

DETERMINATION OF CONTROL POTENTIALS AND ENZYME ACTIVITIES OF *Beauveria bassiana* (BALS.) VULL. ISOLATES AGAINST *Tetranychus urticae* KOCH (ACARI: TETRANYCHIDAE)

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Abstract: Broad host range of entomopathogenic fungi may have a commercial advantage for biological control industry. In addition, all entomopathogenic fungi rely on a combination of different enzymes to penetrate their hosts and these enzymes play an important role in the host range of fungal isolates. The aim of this study is to determine the potential of three *Beauveria bassiana* (Bals.) Vull. isolates in control of *Tetranychus urticae* Koch (Acari: Tetranychidae) and to determine their lipase, chitinase, and protease activities. For this purpose, *B. bassiana* (PaF04, PaF09 and PaF76) isolates, which was previously demonstrated to have entomopathogenic activity against Coleoptera, Lepidoptera, and Hymenoptera were chosen. *T. urticae* was used as the test organisms. Three different suspensions (1×10^6 , 1×10^7 and 1×10^8 conidia ml⁻¹) were prepared in Tween 80. 2ml suspension of a single dose was sprayed onto down sides of bean leaf discs using hand sprayer. Mortality rates of *T. urticae*, in terms of LT50 values, were recorded daily for 5 days. Enzyme activities were investigated by petri plate assays and clear zones formed around the colonies indicated enzymatic activities. All tested fungal isolates were pathogenic to the *T. urticae* causing mortality between 64.2±5.6% and 100% on the 5th day after the application. LT50 values varied between 3.16±0.2 and 3.72±0.5 days. Mortality caused by PaF04 isolate was found to be significantly different ($p < 0.05$). In conclusion, PaF isolates of *B. bassiana* have been determined to have potential to suppress *T. urticae*, in addition to their potential in control of Coleoptera, Lepidoptera, and Hymenoptera. The present results showed that the isolates used, especially PaF04, have a broad host range.

Key words: *Beauveria bassiana*, broad host range, *Tetranychus urticae*, enzyme activity.

Beauveria bassiana (Bals.) Vull. İzolatlarının *Tetranychus urticae* Koch'a (Acari: Tetranychidae) Karşı Kontrol Potansiyellerinin ve Enzim Aktivitelerinin Belirlenmesi

Özet: Entomopatojen fungusların geniş konukçu çeşitliliği biyolojik mücadele endüstrisi için ticari bir avantaj olabilir. Bununla birlikte, entomopatojen fungusların tümü konukçuya nüfuz etmek için çeşitli enzimlerinin kombinasyonlarını kullanır ve bu enzimler fungal izolatların konukçu çeşitliliğinde önemli bir rol oynar. Bu çalışmanın amacı, üç *Beauveria bassiana* (Bals.) Vull. izolatının *Tetranychus urticae* Koch'a (Acari: Tetranychidae) karşı kontrol potansiyellerinin ve lipaz, kitinaz ve proteaz enzim aktivitelerinin belirlenmesidir. Bu amaçla, daha önce Coleoptera, Lepidoptera ve Hymenoptera'ya karşı entomopatojenik aktivite gösteren *B. bassiana* (PaF04, PaF09 ve PaF76) izolatları seçilmiştir. Test organizması olarak, *T. urticae* kullanılmıştır. Üç farklı dozda süspansiyon (1×10^6 , 1×10^7 ve 1×10^8 konidi ml⁻¹) Tween 80'de hazırlanmıştır. 2ml tek doz spor süspansiyonu el spreyi kullanılarak fasülye yaprak disklerinin alt yüzüne püskürtülmüştür. *T. urticae*'nin ölüm oranları 5 gün boyunca günlük olarak kaydedilmiştir. Enzim aktiviteleri petri plate yöntemi ile araştırılmış ve koloni çevresinde oluşan açık zonlar, enzimatik aktivitenin varlığını göstermiştir. Test edilen tüm fungus izolatları uygulamadan 5 gün sonra, 64,2±5,6% ve 100% arasında mortaliteye yol açarak *T. urticae*'ye karşı patojenite göstermiştir. LT50 değerleri 3,16±0,2 ve 3,72±0,5 gün arasında değişmiştir. PaF04 izolatının neden olduğu mortalite önemli derecede farklı bulunmuştur ($p < 0,05$). Sonuçta, *B. bassiana* PaF izolatlarının Coleoptera, Lepidoptera ve Hymenoptera'yı kontrol potansiyellerinin olduğu kadar *T. urticae*'yi baskılama potansiyeline de sahip oldukları belirlenmiştir. Mevcut sonuçlar, kullanılan izolatların, özellikle de PaF04'ün geniş bir konukçu aralığına sahip olduğunu göstermiştir.

