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ORIGINAL PAPER

Possibilities of native endophytic fungi as entomopathogenic biocontrol agents at a local scale: the case of deciduous and non-deciduous Mediterranean forest trees

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Abstract Tree endophytic fungi play an important role in reducing insect herbivory, either by repelling them or killing them directly. Identifying which fungi show such activity could lead to new environmentally friendly pesticides. In this study, the Mediterranean basin climate conditions are projected to harshen in the next decades, will increase vulnerability of tree species to pest invasions. Endophytic fungi were isolated from wood and leaves of *Quercus pyrenaica*, *Q. ilex* and *Q. suber* and tested for virulence against adults of the mealworm beetle, *Tenebrio molitor* L. using a direct contact method. Only 3 of 111 sporulating isolates had entomopathogenic activity, all identified as *Lecanicillium lecanii.* The pathogenicity of *L. lecanii* on *T. molitor* resulted in a median lethal time (TL50) of 14–16 d. Compared with commercial products, *L. lecanii* caused faster insect death than the nematode *Steinernema carpocapsae* and nuclear polyhedrosis virus (no effect on *T. molitor* survival), and slower than *Beauveria bassiana* (TL50 = 5), *Beauveria pseudobassiana* (TL50 = 8d) and *Bacillus thuriengensis* (80% mortality first day after inoculation). Mortality was also accelerated under water stress, reducing TL50 by an additional 33%. Remarkably, water stress alone had a comparable effect on mortality to that of *L. lecanii* isolates. This study confirms *T. molitor* as a good model insect for pathogenicity testing and agrees with management policies proposed in the EU Green Deal.

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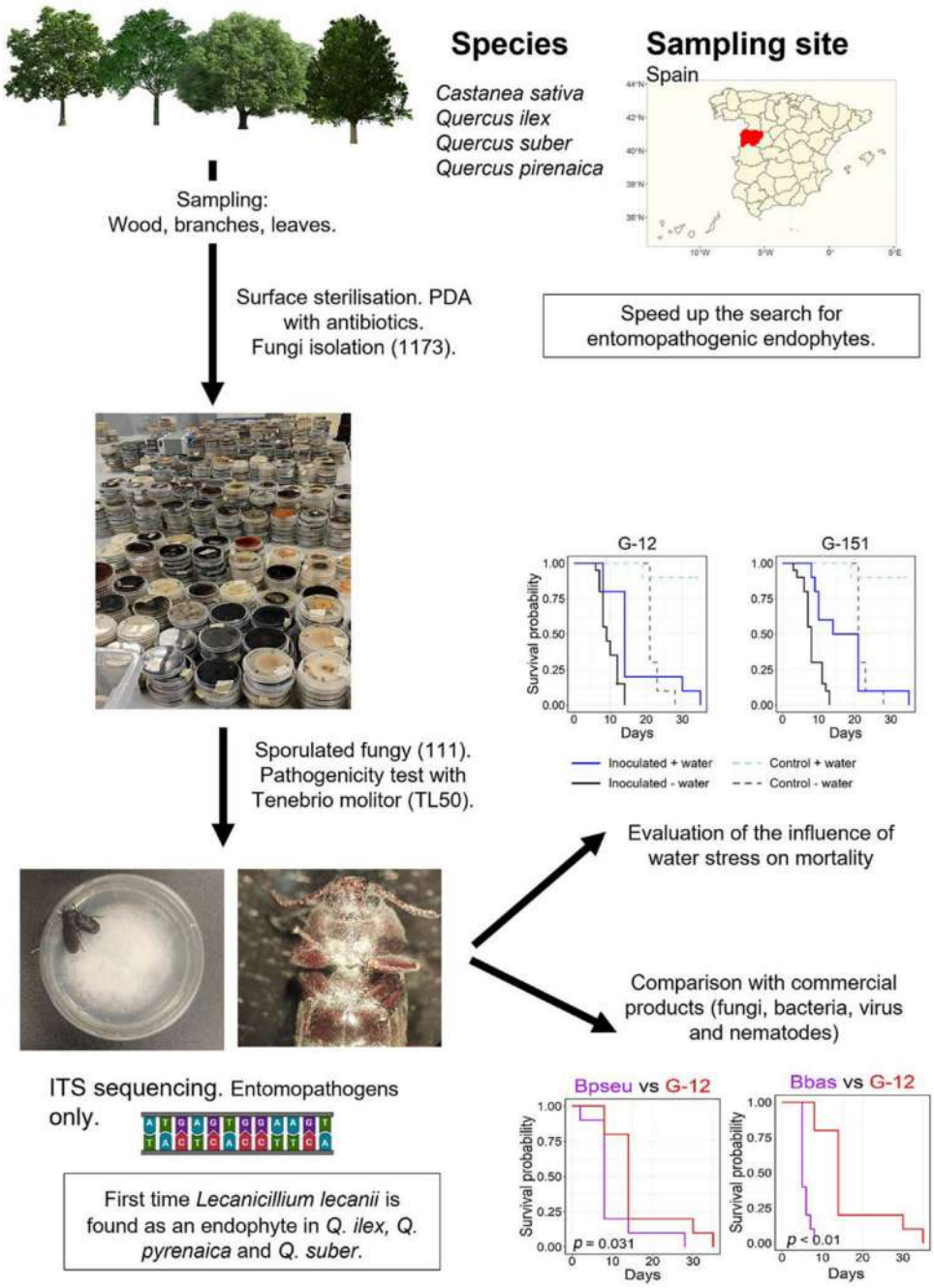
Graphical abstract



Keywords Biocontrol · Pesticide · Tree pest · Native fungi · Climate change

Introduction

The integrity of the biosphere is one of nine planetary boundaries identified by the Stockholm Resilience Centre as one exceeded due to human influence on the planet (Richardson et al. 2023). Invasive insects, especially as pests, and pathogenic organisms can weaken and destroy forest stands. As a result, significant ecological and economic damage can occur with long-lasting or even permanent consequences. New tools that can be integrated into integrated pest management (IPM) strategies are needed to deal with the silent invasion of insects and species that behave as pests.

