

Article

Trapping Entomopathogenic Fungi from Vine Terroir Soil Samples with Insect Baits for Controlling Serious Pests

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Abstract: Fungi are eukaryotic microorganisms with many forms and certain demands about the soil they colonize, which reveal the conditions that prevail therein. Although they can often kill pests without damaging plants or humans, they are not widely used as pesticides as they are not easy to handle and require much time before they act. Nevertheless, insects can be used as baits to trap these entomopathogenic fungi, giving information about their distribution within the terroir. The purpose of this study was to investigate the soil distribution of entomopathogenic fungi species in soil samples from two vine soil terroirs. Nine fungi were identified in total, but *M. anisopliae* and *B. bassiana* showed the greatest frequency, which was expressed by difference in adult bait mortality among species. These fungi had not been previously documented in vine terroirs.

Keywords: terroir; baits; entomopathogenic fungi; soil; Achaia; vine; winery

1. Introduction

Entomopathogenic fungi are microorganisms that specifically infect and often kill insects and other arthropods [1]. Most are nonpathogenic to plants, and relatively nontoxic to humans and animals [2]. Although fungus-infected insects can be commonly found in nature, and epizootics are observed impacting pest populations, mortality from fungal infection rarely occurs naturally at sufficiently high levels or early enough in a pest cycle to prevent economic loss. Despite their many advantages over other biological and chemical products, entomopathogenic fungi remain relatively underutilized. Approximately 60% of insect diseases are caused by pathogenic fungi [3]. The entomopathogenic fungi occurrence in the soil can depend, inter alia, on the soil type [4], the cultivated plant species [5–7], or the agricultural practices [6,8–11]. Contrary to other insect pathogenic microorganisms, entomopathogenic fungi directly infect their host through the exoskeleton.

Entomopathogenic microorganisms such as viruses and bacteria, on the other hand, infect upon ingestion, through the mid-gut. Because of their low resistance to environmental changes, the degree of soil colonization by these fungi can be a reliable indicator of its condition [12]. Soil can provide a suitable environment for entomopathogenic fungi by protecting them from UV light and other biological and nonbiological factors that may limit their distribution [13]. Environmental concerns render the use of entomopathogenic fungi in the myco-biocontrol of pests highly significant. Their application

as insecticides, however, presents a series of constraints, i.e., the short shelf life of the inoculum, a two- to three-week period required until mortality, the need for relatively high humidity during application, and the high cost of commercial formulations. The entomopathogenic fungi genera, including *Beauveria*, *Metarhizium*, and *Isaria*, usually colonize the soil as part of their life cycle [7,14]. Entomopathogenic fungi are usually trapped using the following insects as baits: *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), or *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) [4,7,15–20].

Terroir refers to the link between the properties of a vine (quality, taste, and style), and the natural environment in which it was produced which might influence these properties [21,22]. Vines can be cultivated in a wide range of soils. The soil can variously influence vine behavior and grape composition, as it determines the mineral nutrition and water uptake of the plant as well as the root depth and temperature in the root area [22]. The study of soils can be approached geologically, pedagogically, and agronomically.

The present study represents an examination of vine terroir soils from an agronomic (entomological) point of view. More specifically, the objective was to identify the entomopathogenic fungi distribution in soil samples from vine terroirs. To this end, we used the following stored product insects as baits: *Prostephanus truncatus* Horn (Coleoptera: Bostrychidae), *Sitophilus zeamais* (L.) (Coleoptera: Curculionidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrychidae), *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae), and *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae). Stored product insects were selected for this study, as they are easier to handle, faster in their development, and more cost-effective in their mass rearing, in comparison with grapevine or other pests. Soil samples were randomly collected from different points in two vine terroirs of *Vitis vinifera* L. (Vitales: Vitaceae). The vine terroirs, Koutsoura and Petsakoi, belong to the Rouvalis Winery in the prefecture of Achaia. *V. vinifera* plantations are the most frequent in western Achaia and render a substantial contribution to the local and national economy [23]. Vine terroirs were selected as an interesting case for their economic output and as potentially favourable to entomopathogenic fungi. It is worth noting that there are no data in relation to the terroir soil-inhabiting fungi in this part of Western Greece.

