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EVALUATION AND ENVIRONMENTAL TESTING OF ENTOMOPATHOGENIC FUNGI FOR THEIR EFFECTIVENESS AS BIO-CONTROL AGENTS OF MAJOR VINEYARD PESTS

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ABSTRACT

Entomopathogenic fungi (EPF) are considered as important biological control agents of insect pests. They present many advantages in pest control including environmental safety, selective action and ability to infect their host through the cuticle via contact. However, they are able to be pathogenic against insect pests only under a narrow spectrum of environmental conditions. Several biotic and abiotic factors affect viability and pathogenicity of EPF. The effects of temperature, relative humidity (RH) and thermal stress on *Beauveria bassiana* and *Paecilomyces fumosoroseus* were evaluated in the present assay. Temperature significantly influenced the percentage of spore germination of the fungi. The optimum temperature for conidial germination of both the fungi was 25˚C while *B. bassiana* germinated better in temperature values as high as 30˚C. Spore germination was also significantly affected by the exposure of conidia to low (3˚C) or high (40˚C) temperatures for 1 hour (thermal stress). In this case, *B. bassiana* was negatively affected by the exposure to low temperatures while *P. fumosoroseus* performed adequately. Relative humidity influenced both spore germination and the pathogenic ability of the fungi to cause infection. Third instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) were treated with conidial suspensions to evaluate pathogenicity. *G. mellonella* is considered as an insect vulnerable to infections by microbial pathogens and as such, it can be used as a model organism to estimate behavioural responses of other insects caused by EPF. Evaluation of EPF was aimed to provide important data for present and future studies regarding biological control of vineyard pests such as *Lobesia botrana* (Lepidoptera: Tortricidae) and *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), as well as information about the potential role of EPF within vineyards' micro-ecosystems.

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INTRODUCTION

The European grapevine moth (*Lobesia botrana*) is considered as the most damaging pest of vineyards in the Mediterranean basin and elsewhere, infesting flowers and fruits of *Vitis vinifera* L. (Andreadis et al., 2005; Moreau et al., 2010). In addition to the significant

direct losses, the action of *L. botrana* has been reported to enhance secondary infections by *Botrytis cinerea* and several more fungi (Cozzi et al., 2006; Delbac et al., 2010). The insect has 2-4 generations per year (3 in most viticultural regions). Direct damages caused by *L. botrana* as well as the subsequent fungal infections, have a substantial effect on the quantity and quality of the produced grapes and wines (Ioriatti et al., 2011). Control methods include the mating disruption technique, the use of egg parasitoids, synthetic pesticides and the bacterium *Bacillus thuringiensis*. The vine weevil (*Otiorhynchus sulcatus*) is another economically important viticultural pest. It is native to Europe but is also commonly found in North America. Its feeding activity can destroy primary buds and new shoots during spring, causing substantial damages, especially in newly established vineyards. Its control usually involves azadirachtin (neem oil), imidacloprid, spinosad and pyrethrins but the potential effects of entomopathogenic microbes against the insects have not been adequately investigated. The spotted wing drosophila (*Drosophila suzukii*) and mealybugs (*Planococcus ficus* and *P. citri*) are also considered as important vineyard pests that could be possibly controlled by the action of entomopathogenic organisms. Weather conditions such as temperature, humidity and sunlight significantly affect growth and the ripening process of grapevines. Therefore, such conditions influence the phenological stages of *V. vinifera* and the chemical characteristics of the vines. Furthermore, environmental factors influence life cycle, feeding activity and breeding of the major viticultural pests (Reineke and Selim, 2019).

Entomopathogenic fungi (EPF) are organisms known to be pathogenic to insects and as such, they can provide effective control of the populations of important agricultural pests. They have been reported as substantial bio-control agents against economically important pests in many crops including tree orchards and vineyards. It has been estimated that over 750 species of fungi, belonging to more than 90 genera, present entomopathogenic innate qualities (Rajula et al., 2020; Roy et al., 2010). EPF are found abundantly in most of the soils. Among various genera and species, some fungi appear as non-selective pathogens while others present host-specific properties (Vukicevich et al., 2020). EPF, among other advantages, can infect their hosts via contact and they do not require consumption-digestion by the insect to cause infection. Moreover, the extensive use of synthetic insecticides for controlling *L. botrana* has significant environmental, economic and public health impacts. Many agricultural pests also tend to develop resistance to insecticidal chemical compounds (Civolani et al., 2014) while some insecticides can harm indigenous yeast strains and affect fermentation (Caboni and Cabras, 2010).