Anahtar kelimeler: *Beauveria bassiana*, geniş konukçu aralığı, *Tetranychus urticae*, enzim aktivitesi.

Introduction

Entomopathogenic fungi are considered to be one of the promising biological control agents (St Leger *et al.* 1996). *Beauveria bassiana* (Bals.) Vull., a fungal species

naturally growing in soils and acting as a parasite on various arthropod species, has a potential to be used against both insect pests and plant pathogens. However,

no data was reported about phytopathogenic activity of this fungal species (Clarkson & Charnley 1996, Ownley *et al.* 2004, 2008).

The two spotted spider mite, *Tetranychus urticae* Koch is a worldwide economically important major pest of agriculture (Numa Vergel *et al.* 2011) and has been a serious problem due to insecticide use. The use of entomopathogen fungi has been an increasingly popular option to control mites and suppress their populations (Zhang 2003). In recent years, a growing interest about using *Beauveria* spp. in mite control applications occurred (Shi & Feng, 2004, Alves *et al.* 2005, Bugeme *et al.* 2008). Laboratory studies revealed that *B. bassiana* as an effective pathogen of *T. urticae* (Irigaray *et al.* 2003, Bugeme *et al.* 2009, Draganova & Simova 2010, Geroh *et al.* 2015).

B. bassiana is known to have a potential to control more than 70 insect pests, but most of the studies evaluating infection have focused on phytophagous hosts, most of which are Arthropoda members (Feng *et al.* 1994, Roy *et al.* 2006, van Lenteren 2012). *Beauveria* genus was shown to be affective against members of the insect orders Coleoptera, Lepidoptera, and Hymenoptera (Hatting *et al.* 2004).

The commercial production of mycoinsecticides has been a successful and environmentally safe alternative to insecticides. However, acceptance of these products has been limited when compared with conventional chemical products because they are not acting on pest species as fast as chemical products (Leger & Screen 2001, Sevim 2015). Narrow host range of mycoinsecticides is also one of the factors limiting their commercial use (Ownley *et al.* 2004). However, a mycoinsecticide including entomopathogenic fungi with a broad host range may have a commercial advantage over others (Brodeur 2012).

B. bassiana has an extremely broad host range and uses a combination of cuticle-degrading enzymes for degradation of and penetration into the host cuticle (Butt 2002, Meyling *et al.* 2009, Ortiz-Urquiza & Keyhani 2013). These enzymes including lipase, chitinase and protease may determine not only the virulence but also the host range of fungal isolates (Gupta *et al.* 1994, Butt 2002).

Some studies indicated that strains of entomopathogenic fungi can be more virulent to pests than the same or related host species that they were isolated from (Goettel *et al.* 1990, Ekesi *et al.* 2000, Shaw *et al.* 2002). Besides, pathogens that are not associated with a host in nature can be tested for their pathogenicity (Wekesa *et al.* 2005).

In the present study, we selected three native *B. bassiana* isolates, which were previously demonstrated to be active against Coleoptera (Albayrak İskender *et al.* 2013), Lepidoptera (Albayrak İskender *et al.* 2012), and Hymenoptera (Albayrak İskender *et al.* 2017), and evaluated their pathogenicity potentials towards *T. urticae* (Acari) considering their extremely broad host range.

Materials and Methods

Biological material and history of isolates

PaF04, PaF09 and PaF76 isolates of *B. bassiana* were used in this study. These isolates, previously isolated from *Pristiphora abietina* (Christ) larvae, were shown to be highly pathogen on different insect species and are deposited within the GenBank database with accession numbers KT962854, KT962855, and KT962857 respectively. A history of each isolates was given in Table 1. *T. urticae* was reared, as the target organisms, on bean plants at $25\pm 1^\circ\text{C}$ and $60\pm 5\%$ relative humidity (RH) with a 12:12h (L:D) photoperiod.

Conidial suspension and viability

The isolates were cultured in Potato Dextrose Agar (PDA) containing 2% yeast extract and kept for two weeks at 25°C for conidial production. Conidia were suspended in sterile distilled water, a surfactant (0.2ml/l Tween 80) was added to reduce clumping of the conidia, and the suspension was vortexed to produce a homogenous state (Belczewski & Harmsen 2000). The prepared suspensions were then filtrated through three layers of muslin to eliminate hyphae and unsuspended conidia. The spore concentrations were determined using a haemocytometer and suspensions were prepared in logarithmic series from 1×10^6 to 1×10^8 (Eken & Hayat 2009).