2. Materials and Methods

2.1. Insects

Rearing of *P. truncatus* was carried out on corn. *R. dominica*, *S. oryzae*, and *S. zeamais* were reared on whole wheat, while rearing of *C. ferrugineus* and *T. confusum* was carried out on flour, respectively. All insects were kept in cages at 25 ± 1 °C, relative humidity 60–70% and photoperiod 16:8 h (light-darkness) (PHC Europe/Sanyo/Panasonic Biomedical MLR-352-PE) (Department of Pharmacy, University of Patras, Patra, Greece).

2.2. Sampling—Entrapment of Entomopathogenic Fungi

Ten soil samples were collected in total from the following two vine terroirs of the Rouvalis Winery in the prefecture of Achaia: Koutsoura, Petsakoi (Figure 1). At each site, the surface litter was removed and the soil was dug to a depth of 10 cm with a soil core borer. The samples, 1000 g each from five points in the vineyard, were collected in September 2019. They were then placed in plastic bags and stored at 4 °C, until they were transferred to the laboratory for further processing. We first airdried the soil samples prior to remoistening them, to prevent infection by entomopathogenic nematodes. This was done to avoid possible entomopathogenic nematode (EPN) infestation, as suggested by Quesada-Moraga et al. [10]. The collected soil was placed on a rough cardboard on the laboratory stalls for 24 h to reduce its humidity. Afterwards, the soil was sieved (Metal, 2 mm × 1 mm, Aggelis Equipment, Athens, Greece) and placed in Petri dishes. At this point, one batch of ten adults, per species, was inserted in each of the eight soil samples. This was repeated ten times, which means

that one hundred individuals per species were tested per soil sample. Adults were placed on the soil surface, and either the soil was agitated or the containers were gently inverted/shaken periodically to ensure that the adults remained exposed to the soil. They were then left in dark chambers at 25 ± 1 °C for fourteen days. Every seven days, mortality measurements were made.



Figure 1. Vineyards in the prefecture of Achaia were the soil samples collected (A) Koutsoura ($39^{\circ}13'01''$ B, $23^{\circ}45'84''$ A) and (B) Petsakoi ($38^{\circ}25'46.8''$ B, $22^{\circ}10'26.5''$ A).

2.3. Isolation of Entomopathogenic Fungi and Conidia Identification Method

Dead adults were immersed in 6% NaOCl₂ for 3 s, in order to be sterilized (to avoid developing saprophytic fungi). They were then placed in 9cm sterilized Petri dishes with a No. 1 Whatman paper impregnated with ddH₂O (double distilled water) until mycelia appeared. Insect cadavers showing external mycelial growth were determined by examining each cadaver using a Stemi 2000 stereomicroscope (Carl Zeiss®, Oberkochen, Germany). These samples were kept in dark chambers at a temperature of 25 ± 1 °C, and they were daily observed in the stereoscope to determine possible infestation by entomopathogenic fungi. Conidia from the infected adults were placed in 9 cm sterilized Petri dishes on a layer of Sabouraud Dextrose Agar (SDA), for the isolation of entomopathogenic fungi. The dishes were kept at 25 ± 1 °C in the dark in order to achieve the incubation and development of the fungi. When a fungus was developed, it was isolated again in order to avoid infestation and contaminated cultivation. The above-mentioned process was carried out inside a laminar flow chamber (Equip Vertical Air Laminar Flow Cabinet Clean Bench, Mechanical Application LTD). The isolates were then subcultured several times on plates with Sabouraud Dextrose Agar (SDA) to ensure purity and monosporic cultures, and they were morphologically identified with ZEISS Primo Star (Carl Zeiss Microscopy GmbH, Jena, Germany) at 400 × magnifications. The selected isolates are stored at -20 °C deep freezer, in the microorganism's repository of the EMBIA Laboratory, Department of Pharmacy, School of Health Sciences, University of Patras.

