Pathogenicity of indigenous strains of three entomopathogenic fungi to the sisal weevil, *Scyphophorus acupunctatus* (Gyllenhal) (Coleoptera: Curculionidae)

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Summary The pathogenicity of indigenous isolates of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* was evaluated in the laboratory against larvae and adults of the sisal weevil, *Scyphophorus acupunctatus*. Inoculation was achieved via immersion of individuals into conidia suspensions of different concentrations. All three fungal species proved high pathogenicity against larvae of the weevil, causing 100% mortality in most of the treatments. *Beauveria bassiana* caused the highest mortality of the adults ($86.67 \pm 12\%$), followed by *M. anisopliae* ($46.67 \pm 17.8\%$) and *I. fumosorosea* ($40 \pm 17.5\%$). Mean survival time also differed significantly among treatments and life stages of the weevil. In total, larvae survived significantly fewer days than adults post infection. Results of the present study indicate the potential of indigenous strains of entomopathogenic fungi as biological control agents against the invasive weevil.

Additional keywords: agave, *Beauveria bassiana*, Curculionidae, *Isaria fumosorosea*, *Metarhizium anisopliae*

Introduction

The sisal weevil, *Scyphophorus acupunctatus* (Gyllenhal), is amongst the most severe pests of both cultivated and ornamental agave plants (Pott, 1975; Valenzuela-Zapata, 1994; Camino Lavin *et al.*, 2002). In its place of origin, the Nearctic region, it has been found to cause severe economic losses by strongly damaging *Agave tequilana* (Weber) the tequila producing agave (Solis *et al.*, 2001). The sisal weevil was introduced in Europe around 1980, when it was first recorded on imported *Yucca* sp. in the Netherlands (van Rossem *et al.*, 1981). Since then it has been found in many European countries (i.e. Italy, France, Greece) on ornamental plants (Colombo, 2000; EPPO 2008; Kontodimas and Kallinikou, 2010).

The females use the basal parts of the agave leaves for feeding and as oviposition sites. Furthermore, once the neonate larvae hatch they start to bore galleries within the plant's head and form a cocoon made

by plant's fibers to complete their development (Lock, 1965). In addition to the destructive activities of the larvae, the adults are often natural vectors of plant pathogenic bacteria, such as *Erwinia carotovora* (Dye) (Aquino Bolanos *et al.*, 2011). In Central and South American agave cultivations chemical insecticides are commonly applied to control the sisal weevil, however there are many problems and controversies connected to their use (Solis *et al.*, 2001; Terán-Vargas *et al.*, 2012). Firstly, as the weevil completes its development within a protected habitat, inside the agave leaves, insecticides have a low effectiveness (Figueroa-Castro *et al.*, 2013). Secondly, their extended application harbors the risk of resistance development in the pest species (Terán-Vargas *et al.*, 2012). Finally, there are certain restrictions in the use of synthetic insecticides in urban landscape areas, where the weevil is mainly found, due to their effects on the environment and health risks. Therefore, the development of biological and biotechnical control methods is of great environmental and economic importance.

Although a few arthropod natural enemies of the sisal weevil could potentially be regarded as biological control agents in its

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site of origin (Velazquez *et al.*, 2008), these arthropods are not found in the invaded regions. Entomopathogenic fungi and nematodes have been reported as potential biological control agents of the weevil (Hueso *et al.*, 2005; Velazquez *et al.*, 2008). Both the fungi and the nematodes are of great importance for the biological control of *S. acupunctatus*, as they are able to overcome the barrier of the concealed environment in which the weevil develops. While the nematodes are able to move within the agave leaves and are easily able to reach the concealed pest (Dembilio *et al.*, 2010a), the entomopathogenic fungi infect their hosts by direct contact and subsequent penetration of their cuticle (Dembilio *et al.*, 2010b). This makes insects susceptible both to direct contact with the pathogen, but also to passive, horizontal transmission, between healthy and infected individuals vectoring spores (Klein and Lacey, 1999; Quesada-Moraga *et al.*, 2004).