In Mediterranean forests, decay phenomena are common occurrences that can have significant impacts on ecosystem health and biodiversity. As highlighted by Lazo (2018), decay may stem from a combination of abiotic factors such as prolonged droughts and shifts in precipitation patterns, along with biotic factors, for example, the proliferation of opportunistic pathogens. Insect proliferation in plague episodes weakens the vegetative state of forest stands causing economic losses and ecosystem damage. Among the insects that cause the most damage in Spanish Mediterranean forests are bark beetles such as *Cerambyx welensii* Küster in *Quercus ilex* L. and *Q. pyrenaica* Willd and *Coraebus undatus* (Fabricius) in *Q. suber* L.

Entomopathogenic fungi (EF) constitute the largest known group of insect pathogens (Vega et al. 2012). Insect-infecting fungi are ever-present in nature and their application has proven to be a valid strategy to reduce populations of pests and damaging insects in forest ecosystems (Lacey et al. 2015). Since EF causes destructive diseases in insects as a by-product of their natural life cycle, they are an environmentally friendly pest control method (Shah and Pell 2003). As alternatives to chemical pesticides, EF can help mitigate selective pressure leading to the development of pesticide resistance in pest populations and are able to stop the expression of resistance once it has occurred (Lacey 2017). In recent decades, there has been a marked increase in adopting biological control agents as an alternative to chemical pesticides, driven by growing concerns about the environmental, public health and potential resistance impacts associated with them. This trend is particularly relevant in forest ecosystems, where large-scale application of pest control methods poses significant challenges, especially with regard to biodiversity protection (Lacey et al. 2015).

Several myco-insecticides have been developed from EF due to their relative ease of production on a commercial scale and their ability to infect numerous target organisms. Hyphomycete fungi, such as *Beauveria bassiana* (Bals.-Criv.) Vuill, *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Lecanicillium lecanii* (R. Zare and W. Gams) Viegas, among others, have been employed as inundative biocontrol agents. These isolates have been utilized extensively for managing various insect pest species in both greenhouses and open fields across numerous countries (Bamisile et al. 2021). At the forest level, the fungus *Entomophaga maimaiga* Humber, Shimazu & Soper (Entomophthoromycota: Entomophthorales) has been introduced in the USA to successfully control *Lymantria dispar* L. (Lepidoptera: Erebidae) (Hajek et al. 1996). The fungus *Beauveria brongniartii* (Sacc.) Petch has been applied against European cockchafer beetles, *Melolontha melolontha* L. (Lacey 2017). Other applications for the control of insect pests or disease vectors are being tested for forest crops, e.g., *B. bassiana* Bals. (Vuill) to control *Cosmopolites sordidus* Germar (Coleoptera Curculionidae) in banana crops (Picciotti et al. 2023) or *Trichoderma chlorosporum* P. Chaverri & Samuels for biocontrol of *Philaenus spumarius* L. (Hemiptera: Aphrophoridae), the insect vector of the quarantine bacterium *Xylella fastidosa* Wells (Ganassi et al. 2023).

Insect mortality is due to a combination of factors such as the effects of a fungal toxin, physical impairment of blood circulation, nutrient depletion and organ infiltration (Inglis et al. 2012). Various other factors may determine or influence the susceptibility of a host to infection by these fungi. These include the genetics of the fungal strain, the physiological state of the host, nutrition, defence mechanisms, the presence of other micro-organisms, as well as other factors such as environmental parameters (Inglis et al. 2012). Highly virulent species and strains of native EF can serve as effective bioinsecticides. Native strains of EF are fungi that have co-evolved with their insect hosts and local environmental conditions (Picciotti et al. 2023). Environmental conditions and the adaptation of fungi to the environment are important for the successful infection of insects by EF. Mann and Davis (2021) identified temperature, exposure to ultraviolet light, competition with other microorganisms and secondary plant metabolites as the main factors that may inhibit or promote fungal pathogenicity applied to the control of bark beetles by EF. Moreover, fungi native to each location may be more adaptable to local environmental conditions. For example, Alali et al. (2019) found that *B. bassiana* collected from the hottest areas are the most tolerant in terms of growth, sporulation, and germination at high temperatures. Lovett and St Leger (2018) showed that continuous culturing of *M. anisopliae* at high temperatures increases their growth rate.

Numerous studies have shown that the host range of EF can extend beyond insects. Several have the ability to grow within plants as endophytes, in addition to their presence in the soil, for example, for *B. bassiana*, *Lecanicillium* spp., *M. anisopliae* and *Trichoderma harzianum* Rifai which have been reported as endophytes of plants, not only in herbaceous crops (Vega et al. 2012; Gurr et al. 2018; Jaber and Ownley 2018) but also in forestry species such as *Pinus radiata* D. Don (Reay et al. 2010) and *Pinus monticola* Douglas ex D.Don (Ganley and Newcombe 2006). The endophytic growth of *B. bassiana* in cabbage hinders the growth and development of larvae, leading to a decrease in oviposition by diamondback moth (Zhang 2015). EF may also play an important role in reducing herbivory as endophytes following their colonization of plants, which has been reported to reduce damage caused by several pests in different crops (Jaber and Ownley 2018).

Large-scale global efforts are on-going to maximize the use of these microorganisms in pest control. There is strong commercial interest in sourcing novel microbial actives. Several emerging companies are investing in sequencing, characterization and screening of important plant biomes

and crop soils to discover unique microbes for development. Similarly, large multinational companies such as Bayer, Syngenta, BASF, and others are adding microbial and biochemical pesticides to their portfolios by investing a significant resources (Arthurs and Dara 2019). As a sign of this interest, multinationals invested more than US $2 billion between 2012 and 2015 in purchases and commercial agreements with smaller biopesticide companies (Dunham 2015). In addition to this, the EU's Green Deal, a multifaceted strategy, is committed to achieving climate neutrality by 2050. Integral to this vision is the urgent need to reduce reliance on chemical pesticides. By promoting sustainable agricultural practices and enhancing biodiversity conservation, the Green Deal aims to curb the use of harmful chemicals while fostering resilient ecosystems and ensuring food security for future generations (European Council of the European Union 2020).