2.4. Colony Forming Units of Fungi in Vine Terroir Soil

One gram of soil was weighed out of each sample, to which 9 mL of ddH₂O with 0.05 Triton X-100, which reduces the surface tension, was added. The resulting solution was intensely shaken for 30–40 s. Following this, 0.1 mL of the soil solution was spread out on a selective medium with a glass spatula. The selective medium consisted of 1 L of water, 20 g of glucose, 18 g of agar, and 10 g of peptone [6,12]. After sterilization and cooling of agar, the following selective components were added to the medium: 0.6 g of streptomycin sulfate, 0.005 g of chlortetracycline, 0.05 g of cyclo-heximide, and 0.1 g of dodine. The experiment was performed in ten Petri dishes per sample. The dishes were kept at 25 ± 1 °C in the dark in order to facilitate the incubation and development of fungi. After

14 days, the colonies of individual fungal species were counted. The results were expressed as the number of colony-forming units (CFU) of fungi in 1 g of soil.

$$[\text{CFU g}^{-1}\text{soil}] = 2 \times \text{colonies per plate/dilution} \times 10^3$$

2.5. Statistical Analysis

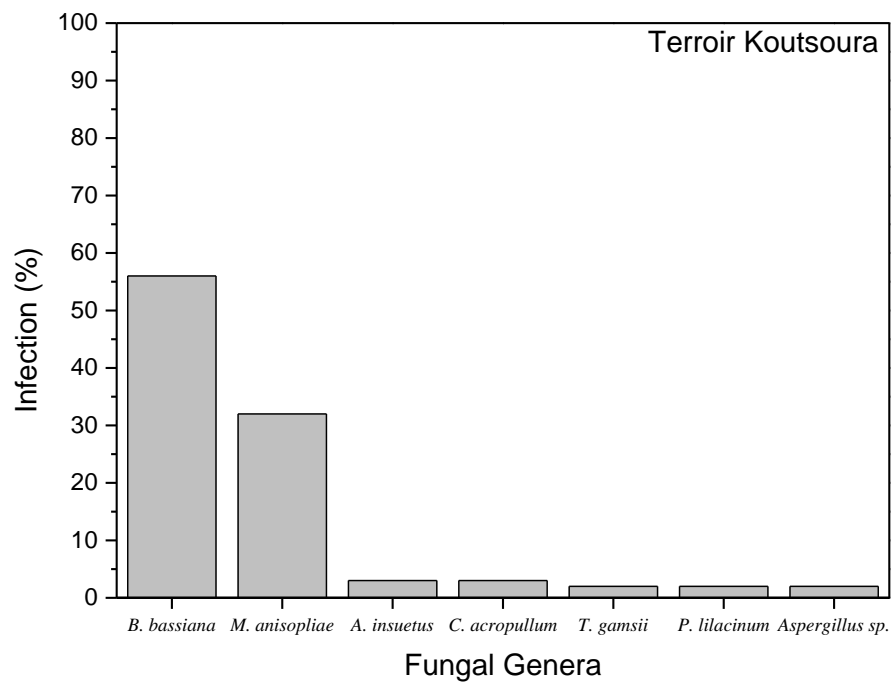
Mean mortality was calculated as value and these values were arcsine transformed prior to analysis. Data were normalized and then analyzed by mortality with three-way ANOVA (Factor: Bait Species, Time of experiment and Terroir), using the general linear model of the SPSS (SPSS Inc., Armonk, NY, USA, version 23) (IBM 2013). In case of significant F values, means were compared using the Bonferroni test. The mean density of colony forming units of fungi was analyzed by CFU with one-way ANOVA (Factor: Terroir) using the compare means model of the SPSS (SPSS Inc., IL, USA, version 23) (IBM 2013).

