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XXXVII CYCLE

INTERACTION OF CORAEBUS OAK BORERS AND OTHER COLEOPTERANS WITH ENTOMOPATHOGENIC FUNGI

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ABSTRACT

The genus *Quercus* plays a crucial ecological and economic role throughout the Mediterranean, particularly in Sardinia, where oak species contribute to biodiversity, carbon storage, and the production of high-value products like cork. Wood-boring beetles, especially *Coraebus* species (Coleoptera: Buprestidae), are posing a rising threat to *Quercus* forests, causing considerable damage to oak trees. The two species present in Italy are *Coraebus florentinus*, which causes desiccation of branches and weakening of trees at high infestations, and *Coraebus undatus*, which attacks cork oak phellogen layer, reducing cork quality by creating galleries imbedded into the growing cork layer. *C. undatus* infestations were previously rare in Sardinia, but since 2016 there has been a significant increase in damaged cork planks, notably in the northeastern regions. This research investigated the distribution, seasonal dynamics, and phenology of *C. undatus* to evaluate its impact on Sardinian cork production and to identify infestation hotspots. Our monitoring data revealed concentrated infestations near industrial cork storage facilities, with attacks varying in frequency and intensity between 2011 and 2021. Emergence data showed that adult beetle activity peaked in mid to late July, emphasizing the importance of seasonal control.

As part of an Integrated Pest Management (IPM) framework, this research also studied the efficacy of a newly isolated strain of the entomopathogenic fungus *Beauveria bassiana* as a biological control agent of *Coraebus* species. *B. bassiana* had significant lethal effects on larvae, pupae, and adult beetles under laboratory conditions. In comparative experiments with other commercially available strains, *B. bassiana* strain UNISS22 had distinct abilities to produce increased biomass and the ability to penetrate and spread within oak plants through the lymphatic system with characteristic endophytic behaviour. In addition, the whole genome of the new strain was sequenced and annotated, revealing its own gene profile with specific adaptations to insecticidal action and endophytism. These findings suggest *B. bassiana* as a viable biocontrol alternative to typical chemical pesticides, which is consistent with sustainable forestry and conservation goals in sensitive Mediterranean environments.

In addition, this study examined the safety profile of *B. bassiana* by conducting experiments on the non-target species *Chrysoperla lucasina*, a widespread beneficial insect predator. The results showed that *B. bassiana* had no significant detrimental effects on *C. lucasina* survival or development, suggesting that it can be used safely in field conditions without reducing beneficial insect populations. However, further research on the potential impacts of *B. bassiana* on non-target species is required to properly establish the ecological safety of this fungusin broader applications in oak forests.

Overall, this research provides important data for the development of sustainable pest management approaches in Mediterranean *Quercus* forests, with a focus on eco-sustainable solutions for effective forest conservation.

Keywords: *Quercus*, *Coraebus*, *Beauveria bassiana, Chrysoperla lucasina*, biological control, Sardinia, non-target effects

CHAPTER I

1.1. Introduction

Forest ecosystems are complex and dynamic systems that provide a wide range of ecological, economic, and social benefits. However, they are under increasing pressure from biotic stresses, mainly pests and diseases. Native and invasive pests have a substantial negative impact on forest health, resulting in tree mortality, biodiversity reduction, and ecosystem functioning changes (van Lierop et al., 2015). Climate change, global trade, and human activity have worsened these challenges by facilitating the spread and establishment of invasive species in new areas. Pest outbreaks are becoming more common and severe, thus threatening tree species across various forest types. In addition, these outbreaks disturb forest ecological balance by influencing nutrient cycling, carbon storage, and habitat availability (McPherson & Erbilgin, 2019).

A prominent example of forest ecosystem vulnerability to pests is the decline of oak trees, which are a crucial component of many Temperate and Mediterranean forests. Oak decline is a multifaceted issue caused by a combination of abiotic and biotic factors, including drought, poor soil conditions, pest infestations, and diseases (Thomas et al., 2002). Among the most damaging biotic factors are insect pests, which infest oak trees, thus compromising their vigour and leading to widespread dieback. Bark and wood-boring insects, such as beetles from the *Coraebus* genus, have emerged as contributors to oak decline in various regions. These insects invade oak trees, tunnelling through their bark and disrupting the flow of nutrients and water, possibly causing tree death (Sallé et al., 2014).