Several biotic and abiotic factors influence viability and pathogenicity of all microbial entomopathogenic organisms. In particular, entomopathogenic fungi are considered as organisms highly dependent on environmental parameters and can provide effective control of pests only under a narrow window of climatic conditions (Copping, 2001; Lord, 2005; Membang et al., 2021). Temperature, relative humidity, solar radiation and wind are considered as the most important environmental factors influencing the efficacy of entomopathogenic fungi against pests, while these factors usually interact with each other regarding their impact on entomogenous organisms (Hong et al., 2002; Inglis et al., 2001). Temperature and humidity conditions on the surface of leaves, flowers and fruits may differ substantially from ambient conditions. However, knowledge related to the optimum conditions for the growth of entomogenous fungi as well as determination of the limits in which they can survive, have been subjects of high scientific interest.

The effects of temperature and water availability on growth of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* have been examined by Hallsworth and Magan (1999). Liu et al. (2003) presented results indicating temperature dependence of conidial germination and hyphal growth. The effects of temperature on 65 isolates of *B. bassiana* were determined by Fargues et al. (1997). Wraight et al. (2000) studied the effects of temperature and ambient humidity on the entomopathogenic fungi *B. bassiana* and *P. fumosoroseus* for the control of whiteflies and Inglis et al. (1996) investigated the effects of temperature and thermoregulation on mycosis by *B. bassiana* in grasshoppers. Moreover, Vu et al. (2007) evaluated the effects of temperature and relative humidity as important parameters for the selection of EPF isolates targeting effective control of aphids *Myzus persicae* and *Aphis gossypii*. Thermal stress can significantly influence germination, viability and pathogenicity of entomogenous fungi. The effects of stress caused by high temperature or excessive water availability to entomopathogenic fungi

were studied in India (Devi et al., 2005).

The accomplishment of the following experiments attempts to provide data and knowledge about the effects of temperature, relative humidity and thermal stress on germination, viability and virulence of entomopathogenic fungi, as these factors must be well-considered prior to the application of EPF in viticulture and other crops.

MATERIALS AND METHODS

Effects of temperature on spore germination of *B. bassiana* and *P. fumosoroseus*

Germination percentages of the isolates *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01, under multiple temperature values, were determined in this assay. Those isolates were selected for the experiments as representatives of the two tested species. The origin of the isolates was also subject of scientific interest as *B. bassiana* IMI-391044 was isolated from a hot habitat (Syria) while *P. fumosoroseus* EBAC-01 was isolated, in a relatively cold region (Reading, UK), using the *Galleria* bait method (Zimmermann, 1986). Both the fungi were initially grown on half-strength PDA agar for 15 days in the dark. The spores were then harvested to make suspensions of 1×10^5 conidia/ml. Spores were harvested by scraping them from the surface of the plates using a microscope slide (Beris et al., 2013). A sterile liquid solution of distilled water with 0.1% Tween 80 was poured into the dishes to carry away most conidia. The liquid suspension containing conidia was stirred using a magnetic stirrer and filtered twice using a sterile nylon membrane (Liu et al., 2003). The spore concentration in the resulting suspension was determined using standard (improved Neubauer) haemocytometers. Simultaneously, half-strength SDA Petri dishes (9 cm Ø) were prepared under sterile conditions. After suspensions were made and agar plates cooled, 100 μl of liquid suspension was added on each plate using a micropipette. Then, it was spread throughout the surface of the plate using a microscope slide. Subsequently, plates were covered with their lids and sealed. Plates were incubated in plant growth chambers (MLR-351H, Sanyo, Osaka, Japan) at 20, 22.5, 25, 27.5, 30 and 32.5ºC in the dark. Conidia were counted every 12, 18, 24 and 30 hours after initial placement in the incubators.

Spore germination was calculated by counting the number of germinated conidia out of 100 conidia measured. For each isolate-temperature combination, two sets of 100 conidia were measured on each plate. Five plates (replicates) were designed for each combination. All measurements were taken under a microscope (400×) and all conidia with visible germ tubes of any length were counted as germinated.

Statistical analyses were conducted using the statistical package SPSS 17.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by the Post-hoc tests Dunnett T3 and Games-Howell were used to compare values. Levene's statistic indicated that variances were not homogenous and therefore, Dunnett T3 and Games Howell were the appropriate Post-hoc tests to determine the significant differences between groups. The time that 50% of conidia required to germinate (GT_{50}) was calculated using Probit analysis.