It has been widely accepted that entomopathogenic fungi show high intra-specific diversity, which results in different levels of pathogenicity among different strains of the same species (Ferron, 1978). Furthermore, the virulence of a specific strain to specific insect genera has been proved to depend upon the host insect identity from which it was isolated (Fargues and Robert, 1983; Maurer *et al.*, 1997). This is attributed to the strong selective pressure of the host insect environment on the pathogen (Roy *et al.*, 2006) through which the origin of the entomopathogens could affect their pathogenicity against pests (Cherry *et al.*, 2005).

Despite the importance of alternative methods for the control of the sisal weevil, research on the evaluation of potential biological control agents of the pest is very limited (Molina-Ochoa *et al.*, 2004; Valdes Estrada *et al.*, 2014). In the present study we aim to evaluate the virulence of locally isolated strains of *Beauveria bassiana* (Balsamo), *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Isaria fumosorosea* (Wize) to adults and larvae of the introduced weevil *S. acupunctatus*. It is expected that the use of in-

digenous isolates of fungal entomopathogens is advantageous, as they are already adapted in specific environmental conditions and could be part of a conservation biological control strategy.

Material and Methods

Entomopathogenic fungi

All entomopathogenic fungi strains used in the laboratory assays were obtained from the entomopathogenic fungi collection of the Benaki Phytopathological Institute. The strain of *B. bassiana* IBB 010 was initially isolated from a *Rhynchophorus ferrugineus* (Olivier) cadaver, found in Ellinikon region (Athens, Greece). Both strains IBB020 of *I. fumosorosea* and TMB04 of *M. anisopliae* were isolated from soil samples with the *Galleria* bait method (Zimmerman, 1986) from the Agios Stefanos and Marathon regions (Attica, Greece), respectively. The molecular identification of the strains of *I. fumosorosea* and *M. anisopliae* was previously conducted by Beris *et al.* (2013). The fungal isolations were cultured on Sabouraud dextrose agar (SDA) at 25°C, under dark regulated conditions. Spore (conidia) suspensions were prepared by scraping the surface of 2 week old cultures into an aqueous solution of 0.2% Tween 80, after which the suspensions were vortexed for 60 s and filtered twice using a sterile nylon membrane to remove mycelium. Subsequently, the concentration of the spore suspensions was determined by the use of a standard haemocytometer. The concentrations of spore suspensions used in bioassays were 2.12×10^7 and 2.12×10^6 spores/ml for *B. bassiana*, 1.27×10^7 and 1.27×10^6 spores/ml for *M. anisopliae* and 7.45×10^6 spores/ml for *I. fumosorosea*. Spore concentrations are not equal due to the different developmental rate of each fungus (Kontodimas and Gkotsi, 2009). Spore viability measured over 96% for all selected strains.

Insects

Both adult and larvae of the sisal weevil were collected from ornamental aga-

ve plants, *Agave americana* L., in the urban landscape area of Ardittos hill (Athens, Greece). The infestation of the agave plants was spotted in the summer of 2012 and no action for controlling the pest was taken prior to the collection of the weevils. The adults were collected by hand at site, while whole plants were cut from the basis and taken to the laboratory for the isolation of 4th instar larvae. The adults were kept in polyester cages (30 x 30 x 30cm) and the larvae in plastic boxes (20 x 20 x 20cm), with an opening covered by mesh for ventilation. All insects were kept at controlled laboratory conditions (25±1°C, 65±5% R.H. and 14:10 L:D) and fed on a natural diet (agave slices; twice per week) until they were used in the bioassays.

