

Article

Microbial Terroir of Nemea Vineyards: Isolation and Characterization of an Endemic *Purpureocillium lilacinum* Genotype with Biocontrol Potential

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Abstract

Mediterranean organic viticulture requires sustainable pest management strategies that leverage local soil biodiversity. This study isolated endemic entomopathogenic fungi from vineyard soils in Nemea, Greece, using a dual-insect baiting system with *Tribolium confusum* and *Sitophilus* spp. The recovered isolates caused complete mortality in bait insects, with mycelial emergence from 93.75% of cadavers. DNA sequencing of the ITS1 region identified the recovered isolates as *Purpureocillium lilacinum*. Phylogenetic analysis revealed that Nemea isolates (TD and TM series) form a monophyletic clade with 100% bootstrap support, showing distinct genetic divergence from the reference strain *P. lilacinum* NRRL 895—evidence of a unique “microbial terroir.” Virulence assays demonstrated species-dependent mortality against stored-product pests: *Sitophilus granarius* was the most susceptible (76.7% mortality; LT₅₀ = 1.9 days), followed by *Sitophilus zeamais* (61.1%; LT₅₀ = 2.7 days), *Tribolium confusum* (56.7%; LT₅₀ = 2.8 days), and *Sitophilus oryzae* (50.0%; LT₅₀ = 3.3 days). Mycosis confirmation (65–83%) and 0% control mortality confirmed pathogenicity. As locally adapted biological control agents, these endemic *P. lilacinum* strains are highly suitable for protecting crops from major insect pests.

Keywords: organic viticulture; Nemea terroir; entomopathogenic fungi; biological control; phylogenetic analysis; Agiorgitiko



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1. Introduction

Global wine production is transforming as growers move away from synthetic chemicals and pursue sustainable methods. In Europe, this adjustment follows European Union Regulation 2018/848. The purpose of this adjustment is to protect biodiversity and maintain ecological balance in farming systems [1]. Organic vineyards depend on soil microbes to control pests and diseases. This approach simultaneously improves vine health and fruit quality [2,3]. Everyday farming relies on synthetic fungicides, herbicides, and fertilizers. These chemicals damage soil structure and microbial diversity, while promoting pathogen resistance [4,5]. Finding biological control agents (BCAs) that live naturally in vineyards helps prevent these problems. Entomopathogenic fungi (EPF) are part of this biodiversity, offering a means to manage pests without harming the environment [6,7].

Insect pathogens like fungi, bacteria, and nematodes work well with other management plans, making them practical substitutes for chemical control [8–12]. Many studies investigate using these organisms, specifically fungi, to target storage pests [13–19]. These fungi occur naturally, are safe for the environment, and have low toxicity to mammals [19,20]. The fungi grow and produce spores on the outside of the insect cadavers. This releases a large quantity of fresh spores back into the crop environment. The new spores spread naturally, which starts and maintains an outbreak of the disease among the remaining pests. They also grow on the host, which puts more of the pathogen back into the system. This makes their ability to stay active a benefit, unlike the residues of standard pesticides, which are usually a problem [13]. Mycopesticides help manage insects without the side effects of standard chemicals and are now used globally [21–24]. Even though these diseases kill many storage insects, people rarely use them as tools in warehouses [9,25].

The most studied EPF species against stored-product pests is *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae). Several laboratory and field studies have demonstrated efficient insecticidal action against postharvest insects [11,17,26–31]. Unlike *B. bassiana*, there are disproportionately fewer data on the use of other common EPF species such as *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Isaria fumosorosea* (Wize) (Hypocreales: Clavicipitaceae) despite the fact that there is strong evidence that they can be successfully used for the protection of stored grains against several insect pests [11,24,25,32,33].

Purpureocillium lilacinum (syn. *Paecilomyces lilacinus*) is a common fungus with many ecological functions. It works against plant-parasitic nematodes, several insect pests and other fungi [32,33]. Latest research shows it helps plants grow and lives inside plant tissues as an endophyte. Viticulture defines “terroir” through local geology and climate, but the specific “microbial terroir” is just as important [34]. Local EPF strains that evolved alongside specific vine varieties and soil types often survive better and work more effectively than commercial products brought in from outside [35,36]. Collecting and identifying native species in wine regions is necessary for creating pest control plans adapted to specific sites.

Nemea, in the Peloponnese region of Greece, is a wine-producing area with a long history of *Vitis vinifera* L. cv. ‘Agiorgitiko’ farming. We do not yet know the variety of EPF in Nemean soils. This study uses insect baits to find and identify local strains of EPF from vineyards in Nemea. By confirming EPF presence, the research provides a basis for using local fungi in organic viticulture.

2. Materials and Methods

2.1. Study Site and Soil Sampling

We collected soil from two vineyards in Nemea, Peloponnese (37.810361°, 22.659250° and 37.837306°, 22.654083°) (Figure 1). These sites experience Mediterranean weather and produce the local ‘Agiorgitiko’ grape variety. At each location, we cleared surface litter and took soil from the vine rhizosphere at a depth of 10 cm. We followed a fully randomized pattern to gather five sub-samples from each vineyard, which were then mixed into a single 1 kg composite sample per site. Samples were kept in sterile polyethylene bags during transport to the lab.