Effects of thermal stress on spore germination of *B. bassiana* and *P. fumosoroseus*

In this bioassay, the influence of different types of thermal stress on conidial germination of entomopathogenic fungi was investigated. Again, the isolates *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01 were tested. Fungi were grown on half-strength SDA agar for 20 days in darkness. The spores were harvested to produce suspensions of 1×10^5 conidia/ml (in sterile distilled water with 0.1% Tween 80). In addition, new half-strength SDA Petri dishes were prepared (Zimmermann, 2007). After suspensions were made and agar plates were cooled, 100 μl of liquid suspension was added on each plate using a micropipette. It was then spread throughout the surface of the plate using a microscope slide. Subsequently, plates were covered with their lids and sealed. Thermal stress was applied to conidia in four ways: 1) Initial exposure at 40ºC (I40), where conidia were kept for the first hour of incubation at 40ºC and then at 25ºC for 18 hours. 2) Initial exposure at 3ºC (I3) where conidia were placed for the first hour of incubation at 3ºC and then remained for 18 hours at 25ºC. 3) Mid-time exposure of conidia at 40ºC (M40), where conidia were incubated for 9 hours at 25ºC then turned to 40ºC for one hour and then again to 25ºC for 9 more hours. 4) Mid-time exposure of conidia at 3ºC (M3), where conidia were incubated for 9 hours at 25ºC then turned to 3ºC for one hour and then again to 25ºC for 9 more hours. Moreover, control plates of both the fungi were kept at 25ºC for 19 hours. This bioassay was carried out by programming the plant growth chambers to the above time-temperature combinations. All trials were conducted in

darkness. Conidial germination was counted 19 hours after the initial placement of plates into the incubators.

Spore germination was calculated by counting the number of germinated conidia out of 100 conidia measured. Two sets of 100 conidia were measured on each plate. Five plates (replicates) were designed for each treatment. All measurements took place under a microscope (400×) and all conidia with visible germ tubes of any length were counted as germinated.

Statistical analyses were conducted using the statistical package SPSS 17.0. One-way analysis of variance (ANOVA) was used to show if there were significant differences among groups. In the case of *P. fumosoroseus*, variances were not assumed homogenous (Levene's test) and therefore, the Post-hoc tests Dunnett T3 and Games-Howell were used to determine significant differences among treatments, while in the case of *B. bassiana* variances were proven to be homogenous, therefore, LSD and Tukey HSD Post-hoc tests were used.

Effects of relative humidity on spore germination of *B. bassiana* and *P. fumosoroseus*

In this study, conidial germination of the isolates *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01 was assessed at different RH values. Fungi were grown on half-strength PDA for 16 days in the dark. Liquid spore suspensions of 1×10^5 conidia/ml, as well as half-strength SDA plates were prepared (sterile distilled water with 0.1% Tween 80). Afterwards, 100 μl of the suspension was added on each plate using a micropipette. The liquid was spread throughout the surface of the plate using a microscope slide. Plates were covered with their lids but not sealed. The conidia were exposed to the ambient humidity ratio that was set each time. The MLR-351H chambers can set relative humidity 60 to 90% when lights are off and 55 to 85% RH when on. In the present bioassay, temperature was set at 25ºC and lights were off. When no humidity control was set, RH inside the chamber was 35±5% (at 25ºC and dark). This humidity value was used as control. Moreover, treatments were set at 60, 70, 80 and 90% RH. Hydrometers were used to confirm the accuracy of RH values in the chambers.

Plates remained in the cabinets for 18 hours and then germination of conidia was counted. Germination percentages were calculated by counting the number of germinated conidia out of 100 conidia measured. Two sets of 100 conidia were measured on each plate. Ten replicates were designed for each treatment. Measurements were carried out under a microscope (400×) and all conidia with visible germ tubes of any length were counted as germinated.

Analyses were conducted using the statistical package SPSS 17.0. One-way analysis of variance (ANOVA) followed by the Post-hoc tests Dunnett T3 and Games-Howell (variances not assumed homogenous by Levene's statistic) were used to compare mean values.

Effects of relative humidity on the pathogenic ability of *B. bassiana* and *P. fumosoroseus* against *Galleria mellonella*

In this bioassay, effects of different RH values on pathogenicity of entomopathogenic fungi *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01 against larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) were determined. Third instar larvae of *G. mellonella* were purchased from Livefoods Direct Ltd. (Sheffield, UK). Fungi were grown on half-strength SDA agar for twenty days at dark. A liquid suspension of 1.35×10^7 conidia/ml was made for each fungus (sterile distilled water with 0.1% Tween 80). Insects were treated by spraying them in plastic boxes (27 × 20 × 14 cm) which were totally covered with filter paper (bottom and walls). Spore suspensions were sprayed to the larvae using micro-sprayers. Control larvae were sprayed with sterile distilled water solution of 0.1% Tween 80.

