses of conidial suspension of the isolate (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}). The control group was treated with sterile water including 0.1% Tween 80. For each replicate, 10 last instar larvae were used in all experiments which were repeated three times. Corn stems were collected from fields and cut into 10 cm length by a scalpel. Then, these 10 cm pieces were longitudinally divided into two parts in equal, and a rectangle shaped hole was dug by a blade in the middle of both pieces of divided stems. Larvae were treated by dipping into 10 ml of conidial suspension, and then put into these holes. After that, other piece of stem was closed on the first and sealed with a rubber band to keep them tight. Finally, all stems were put into plastic boxes (15 mm) and incubated at room temperature. Mortalities were checked every 24 h. Dead larvae were taken and subjected to surface sterilization by dipping into 2% hypochlorite followed by 70% ethanol and sterile water. Finally, they were put into the moisture chamber to encourage fungal sporulation outside of the cadaver. All experiments were repeated three times on different days using different fungal cultures.

Data analysis

Sequences were assembled and edited with BioEdit and aligned (Hall, 1999). Cluster analyses of the sequences was performed using BioEdit (version 7.09) with Clustal W followed by Kimura-2 parameter analysis (Kimura, 1980) with neighbor joining analysis on aligned sequences was performed with MEGA 4.0 software (Tamura et al., 2007). Alignment gaps were treated as missing data. Reliability of phylograms was tested by bootstrap analysis with 1000 replicates using MEGA 4.0.

Mortality values were corrected according to Abbott's formula (Abbott, 1925). LC_{50} value was calculated with probit analysis by using SPSS 15.0 software.

Nucleotide sequence GenBank accessions number

The accession number of the sequences of ITS and EF1-α are HQ454299 and HQ454300, respectively.

Results

This study was conducted in the Eastern Black Sea Region of Turkey between 2008 and 2010 to examine the fungal infection of *O. nubilalis* larvae. After collecting larvae from several regions, only one isolate from larvae which was covered with white mycelia were observed. The fungus was isolated and cultivated on SDAY medium to determine some of morphological features. The conidia were like globose and diameter of the conidia was measured as 2.93 ± 0.24 µm. The conidial chains were long and conidial

heads diffuse. The colony color was white on SDAY medium after two weeks. Based on its morphological features, it was identified as *Beauveria bassiana*. Additionally, we sequenced ITS1-5.8S-ITS2 and partial sequence of EF1-α to confirm isolate identification. Molecular characteristics also showed that this isolate was similar to *B. bassiana* and was named as Ost3. According to the dendrograms based on ITS and EF1-α sequences, the isolate Ost3 was very similar to Brazilian isolate *B. bassiana* ARSEF937 (Figure 1; 2).

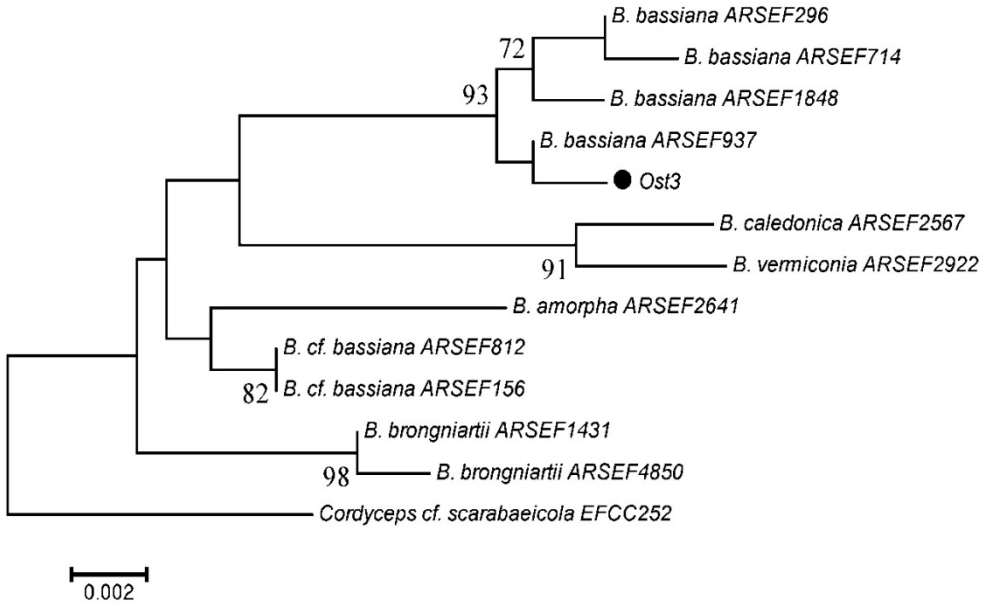

Figure 1. Pyhlogenetic position of the isolate Ost3 within *Beauveria* genus based on ITS1-5.8S-ITS2 sequence. The phylogenetic tree construction were conducted with neighbour-joining method packaged in software MEGA 4.0. Representative strains were taken from the study of Rehner and Buckley (2005). Bootstrap value is shown next to nodes are base on 1000 replicates. Bootstrap values C≥70% are labeled. The tree was rooted using isolate *Cordyceps* cf. *scarabaeicola* as the outgroup.