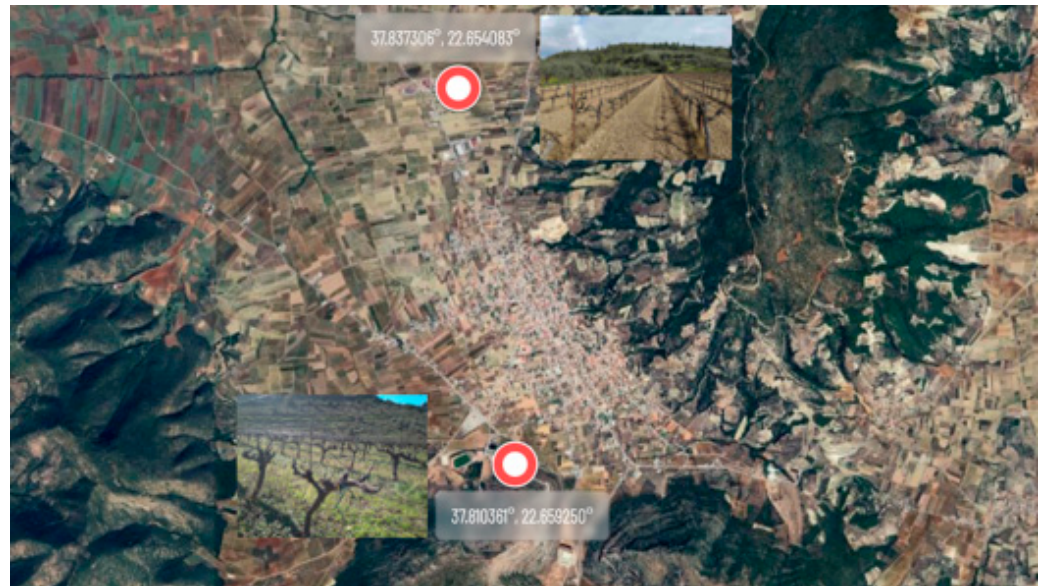


Figure 1. Geographical mapping of soil sampling sites in the Nemea region (Peloponnese, Greece). Locations for vineyard soil series TD (37.810361°, 22.659250°) and TM (37.837306°, 22.654083°) are indicated. Geographic coordinates were recorded using a Garmin eTrex 10 handheld GPS receiver (Garmin Ltd., Olathe, KS, USA), with a reported accuracy of $\pm 3\text{--}5$ m under open sky conditions. Coordinates are referenced to the WGS84 datum.

2.2. Insect Rearing

We used adult beetles as baits to trap fungi. These included *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae), *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), and *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae). We reared these insects at the University of Ioannina, Arta Campus, in the Laboratory of Agricultural Entomology. *T. confusum* was fed wheat flour mixed with 5% brewer's yeast, while the *Sitophilus* species were maintained on whole wheat grains. All colonies were kept in climatic chambers set to 25 ± 1 °C and 60–70% relative humidity. The photoperiod followed a 16:8 h (L:D) cycle [37]. For Virulence Estimation, we used third-instar larvae of *Tribolium confusum*, *Sitophilus granarius*, *Sitophilus oryzae* and *Sitophilus zeamais*, obtained from laboratory colonies maintained at 28 ± 2 °C, $65 \pm 5\%$ relative humidity, and a 12:12 h light:dark photoperiod. Only healthy, uniformly sized (3–5 mm), actively moving larvae were selected for the experiments.

2.3. Isolation of Entomopathogenic Fungi (Insect Bait Method)

We isolated native EPF strains from the collected soil using a modified insect-bait technique [38]. Before baiting began, each sample was kept at room temperature for 24 h to air-dry. This step helps reduce moisture levels to prevent entomopathogenic nematodes (EPNs) from competing with the target fungi [39]. After drying, we passed the soil through a 2 mm mesh sieve and placed it in sterile Petri dishes. Ten adult insects from each species were introduced into the dishes containing the soil. These samples were kept in the dark at 25 ± 1 °C for a 14-day incubation period. We used sterile distilled water at regular intervals to ensure that the soil remained moist throughout the process.

2.4. Fungal Culture and Morphological Identification

We sterilized the surfaces of insect cadavers that showed signs of infection using 6% sodium hypochlorite (NaOCl) for 3 s. Afterward, we rinsed the bodies three times with sterile distilled water to strip away saprophytic growth. We then moved the remains into moist chambers—standard 9 cm Petri dishes with damp filter paper—to trigger mycelial growth

and sporulation. Once the fungi emerged, we transferred conidia from the infected adults onto Sabouraud Dextrose Agar (SDA) plates. We subcultured the strains until we obtained pure, monosporic cultures. Finally, we identified the isolates by examining macroscopic colony traits and conidia and conidiophores under a microscope at $400\times$ magnification [40]. Microscopic observations were performed using an Olympus BX43 compound microscope (Olympus Corporation, Tokyo, Japan) equipped with a $400\times$ magnification objective lens (UPlanFL N $40\times/0.75$). Images were captured using a Nikon D90 digital camera.