Bioassays

The application of the spore suspensions was achieved through direct cuticle contact. Groups of five, 4th instar larvae and adults were immersed in the prepared aqueous suspensions for 30 sec after which each group of insects was transferred in 1L rearing boxes and provided with fresh natural diet. The treatment was repeated 15 times (replicates) for each fungus strain and concentration per life stage. Furthermore, a control treatment was included for both adults and larvae, where insects were immersed into an aqueous solution of 0.2% Tween 80. All bioassays were carried out in controlled environment conditions (25±1°C, 65±5% R.H. and 14:10 L:D). The mortality was recorded daily for up to 11 and 21 days for larvae and adults, respectively. When a weevil died, the cadaver was transferred individually to a Petri dish lined with a moistened sterile filter paper to ascertain the involvement of the entomopathogens to the death of weevils. The Petri dishes were sealed with parafilm and kept at room temperature, in darkness, to monitor for external signs of fungal infection.

Statistical analyses

General Linear Model (GLM) with a binomial distribution was used to obtain Analysis of Deviance for a level of significance

$\alpha=0.05$. The analysis included life stage (larval and adult), species of fungi (*B. bassiana*, *I. fumosorosea* and *M. anisopliae*), and concentration of spore suspensions (10^6 and 10^7 spores/ml) as variables. Additionally, the average survival times were calculated and compared by the Kaplan-Meier survival analysis (Kaplan and Meier, 1958). All the GLM procedures and survival analysis were carried out in R (v. 2.1; R Foundation for Statistical Computing, Vienna, AT).

Results

The examined strains of entomopathogenic fungi caused different levels of mortality to *S. acupunctatus*. The mean mortality of weevils differed significantly at different life stages, and fungus species, but it did not differ between different concentrations of spore suspensions ($X^2=22.939$, $df=1$, $P<0.0001$; $X^2=9.5758$, $df=2$, $P<0.0001$ and $X^2=2.5984$, $df=1$, $P=0.1070$, respectively). No mortality was recorded for adult weevils or larvae when treated with the aqueous solution of 0.2% Tween 80 (i.e. control treatment). Interactions among the variables did not affect the mortality levels, thus, they were omitted from the initial model.

All treatments with fungi resulted in almost all concentrations at 100% mortality of larvae (Figure 1a). Mortality in adults depended on fungus species but not on the concentration of the suspensions ($X^2=11.4223$, $df=2$, $P=0.0033$ and $X^2=2.9638$, $df=1$, $P=0.0851$, respectively). The treatment with *B. bassiana* at the concentration of 2.12×10^7 spores/ml caused the highest mortality to adult weevils (86.67±12%) compared to *M. anisopliae* and *I. fumosorosea* treatments (Figure 1b). The lowest mortality levels to adults were caused by *M. anisopliae*, when applied at the low spore concentration (26.67±15.8%).

The examined strains of *B. bassiana*, *M. anisopliae* and *I. fumosorosea* developed external fungal growth and sporulated on all treated cadavers of both adults (Figure 2) and larvae, regardless the spore concentration.

Figure 1. Mean mortality $\pm$ SE of (A) larvae and (B) adults of *Scyphophorus acupunctatus* after immersion into spore suspensions of entomopathogenic fungi.

*Different letters within the same chart indicate significant differences (LR test, $\alpha=0.05$).

Figure 2. Development of external fungal mycelium on cadavers of *Scyphophorus acupunctatus* adults: *Beauveria bassiana* (a), *Metarhizium anisopliae* (b) and *Isaria fumosorosea* (c).

The mean survival time of the treated larvae differed significantly at different fungi species and different concentrations, namely the low concentration of *B. bassiana* (6.80 ± 0.251 days) compared to the other treatments (4.73 - 5.67 days) ($\chi^2=8.3$, $df=4$, $P=0.0807$; Table 1).

The mean survival time differed significantly for adult weevils that received different treatments ($\chi^2=17.5$, $df=4$, $P=0.0016$; Table 1). Adult weevils treated with the high concentration of *B. bassiana* exhibited the shortest survival time (10.20 ± 1.055 days) (Table 1). Overall, the survival time of larvae was significantly shorter than that of adults ($\chi^2=105$, $df=1$, $P<0.001$).