3. Results

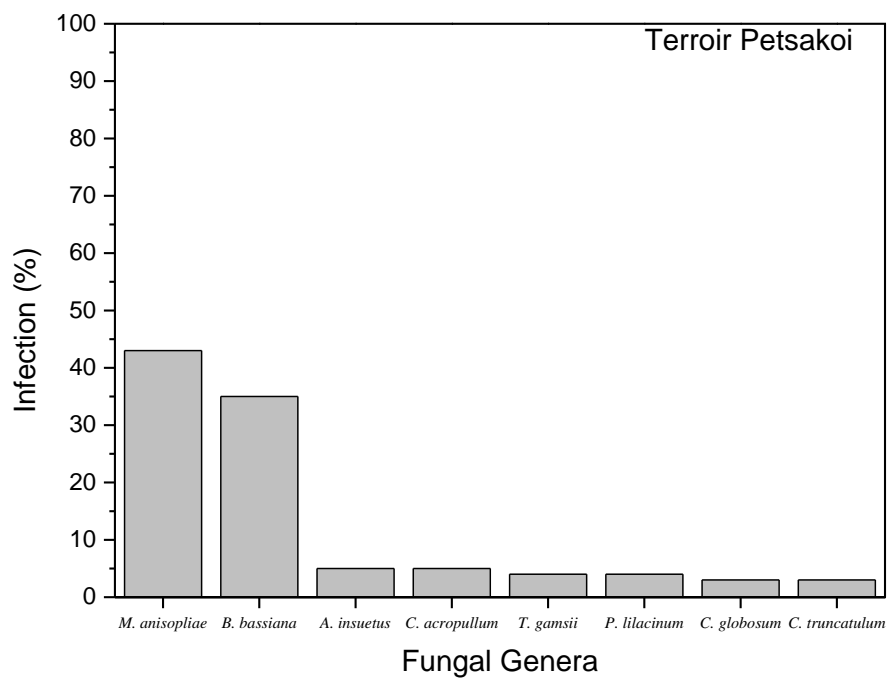
A total of nine isolates were morphologically identified from the two vine terroirs as *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae); these were the fungi with the highest frequency. We also identified *Aspergillus insuetus* (Bainier) Thom and Church (Eurotiales: Trichocomaceae); *Aspergillus* sp. Micheli (Eurotiales: Trichocomaceae); *Chaetomium acropullum* X. Wei Wang (Sordariales: Chaetomiaceae); *Chaetomium globosum* Kunze (Sordariales: Chaetomiaceae); *Chaetomium truncatulum* Kunze (Sordariales: Chaetomiaceae); *Trichoderma gamsii* Samuels and Druzhinina (Hypocreales: Hypocreaceae); and *Purpureocillium lilacinum* (Thom) Luangsa-ard, Hou-braken, Hywel-Jones and Samson (Hypocreales: Ophiocordycipitaceae).

From the vine terroir Koutsoura, we isolated *B. bassiana* at 56% and *M. anisopliae* at 32%. The fungi with unidentified entomopathogenic abilities were *A. insuetus* at 3%; *C. acropullum* at 3%; *T. gamsii* at 2%; *P. lilacinum* at 2%; and *Aspergillus* sp. at 2% (Figure 2). From the vine terroir Petsakoi, two fungal isolates were identified as *M. anisopliae* at 43% and *B. bassiana* at 35%. Fungi of unknown entomopathogenic abilities were *A. insuetus* at 5%; *C. acropullum* at 5%; *T. gamsii* at 4%; *P. lilacinum* at 4%; *C. globosum* at 3%; and *C. truncatulum* at 3% (Figure 2). In the soil samples from the two vine terroirs, the Hyphomycetes *B. bassiana* and *M. anisopliae* were isolated from *P. truncates*, *R. dominica*, *S. zeamais*, *C. ferrugineus*, *T. confusum*, and *S. oryzae*. In the soil sample from the Koutsoura vine terroir, *B. bassiana* caused the most fungal infestations of all insect baits while in the Petsakoi vine terroir and *M. anisopliae* was the most entomopathogenic fungus (Tables 1 and 2).