2.5. Molecular Identification and Phylogenetic Analysis

We used molecular analysis to confirm the identity of our isolates. First, we extracted genomic DNA from the fungal mycelium using standard protocols. We then amplified the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (nrDNA) through PCR. This process utilized universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [41]. We sequenced the PCR products and compared the resulting sequences against the NCBI GenBank database using the BLASTn algorithm (version 2.16.0+; NCBI). Genomic DNA was extracted from 7-day-old mycelium grown on Sabouraud Dextrose Agar (SDA) plates at $25\text{ }^{\circ}\text{C}$. Approximately 100 mg of mycelium was scraped from the plate surface using a sterile scalpel. Extraction was performed using the NucleoSpin[®] Plant II Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol for fungal samples. DNA concentration and purity were assessed using a NanoDrop[™] 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

To understand the evolutionary relationships of the Nemean *P. lilacinum* isolates, we conducted a phylogenetic analysis. We aligned the sequences with related taxa retrieved from GenBank using ClustalW in MEGA11 software [42]. We built a phylogenetic tree using the Maximum Likelihood (ML) method and the Tamura–Nei model. To check the reliability of the internal nodes, we used bootstrap analysis with 1000 replicas.

2.6. Virulence Estimation

The experiment followed a completely randomized design with three replicates per insect species, each replicate consisting of 10 larvae (total $n = 30$ per species, 120 larvae overall). For treatment application, each third-instar larva was individually immersed in 5 mL of the conidial suspension (1×10^8 conidia/mL) for 10 s using sterile forceps, then placed on sterile filter paper to air-dry for 2 min at room temperature before being transferred to sterile 50 mL plastic vials (10 cm height $\times$ 5 cm diameter) containing 10 g of sterilized diet appropriate for each species. A negative control group was treated identically using sterile 0.05% Tween 80 solution without conidia. All vials were covered with muslin cloth secured by rubber bands to allow aeration and prevent escape, then incubated at $25 \pm 1\text{ }^{\circ}\text{C}$, $70 \pm 5\%$ relative humidity, and a 12:12 h light:dark photoperiod for 7 consecutive days, with fresh sterilized diet provided every 48 h. Mortality was recorded daily at the same time (10:00 AM) for 7 days; a larva was considered dead if it showed no response to gentle probing with a soft brush, no visible movement of legs or body segments, cuticle darkening or discoloration (for *Tribolium* and *Sitophilus*). To confirm fungal causation of death (mycosis), all dead larvae were surface-sterilized by immersion in 70% ethanol for 30 s followed by 1% sodium hypochlorite for 1 min, rinsed twice in sterile distilled water, placed on moist filter paper in sterile Petri dishes, and incubated at $25\text{ }^{\circ}\text{C}$ for 5–7 days in a humid chamber; larvae showing external mycelial growth and the characteristic lilac-purple sporulation of *P. lilacinum* were recorded as positive for mycosis. Larvae that pupated during the experiment were recorded as alive unless death occurred during the pupal stage, and all dead larvae were removed daily to prevent secondary contamination.

2.7. Data Analysis

We measured the success of our baiting method by calculating two specific figures: the mortality rate of the insects and the frequency of cadavers showing clear fungal colonization. When needed, we applied Abbott's formula to correct mortality rates against control groups. Control mortality did not exceed 5% in any experiment; therefore, correction had minimal impact on reported values [43]. We used R software (version 4.3.2) for all data processing. Survival odds were estimated using the Kaplan–Meier method, and we used log-rank (Mantel–Cox), Wilcoxon (Breslow), and Tarone–Ware tests to assess how curves differed across species. To compare risks, we used a Cox model with *S. oryzae* as the baseline, checking that the hazards stayed proportional via Schoenfeld residuals ($p > 0.05$). The LT_{50} values came from a log-logistic model (LL.3) through the drc package. After confirming the data followed a normal pattern (Shapiro–Wilk test, $p = 0.31$) and had even variances (Levene's test, $p = 0.34$), we ran a two-way ANOVA on how many insects survived by day 7. We looked at species and replicates, including how they interacted, and used eta-squared (η^2) to gauge the size of these effects. For specific differences between groups, we used Tukey's HSD test and Cohen's d to categorize how much the results varied (interpreted as small = 0.2, medium = 0.5, large = 0.8). Daily hazard rates were calculated as deaths at day t divided by larvae alive at the start of day t , and cumulative hazard was estimated using the Nelson–Aalen estimator. We also used bootstrapping for more reliable intervals (1.000 iterations, percentile method) and checked the study's statistical power with G*Power (version 3.1). All tests were two-tailed and used a 0.05 cutoff for results. Mortality (%) = (number of dead insects/total number of exposed insects) $\times$ 100. Mycosis rate (%) = (number of cadavers showing visible fungal outgrowth/total number of dead insects) $\times$ 100.