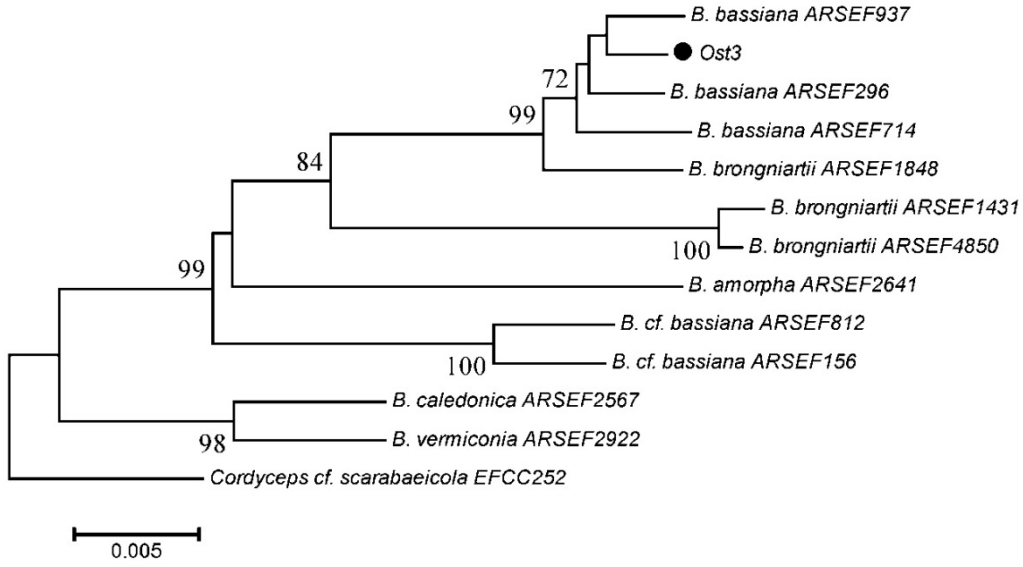

Figure 2. Pyhlogenetic position of the isolate Ost3 within *Beauveria* genus based on EF1-α sequence. The phylogenetic tree construction were conducted with neighbour-joining method packaged in software MEGA 4.0. Representative strains were taken from the study of Rehner and Buckley (2005). Bootstrap value is shown next to nodes are base on 1000 replicates. Bootstrap values C≥70% are labeled. The tree was rooted using isolate *Cordyceps* cf. *scarabaeicola* as the outgroup.

Dose – response test was also performed to calculate LC_{50} value. Based on the probit analysis, the LC_{50} of this fungus against larvae within13 days after treatment was 4.8×10^5 conidia ml^{-1} (Table 2).

Table 2. Summary of Probit analysis parameters from the multiple-concentration bioassays performed with the *B. bassiana* isolate Ost3 against larvae of *O. nubilalis*

Bioassay	Intercept	Slope (±SE)[a]	LC_{50} (95% Fiducial limits)	$\chi 2$[b]	df
Larvae	-2.3	0.404 ± 0.139	4.8×10^5 (1.6×10^4 – 5.6×10^6)	0.105	3

[a]Slope of the concentration (±standard error) response of larvae of *O. nubilalis* to the *B. bassiana* isolate Ost3.
[b] Pearson chi-square goodness-of-fit test on the Probit model (α= 0.05).

Discussion

There is an increasing demand to find out more effective and safe biocontrol agent against insect pests in both agriculture and forest industry to decrease the hazardous effects of chemical insecticides to environment. Since entomopathogenic fungi are able to infect their host by attaching on the host integument *B. bassiana* could be considered an candidate for controlling *O. nubilalis* populations. In the current study, we investigated fungal pathogens of *O. nubilalis* as a possible biocontrol agent that can be used in the Eastern Black Sea Region of Turkey. We were able to isolate one isolate and it was identified as *B. bassiana*.

Numerous entomopathogenic fungi are currently used to control insect pests. The entomopathogenic fungus *B. bassiana* is a well-known, naturally occurring and widely distributed natural pathogen of various lepidopteran pests worldwide and products based on this species are available for use against a very wide variety of insect pests, from banana weevils (*Cosmopolites sordidus*) in Brazil, pine caterpillars (*Dendrolimus* spp.) in China, to the European corn borer, and greenhouse aphids in the Western world (Goettel et al., 2005; Sevim et al., 2010b; Wraight et al., 2010; Zurek & Keddie, 2000; Legaspi et al., 2000). Although many authors provide experiments in an interaction between *O. nubilalis* population and *B. bassiana*, and pathogenicity of *B. bassiana* to *O. nubilalis* in the field and laboratory conditions (Carruthers et al., 1985; Feng et al., 1985, 1988; Bing & Lewis, 1993; Cagan & Uhlik, 1999; Wagner & Lewis, 2000), this is the first study of molecular characterization *B. bassiana* from *O. nubilalis*. It is known that the success of control strategy might be higher when native isolates are used against the target insect because of the ecological compatibility of the fungal species with certain environmental conditions. Therefore, isolate Ost3 could be considered as a possible biocontrol agent against *O. nubilalis* in the Eastern Black Sea Region of Turkey.

Isolate Ost3 produced 70% mycosis on dead cadavers after inoculation of 1×10^8 conidia ml^{-1}. Since sporulation on the host is an important factor for fungal dissemination in the field, isolate Ost3 should be further investigated as a microbial control agent against *O. nubilalis*.

Environmental factors have great effects on efficacy of entomopathogenic fungi. In general, entomopathogenic fungi require high humidity for fast propagule germination and growing, high rainfall for transmission of propagules and to disperse conidia and, suitable temperature (e.g. the optimum temperature for Hypomycetous fungi is between 20-25ºC) for successful control (Inglis et al., 2001). In this content, the Eastern Black Sea Region has a favorable climatic conditions for applying entomopathogenic fungi because annual temperatures are lower and rainfall and moisture are higher in this region. Isolate Ost3 seems to be a good candidate as a possible biocontrol agent against *O. nubilalis* in this region in terms of climatic conditions.

In conclusion, we showed that *B. bassiana* Ost3 has a good potential for further investigation as a possible biological control agent against *O. nubilalis*. Further studies are needed to determine the effectiveness of the isolate in the field. Mass production studies are also warranted.

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