The insect *Tenebrio molitor* L. (Coleoptera: *Tenebrionidae*, also known as mealworm beetle) is an insect that is susceptible to entomopathogenic micro-organisms. *T. molitor* is easily reared in large numbers with simple population and age controls. Numerous studies use it as a model insect to confirm the pathogenicity of fungi (Reay et al. 2010; Kim et al. 2018; Maistrou et al. 2018; Karaborklu et al. 2019; Bamisile et al. 2021; Eski and Murat Gezgin 2022; Shin et al. 2022). Therefore, this insect emerges as a valuable model for studying the effects of entomopathogenic fungi for pest control at a laboratory scale.

The objective of this study was to search for local endophytic EFs in Mediterranean forest species (*Q. ilex*, *Q. suber*, *Q. pyrenaica* and *Castanea sativa* Mill.) that could be used to control their pest species. It reports the first time that *L. lecanii* (Zimmermann) Zare & Gama has been found as an endophyte in these three species of *Quercus.* The increasing vulnerability of Mediterranean forests to pest outbreaks, intensified by climate change, underlines the importance of the discovery of *L. lecanii* as a native endophyte in *Quercus* species. Studies usually focus on the search for entomopathogenic fungi in soil and their mass application by flooding in a similar way to the use of conventional chemicals. Pathogenicity tests were carried out on the model insect *T. molitor* and compared with other commercial biological products. The influence of water stress on the survival of *T. molitor* once it has been infected by the native endophytic fungi found is also analysed. Even though this assay does not guarantee biological selectivity, as possibly its entomopathogenic power may be similar across pest insect species in these ecosystems, ecological selectivity is ensured, as only defoliating or boring insects would be affected by the endophytic EF.

Materials and methods

Isolation of fungi from plant tissues

Wood, bark, twigs and leaves of two deciduous species and two non-deciduous species from four areas of the province of Salamanca (eastern Spain) were sampled. Selected species were *Q. suber* (Valdelosa), *Q. pyrenaica* (Cubo de Don Sancho), *Q. ilex* (La Alamedilla) and *Castanea sativa* (Linares de Riofrío). Thirty trees were sampled from each area.

Fungi were isolated from plant tissues following Inglis et al. (2012). Tissue samples were cut into 0.5 cm Sects. (4 fragments/sample). For bark samples, the outer bark was removed so that only the xylem and the inner bark layer (phloem) remained. Fragments were sterilized and cultured on a general potato dextrose agar (PDA) medium with antibiotics (streptomycin (0.100 g L^{-1}) and ampicillin (0.050 g L^{-1})). Sterilization consisted of four steps: Tissue sections were washed for 30 s in autoclaved distilled water, immersed for 30 s in 3% NaOCl, immersed for 1 min in 70% ethanol and finally washed three times for 30 s in autoclaved distilled water. The last water rinse was plated on PDA medium and incubated to ensure surface sterilization. The plates were incubated at 23 ℃.

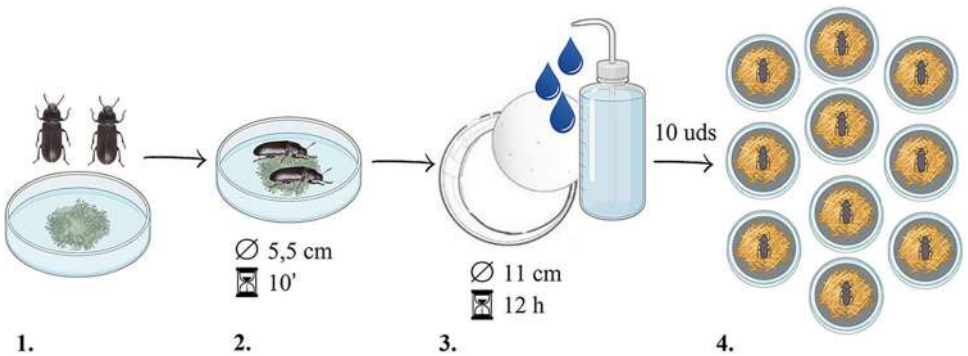


Fig. 1 Inoculation by direct contact: 1. Sporulated fungi 12–20 d old and two *T. molitor* per plate (10 per fungi). 2. 10 min with the insects walking on the plate (two manual shakings). 3. Humid chamber for 12 h with all 10 T. *molitor* together. 4. Individual petri dishes with sterilized wheat bran as food and a bed of self-washed filter paper

As the different fungi emerged from the sterilised tissues, they were isolated on individual plates and stored in 13% glycerol. Of the isolated fungi, those that produced sporulation on PDA were selected for use in pathogenicity tests.

Several samples were used for metabarcoding of DNA/RNA to identify fungal diversity and published by Diez-Hermano et al. (2024).

Laboratory rearing of *T. molitor*

A viable population of *T. molitor* was established in the laboratory of the Forest Pathology Group at La Yutera Campus, Palencia, University of Valladolid. The initial specimens were purchased locally and reproduced under laboratory conditions. The colony was maintained and propagated on trays in a growth chamber at 27 ± 1 °C, 70% ± 5% relative humidity and 16:8 h photoperiod (light: dark) with wheat bran feeding and PDA every two days as a water source (Deruytter et al. 2021).

Survival test on *T. molitor*

Virulence tests were performed on *T. molitor* beetles, which are susceptible to entomopathogenic microorganisms and easy to handle for insect bioassays (Kim et al. 2019; Shin et al. 2022). One hundred and twenty sporulating fungal isolates were used for testing.

Entomopathogenic activity was assessed following Güerri-Agulló et al. (2010) with minor modifications. Adults of *T. molitor* (10–15 d) were placed in pairs in 5.5 cm diameter Petri dishes containing a culture of the fungi to be tested. Ten insects were used per fungus. Fungi were 12–20 d, grown on PDA and had sporulated. The control consisted of insects on Petri plates with only PDA. Insects were allowed to move freely on the plate for 10 min, with two 5-s manual shaking times, and were then placed in 11 cm diameter Petri dishes with wet filter paper bottoms that acted as humidity chambers at 23 ℃ for 12 h. Insects were then transferred to individual dishes with sterilized wheat bran as food and a bed of self-washed filter paper (Fig. 1). Every second day a piece of autoclaved PDA (0.3 cm^3) was provided as a water source, as suggested by Deruytter et al. (2021). Mortality was checked daily. After the death of the insect, the fungi that were pathogenic to *T. molitor* were re-isolated on PDA.