In Italy, oak tree decline has become a major ecological concern, especially in Sardinia, where the distinctive Mediterranean environment makes these trees more vulnerable. Sardinia’s Island environment and its Mediterranean climate, with hot, dry summers and unpredictable rainfall, increase the stress on oak trees and make them more susceptible to infestation (Sallé et al., 2014).

More generally, the threat posed by *Coraebus* species, including *Coraebus florentinus* Herbst and *Coraebus undatus* Fabricius, has become a critical concern in regions like the Mediterranean, where oak forests are already vulnerable due to climate stressors (Brasier, 1996) (Figure 1).

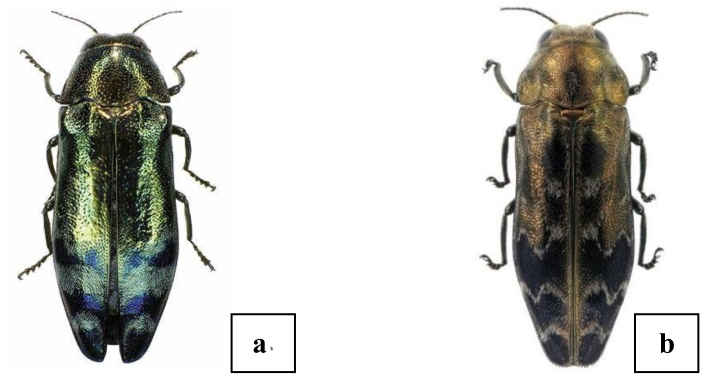

Figure 1 - *Coraebus florentinus* (a), *Coraebus undatus* (b) (Olejnicek, S. and Regione Autonoma della Sardegna).

These beetles can target weakened trees, accelerating their decline. Once established, *Coraebus* species can cause extensive damage, leading to large-scale forest degradation and loss of biodiversity. The damage caused by these pests also has economic implications, as oak forests provide valuable timber, cork, and ecosystem services (Sallé et al., 2014). The estimated economic losses to the cork industry in the Mediterranean region due to *Coraebus* infestations are difficult to quantify accurately, but some reports suggest that millions of euros are lost each year, particularly in Spain, where both cork oak and holm oak are economically important (Branco et al., 2014). Therefore, addressing the challenges posed by *Coraebus* species and other pests such as lepidopteran defoliators is essential for the conservation and management of oak forests, which are vital for maintaining biodiversity and ecological stability (Buse et al., 2007).

Coraebus species, particularly *C. florentinus* and *C. undatus*, have complex life cycles that are closely linked to the oak trees they attack. These species are metallic wood-boring beetles from the Buprestidae family that typically attack oak trees, particularly during times of stress and are considered to be a major contributor to oak decline, with their life cycles being strongly related to the physiological condition of the tree. *Coraebus undatus* life cycle lasts from two to three years, beginning when mature females deposit eggs on the bark of weakened oak trees, during the summer months. Once the eggs hatch, the larvae of *C. undatus* penetrate the bark and begin to tunnel through the cambium, disrupting nutrient and water transport within the tree. This larval phase is the most destructive, as the burrowing larvae create extensive galleries that severely weaken the tree (Sallé et al., 2014). The larvae typically overwinter inside the tree and resume feeding in the following spring and summer, after which they pupate in the cork. Adults are reported to emerge after pupation in late spring or early summer to continue the cycle. On the other hand, *C. florentinus* lays its eggs near the apex of new shoots, taking advantage of vulnerable, nutrient-rich plant regions. When the eggs hatch, the larvae tunnel into the wood, form wide galleries and pupate in 4- to 5-year-old branches. This digging damages vascular tissue and reduces water and nutrient transport, resulting in branch desiccation and dieback (Cardenas et al., 2013).

The preference of *Coraebus* species for already stressed or weakened trees makes them particularly damaging in regions experiencing environmental stressors such as drought (Sallé et al., 2014). Furthermore, their endophytic behaviour often leads to insidious infestations that go unnoticed until substantial damage has occurred, complicating management efforts (Buse et al., 2007) (Figure 2).