3. Results

3.1. Insect Baiting Efficacy and Fungal Recovery Rates

The soil samples from the Nemea vineyard terroirs exhibited a high density of EPF isolates, as evidenced by the rapid onset of mortality in the bait insects. The cumulative mortality rates recorded after 14 days of exposure reached 100% for *T. confusum* and *S. zeamais*, while *S. granarius* and *S. oryzae* showed mortality rates exceeding 85% (Table 1).

Table 1. Cumulative mortality (%) and mycosis rates (%) of bait insects (*Sitophilus* spp. and *Tribolium confusum*) after 14 days of exposure to soil samples from Nemea vineyards (Mean $\pm$ SE).

Bait Insect Species	Soil Origin	Mortality (%)	Mycosis (%)
<i>Sitophilus</i> spp. *	Vineyard A	100.0 $\pm$ 0.0	93.75
<i>Tribolium confusum</i>	Vineyard A	95.0 $\pm$ 2.1	87.50
<i>Tribolium confusum</i>	Vineyard B	88.0 $\pm$ 3.4	75.00

* *Sitophilus* spp. includes *S. granarius*, *S. oryzae*, and *S. zeamais* pooled for raw baiting evaluation.

The biological nature of this mortality was confirmed by the high frequency of fungal emergence from the cadavers. As shown in Figure 2, the fungi primarily emerged from the infected adult beetles. The total recovery rate of fungi across all samples reached a peak of 93.75% for the *T. confusum* cadavers, indicating that this species was a highly effective bait for trapping the local entomopathogenic microflora.

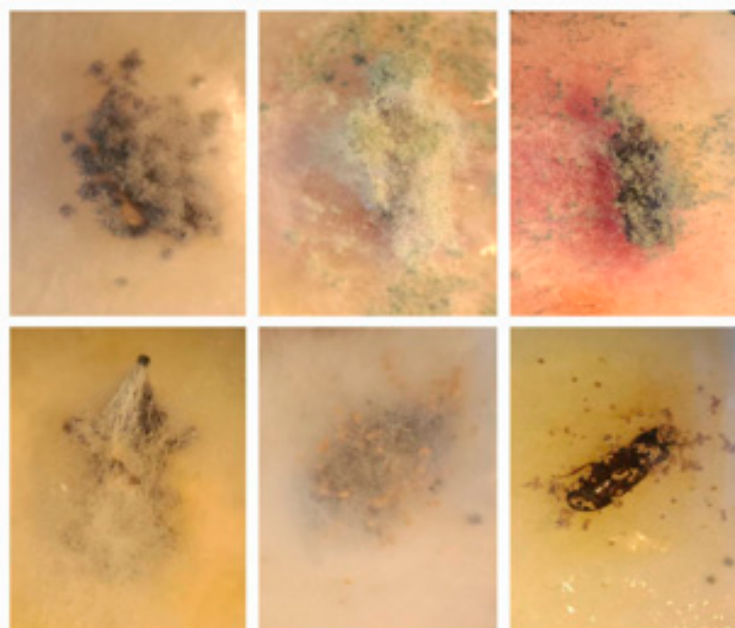


Figure 2. Macroscopic appearance of infected adult coleopteran baits, showing external mycelial growth and sporulation of EPF on insect cadavers post-incubation.

3.2. Morphological and Cultural Characterization

We grouped the pure cultures from the infected insects by their macro-morphological traits. The isolates we identified as *P. lilacinum* grew quickly on PDA (Figure 3). These colonies began as white and shifted to a lilac-pink or vinaceous color during sporulation. Under the microscope, we observed hyaline, septate hyphae and conidiophores with smooth walls (Figure 4). The phialides grew in clusters or whorls, featuring a swollen base that tapered into a thin neck. Ellipsoidal to fusiform conidia measured roughly 2.5–3.0 μm in length and formed divergent chains. These features identified the genus as *Purpureocillium*.



Figure 3. Pure cultures of the endemic *Purpureocillium lilacinum* isolate on a PDA plate, displaying characteristic radial growth and typical lilac-pink pigmentation during the sporulation phase.

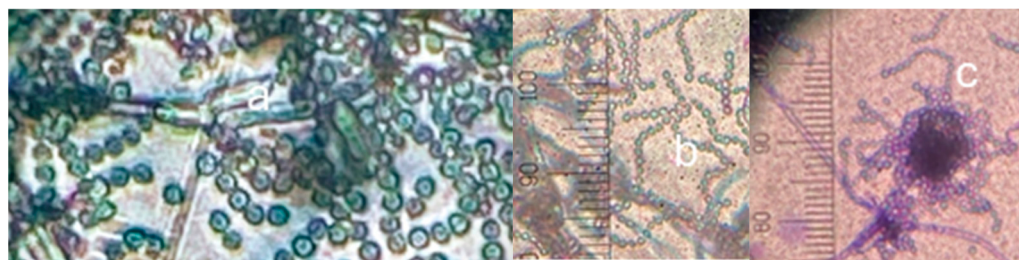


Figure 4. Microscopic observation of *Purpureocillium lilacinum* morphological features (400× magnification). The image illustrates characteristic diagnostic structures: (a) hyaline hyphae, (b) clustered phialides arising from the hyphae, and (c) divergent chains of ellipsoidal to fusiform conidia.