Two hours after treatment, twenty larvae plus 0.5 g of the material they fed on (wax flakes) were placed into each Petri dish (14 cm Ø). Petri dishes were covered with their lids but not sealed. Treated *Galleria* larvae were then placed into the chambers set at 25ºC and 12:12 photoperiod. Under the above conditions, and without setting humidity control, humidity inside the chambers was 40±5%. Control insects and larvae treated with *B. bassiana* and *P. fumosoroseus* were placed at chambers with 40, 60 and 80% RH. Ten replicates of 20 insects were set for each treatment-RH combination. Insects remained at the above humidity values for seven days.

Mortality measurements were conducted daily from third to seventh day after incubation. Dead larvae were recorded, removed from the plates, placed into sterile Petri dishes in groups, and incubated at 25ºC. Any larva that was rotten, covered by mycelium, or remained still after touching was counted as dead. One day after the completion of the last mortality measurement, all dead larvae were examined to determine the percentage of mycosis for each treatment.

Analyses were conducted using SPSS 17.0. One-way

analysis of variance (ANOVA) followed by the Post-hoc tests Dunnett T3 and Games-Howell were used to compare values. Levene's statistic indicated that variances were not homogenous and therefore, Dunnett T3 and Games Howell were the appropriate Post-hoc tests to determine significant differences between groups. Lethal time 50% (LT_{50}) was calculated using Probit analysis.

RESULTS

Effects of temperature on spore germination of *B. bassiana* and *P. fumosoroseus*

Temperature had a significant effect on spore germination of the entomopathogenic fungi *P. fumosoroseus* EBAC-01 and *B. bassiana* IMI-391044 ($P < 0.05$, $F = 56.2$, $df = 11, 48$). The Post-Hoc tests Dunnett T3 and Games-Howell were used to indicate significant differences among groups (Figures 1 and 2). Both tests showed equivalent results in all cases. Significant differences in spore germination, between the two species at each temperature value, occurred only at 22.5 and 30ºC. The time needed for 50% of conidia to germinate (GT_{50}) for each fungus and under each temperature value, was calculated by Probit analysis and is shown in Tables 1 and 2.

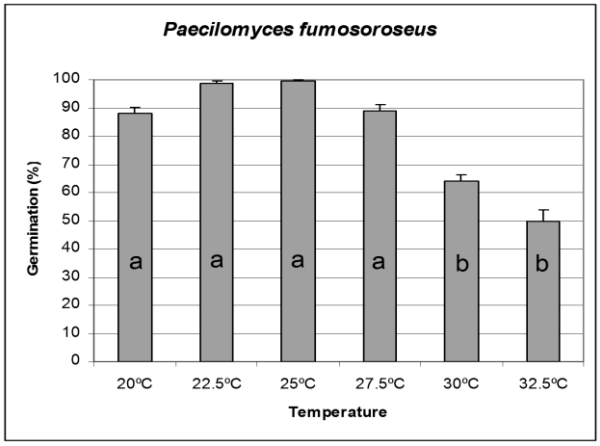

Figure 1. Mean germination percentages (+S.E.) of the entomopathogenic fungus *P. fumosoroseus*, 30 hours after incubation, under different temperature values.

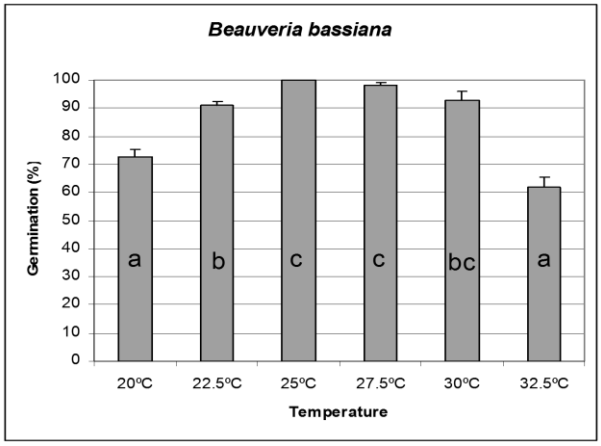

Figure 2. Mean germination percentages (+S.E.) of the entomopathogenic fungus *B. bassiana*, 30 hours after incubation, under different temperature values.

Different letters indicate significant differences (for $P = 0.05$) among treatments.