Figure 2 - Oak tree with severe *Coraebus florentinus* infestation (a); galleries of *C. undatus* larvae on tree trunk and extracted plank (b).

Luciano (2008) highlights that the combination of environmental stressors and pest pressure has a significant impact on oak health in the Sardinian region. *Coraebus florentinus* and *C. undatus* attack already stressed oaks, tunnelling through the bark and disrupting crucial nutrient and water supply, eventually causing widespread dieback and mortality (Buse et al., 2007). The fast spread of these invasive pests, supported by environmental change and human activities, threatens not only the health of Sardinian oak forests, but also the wider biodiversity and ecosystem services that these woods supply (McPherson & Erbilgin, 2019). Therefore, addressing the problems posed by *Coraebus* species and other pests is crucial for the conservation and long-term management of Italian oak forests.

The management of these beetles is therefore challenging. In addition, the use of chemicals in forest ecosystems has come under increasing examination due to their potential harmful effects on biodiversity, water quality, and soil health. Several pesticides, including herbicides and insecticides, once widely used for pest control and forest management, have been banned or restricted due to their negative environmental impact. Several pesticides previously used to control forest pests have been banned globally under the Stockholm Convention on Persistent Organic Pollutants due to their long-lasting effects on wildlife, particularly birds, and their persistence in the environment (UNEP, 2009). Other pesticides have been restricted in several regions, including the European Union, because of their detrimental impact on pollinators like bees, which play a crucial role in forest ecosystems (European Commission, 2018). Forest management practices are shifting towards integrated pest management (IPM) strategies that prioritize biological control and reduce reliance on harmful chemicals, thus mitigating the negative ecological consequences of chemical use (Thompson et al., 2011). These bans reflect a growing recognition of the need to protect the ecological balance of forests by managing pests and diseases sustainably.

Therefore, it is crucial that we develop more ecologically friendly and effective ways of protecting our forests. Adapting forest management to have forest stands and landscapes that are more resilient and resistant to insect disturbances is one promising strategy.

A successful alternative to the use of chemical compounds against pests is the enhancement of their natural enemies that normally inhabit the forest ecosystem. This can be achieved through appropriate forest management practices that favour an overall increase in forest biodiversity and in the abundance of pest antagonists. The latter includes biological control agents such as predators and parasitoids, as well as microbial control agents.

The use of entomopathogens, which are microorganisms that cause disease in insects, has become increasingly important in the management of forest pests. These natural enemies of insect pests offer a sustainable and ecologically friendly alternative to chemical pesticides, reducing the risk of non-target effects and environmental contamination. Entomopathogens, including bacteria, fungi, viruses, and nematodes, have been extensively studied for their potential to control pest populations. Among these, entomopathogenic fungi are particularly promising due to their ability to infect and kill a wide range of insect pests in different environments, making them a resourceful tool in IPM programs (Goettel et al., 2010).

One of the most effective and studied fungal entomopathogens is *Beauveria bassiana* (Bals.-Criv.) Vuill., which has shown high efficacy against many forest pests, including wood-boring beetles. *B. bassiana* is a soil-borne fungus that occurs naturally in many ecosystems and infects insects by direct contact. The infection process begins when fungal spores, or conidia, attach to the cuticle of the insect host. Once attached, the spores germinate and penetrate the insect cuticle using mechanical pressure and enzymatic degradation. Once inside the host, *B. bassiana* proliferates, producing toxins and virulence factors that disrupt the insect’s immune system and metabolic processes. The fungus spreads throughout the insect's body, eventually leading to death through tissue destruction, dehydration, or sepsis (Ortiz-Urquiza & Keyhani, 2013) (Figure 3).