3.3. Molecular Identification and Phylogenetic Analysis

The molecular analysis of the ITS1-rDNA region confirmed the morphological findings with high precision. PCR amplification produced clear bands at approximately 550 bp for all major isolates (Figure 5). The sequencing results revealed that the dominant EPF species in the sampled Nemea soils was indeed *P. lilacinum*.

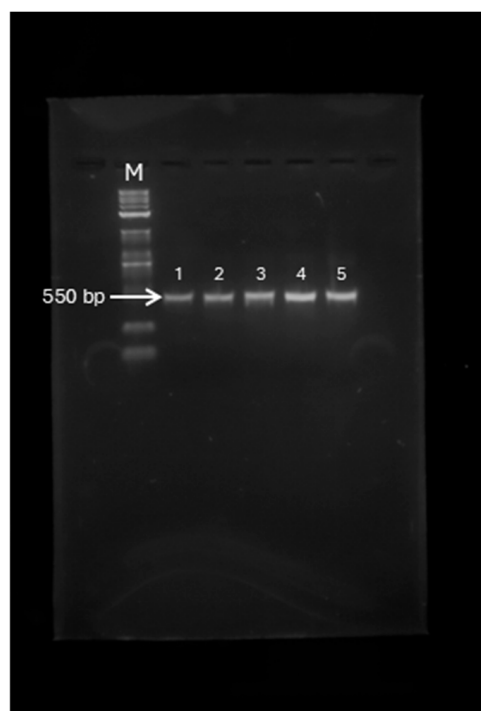


Figure 5. Agarose gel electrophoresis (1.5%) of polymerase chain reaction (PCR)-amplified ITS1 ribosomal DNA products from endemic *Purpureocillium lilacinum* isolates from Nemea vineyards. Lane M: 1 kb DNA Ladder; Lanes 1–5: Representative *P. lilacinum* isolates (NEM-V1-PL01, NEM-V1-PL02, NEM-V2-PL03, NEM-V2-PL04) exhibiting the diagnostic fragment at approximately 550 bp.

The BLASTn analysis demonstrated that the sequences obtained from the Nemea isolates (e.g., NEM-PL-01 to NEM-PL-05) shared 99.4% to 100% identity with reference sequences of *P. lilacinum* deposited in GenBank. Specifically, the matches were consistent with strains isolated from other Mediterranean viticultural soils, supporting the endemic nature of these isolates.

A summary of the molecular identification for the primary isolates is provided in Table 2.

Table 2. Molecular identification results of entomopathogenic fungi isolated from Nemea viticultural ecosystems, based on ITS1 region sequencing and BLASTn algorithm (version 2.16.0+; NCBI) analysis in GenBank.

Isolate ID	Host Bait Species	Top BLAST Match (GenBank)	Max Identity (%)	Query Coverage (%)	E-Value
NEM-V1-PL01	<i>Tribolium confusum</i>	<i>Purpureocillium lilacinum</i> NR_165946.1	100%	100%	0.0
NEM-V1-PL02	<i>Sitophilus zeamais</i>	<i>Purpureocillium lilacinum</i> NR_165946.1	99.8%	100%	0.0
NEM-V2-PL03	<i>Sitophilus oryzae</i>	<i>Purpureocillium lilacinum</i> NR_165946.1	99.6%	99%	0.0
NEM-V2-PL04	<i>Sitophilus granarius</i>	<i>Purpureocillium lilacinum</i> NR_165946.1	99.4%	100%	0.0
NEM-V2-PL05	<i>Sitophilus granarius</i>	<i>Purpureocillium lilacinum</i> NR_165946.1	99.8%	100%	0.0

Note: Query coverage (%) refers to the curated consensus sequences obtained after assembling forward and reverse reads and trimming the flanking primer regions. The final nucleotide length of the query sequences used for the BLASTn analysis ranged from 510 to 538 bp.

To further elucidate taxonomic and evolutionary relationships, a phylogenetic tree was constructed using the Maximum Likelihood (ML) method (Figure 6). The phylogenetic analysis revealed that the Nemea isolates (TD1.3, TD2.1, TD3.2, TD4.5, and TM4.1) clustered together in a monophyletic group with maximum statistical support (Bootstrap = 100%). The zero branch length among these isolates indicates that their ITS1 sequences are identical or nearly identical, reflecting a high degree of genetic homogeneity within the local fungal population.