Table 1. Spore germination of *P. fumosoroseus* EBAC-01, under different temperature values, 12, 18 and 24 hours after initial incubation of conidia. Germination time 50% (GT_{50}), for each temperature value is also presented.

Spore germination	Temperature					
	20ºC	22.5ºC	25ºC	27.5ºC	30ºC	32.5ºC
At 12 h	25.6%	48.2%	59.6%	21%	21%	8%
At 18 h	80.8%	98%	98.6%	80.6%	42%	21.8%
At 24 h	87.5%	99%	99.4%	87.4%	57%	36.4%
GT_{50} (hours)	14.63c	12.49b	9.59a	14.83c	22.24d	27.69e

Table 2. Spore germination of *B. bassiana* IMI-391044, under different temperature values, 12, 18 and 24 hours after initial incubation of conidia. Germination time 50% (GT_{50}), for each temperature value is also presented.

Spore germination	Temperature					
	20ºC	22.5ºC	25ºC	27.5ºC	30ºC	32.5ºC
At 12 h	6.2%	23%	59.8%	41.8%	20.2%	4.6%
At 18 h	65.4%	86%	92.2%	91%	57.6%	28.4%
At 24 h	70%	89%	99%	96%	65.4%	49%
GT_{50} (hours)	16.23b	14.46ab	10.86a	12.7a	18.42c	25.07d

Effects of thermal stress on spore germination of *B. bassiana* and *P. fumosoroseus*

Thermal stress significantly affected spore germination of entomopathogenic fungus *P. fumosoroseus* EBAC-01 ($P < 0.05$, $F = 16.39$, $df = 4, 20$). In this case, exposure of conidia to high temperatures decreased the germination percentage to a higher extent than the exposure to low temperatures (Figure 3). Levene's statistic showed that variances were not assumed as homogenous, therefore the Post-Hoc tests Dunnett T3 and Games-Howell were used to indicate which of the following treatments differed significantly (Figure 3).

In the case of *B. bassiana* IMI-391044, thermal stress significantly influenced the conidial germination percentage ($P < 0.05$, $F = 21.13$, $df = 4, 20$). In contrast to *P. fumosoroseus*, exposure of conidia to low temperatures decreased the germination percentage to a higher extent than the exposure to high temperatures (Figure 4). Levene's statistic showed that variances within groups were homogenous. Therefore, in this case the Post-Hoc tests Tukey HSD and LSD were used to indicate which of the following treatments differed significantly.

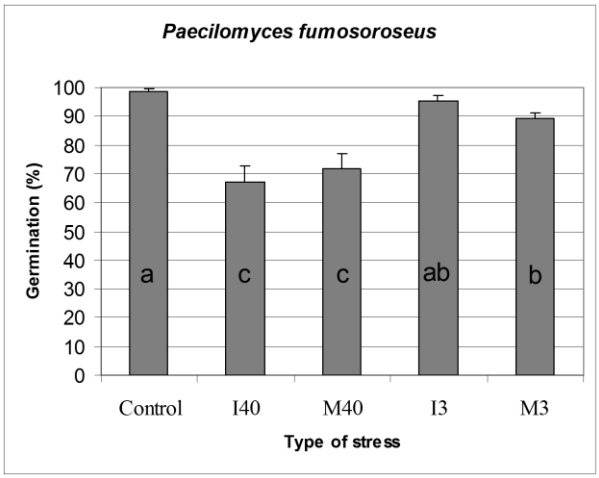

Figure 3. Mean germination percentages (+S.E.) of *P. fumosoroseus* after exposure of conidia to different thermal stress conditions. Different letters indicate significant differences (for $P = 0.05$) among treatments.

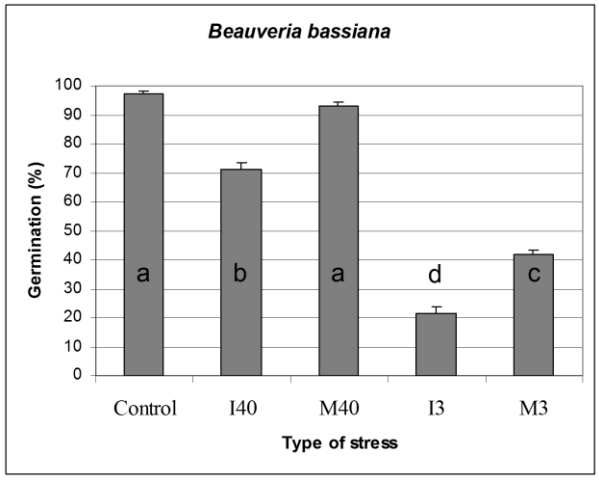

Figure 4. Mean germination percentages (+S.E.) of *B. bassiana* after exposure of conidia to different thermal stress conditions. Different letters indicate significant differences (for $P = 0.05$) among treatments.