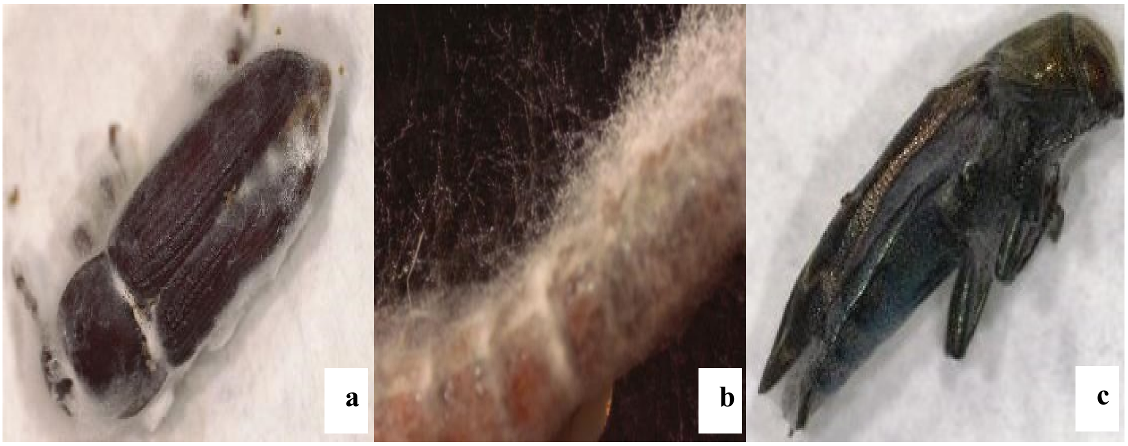

Figure 3 - *Beauveria bassiana* infection on *Tenebrio molitor* adult (a) and larva (b) and on *Coreabus undatus* adult (c).

In addition to its lethal effects, *B. bassiana* can restrict insect behaviour and reproduction, thereby reducing pest populations over time. *B. bassiana* adaptation and wide host range make it an important tool for forest pest management, especially against pests such as *Coraebus* species, which are difficult to control using conventional methods. Furthermore, *B. bassiana* is compatible with other biological control agents and can be used in IPM methods to provide long-term, sustainable pest control with limited ecological impact (Zimmermann, 2007). Early signs of infection include reduced activity and lethargy as the fungus invades and disrupts essential physiological systems (Goettel & Hajek 2001). Infected insects often stop feeding and may exhibit unusual behaviour (Zimmermann, 2007). As the fungal infection progresses, a distinctive white powdery appearance develops on the insect body, especially around joints and joints with weaker cuticles. This growth is known as external mycelial growth (Vega et al., 2012). After the insect dies, this external growth is followed by aerial conidia, which *B. bassiana* releases from the cadaver to infect new hosts (Goettel et al., 2010) (Figure 4).

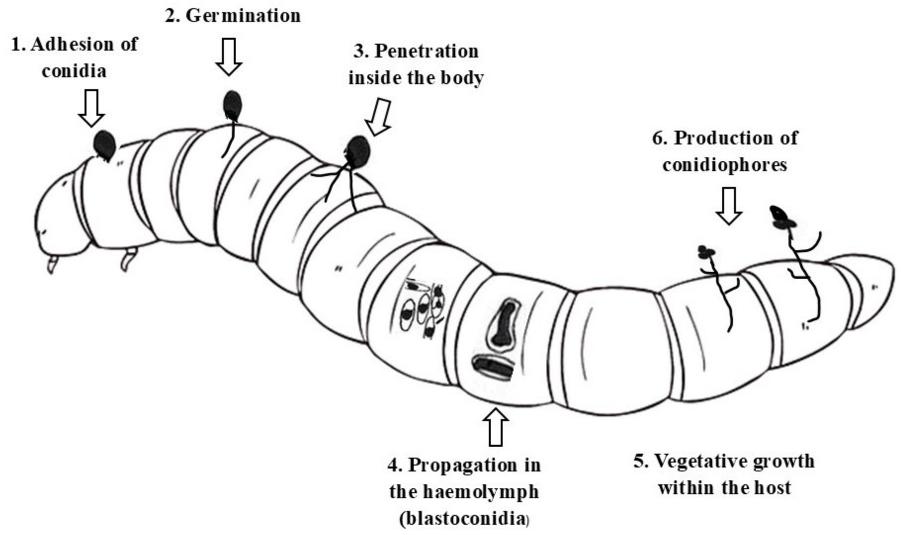

Figure 4 - *Beauveria bassiana* natural pathogenesis process.