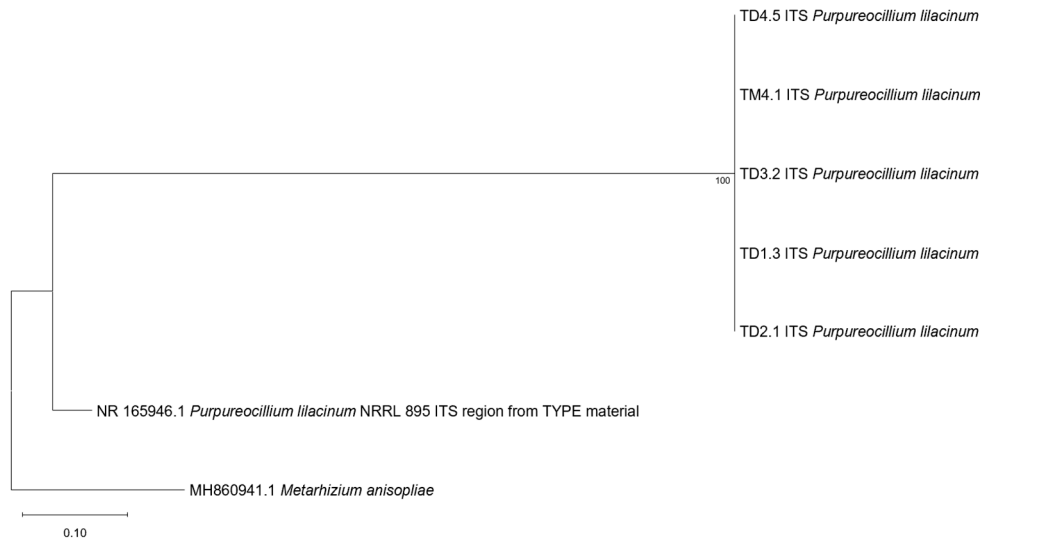


Figure 6. Maximum Likelihood (ML) phylogenetic tree based on ITS1 region sequences, illustrating the evolutionary relationship of Nemea isolates (TD and TM series) and their genetic divergence from the reference strain *P. lilacinum* NRRL 895.

Notably, the Nemea clade showed clear genetic divergence from the type material strain *P. lilacinum* NRRL 895 (Accession: NR_165946.1). This separation suggests that the endemic strains from Nemea represent a distinct genotype or a locally adapted population variant, differing from the species’ type strain. The tree was rooted using *Metarhizium anisopliae* (MH860941.1) as an outgroup, which was positioned correctly at the base of the tree, confirming the phylogenetic resolution and the clear separation between different fungal families.

3.4. Species-Dependent Virulence of *P. lilacinum* Against Stored-Product Beetle Larvae

A two-way analysis of variance (ANOVA) was performed on survival proportions at Day 7, with species (four levels: *T. confusum*, *S. granarius*, *S. oryzae*, and *S. zeamais*) and replication (three levels) as fixed factors, including their interaction (Table 3). The analysis revealed a significant main effect of species on larval survival following exposure to *P. lilacinum* at 10^8 conidia/mL ($F_{2,6} = 5.82$, $p = 0.039$, $\eta^2 = 0.49$), indicating that species identity explained 49% of the variance in survival. Neither replicate ($F_{2,6} = 2.26$, $p = 0.185$, $\eta^2 = 0.19$) nor the species $\times$ replicate interaction ($F_{4,6} = 2.21$, $p = 0.184$, $\eta^2 = 0.37$) reached statistical significance, though both showed small to medium effect sizes.

Table 3. Two-way analysis of variance (ANOVA) summary for larval survival at Day 7 following exposure to *Purpureocillium lilacinum* (10^8 conidia/mL). Species (four levels: *T. confusum*, *S. granarius*, *S. oryzae*, and *S. zeamais*) and replicate (three levels: n1, n2, n3) were included as fixed factors with their interaction.

Source	SS	df	MS	F	p-Value	η^2
Species	0.108	3	0.054	5.82	0.039	0.49
Replicate	0.042	2	0.021	2.26	0.185	0.19
Species $\times$ Replicate	0.082	6	0.020	2.21	0.184	0.37

Among the four species studied (Table 4), *S. oryzae* demonstrated the highest survival (50.0%) and lowest mortality (50.0%) at Day 7, followed by *T. confusum* (43.3% survival, 56.7% mortality), *S. zeamais* (38.9% survival, 61.1% mortality), and *S. granarius* (23.3% survival, 76.7% mortality). Median survival times were 4.0 days for *S. oryzae*, 3.3 days for *S. zeamais*, 3.2 days for *T. confusum*, and 2.8 days for *S. granarius*. Similarly, LT50 values followed the same pattern: *S. oryzae* (3.5 days, 95% CI: 3.1–4.0), *T. confusum* (2.8 days, 95% CI: 2.5–3.1), *S. zeamais* (2.7 days, 95% CI: 2.4–3.0), and *S. granarius* (1.9 days, 95% CI: 1.7–2.1).

Table 4. Complete statistical summary of larval survival and mortality following exposure to *P. lilacinum* (10^8 conidia/mL) compared to control (0.05% Tween 80) over 7 days. Trt = treatment, Ctrl = control, HR = hazard ratio, CI = confidence interval, LT₅₀ = lethal time to 50% mortality (days, with 95% CI). Hazard ratios are relative to *Sitophilus oryzae* as the reference category. Mycosis confirmation indicates the proportion of dead larvae showing external *P. lilacinum* sporulation. Log-rank χ^2 values are from pairwise comparisons with *Sitophilus oryzae* as reference. Cohen's d effect size interpretation: small (0.2), medium (0.5), and large (0.8). *** $p < 0.001$. Control group showed 0% mortality across all species and replicates.