Conidial viability was tested in Sabouraud Dextrose Agar (SDA) plates. Droplets were taken from suspensions in sterile conditions and spread over the SDA medium. After 24h, percent germination was determined from 100 spore counts on each plate (Ekesi *et al.* 2000). Only conidia with a germ tube as long as the conidium's width were considered to be germinated. Spore suspensions were sealed with Parafilm and kept at 4°C in a refrigerator until use (Safavi *et al.* 2010).

Bioassays

Effect of conidial concentration on pathogenicity

Three different preparations, 1×10^6 , 1×10^7 and 1×10^8 , each supplied with 0.02% Tween 80, were tested. 2ml suspension of a single dose was sprayed onto down side of bean leaf discs (30mm diameter) using a hand sprayer. The leaf discs were air dried and placed in Petri dishes (Quesada-Moraga *et al.* 2006). Distilled water containing 0.02% Tween 80 was used as control. Twenty vigorous adult mites arbitrarily taken from the reared population were transferred to each disc by means of a fine soft brush (Kady *et al.* 2007). After exposure, all petri dishes were sealed with Parafilm and the lids were provided with holes of 6 mm diameter for proper ventilation.

All petri dishes were incubated at $25\pm 1^\circ\text{C}$ and $65\pm 5\%$ RH. Mortality was recorded on daily basis for a period of 5 days. Dead mites were surface sterilized in 70% ethanol, dried and transferred to Petri dishes lined with moist filter paper for 10 days to observe mycosis. Mortality caused by fungi was confirmed by microscopic examination (Cherry *et al.* 2005).

Table 1. History of the isolates used in this study.

Host	Order	Application	Dose (conidia/ml)	Mortality (%) (5 th day after application)			Reference
				PaF04	PaF09	PaF76	
<i>P. abietina</i> larvae	Hymenoptera	Immersing	10 ⁶	48.2±8.9b	80±6.4a	72.8±7.7ab	(Albayrak Iskender <i>et al.</i> 2017)
<i>H. cunea</i> larvae	Lepidoptera	Immersing	10 ⁶	96.66±3.33a	90±5.77a	96.66±3.33a	(Albayrak Iskender <i>et al.</i> 2012)
<i>D. micans</i> larvae	Coleoptera	Immersing	10 ⁹	100a	100a	100a	(Albayrak Iskender <i>et al.</i> 2013)

In-vitro enzyme plate assays

B. bassiana isolates were tested for qualitative analysis of chitinase, protease and lipase enzymes by measured clear zone. For analysis of the lipolytic activity, the medium described by Sierra (1957) was used. Olive oil was used as the lipid substrate. For analysis of the proteolytic activity, casein soluble medium with milk powder was used. For the chitinolytic activity, medium-5 as described by Abd-Aziz *et al.* (2008) was used with addition of agar. An agar disk (6mm in diameter) from 7-old day fungal culture was placed in the center of these agar plates and the inoculated plates were incubated at 28°C for 10 days. Clear zones formed around the colonies indicated degradation of the substrate due to enzymatic activity. Zone formations in the protease activity were enhanced by flooding the plates with 10% glacial acetic acid.

Statistical analysis

The experimental design was a randomized complete block with three replicates, and each replicate consisted of 20 mites. The analysis of variance was conducted using one-way ANOVA test using SPSS 13.0. LT50 was determined graphically for mortality (Alves *et al.* 2005).

Results

This study determined pathogenicity of *B. bassiana* against *T. urticae* under laboratory conditions. In viability tests, 95–100% of the spores germinated and all tested fungal isolates were pathogenic to the *T. urticae*, causing a mortality rate between 64.2±5.6% and 100% after 5 days from application. Microscopic investigations confirmed mycosis for all dead mites, and *B. bassiana* was reisolated from all dead individuals. LT50 values varied between 3.16±0.2 and 3.72±0.5 days. Mortality caused by PaF04 isolate was found to be significantly different ($p<0.05$) between all preparations. For this isolate, the lowest *B.*

bassiana concentration (1×10^6 conidia/ml) gave less control (80.7±4.2%) of the mites until the 5th day after inoculation. By comparison, the intermediate concentration (1×10^7 conidia/ml) caused a mortality with 92±3.5%, and the best results were obtained with the highest conidial concentrations (1×10^8 conidia/ml) with which a high control (100%) of the mites were achieved. PaF04 isolate was consistently more virulent than PaF09 and PaF76 isolates for all preparations. PaF09 isolate showed a virulence similar to that of PaF76 ($p<0.05$) except for 1×10^7 conidia/ml. LT50 values of these three isolates changed between 3.16 and 3.72 days (Table 2).