Endophytic EF that were found were also tested when *T. molitor* was subjected to water stress conditions induced by not supplying any source of moisture during the assay.

Native fungi were compared against commercial products and fungi with known insecticidal capability (Table 1). The non-fungal commercial products were applied according to the manufacturer's application as indicated on the product.

Table 2 Total number of single isolates and sporulated isolates from samples on potato dextrose agar (PDA) medium

		Leaf	Twigs	Wood	Total
Total isolates	*Quercus ilex*	70	149	62	281
	Quercus pyrenaica	174	108	43	325
	Quercus suber	61	85	123	269
	Castanea sativa	82	123	93	298
Total		387	465	321	1173
Sporulated PDA isolates	*Quercusilex*	7	13	6	26
	Quercus pyrenaica	11	8	6	25
	Quercus suber	4	5	21	30
	Castanea sativa	11	11	8	30
Total		33	37	41	111

Identification of fungi

Fungi that performed best in the pathogenicity test were identified by sequencing of their ribosomal internal transcribed spacer (ITS) region. Total DNA was extracted using E.Z.N.A.® Plant and Fungal DNA Ki (Omega Bio-tekinc., USA) and sent to STAB Vida (Portugal) for sequencing. Primers used were ITS1-F (forward): 5′- CTTGGTCATTTA GAGGAAGTAA -3′ (Gardes and Bruns 1993) and ITS4 (reverse): 5′- TCCTCCGCTTATTGATATGC -3′ (White et al. 1990). Sequences were BLASTed against nr/nt database at NCBI and the top result with highest similarity and lowest e-value was selected as identity.

Statistical analysis

All analyses were conducted in R programming (R Core Team 2022). Lethal median time (TL_{50}) was estimated as the

Table 1 Commercial products tested on *Tenebrio molitor* for comparison with isolated endophytic EF

Product	Commercial brand	Strain
Beauveria bassiana (Bbas)	Botanigard® Certis Belchim	GHA
Nematode *Steinernema carpocapsae* (Scar)	Agrobío	–
Nuclear polyhedrosis virus (NPV)	Helicovex®	DSMZ: BV-0003
Bacillus thuringiensis (Bthu)	Costar® Syngenta	SA-12
Beauveria pseudobassiana (Bpseu)	Laboratory strain	MG-BU-17–001

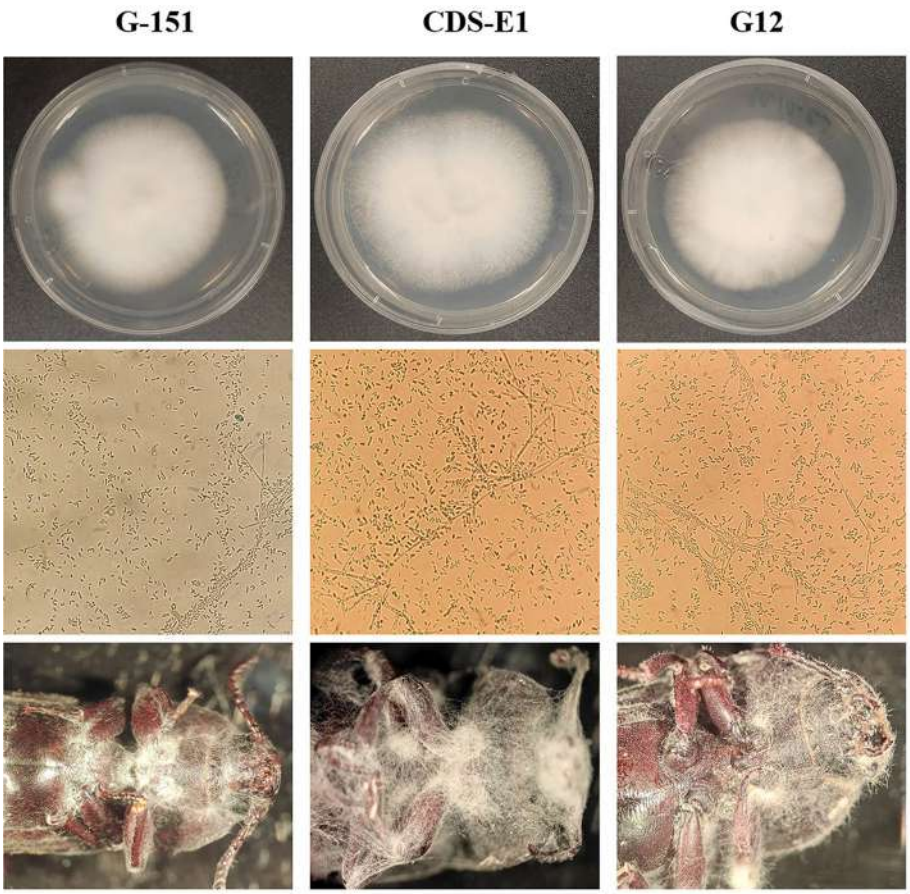


Fig. 2 Morphology and entomopathogenic activity of isolated endophytes. Each column corresponds to one fungus. Top row: culture plates after 7 d; middle row: spore suspensions viewed under a 40× light microscope, bottom row: emerged mycelium covering the surface of *T. molitor* specimens 7 d after death and growing in a humid chamber at 23 ℃ (1.6× magnifying glass)

Table 3 Location and source tissue of reported EF

Code	Host species	Tissue	Identity
G-151	*Quercus suber*	Wood	*Lecanicillium lecanii*
CDS-E1	*Quercus pyrenaica*	Leaves	*Lecanicillium lecanii*
G-12	*Quercus ilex*	Leaves	*Lecanicillium lecanii*

number of days by which half of the insects had died. Survival curves were estimated using the Kaplan–Meier method for censored data as implemented in the *survival package* (Therneau 2022). Confidence intervals were estimated by log transformation. Differences between pairs of curves were determined using the G-rho family of tests (rho = 0, equivalent to a log-rank test) using the *survminer package* (Kassambara et al. 2021). Benjamini–Hochberg correction for multiple comparisons was applied. Differences were declared significant for corrected p-values < 0.05.