1.2. General and specific objectives of the thesis

This study aimed to contribute to the development of new approaches to address the critical issue of *Coraebus* species infestation in oak forests, with a focus on the Mediterranean region. These pests not only threaten the ecological integrity of the forest but, in the case of *C. undatus,* can also affect the cork industry with serious economic consequences. In view of the increasing concerns about the use of chemical pesticides and their negative impact on the environment, this study explored the potential of the biological control agent *Beauveria bassiana* as an environmentally friendly alternative for managing *Coraebus* populations. A major achievement of this work was the isolation and characterization of a novel strain of the fungus from a naturally infected *C. florentinus* larva collected inside a cork tree in Sardinia. In addition to studying the pathogenicity of this fungal strain on a mealworm-based laboratory model, efficacy against the target *Coraebus* pests was also determined. In parallel, this study investigated the ability of the fungus to interact with the plant through an endophytic behaviour, potentially reaching *Coraebus* endophytic larvae inside plant tissues, as a result of an external treatment. Finally, the safety of the fungus on non-target species was investigated using chrysopid larvae as a predatory model. A preliminary genomic analysis of the fungus was also conducted. The ultimate goal of this work was to develop a comprehensive, eco-friendly pest management strategy to maintain forest health and biodiversity.

The **specific objectives** of this research were:

1. Investigating the biology, phylogenetics and infestation history of *Coraebus undatus* as an emerging pest in Sardinian cork oak forests, including the study of its life cycle and ecological habits.
2. Isolating an effective entomopathogen naturally occurring in *Coraebus* larvae ecosystem, and studying its potential against these coleopteran pests, also in comparison with already known microbial control agents.
3. Studying the behaviour of the selected entomopathogen (*Beauveria bassiana*) in the environment, with a special focus on its endophytic potential.
4. Characterizing phenotypically and genotypically the newly isolated *Beauveria bassiana* strain, including original sequencing and analysis of its genome.
5. Studying the safety profile of the species *Beauveria bassiana* in respect to common non-target species.

Based on these objectives, this research was consequently divided into several lines of work, which are presented in the following three logically organized chapters based on scientific articles already published in scientific journals or on manuscripts ready for submission to the pre-publication review process.

1. Lentini Andrea, Roberto Mannu, Maurizio Olivieri, Luca Ruiu, Ana H. Dias Francesconi, Walaa Morda, Antonio Alberto Mulas, Giuseppino Pira, Pino Angelo Ruiu, 2023. *Coraebus undatus*: an emerging pest in Sardinian cork oak forests. IOBC-WPRS Bulletin,168, 73-78.
2. Morda Walaa et al. 2024. Potential of endophytic *Beauveria bassiana* for the management of *Coraebus* (Coleoptera: Buprestidae) species in cork oak forests. Manuscript in preparation.
3. Morda Walaa, Maria Tiziana Nuvoli, Luca Ruiu. 2024. Safety of the entomopathogenic fungus *Beauveria bassiana* for wild and laboratory-reared *Chrysoperla lucasina* strains. Insects, 15(8), 576.

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CHAPTER II - Coraebus undatus: an emerging pest in Sardinian cork oak forests

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2.1. Abstract

Coraebus undatus (Fabricius) (Coleoptera: Buprestidae) is considered as a major concern for cork production in Mediterranean regions. Larvae feed on the phellogen layer and excavate long galleries that are gradually incorporated into the cork layer as cork grows. Infested cork shows a change in physical and mechanical characteristics, with a considerable reduction in quality. In Sardinia (Italy), infestations of *C. undatus* have been recorded only sporadically, but since 2016 damaged cork planks have dramatically increased in the northeastern part of the island. This work assessed the real threat of *C. undatus* to cork production in Sardinia by evaluating the distribution area of the infestations, the pest season phenology, and the dynamics of attacks between two debarking seasons (period 2011-2021). The temporal dynamics of *C. undatus* attacks were estimated by observing cork strips and dating the feeding galleries based on their positions between annual cork-rings. Based on our observations, attacks of *C. undatus* were concentrated in the cork oak forests closest to the industrial cork storage areas. Infestations decreased moving away from this focus of infestation to reach near-zero levels at 2 km in the northwest direction and 10 km in the southeast direction. The frequency of *C. undatus* attacks were relatively low in 2011-2015, increased exponentially from 2016 until 2019, and decreased dramatically afterwards. The flight curve estimated in 2021 on the basis of trap catches, showed the presence of adults starting from the first week of July, a peak at the second half of July, and the end of the flight at the end of August. In 2022, after placing the traps in mid-July, a peak likely occurred in the second decade of July, followed by a gradual decline and the end of adult flight in mid-August. The findings of this preliminary study need to be confirmed by multi-year observations.