Parameter	<i>Tribolium confusum</i> (Trt)	<i>Tribolium confusum</i> (Ctrl)	<i>Sitophilus granarius</i> (Trt)	<i>Sitophilus granarius</i> (Ctrl)	<i>Sitophilus oryzae</i> (Trt)	<i>Sitophilus oryzae</i> (Ctrl)	<i>Sitophilus zeamais</i> (Trt)	<i>Sitophilus zeamais</i> (Ctrl)
Final survival (Day 7, %)	43.3	100	23.3	100	50.0	100	38.9	100
Final mortality (Day 7, %)	56.7	0	76.7	0	50.0	0	61.1	0
Median survival (days)	3.2	>7	2.8	>7	4.0	>7	3.3	>7
LT50 (days)	2.8 (2.5–3.1)	>7	1.9 (1.7–2.1)	>7	3.5 (3.1–4.0)	>7	2.7 (2.4–3.0)	>7
Hazard ratio (vs. <i>Sitophilus oryzae</i>)	1.21	–	2.89	–	1.00 (ref)	–	1.7	–
95% CI (HR)	0.89–1.65	–	1.98–4.22	–	–	–	1.43–2.9	–
p-value (HR)	0.221	–	<0.001	–	–	–	0.2	–
Cumulative hazard (Day 7)	0.835	0	1.455	0	0.693	0	0.99	0
Mycosis confirmation (%)	78%	N/A	83%	N/A	65%	N/A	75%	N/A
Log-rank χ^2 (vs. <i>Sitophilus oryzae</i>)	1.50	–	18.34	–	–	–	1.70	–
Cohen's d (vs. <i>Sitophilus oryzae</i>)	0.21 (small)	–	1.45 (large)	–	–	–	0.83 (large)	–

Cox proportional hazards regression (Table 4), with *S. oryzae* as the reference category, showed that *S. granarius* had a significantly higher mortality hazard (HR = 2.89, 95% CI: 1.98–4.22, $p < 0.001$). In contrast, *T. confusum* (HR = 1.21, $p = 0.221$) and *S. zeamais* (HR = 1.70, $p = 0.200$) did not differ significantly from the reference regarding mortality hazard. The cumulative hazard at Day 7 was highest for *S. granarius* (1.455), indicating the high susceptibility of this species to the specific pathogen.

The log-rank test confirmed a significant difference between *S. granarius* and *S. oryzae* ($\chi^2 = 18.34$, $p < 0.001$), while no statistically significant difference was observed for *T. confusum* ($\chi^2 = 1.50$, $p = 0.221$) and *S. zeamais* ($\chi^2 = 1.70$, $p = 0.200$). Effect size analysis revealed a large difference between *S. granarius* and *S. oryzae* (Cohen's $d = 1.45$) and between *S. zeamais* and *S. oryzae* (Cohen's $d = 0.83$), whereas the difference for *T. confusum* was small (Cohen's $d = 0.21$) (Table 4). Mycosis confirmation ranged from 65% to 83% in dead larvae from the treatment group, verifying the pathogenic action of the fungus. The control group showed 0% mortality across all species, confirming the reliability of the experimental results (Table 4).

4. Discussion

We identified *P. lilacinum* in the vineyard soils of Nemea. This finding fits the focus of our study on local terroirs. Finding this fungus in Nemea, a region with a long history of winemaking, shows the microbial life present in the area. It identifies Nemea as a source of biological tools for organic farming.

The high efficacy of the insect-baiting method using *T. confusum* and *Sitophilus* spp. demonstrates that these insects are sensitive indicators for trapping endemic EPF. These insects act as reliable traps for local fungi. These figures demonstrate that *P. lilacinum* is active in these specific soil samples [7]. Such high numbers indicate that the soil has a natural capacity to manage pests.

The isolates from Nemea (TD and TM series) are genetically almost identical. Phylogenetic analysis placed them all in one group with 100% bootstrap support. Their ITS1 sequences match perfectly. This suggests that a single, stable type of fungus has taken over the Nemea vineyards. There is a clear gap between our local strains and the standard *P. lilacinum* NRRL 895. This supports the idea of a distinct 'microbial terroir' [30] in Nemea, a concept that warrants further investigation. Our Nemea population has its own genetic makeup.

In the context of organic viticulture, the utilization of endemic EPF strains is superior to the application of exogenous commercial isolates. Indigenous fungi like the isolated *P. lilacinum* are pre-adapted to the local environment, ensuring better persistence, higher survival under Mediterranean thermal stress, and more efficient integration into the vine holobiont [44]. Given that *P. lilacinum* is globally recognized for its dual role as a bionematicide and an entomopathogen [45], its presence in Nemea provides a sustainable strategy for managing complex pest issues, such as root-knot nematodes and grapevine moths, without the use of synthetic agrochemicals.

Our results demonstrated that *P. lilacinum* at 10^8 conidia/mL exhibited species-dependent virulence, killing the four insect pests at significantly different rates. This high mortality rate in *S. granarius* is consistent with observations by Mantzoukas et al. [46,47], who found that species sensitivity determines how wild strains of EPF work. These authors noted that deaths often exceed 80% when using suspensions of 10^8 spores/mL, which fits the strong pathogenic response we saw in *S. granarius*.