Results

Isolated fungi

A total of 1173 individual isolates were obtained on PDA plates. Of these, 111 produced sporulation on PDA. Tree and tissue distribution of the different isolates is shown in Table 2.

Most fungi were isolated from *Q. pyrenaica* (325), followed by *C. sativa* (298), *Q. ilex* (281) and *Q. suber* (269). Regarding the type of tissue of origin, the highest number of fungi was isolated from twigs (465), followed by leaves (387) and wood (321). As to sporulating endophytic fungi, *Q. suber* and *C. sativa* (30) were the species from which the highest number was isolated, followed by *Q. ilex* (26) and *Q. pyrenaica* (25). For the tissue of origin, the highest number of fungi showing sporulation was isolated from twigs (37), followed by leaves (33) and wood (31).

Endophytes with entomopathogenic activity

Three of 111 (2.7%) sporulating fungi isolated from plant samples caused mortality in *T. molitor* (Fig. 2). Each isolate belonged to a different *Quercus* species (Table 3). All three

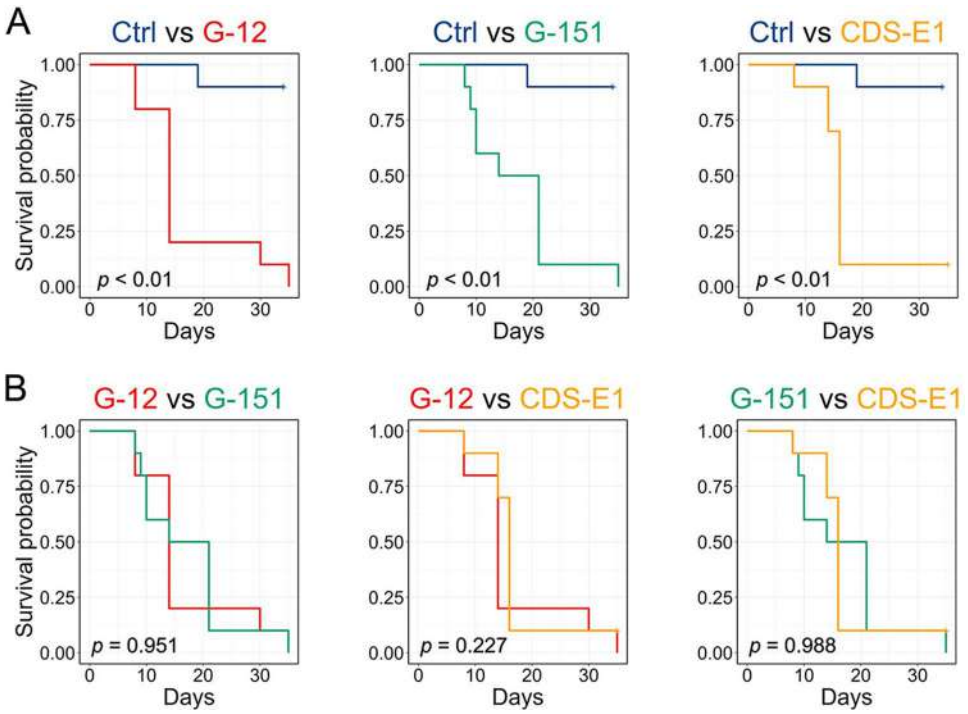


Fig. 3 Survival pairwise comparisons between control and *L. lecanii* field isolates. **A** Field isolates against control. **B** Comparisons between *L. lecanii* field isolates. The vertical axis is the probability of survival; the horizontal axis represents time in days. Colours indicate relationship between survival curves and treatment. Two survival curves are considered different if p-value < 0.05 (bottom left corner of each graph). Ctrl: Control group, G-12: *L. lecanii* isolated from *Q. ilex*, G-151: *L. lecanii* isolated from *Q. suber*, CDS-E1: *L. lecanii* isolated from *Q. pyrenaica*

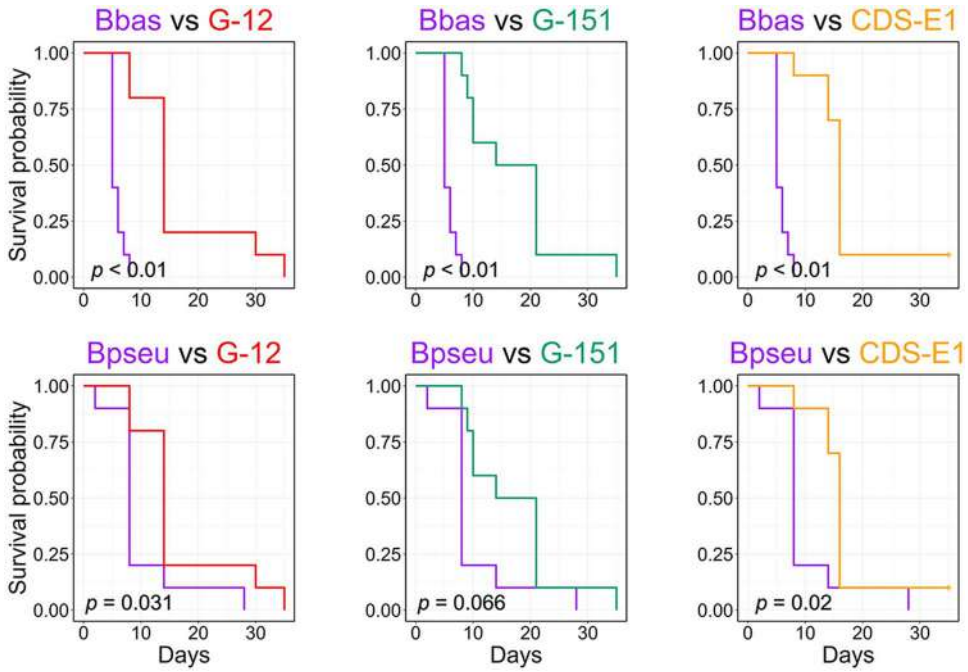


Fig. 4 Survival pairwise comparisons between commercial fungi and *L. lecanii* field isolates. Top row: *B. bassiana.* Bottom row: *B. pseudobassiana.* The vertical axis is the probability of survival and the horizontal represents time in d. Colours indicate the relationship between survival curves and treatment. Two survival curves are considered different if the p-value < 0.05 (bottom left corner of each graph). G-12: *L. lecanii* isolated from *Q. ilex*, G-151: *L. lecanii* isolated from *Q. suber*, CDS-E1: *L. lecanii* isolated from *Q. pyrenaica*

were identified as *L. lecanii* following ITS sequencing. No EF were isolated from *Castanea sativa*.