Key words: xylophagous beetles, *Quercus suber*, Mediterranean forests

2.2. Introduction

Coraebus undatus (Fabricius) (Coleoptera Buprestidae) is an oligophagous species that feeds on *Quercus* spp., being one of the main insects damaging cork productions in Mediterranean regions (Du Merle & Attié, 1992; Jiménez et al., 2012). Soria et al. (1992) stated that this species is particularly harmful in declining cork oak forests, especially when infestations by defoliating lepidopterans, prolonged drought periods or improper anthropogenic interventions, such as soil tillage for grass seeding or excessive livestock pressure, occur. Damage by *C. undatus* can be seen during debarking and consists of the presence of feeding galleries in the phellogen layer that are gradually incorporated into the cork layer during cork growth (Martín, 1964). The presence of galleries inside the cork determines changes in physical and mechanical characteristics of the product with a consequent depreciation in the market (Oliveira et al., 2015). Furthermore, damaged cork planks are difficult to extract from the plant and during debarking portions of phellogen are also removed, thus irreparably damaging the future productivity of the plants (Tiberi et al., 2016; Suñer & Abós, 1994).

In Sardinia (Italy), infestations by *C. undatus* were recorded only sporadically until 2015, but since 2016 damaged cork planks have dramatically increased in the north-eastern part of the island. This work assessed the real threat of *C. undatus* to cork production in Sardinian infested area by evaluating the distribution, the seasonal phenology, and the dynamics of attacks between two debarking seasons (period 2011-2021).

2.3. Materials and methods

2.3.1. Study sites

The study was conducted in 2021 and 2022 in the cork district of Gallura (northeastern part of Sardinia), which had reports of severe *C. undatus* attacks. The spread of infestation was estimated in the summer of 2022 by observing the exposed trunk after debarking in cork oak forests subjected to cork extraction. In all the monitored cork oak forests (n = 47), 40 randomly selected trees per site were checked for the presence of larval galleries of *C. undatus*. Based on these observations, distribution maps indicating the percentage of infested plants were made.

2.3.2. Monitoring

To determine the seasonal dynamics of *C. undatus*, adult flight was monitored using purple-colored prism traps baited with a mixture of green leaf volatiles (GLVs) as described by Fürstenau et al. (2015). The prism traps consisted of a panel of purple-colored corrugated polypropylene cardboard (100 cm long × 53 cm wide) folded crosswise to obtain a 3-faced prism (33 × 53 cm each side). The outer surface of every side was covered with entomological glue. In 2021, the traps were placed in a cork oak forest located in Tempio Pausania (Province of Sassari) on May 28th and were removed on September 16th. In 2022, the traps were placed in a cork oak forest located in Calangianus (Province of Sassari) in mid-July and were removed in late September. Traps were monitored weekly. Replacement of the lures took place whenever the dispenser was empty or, at most, every 15 days. All adults found in the traps were collected and identified in the laboratory of the Department of Agricultural Sciences of the University of Sassari.

2.3.3. Evaluation of the attacks of by C. undatus

The dynamics of attacks between two successive debarking seasons (10-year extraction interval) was studied in two cork oak forests, one located in the "Cusseddu-Miali-Parapinta" Experimental Farm of AGRIS Sardegna (Tempio Pausania), which was subjected to debarking in 2021 (previous debarking was in 2011), and the other located in the private "La Talga" Farm (Calangianus), subjected to cork extraction in the summer of 2022 (previous debarking was in 2012). In each study area, approximately 20 infested cork planks were taken to date the year of attacks. To count the number of galleries incorporated into the cork layer, each plank was cross-sectioned to obtain a strip with a width of 3-4 cm and a length equal to the length of the plank. All the obtained cork strips were sanded in the cross-sections with sandpaper at different weights before observation in the transverse section of the galleries under a binocular microscope. The feeding galleries of *C. undatus* were classified based on their width to estimate the year of attack which allows identification of whether the damage is due to a 1- or 2-year-old larva and, their position relative to cork growth rings (Figure 1). The incidence of attack was assessed as the number of galleries (i. e., attacks) encountered per linear meter of cork strip.