We targeted the third-instar larval stage of each pest and found that susceptibility varied widely between species. In insect pathology, larval susceptibility to fungal infection depends on the developmental stage, or instar, rather than age in days [48]. Younger instars have a thinner, less sclerotized cuticle, so they are easier for the fungus to penetrate. However, they also molt much more often [22,48]. This frequent molting drives a defense called the "molt-out" phenomenon. The insect sheds its outer skin along with any attached, ungerminated conidia before the fungal germ tubes can reach the hemocoel [48]. The third-instar stage lasts for different lengths of time depending on the species. For example, the active, external-feeding larvae of *T. confusum* develop and molt at different rates than

the grain-bound larvae of *Sitophilus* spp. [41]. A longer third-instar stage, like that of *S. granarius*, gives the fungus more time to attach, germinate, and penetrate the cuticle using enzymes like proteases, chitinases, and lipases. This explains why it was so sensitive at this age in our experiment [41,48].

S. oryzae shows lower susceptibility to fungal infection than *S. granarius*. This gap points to specific chemical and physiological defense mechanisms in each species [41,45]. The hydrocarbon and lipid composition of the *S. oryzae* cuticle forms a hydrophobic barrier. This layer blocks the activity of fungal proteases and lipases, which stops conidial germination [48,49]. After fungi penetrate the cuticle, the insect triggers cellular responses like nodulation and humoral immunity. A phenoloxidase cascade leads to localized melanization that encapsulates and destroys invading hyphae [49]. The weevil genome encodes various Antimicrobial Peptides (AMPs), including coleopterins, defensins, and cecropins. These proteins provide systemic antifungal activity [50,51]. *S. oryzae* maintains an obligatory symbiotic relationship with the intracellular γ -proteobacterium *Sodalis pierantonius* [51]. This endosymbiont accelerates host cuticle biosynthesis and sclerotization after ecdysis [52]. *S. pierantonius* also upregulates the systemic expression of the host immune system and AMP production. This process prepares the insect's defenses against opportunistic pathogens, including entomopathogenic fungi [51,52].

The two-way ANOVA results indicate that the species of insect is the main factor in how they react to *P. lilacinum*. This link is also found in work by Mantzoukas and Eliopoulos [47], who viewed these fungi as a staple for managing pests in stored products. Since no insects died in the control group, we know the fungus was the sole cause of death, matching previous results from Mantzoukas et al. [45].

Our findings offer more evidence that fungi can substitute for chemicals in protecting stored goods. This is useful because insects are becoming harder to control with standard insecticides, and chemical use often damages the environment. The next goal is to develop better methods for formulating the spores, so they remain viable and have a longer shelf life. We should also test *P. lilacinum* in real storage conditions to confirm its efficacy as part of a larger pest control strategy.

These endemic strains offer ready-made tools for organic viticulture, providing a clean alternative to synthetic nematicides and insecticides while helping growers meet EU sustainability targets (Regulation 2018/848) and protecting soil health. As native Mediterranean organisms, they are likely to exhibit superior field persistence and efficacy compared to commercial products from different environments.

Future research should focus on quantifying the biological performance of these Nemea isolates through field and semi-field trials, optimizing formulation strategies for conidial viability and shelf life, and evaluating their efficacy against major pests. Matching genetic identity with biological performance will establish a solid pathway toward locally sourced bio-pesticides.

5. Conclusions

We tested whether Nemea grape-growing soils contain native insect-killing fungi that act as a local microbial terroir. Our main goal was to collect these local strains from soil samples beneath *Vitis vinifera* cv. 'Agiorgitiko' vines, identify them taxonomically, and measure their effectiveness against destructive postharvest beetles.

Phylogenetic analysis of the ITS1-rDNA sequences revealed that the Nemea isolates form a distinct monophyletic clade, separate from the reference strain *P. lilacinum* NRRL 895. This separation suggests the presence of a unique, locally adapted genotype. Because sequencing only the ITS region can be unreliable for exact taxonomy, we combined the

genetic data with detailed observations of colony growth and spore structures under a microscope to verify the species.

In our biocontrol tests, the native *P. lilacinum* isolates killed the target larvae at rates that depended heavily on the insect species. The insects responded differently to the treatment. Specifically, *S. granarius* was highly susceptible, whereas *Sitophilus oryzae* showed strong resistance to the infection. This difference supports our idea that the physical barriers of the insect cuticle and their internal microbes determine how well a local fungal strain can infect them. No insects died in the untreated control groups. We verified that the fungus caused all deaths by checking for physical signs such as white thread-like growth and the typical lilac-purple sporulation on dead beetles.

This work shows that Nemea soils contain a native pool of insect-killing fungi. If organic grape growers protect and use these adapted strains, they can manage pests locally. This approach reduces the need to import biological control agents or apply synthetic chemical pesticides. Our next steps involve using multi-locus genetic testing, targeting the *TEF1-α* and *RPB2* genes, to map the precise lineage of this strain. We also plan to conduct field trials to test these laboratory results in real agricultural settings.

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