Discussion

All species of fungi proved to be highly effective on larvae of *S. acupunctatus*. Further wider dose range tests would be necessary to show the dose effect, the differences among strains and the potential of the tested strains in lower concentrations. However, our results showed that the examined strains of entomopathogenic fungi are able to infect larvae and adults of the sisal weevil.

Although all strains managed to fully complete their life cycle by sporulating on *S. acupunctatus* cadavers, *B. bassiana* proved to be the most pathogenetic to adult weevils. Several studies have pointed out the relation between strains of entomopathogens isolated from specific hosts and the high virulence towards the host genera (Fargues and Robert, 1983; Maurer et al., 1997). Therefore, the high virulence of the strain IBB010 of *B. bassiana* might be related to its source of isolation, an infected red palm weevil cadaver, as both the red palm weevil and the sisal weevil belong to the same tribe of Rhynchophorini.

Until now, *B. bassiana* is the only entomopathogenic fungus species mentioned as a natural enemy of the weevil in its place of origin (Aquino Bolanos et al., 2011). Our results showed that the concentration of 2.12×10^7 spore suspension of *B. bassiana* caused a quicker death of adults compared to spore suspensions of *M. anisopliae* and *I. fumosorosea*. Moreover, a 10-fold increase of the diluted spore suspension of the *B. bassiana* strain from 2.12×10^6 to 2.12×10^7 decreased the mean survival time for adults by almost 4 days.

The sisal weevil has identical life hab-

Table 1. Mean survival time $\pm$ SE (days after treatment) for larvae and adults of *Scyphophorus acupunctatus* treated with *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* spore suspensions.

Treatment	Concentration (spores/ml)	Mean survival time $\pm$ SE (days)	
		Larvae	Adults
<i>B. bassiana</i>	2.12×10^7	5.47 ± 0.351^a	10.20 ± 1.055^a
	2.12×10^6	6.80 ± 0.251^b	14.74 ± 0.999^b
<i>M. anisopliae</i>	1.27×10^7	5.00 ± 0.359^a	15.65 ± 1.031^{bc}
	1.27×10^6	5.67 ± 0.308^{ab}	17.20 ± 1.210^c
<i>I. fumosorosea</i>	7.45×10^6	4.73 ± 0.314^a	17.50 ± 1.025^c

* Mean survival times within columns followed by the same letter are not significantly different (LR test, $\alpha=0.05$)

* Mortality was recorded for up to 11 and 21 days for larvae and adults, respectively

its with the red palm weevil and its development is completed within its host plant, adults are the only exposed stage to entomopathogenic fungal treatments (Gindin *et al.*, 2006; Dembilio *et al.*, 2010b). Therefore, the longer survival of an infected host increases the possibility of interacting with other individuals and probably of transmitting the pathogen. In this sense, a lower spore concentration of *B. bassiana* might be more advantageous in a field application, especially because our study showed that the mean mortality did not differ significantly between the two concentrations. However, other aspects of the application of these entomopathogenic fungi are not yet fully studied. For example, potential horizontal transmission between adults or from adults to larvae and vertical transmission from infected adults to their offspring should be elucidated (Glare *et al.*, 2002; Gindin *et al.*, 2006; Dembilio *et al.*, 2010b). Furthermore, *M. anisopliae* and *B. bassiana* have been found to reduce the fecundity of *R. ferrugineus* (Gindin *et al.*, 2006; Dembilio *et al.*, 2010b). If a similar effect can be proved on *S. acupunctatus*, then even strains that caused lower levels of mortality might be a useful tool in the control of the weevil.