I40 = Initial exposure of conidia at 40ºC for 1 hour and then incubated at 25ºC for 18 hours.
M40 = Conidia kept at 25ºC for 9 hours then exposed to 40ºC for 1 hour and again at 25ºC for 9 hours.
I3 = Initial exposure of conidia at 3ºC for 1 hour and then incubated at 25ºC for 18 hours.
M3 = Conidia kept at 25ºC for 9 hours then exposed to 3ºC for 1 hour and again at 25ºC for 9 hours.

Effects of relative humidity on spore germination of *B. bassiana* and *P. fumosoroseus*

Relative humidity had a significant effect on spore germination of entomopathogenic fungi *B bassiana* and *P. fumosoroseus* ($P < 0.05$, $F = 75.2$, $df = 9, 90$). Both the fungi germinated very well at 80% RH where they reached their highest values, which were 96.1 and 97.6% for *B bassiana* and *P. fumosoroseus* respectively. Germination of conidia of both the fungi was reduced at lower RH values (Figure 5). Two Post-Hoc tests (Dunnett T3 and Games-Howell) indicated which of the following percentages differed significantly. Both Post-Hoc tests showed identical results in terms of significant differences among groups.

Effects of relative humidity on the pathogenic ability of *B. bassiana* and *P. fumosoroseus* against *Galleria mellonella*

There were significant differences in larval mortality of *G. mellonella* caused by *P. fumosoroseus* EBAC-01 and *B. bassiana* IMI-391044 ($P < 0.05$, $F = 195.036$, $df = 8, 81$). Results of mortality of *G. mellonella* seven days after treatment are presented in Figure 6. Significant differences among treatments were estimated using Dunnett T3 and Games-Howell Post-Hoc tests. Both the tests showed equivalent results as shown in Figure 6. A high percentage of dead insects developed mycelium (Table 3). The percentage of mycosis differed significantly among different treatments ($P < 0.05$, $F = 61.7$, $df = 8, 81$).

Time required to kill 50% of the insects (LT_{50}) for each fungus was estimated by Probit Analysis (Table 3).

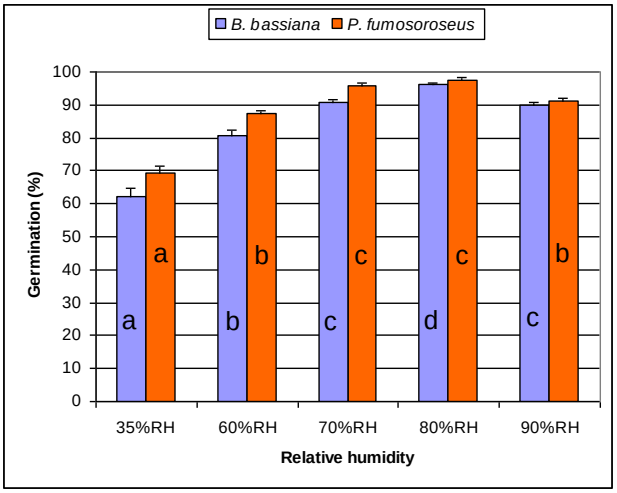

Figure 5. Mean germination percentages (+S.E.) of *B. bassiana* and *P. fumosoroseus* conidia after 18 hours of incubation at various relative humidity values. Different letters indicate significant differences for each fungus separately.

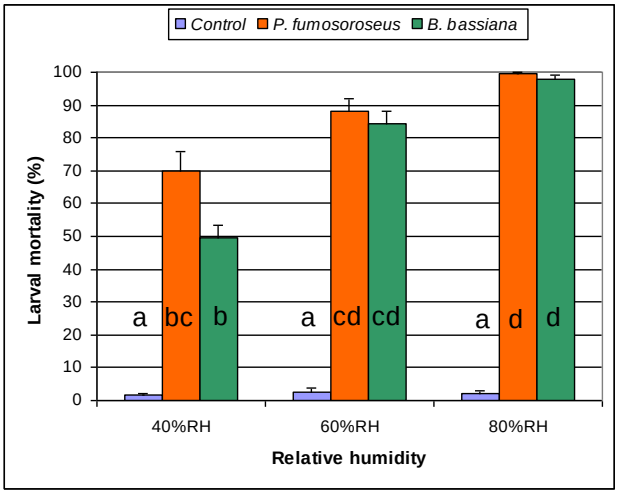

Figure 6. Percentage mortality of *G. mellonella* larvae (+S.E.) seven days after treatment with the isolates *P. fumosoroseus* EBAC-01 and *B. bassiana* IMI-391044, under incubation at three different values of ambient relative humidity. Different letters indicate statistically significant differences among treatments.