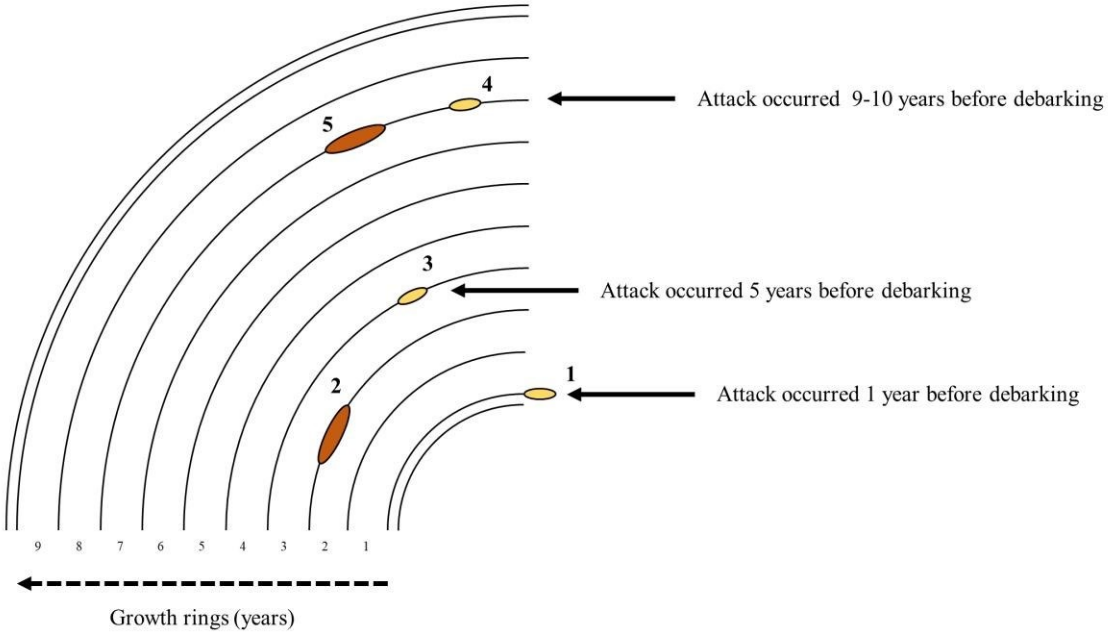

Figure 1 - Schematic representation of cork growth rings and the method used to attribute the year of *Coraebus undatus* infestation based on the size and location of galleries.

2.4. Results and discussion

Infestation data allowed to show that the spread of the insect is for now limited to an area between the municipalities of Tempio Pausania, Calangianus, Luras, and Aggius (Figure 2). The highest attacks were located near cork processing industries where planks are normally stacked for months before processing. The percentages of infested plants throughout the infested area ranged from 2.4 to 57 %, and infestations decreased moving away from the focus of infestation to reach near-zero levels at 2 km in the northwest direction and 10 km in the southeast direction. With respect to these findings, we hypothesize that in the areas near cork processing industries there could be a higher density of adults emerging from infested planks, which were extracted before adult emergence. According to our hypothesis, a small outbreak was also recorded in a cork oak forest in the

Monti municipality (Figure 2). This infested area, which was approximately 20 kilometers far from the Tempio Pausania area and located near a cork deposit, showed only 4 % of infested plants. In Tempio Pausania in 2021, *C. undatus* adults were caught by traps from the first week of July to mid-August, with a peak of captures in the second half of July (Figure 3). In Calangianus in 2022, the peak of captures was observed in the second decade of July (i. e., one week after traps installation), after which the number of *C. undatus* gradually decreased and no adults were captured after mid-August (Figure 4). In both survey years, the traps captured only females in agreement with Fürstenau et al. (2015). Our results, despite being limited to a single environmental context, are in line with those obtained in Catalonia (Spain), where adults flight from mid-June to mid-August (López et al., 2022).