Comparison between isolated EF and commercial products

Pairwise comparisons of time to death (survival curves) for the inoculated insects are shown in Fig. 2 *(L. lecanii* isolates against control), Fig. 3 (*L. lecanii* isolates against

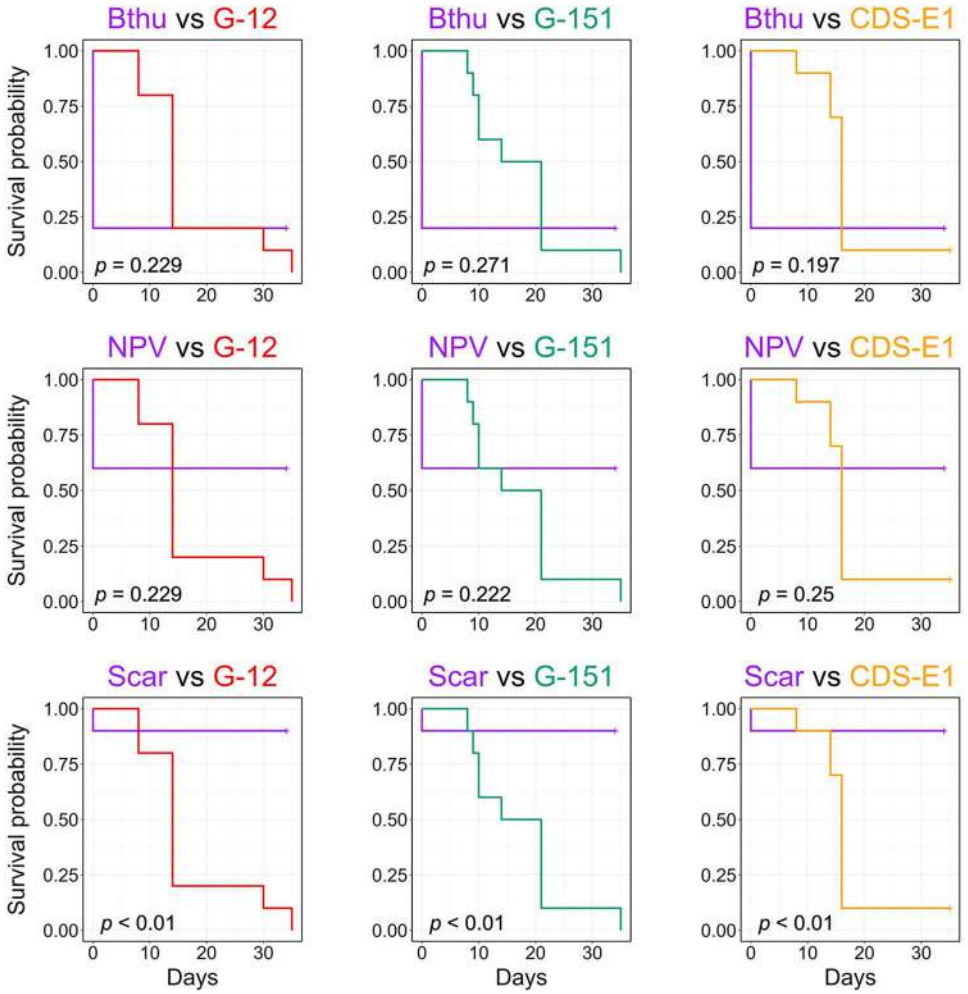


Fig. 5 Survival pairwise comparisons between commercial non-fungal products and *L. lecanii* field isolates. Top row: *Bacillus thuringiensis.* Middle row: nuclear polyhedrosis virus. Bottom row: *S. carpocapsae.* The vertical axis represents the probability of survival, and the horizontal is time in d. Colours indicate the relationship between survival curves and treatment. Two survival curves are considered different if the *p*-value < 0.05 (bottom left corner of each graph). G-12: *L. lecanii* isolated from *Q. ilex*, G-151: *L. lecanii* isolated from *Q. suber*, CDS-E1: *L. lecanii* isolated from *Q. pyrenaica*

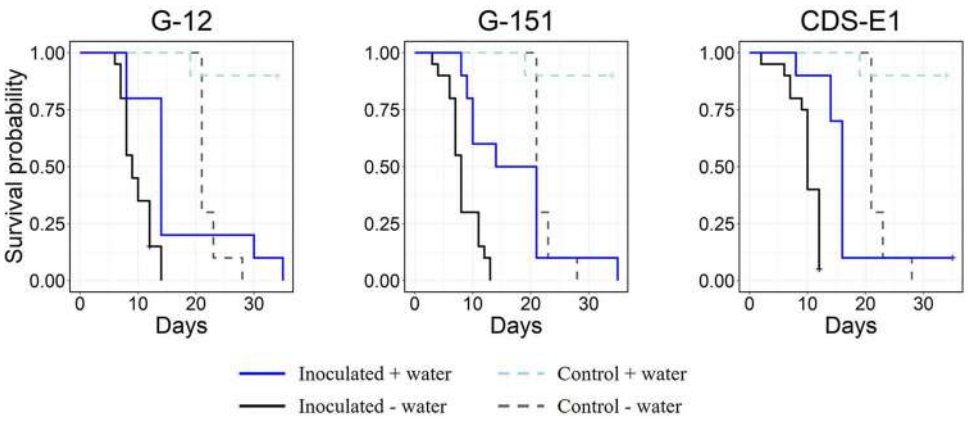


Fig. 6 Comparisons between survival curves with and without a source of water; dashed lines: controls; solid lines: application of *L. lecanii* field isolates. Black: no water source. Blue: with water source (PDA) every second day during the experiment

commercial fungi) and Fig. 4 (*L. lecanii* isolates against commercial non-fungal products). Only one of the control insects (10 repetitions) died during the 34-day follow-up of the experiment (Fig S1).