Statistical analysis produced significant differences in terms of mortality between insect species, time of experiment, and terroir (Table 2). In the Koutsoura terroir, *B. bassiana* caused the highest mortality to the *P. truncates*, *R. dominica*, *T. confusum*, and *C. ferrugineus* baits, while it caused the lowest mortality to *S. zeamais* and *S. oryzae* (Table 1). The mortality of *P. truncates*, *R. dominica*, *T. confusum*, and *C. ferrugineus* baits was also caused by fungi of unknown entomopathogenic abilities such as *A. insuetus*, *Aspergillus* sp., *C. acropullum*, *T. gamsii*, and *P. lilacinum* but at low rates (Table 1). In the same terroir, the mortality caused by *M. anisopliae* was the highest for *S. zeamais* and *S. oryzae*. In the Petsakoi terroir, *B. bassiana* was responsible for mortality in *P. truncates*, *R. dominica*, and *T. confusum*. However, in the same terroir, *M. anisopliae* proved to be more pathogenic than *B. bassiana* (Table 1). The highest mortality caused by *M. anisopliae* was recorded in *C. ferrugineus*, *S. zeamais*, and *S. oryzae* (Table 1). In the Petsakoi terroir too, the mortality of *C. ferrugineus*, *S. zeamais*, and *S. oryzae* baits was caused by fungi of unproved entomopathogenic abilities such as *A. insuetus*, *C. acropullum*, *C. globosum*, *C. truncatulum*, *T. gamsii*, and *P. lilacinum*, albeit at low rates (Table 1).



(A)



(B)

Figure 2. Frequency of entomopathogenic fungal species isolated from the vine terroir Koutsoura (A) and the vine terroir Petsakoi (B).

Table 1. Adult bait mortality (%) in soils from the vine terroirs in Achaia. Mean \pm sd values with the same letter within a column are not significantly different ($P < 0.05$). Pt = *P. truncatus*, R.d = *R. dominica*, T.c = *T. confusum*, C.f = *C. ferrugineus*, S.z = *S. zeamais*, S.o = *S. oryzae*.

Mortality Factor	Vine Terroir											
	Koutsoura						Petsakoi					
	Insect baits											
	P.t	R.d	T.c	C.f	S.z	S.o	P.t	R.d	T.c	C.f	S.z	S.o
Entomopathogenic fungi												
<i>B. bassiana</i>	83 ± 2 ^a	80 ± 3 ^a	86 ± 3 ^a	82 ± 2 ^a	53 ± 3 ^d	53 ± 7 ^d	34 ± 5 ^e	32 ± 2 ^e	28 ± 4 ^e	0	0	0
<i>M. anisopliae</i>	0	10 ± 2 ^g	0	18 ± 4 ^f	45 ± 2 ^d	36 ± 2 ^e	62 ± 1 ^c	56 ± 3 ^d	65 ± 4 ^c	72 ± 2 ^b	78 ± 2 ^a	72 ± 3 ^b
Fungi of unproved entomopathogenic abilities												
<i>A. insuetus</i> .	3 ± 3 ^g	2 ± 2 ^g	4 ± 1 ^g	0	0	1 ± 3 ^g	0	4 ± 2 ^g	0	3 ± 5 ^g	6 ± 1 ^g	5 ± 1 ^g
<i>C. acropullum</i> .	3 ± 3 ^g	0	2 ± 1 ^g	0	2 ± 4 ^g	2 ± 3 ^g	0	0	2 ± 3 ^g	4 ± 2 ^g	2 ± 3 ^g	5 ± 4 ^g
<i>T. gamsii</i>	4 ± 2 ^g	2 ± 2 ^g	3 ± 3 ^g	0	0	3 ± 3 ^g	0	0	0	4 ± 2 ^g	0	5 ± 4 ^g
<i>P. lilacinum</i> .	6 ± 1 ^g	5 ± 3 ^g	2 ± 2 ^g	0	0	4 ± 3 ^g	0	0	2 ± 2 ^g	0	2 ± 1 ^g	6 ± 2 ^g
<i>C. globosum</i>	0	0	0	0	0	0	2 ± 4 ^g	6 ± 3 ^g	3 ± 5 ^g	4 ± 6 ^g	2 ± 3 ^g	3 ± 2 ^g
<i>C. truncatulum</i>	0	0	0	0	0	0	2 ± 2 ^g	2 ± 1 ^g	0	2 ± 4 ^g	2 ± 1 ^g	3 ± 3 ^g
<i>Aspergillus</i> sp.	1 ± 3 ^g	1 ± 1 ^g	1 ± 3 ^g	0	0	1 ± 1 ^g	0	0	0	0	0	0
Other causes												
Unspecified causes	0	0	4 ± 3 ^g	0	0	0	0	0	0	1 ± 4 ^g	8 ± 2 ^g	1 ± 4 ^g