Table 3. Percentage of mycosis of dead *G. mellonella* larvae (±S.E.) eight days after treatment with *P. fumosoroseus* and *B. bassiana* under 40, 60 and 80% RH. The time that fungi required to kill 50% of the population of the insects (LT_{50}), for each treatment, is also presented.

	Paecilomyces fumosoroseus EBAC-01			*Beauveria bassiana* IMI-391044		
	40% RH	60% RH	80% RH	40% RH	60% RH	80% RH
Percent Mycosis	7.14±1.7a	42±8.4ab	83.8±5.2b	3±1.4a	27.6±6.6a	73±7.2b
LT_{50} (Days)	6.85	4.36	2.43	7.27	4.94	2.86

*Different letters indicate significantly different mycosis percentages.

DISCUSSION

Spore germination, hyphal growth and the pathogenic ability of entomogenous fungi to penetrate and infect their host are highly dependent on environmental conditions (Inglis et al., 2001; Lord, 2005). Therefore, systematic study is needed in order to determine the optimum climatic conditions as well as the range, in which these organisms germinate, develop and infect insects. This knowledge is considered as necessary before entomopathogenic fungi are used as biological control agents of crop-damaging insect pests. However, EPF can target almost every stage of insect cycle, making them a unique component in the integrated pest management programmes (Rajula et al., 2020) Insect characteristics, fungus specificity as well as environmental conditions influence the ability of fungus to infect the insect (Cheung and Grula, 1982).

In the present study, temperature had a significant effect on both conidial germination and speed of germination. However, germination occurred under all temperature values tested (20, 22.5, 25, 27.5, 30 and 32.5ºC). Both the fungi showed their highest germination percentage at 25ºC. This value is probably a constant temperature for most of the entomopathogenic fungi as many publications indicated the same or similar results (Hallsworth and Magan, 1999; Liu et al., 2003; Mishra et al., 2015; Walstad et al., 1970). The isolate *P. fumosoroseus* EBAC-01 germinated better and faster at

lower temperatures compared to *B. bassiana* IMI-391044. This is probably a consequence of geographical origin as *P. fumosoroseus* EBAC-01 was isolated in England while *B. bassiana* IMI-391044 is native to Syria. Consequently, conidia of *B. bassiana* isolate germinated better than those of *P. fumosoroseus* EBAC-01 at high temperatures. Spore germination of *B. bassiana* was found to be temperature dependant (Devi et al., 2005). Liu et al. (2003) reported that *B. bassiana* germinated well at 22 and 28ºC while *Metarhizium anisopliae* germinated at 35ºC. Moreover, Valverde-Garcia et al. (2018) found that *Metarhizium acridum* germinated and grew better at temperatures above 27°C, while *B. bassiana* was adapted to the temperature range of 10-25°C. Walstad et al. (1970) observed optimum germination, growth, and sporulation of *B. bassiana* and *M. anisopliae* at 25-30°C and under 100% RH. Hallsworth and Magan (1999) determined the optimum growth temperatures of *B. bassiana, M. anisopliae,* and *P. farinosus* which were 25, 30, and 20°C respectively. Thermal stress had also a significant effect on conidial germination compared to control (constant 25ºC) in some cases. Again, *P. fumosoroseus* EBAC-01 was more affected by stress caused by high temperatures while germination of *B. bassiana* IMI-391044 was much reduced at low temperature stress conditions. Decrease in spore germination of *B. bassiana* under stress conditions caused by high temperatures was reported by Devi et al. (2005). A significant effect of temperature was also recorded by Fargues et al. (1997) who studied the effects of temperature and thermoregulation on 65 isolates of *B. bassiana* from different origins. The influence of fluctuating temperature was also determined by Inglis et al. (1999) who measured its effect on *B. bassiana* in four environments with the same mean daily temperature (25°C) but differing in the degree to which they oscillated daily (constant 25°C, 20 to 30°C, 15 to 35°C, or 10 to 40°C). Membang et al. (2021) found thermotolerant isolates of *B. bassiana* and *M. anisopliae* for the control of the banana root borer (*Cosmopolites sordidus*) in Central Africa.