The three *L. lecanii* field isolates reduced survival of *T. molitor* in comparison to the control (Fig. 3A). TL_{50} was 14 d for both G-12 and G-151, reaching 100% mortality at 34 d of the trial. TL_{50} was 16 d for CDS-E1, with 90% of the insects dying at 16 d and 10% surviving at the end of the experiment. No statistical differences were found between the effects of the three isolates of *L. Lecanii* (Fig. 3B).

With regards to commercial fungi, both *B. bassiana* (TL50 = 5 d) and *Beauveria pseudobassiana* Rehner and Humber (TL50 = 8 d) had faster death rates than *L. lecanii*

Table 4 Median lethal time under hydric stress and survival curve comparison tests

Field isolate	Water source	TL50 (d)	*p*-value (vs Ctrl + Water)	*p*-value (vs Ctrl− Water)	*p*-value (Yes vs No)
G-12	Yes	14	3e−4	0.28 (NS)	0.002
	No	8	2e−5	2e−5	
G-151	Yes	14	7e−4	0.17 (NS)	7e−4
	No	10	1e−6	1e−6	
CDS-E1	Yes	16	2e−4	0.02	2e−5
	No	10	2e−5	2e-5	

NS: Not significant

p-value > 0.05

isolates (Fig. 4), with *B. bassiana* the most lethal of the two (*p*-value = 0.002, Fig. S2).

For commercial non-fungal products, *Bacillus thuringiensis* Berliner had the fastest effect on *T. molitor* survival among all treatments, with 8 kills on the first day of inoculation (TL_{50} = 0 d), after which there was no further mortality. However, *B. thuringiensis* had no statistically significant differences with respect to *B. bassian*a (*p*-value 0.323, Fig. S2), *B. pseudobassiana* (*p*-value 0.39, Fig. S2) and the different field isolates of *L. lecanii* (*p*-value 0.271–0.197, Fig. 5, top row). The nematode *Steinernema carpocapsae* (Weiser) and the Nuclear polyhedrosis virus (NPV) were not significantly different from the controls (*p*-value = 0.988 and 0.192, respectively, Fig. S2). NPV showed no differences against *L. lecanii* isolates (*p*-value 0.25–0.22, Fig. 5, middle row), whereas *S. carpocapsae* did (*p*-value < 0.01, Fig. 5, bottom row).

Survival test under water stress

Survival of *T. molitor* inoculated with the field isolates of *L. lecanii* was tested in the presence and absence of a water source (PDA). Overall, the absence of a source moisture had a deleterious effect on *T. molitor*'s survival, reducing the TL50 by 30–40% (Fig. 6 and Table 4). However, trials with G-12 and G-151 when the insect was provided with a source of water resulted in similar mortality to that which occurred due to the absence of water alone, without entomopathogen inoculation (Table 4, *p*-values = 0.28 and 0.17, respectively). TL50 was 21 d for the controls without a moisture source, with 100% mortality at 28 d (Fig. S3). When both effects were present, entomopathogenic fungus and water stress, the TL was reduced compared to the effect of the entomopathogenic fungus alone.

Discussion

The fungus *L. lecanii* was found as an endophyte on leaves of *Q. pyrenaica* and *Q. ilex* and on *Q. suber* wood. This study is the first in which *L. lecanii* has been isolated as an endophyte from oak tissues. Of the genus of *Lecanicillium,* we are only aware of the detection of *L. muscarius* as an endophyte on *Q. robur* (Nicoletti and Becchimanzi 2020). Other studies show the presence of *L. lecanii* as an endophyte fungus in tissues of other species, mainly crops, in wild contexts (Nicoletti and Becchimanzi 2020) and in forests (Jaber and Ownley 2018). *L. lecanii* seems to be a common endophyte on a wide variety of plants.

*L. Lecanii, a*s part of the endophytic fungi community of *Q. ilex*, *Q. pyrenaica* and *Q. suber,* could be used for the control of many of their pests. Its presence was independent of deciduous and non-deciduous species, occurring in both *Q. ilex* and *Q. pyrenaica* leaves. The microbiota naturally present in the trees or its inoculation and establishment as an endophyte could help control pest species. Gómez-Vidal et al. (2006) showed how entomopathogenic fungi colonized date palm tissues after petiole wounding by *B. bassiana* and *Lecanicillium dimorphum* (J.D. Chen) Zare & W. Gams. However, a challenge to considering endophytic fungi as a plant protection strategy is to manage their reproducible introduction into crops and to predict the outcome (Santamaría et al. 2011). Shin et al. (2022) reported that entomopathogenic fungi (*B. bassiana*) injected into palm trees showed insecticidal activity and survived in the tree for at least one month. Despite this, Alfina and Haneda (2022) suggest that the existence of the fungi in the cambium layer may still be reduced by antifungal compounds produced by plant tissues, such as pinene and turpenol. The way the entomopathogenic endophytic fungus acts seems to involve the production of chemicals that are harmful to an herbivore (Vega et al. 2008; Cory and Ericsson 2010). Feeding deterrence or antibiosis due to fungal metabolites released into the plant has been widely suggested as the mode of action in several studies investigating interactions between

entomopathogenic endophytic fungi and herbivorous insects. However, only a limited number of studies have identified and quantified fungal secondary metabolites produced in tissues that have been colonized by fungal entomopathogens. (Jaber and Ownley 2018). Cory and Ericsson (2010) propose that fungal entomopathogens with relatively wide host ranges should be the most suitable candidates for becoming host guardians. There is now substantial evidence that some endophytic entomopathogenic fungi, in particular *Lecanicillium* spp. can also demonstrate antagonistic activity against plant pathogens. This suggests that these entomopathogens have potential to be developed as biopesticides for multiple purposes in integrated pest management (IPM) strategies (Jaber and Ownley 2018). However, scaling up their application faces significant challenges. For example, the efficacy of endophytic fungi can vary significantly depending on environmental conditions, which could limit their consistency in different regions and ecosystems (Busby et al. 2016). Furthermore, integrating these fungi into IPM programmes requires a detailed understanding of their interactions with other biological control methods and their long-term impact on microbial and insect communities. Therefore, although the potential of native endophytic fungi is considerable, their effective implementation needs careful evaluation backed by further research to ensure their feasibility and sustainability.