Table 2. Three-way ANOVA post hoc bonferroni test with mortality as variable.

Dependent Variable: Mortality			
Source	df	F	p-Value
Bait Species	5	21,758	0.000
Terroir	1	3693	0.001
Time of experiment	6	11,349	0.000
Bait * Terroir	5	2754	0.001
Bait * Time of experiment	30	4749	0.000
Terroir * Time of experiment	6	1976	0.044
Bait * Terroir * Time of experiment	30	1668	0.010
Error	190		
Total	289		
Corrected Total	288		

In the soil samples, fungi on average formed 2.1×10^3 g⁻¹ CFU in 1 g of soil in the Koutsoura terroir and on average formed 2.3×10^3 g⁻¹ in the Petsakoi terroir (Table 2). *M. anisopliae* and *B. bassiana* occurred in significantly higher density in the soil samples from both terroirs, forming 3.2×10^3 g⁻¹ and 7.7×10^3 g⁻¹ CFU in the Koutsoura terroir, and 7.8×10^3 g⁻¹ and 5.8×10^3 g⁻¹ CFU in the Petsakoi terroir, respectively. Statistical analysis also produced significant differences in terms of colony-forming units of fungi in soils between terroirs (Table 3).

Table 3. The density of colony forming units of fungi (CFU $\times 10^3$ g⁻¹) in soils from vine terroirs in Achaia ($F = 2.396$, $df = 5$, $P = 0.032$). Mean \pm sd values with the same letter within a column are not significantly different ($P < 0.05$).

Fungal Species	Vine Terroir	
	Koutsoura	Petsakoi
<i>B. bassiana</i>	7.7 \pm 2.1 ^a	5.8 \pm 1.2 ^a
<i>M. anisopliae</i>	3.2 \pm 2 ^a	7.8 \pm 1.6 ^a
<i>A. insuetus</i>	1.4 \pm 0.8 ^b	1 \pm 1 ^b
<i>C. acropullum</i>	0.5 \pm 0.2 ^b	0.7 \pm 0.2 ^b
<i>T. gamsii</i>	0.5 \pm 0.4 ^b	0.6 \pm 0.2 ^b
<i>P. lilacinum</i>	1.2 \pm 0.8 ^b	1.1 \pm 0.2 ^b
<i>C. globosum</i>	Not present	0.7 \pm 0.3 ^b
<i>C. truncatulum</i>	Not present	0.7 \pm 0.3 ^b
<i>Aspergillus</i> sp.	0.2 \pm 0.4 ^b	Not present

4. Discussion

In the present study, we isolated fungi that had not been previously documented in vine terroirs. Such fungi were *M. anisopliae*, *B. bassiana*, *A. insuetus*, *Aspergillus* sp., *C. acropullum*, *C. globosum*,

C. truncatulum, *T. gamsii*, and *P. lilacinum*. The occurrence, development, and pathogenicity of entomopathogenic fungi in the soil are conditioned by several biotic and abiotic factors in the environment, as well as the agricultural and nonagricultural human activity. Vine terroir soils have unique biotic and abiotic factors due to the C/N ratio of soil organic matter and organic matter turnover, which plays a critical role in the occurrence, development, and pathogenicity of fungi [24,25].