There was a significant difference in spore germination of entomopathogenic fungi *B. bassiana* IMI-391044 and *P. fumosoroseus* EBAC-01 when incubated at different levels of ambient relative humidity. Both the fungi showed their highest germination ratio when incubated at 80% RH, and this was 96.1 and 97.6% for *B. bassiana* and *P. fumosoroseus* respectively. It is often considered that high ambient humidity contributes to spore germination and infectivity of entomopathogenic fungi. However, it is believed that temperature and humidity conditions at the leaf surface may differ substantially from ambient conditions. In control of whitefly, laboratory bioassays in Texas demonstrated the capacity of both *B. bassiana* and *P. fumosoroseus* to infect *Bemisia argentifolii* nymphs on excised hibiscus leaves incubated at relative humidity as low as 25% at 23±2°C (Wraight et al., 2000).

In the present assay, higher germination occurred at 70 and 80% RH compared to control (35% RH). However, both the fungi germinated better at 80% than at 90% RH. According to Wraight et al. (2000), an increasing number of studies indicated that, while fungi do require moisture for development, sufficient moisture exists within the microhabitat of many insect hosts or within the microenvironment of the host's body surface to support infection essentially independent of ambient moisture conditions. The importance of water availability and activity on growth of *B. bassiana* and *P. farinosus* has also been reported by Hallsworth and Magan (1999), Fargues et al. (2003) and Hong et al. (2005). Relative humidity had also a significant effect on the lethal action of entomopathogenic fungi against the model Lepidoptera insect. Mortality caused by *P. fumosoroseus* EBAC-01 to *G. mellonella* larvae was 70, 88 and 99.5% at 40, 60 and 80%RH respectively. *B. bassiana* IMI-391044 caused mortality equal to 49.5, 84.5 and 98% at 40, 60 and 80% RH respectively. In a previous study, when adult females of *Rhyzopertha dominica* were introduced onto wheat kernels with 200 mg/kg of *B. bassiana* and/or 100 mg/kg of diatomaceous earth and incubated at 26, 30, 32.5, or 34 °C, there was significantly lower emergence of adult progeny at 43% RH than at 75% (Lord, 2005). Anderson et al. (2011) suggested that variation in virulence (speed of kill) between isolates, species and doses was determined more by quantitative rather than qualitative differences in fungal growth kinetics.

The efficacy of entomopathogenic fungi as biological control agents depends on various biotic and abiotic parameters. Environmental factors such as temperature and relative humidity, as well as biotic factors (population dynamics, synchronization of life cycles of entomopathogenic fungi to their hosts and behavioural responses) affected pest control using fungal entomopathogens (Baverstock et al., 2010; Lacey et al.,

2001; Shah and Pell, 2003). The evaluation of entomopathogenic fungi and data derived from the present study will be used in future pest control bioassays regarding economically important vineyard pests. The fungal isolates tested in the current study, successfully controlled the populations of a major polyphagous pest (*Ceratitis capitata*) in laboratory experiments (Beris et al., 2013). *C. capitata* was found to infest wine grapes in vineyards of central Greece. *P. fumosoroseus* EBAC1 also presented remarkable entomopathogenic action against the European Grapevine Moth (*Lobesia botrana*), in recent studies conducted in Greece, involving the control of larval populations of *L. botrana* with the use of EPF under various temperature regimes (Beris et al., - unpublished data). Entomopathogenic fungi show great potential as bio-control agents of *L. botrana*. However, no research related to the efficacy of entomopathogenic fungi against *L. botrana* had been published until recently (Altimira et al., 2019; Sammaritano et al., 2018). Entomopathogenic fungi may contribute to Integrated Pest Management programs designed for vineyards. However, abiotic factors such as temperature and relative humidity must be highly taken into consideration in future control strategies. Further research must be conducted to investigate the effectiveness of entomopathogenic fungi against grapevine pests in field conditions. The fungal isolates *P. fumosoroseus* EBAC-01 and *B. bassiana* IMI-391044 are currently considered as highly pathogenic and they shall be tested in future bioassays against *L. botrana* and the vine weevil (*Otiorhynchus sulcatus*). Overall, Entomopathogenic fungi can be included in control strategies against vineyard pests. However, environmental factors must be taken into consideration to increase their efficacy on insect pests. Therefore, release schedules for EPF should be designed according to weather conditions and target-pest's life cycle. Further research is needed to investigate all aspects of entomopathogens' physiology and ecology withing agricultural ecosystems.

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CONTRIBUTION OF THE AUTHOR

The author designed the study, conducted the experiments, collected experimental data, analyzed the data statically and wrote the manuscript.

CONFLICT OF INTEREST

The author declares that he has no conflict of interest.

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