The strain with the highest mortality in the shortest period of time was the commercial *B. bassiana* with a TL50 of 5 d, compared to the native *L. Lecanii* with a TL50 of 14–16 d. In addition to the effect as a result of being different species, it is worth noting that creating a commercial fungal product requires several modifications. Different fungal species and isolates within each species vary in their tolerance to environmental factors, as well as in virulence and potential for mass production (Devi et al. 2005). Typical commercial entomopathogenic fungal product development programs seek to identify the fungus with the greatest potential for a particular use and target, and formulations are designed to accommodate the fungi deficiencies. Considerable efforts are devoted to selecting candidate fungi, emphasizing virulence, spore production and shelf life, and optimizing fermentation variables to maximize spore production (Wraight and Carruthers 2003; Abalo et al. 2023). Under laboratory conditions, it is expected that the commercially selected strain will have a higher virulence than field isolates, although this need not be the most important characteristic for effective pest control. Native species of fungi are better adapted to the local conditions where they were isolated. This is especially important in the highly unstable environment of a cultivated field or a forest, where severe weather conditions or extremely rapid increases in pest populations can limit the effectiveness of pathogenic fungi. These could be similar to the case where Faye et al. (2013), Kouadio et al. (2017) and Koziol et al. (2022) observed that native fungi have the potential to outperform commercial fungi in field applications.

In our study, 2.7% of the endophytic fungi isolated on potato dextrose agar that sporulated had entomopathogenic effects on *T. molitor*. The methodology in this study could be used to search for endophytes EF to speed up their evaluation without the need for prior identification. Kim et al. (2018) and Eski and Murat Gezgin (2022) also used *T. molitor* to isolate entomopathogenic fungi from soil samples. They raised the insects above the soil where the fungi could potentially be and isolated the fungi from the dead insects. Typically, the search for endophytic EFs is done by morphological and/or molecular identification (Bamisile et al. 2021; Jamunarani et al. 2022). This study describes a methodology that could simplify this process and save resources by using *T. molitor*, as only a small proportion of cultivable endophytic fungi are usually entomopathogenic.

Non-fungal commercial products that had an entomopathogenic effect caused instant mortality within one day of application. Mortality caused by the fungal products developed over several days and did not cause mortality at the initial time of application. *L. lecanii* isolates took the longest to take effect and allowed the insect to survive the longest before causing death. This longer time needed to cause mortality could be of interest to combat those insects that have aggregation behaviour patterns. These patterns could enable horizontal transmission, where susceptible individuals meet the next generation of fungus produced on mycosed cadavers. Cheraghi et al. (2012) found that social behaviour of the termite, *Microcerotermes diversus* Silvestri (Termitidae), such as grooming, can be effective in promoting epizootic outbreaks in a colony. Lerche et al. (2004) reported that the population density of western flower thrips (*Frankliniella occidentalis* Pergande) influenced the dissemination of fungal spores of *L. lecanii*. Self-dissemination of fungal spores is the basis of biological control and is a tool for IPM strategies for insect pests with aggregating behaviour and offers several advantages, such as reduced application volume and minimised adverse effects on non-target organisms.

Mortality of adult *T. molitor* is accelerated over time when the insect is subjected to water stress, having the same effect as inoculation with two of the three field isolates of *L. lecanii.* According to Hansen et al. (2004, 2006)*, T. molitor* is a drought-resistant insect which can survive by feeding solely on dry feed such as wheat bran using metabolic water. However, when there is a wet food source, larvae of *T. molitor* grow faster and with a better rate of conversion of food into larvae (Urs and Hopkins 1973). Nevertheless, this study shows the importance of moisture on the survival time of adult *T. molitor.* Moisture eliminates the influence of unavailable water and correctly assesses the entomopathogenic potential of the fungi tested, which could otherwise be camouflaged by the effect of accelerated mortality due to

lack of water. Mortality of *T. molitor* was also accelerated under water-stress conditions when the *L. lecanii* fungus was inoculated, reducing TL50 by 33% compared to when a water source was available. According to Hesketh et al. (2010), entomopathogenic fungal-induced insect deaths is caused by physical damage and loss of normal functions following colonisation of tissues and organs, the effect of fungal metabolites, water loss and starvation. The time to death of the insect depends on several factors, including: the arthropod species; the physiological state of the host; the dose of spores received; the species and strain of fungus; and environmental conditions. Despite the drought-resistance of *T. molitor,* in this study there was an acceleration of the lethal effect of *L. lecanii* due to stress conditions caused by lack of moisture and a lethal effect of water starvation caused by 2/3 of the *L. lecanii* isolates used.

This study presents the discovery of *L. lecanii* as an endophyte within tissues of *Q. ilex*, *Q. pyrenaica*, and *Q. suber*, the first documented occurrence of this fungus in these species. Future investigations are warranted to clarify the specific role of this endophyte within the host tissues and its potential as a defence mechanism against insect pests. An essential aspect in incorporating endophytic fungi into plant protection strategies lies in the reproducible introduction of these organisms into crops and the ability to anticipate their effects. Research on the use of *L. lecanii* inocula to protect the early stages of plants from insect herbivory will be of interest, as well as exploring the potential for protection in mature trees after inoculation. The methodology in this study accelerates the identification of entomopathogenic fungi while challenging the suitability of *T. molitor* as a model insect for evaluating the pathogenicity of native fungal isolates. Given that the health status of *T. molitor* depends on moisture levels, the use of a water source is advised to prevent misinterpretation of entomopathogenic effects when using this insect as a test organism.

The results reported here not only bring valuable knowledge to the field, but also open the door to the development of localised and sustainable pest control strategies that are adapted to the specific conditions and needs of Mediterranean forests through the use of indigenous endophytic fungi.

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