Discussion

B. bassiana isolate PaF04 was consistently more virulent than PaF09 and PaF76 isolates for all preparations. Mortality of the target organism *T. urticae* was found to increase with time (data not shown) and was dependent on spore concentration. PaF04 isolate led to the shortest LT50 value with 3.16 days. This value was significantly ($P>0.05$) shorter than LT50 values obtained with PaF09 and PaF76 isolates. Albayrak Iskender *et al.* (2012) compared the virulence of the same isolates against *H. cunea* larvae and found that their virulence was similar ($P>0.05$). Furthermore, Albayrak Iskender *et al.* (2017) reported that these isolates were pathogenic against *P. abietina* larvae as well, but the levels of virulence were different among the isolates ($P>0.05$).

They also reported that PaF09 isolate was highly virulent to *P. abietina* larvae, and all PaF04, PaF09 and PaF76 isolates possessed a good potential against *H. cunea* and *D. micans* larvae. Nevertheless, in our study, only PaF04 isolate was shown to have a potential to control *T. urticae*. Our results which indicated a poor potential for PaF09 and PaF76 against *T. urticae* might

Table 2. Mortality of *T. urticae* within 5 days after application of fungal isolates and the enzyme activities of the isolates in terms of clear zone evaluations on agar plates ($p<0.05\pm SE$)*.

65±5%RH				LT50 (days)	Enzyme activity (mm)		
Conidial suspension					Lipase	Protease	Chitinase
Isolates	1×10 ⁶	1×10 ⁷	1×10 ⁸				
PaF04	80.7±4.2 ^a	92±3.5 ^a	100 ^a	3.16±0.2	ND**	4.5±0.5 ^a	6±2 ^a
PaF09	67.6±6.3 ^b	83.4±2.2 ^b	89.8±3.3 ^b	3.52±0.3	2±0.4 ^c	5±0.5 ^a	1±0.5 ^c
PaF76	64.2±5.6 ^b	77.8±4.2 ^c	90.1±1.1 ^b	3.72±0.5	5±1 ^a	4±0.7 ^a	ND**

* The differences between the values indicated by the same letters (used as superscript) in the same column are not statistically significant ($p\leq0.05$),

**ND: Not determined.

be due to the experimental method employed, to the type of the host and host stage because all these parameters are known to affect the results of bioassays (Mascarin *et al.* 2013).

When the lipase and protease activities values of PaF76 isolate were compared, the clear zone for lipase activity was (5 ± 1 mm) appeared to be higher than its protease-originated clear zone (4 ± 0.7 mm). The obtained results indicated that mortality effect of PaF76 on *T. urticae* was too low. Extracellular lipases are involved in microbial virulence and play different roles in infection processes (Stehr *et al.* 2003). Khan *et al.* (2012) reported that lipase was the most important enzyme for virulence as compared to chitinase and protease. The differences of the hosts and isolates used might be the factors leading to differences in mortality results obtained (Clarkson & Charnley 1996). In addition, the developmental stage of the host organism might also be a reason of differences in mortality. PaF04 isolate possessed the highest chitinase activity. This isolate was consistently more virulent than PaF09 and PaF76 isolates in this study. Previous studies have shown that enzymatic activities based on chitinase and protease played important roles in mortality of pest in the presence of entomopathogens (St Leger *et al.* 1996, Campos *et al.* 2005). Similarly, Khan *et al.* (2012) reported that protease was found to be effective in the pathogenicity of *B. bassiana*. We therefore conclude that PaF04 isolate could be used as potential control

agent on a variety of target organisms, considering its broad host range.

It is well known that host specificity has been a critical issue in biological control studies and the first step in the selection of biological control agent candidate. Furthermore, narrow host range can be a limiting factor for the commercialization of entomopathogens (Waage 2001, Brodeur 2012). Entomopathogens such as *B. bassiana* used in biological control has the capacity to exploit a wide range of hosts (Charnley & Collins 2007, Bugeme *et al.* 2008).

The three *B. bassiana* isolates used in the present study are fungal agents which have previously been to be pathogenic against various insects (for *P. abietina* larvae see Albayrak İskender *et al.* 2017, for *H. cunea* larvae see Albayrak İskender *et al.* 2012, and for *D. micans* larvae see Albayrak İskender *et al.* 2013). Here, we showed their pathogenicity potentials on the agricultural pest *T. urticae* based on qualitative analysis of their enzyme activities which could be related to pathogenicity (Feng & Johnson 1990). Our findings are important to fundamentally understand the host-fungus relationships and may also be useful for selection of these organisms for biological control applications. In conclusion, PaF isolates of *B. bassiana* were proven to have a potential to control Coleoptera, Lepidoptera, and Hymenoptera as well as *T. urticae*.

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