The vine terroirs exhibit a richness of arthropods, especially insects, in the area of the crop; this is bound to affect the species diversity of their natural enemies, including entomopathogenic fungi. In our study, *M. anisopliae* and *B. bassiana* occurred at the highest frequency in the vine soil samples from both terroirs, which suggests that the respective areas possibly presented high densities of these fungi as natural enemies of the terroir insect fauna.

B. bassiana was the fungal species that infected most bait adults and formed the largest number of CFUs in the soil from the Koutsoura terroir. This fungus is codominant with *M. anisopliae* in the Koutsoura vine terroir soil samples. The dominance of *B. bassiana* in vine terroir soils could be attributed to the fact that these soils are much richer in organic matter and *B. bassiana* has the ability to grow in the saprophagous phase [18,26,27]. The survival of *B. bassiana* conidia is dependent on temperature and the soil water content [28]. It should also be noted that the survival of entomopathogenic fungi in the soil largely depends on the stability of environmental conditions and the related continuous or at least frequent presence of potential insect hosts. *B. bassiana* exhibits more dominance and viability than *M. anisopliae*, perhaps be due to some environmental factors; *B. bassiana* can infect many species of insects and can grow on artificial media or some kinds of soil [29]. *M. anisopliae* conidia were able to survive in the soil longer than *B. bassiana*, because it seems that the latter was more sensitive to the soil microbiota [30]. These host insects are found in large masses in the terroirs. In the Petsakoi terroir, *M. anisopliae* infected the most bait adults and formed the largest number of CFUs in the soil. The conidia of *M. anisopliae* are pathogenic in a wide range of temperature and soil moisture conditions [31]. *M. anisopliae* could also survive for a long time in the soil [32,33]. Therefore, survival outside the host may be critical for the ability of *M. anisopliae* to control insect pests in the soil [34].

Based on data presented in Tables 1–3, species with increased number of CFUs caused noteworthy insect mortality. Another finding is that although there are similar numbers of CFUs of *B. bassiana* and *M. anisopliae* at CFU level (Table 3), the infection rate (insect mortality) of bait insects was different (Table 1). This may be attributed to the complicated process of insect pathogenicity of entomopathogenic fungi, where several factors other than number of CFUs (cuticle degrading enzymes, strain genetic variation, gene expressions, etc.) play an important role on the fungus virulence [13,32].

Natural pest enemies can be incorporated in a conservation biological control policy, within the framework of management strategies, to create optimal soil conditions for the entomopathogenic fungi [35–40]. Eilenberg et al. [35] defined this strategy as “modifications of the environment or existing practices to protect and enhance specific natural enemies or other organisms to reduce the effects of pests”. Therefore, knowledge of the community of natural enemies in the vine terroir, as well as the effect of agronomical practices on these organisms, is essential to use a conservation biological control strategy.

5. Conclusions

This is the first time that terroir soils in Western Greece have been examined in terms of their entomopathogenic fungi population. The tested terroir soils that expressed the highest frequency of fungal isolates possibly provide suitable environmental conditions for these microorganisms. Future research plans involve assessing terroirs in relation to other types of soils. Moreover, our results highlighted that the diversity of the entomopathogenic fungi fauna is interrelated with the terroir. The terroir as a factor plays a very important role in the occurrence of the entomopathogenic fungi inhabiting the cultivated Petsakoi and Koutsoura. Through specific management strategies that provide optimal conditions for the entomopathogenic fungi in the soil, these natural pest enemies can be included in the suppression of pests in a conservation biological control strategy.

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