Another important finding of the present study is the high mortality of larvae caused by all the strains tested. In most of the cases, mortality in the larval stage reached 100%. Although our methodology of immersing the larvae differs considerably

from the natural infection, such high mortality levels in controlled conditions support these strains as candidate biological control agents. While the larvae of the weevil are the most destructive stage they are also the most concealed life stage (Camino Lavin *et al.*, 2002; Lopez-Martinez *et al.*, 2011), challenging the application of the entomopathogenic fungi. It is, however, possible that entomopathogenic fungal endophytes are effective against larval stages of the weevil. Among many others, the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *I. fumosorosea* have been reported as endophytes of several plants (Gomez-Vidal *et al.*, 2006; Quesada-Moraga *et al.*, 2006; Vega, 2008) and attempts have been made to introduce entomopathogens into host plants for the control of pests (Gomez-Vidal *et al.*, 2006; Posada *et al.*, 2007; Valdes Estrada *et al.*, 2014). If the strains studied here could be introduced into agave plants they could possibly act as successful biocontrol agents due to their high pathogenicity against larvae.

In conclusion, our results prove that the strain IBB 010 of *B. bassiana* is highly pathogenic to both adults and larvae of *S. acupunctatus* and could be considered as a biological control agent of the weevil. Additionally, application of lower concentrations of IBB 010 was shown to prolong the adults' survival time, without reducing the mortality levels. This should be taken into consideration, as a positive effect for strategies

based on attracting and infecting adults. Strains IBB020 and TMB04 of *I. fumosorosea* and *M. anisopliae*, respectively, caused lower mortality levels to adults, but were equally effective on larvae. Hence, they could be considered as potential control agents into an IPM plan against the pest.

Despite the lack of field data on the effectiveness of these strains, to the best of our knowledge, this is the first attempt of screening locally isolated entomopathogenic fungi as biological control agents against the introduced *S. acupunctatus*. Further research in field applications and possibility of transmission is needed in order to strengthen current findings.

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Παθογένεια ιθαγενών στελεχών τριών εντομοπαθογόνων μυκήτων επί του εντομολογικού εχθρού της αγαύης *Scyphophorus acupunctatus* (Gyllenhal) (Coleoptera: Curculionidae)

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Περίληψη Η παθογένεια ιθαγενών απομονώσεων των εντομοπαθογόνων μυκήτων *Beauveria bassiana*, *Metarhizium anisopliae* και *Isaria fumosorosea* αξιολογήθηκε στο εργαστήριο εναντίον προνυμφών και ακμαίων του εντομολογικού εχθρού της αγαύης *Scyphophorus acupunctatus*. Ο πειραματισμός διεξήχθη με εμβάπτιση απόμων του εντόμου σε διαφορετικές συγκεντρώσεις εναιωρημάτων κοινιδίων των μυκήτων. Και τα τρία είδη μυκήτων έδειξαν υψηλή παθογένεια εναντίον των προνυμφών του σκαθαριού, προκαλώντας 100% θνησιμότητα στις περισσότερες επεμβάσεις. Ο μύκητας *Beauveria bassiana* προκάλεσε την υψηλότερη θνησιμότητα στα ακμαία ($86.67 \pm 12\%$), ακολουθούμενος από τον *Metarhizium anisopliae* ($46.67 \pm 17.8\%$) και τον *Isaria fumosorosea* ($40 \pm 17.5\%$). Παρατηρήθηκαν στατιστικά σημαντικές διαφορές στο μέσο όρο του χρόνου επιβίωσης μεταξύ των επεμβάσεων και των βιολογικών σταδίων του σκαθαριού. Σε όλες τις επεμβάσεις οι προνύμφες επιβίωσαν σημαντικά λιγότερες ημέρες συγκριτικά με τα ακμαία. Τα αποτελέσματα της παρούσας μελέτης δείχνουν τη δυνατότητα χρήσης αυτών των ιθαγενών στελεχών ως παράγοντα βιολογικής αντιμετώπισης του *S. acupunctatus*.

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