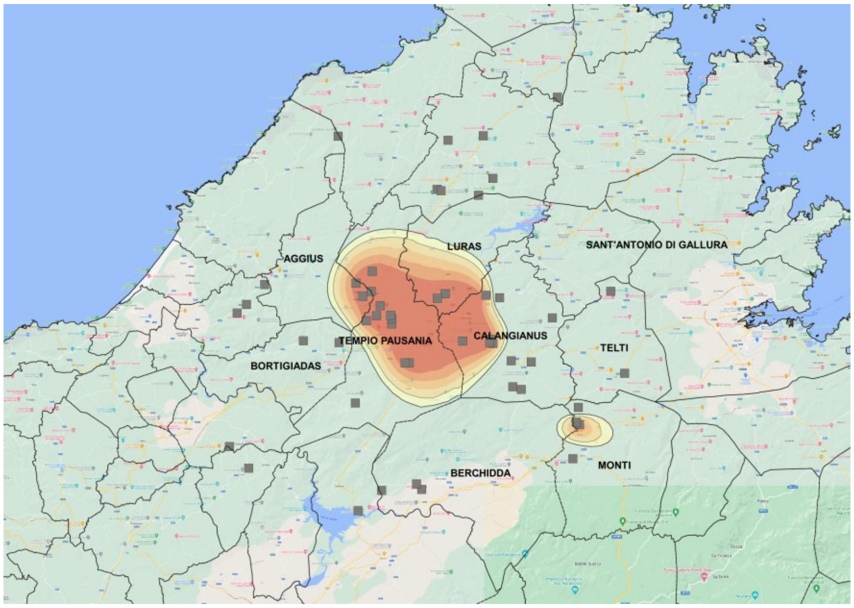

Figure 2 - Area of northern Sardinia affected by infestations of *Coraebus undatus*. The dots represent the cork forests debarked in 2022 where observations were made. Curves with different shades of color delimit the infested areas.

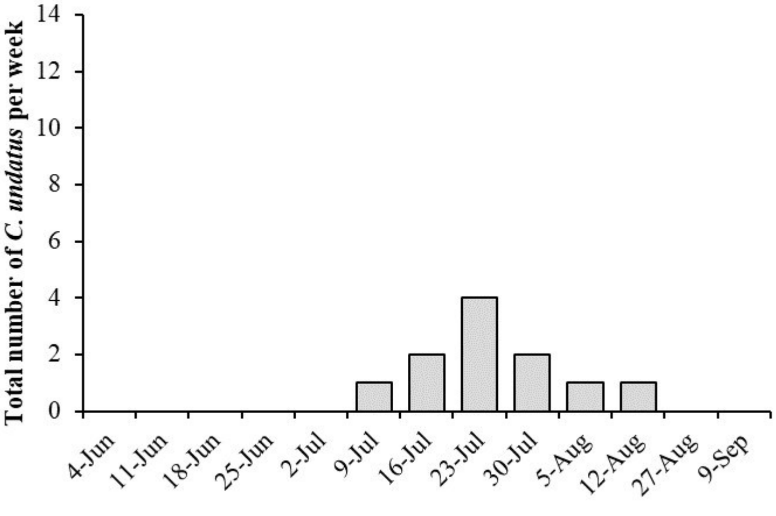

Figure 3 - Total number of *Coraebus undatus* adults caught weekly at 12 purple traps baited with green leaf volatiles (GLVs) ((E)-2-hexenal, (E)-2-hexenol, 1-hexanol, and (Z)-3-hexenyl acetate e n-hexyl acetate) in Tempio Pausania (Sardinia) in 2021.

The analysis of *C. undatus* galleries found on transverse sections of debarked cork made it possible to identify the dynamics of infestations in the Tempio and Calangianus cork oak forests. In the cork oak forest of Tempio, the insect was already present since 2011, remained at low level until 2015, and increased significantly since 2016, reaching average values close to 4 galleries/m in 2019. In the cork oak forest of Calangianus, attacks started from 2015, gradually increased in the following years and reached a peak of approximately 3 galleries/m in 2020 (Figure 5). The attacks declined in the year before debarking in both areas. These results indicate that *C. undatus* have been present for a long time in Sardinia, but the balance between this insect and its natural regulating factors might have been disrupted by conditions that triggered an increase in *C. undatus* population.

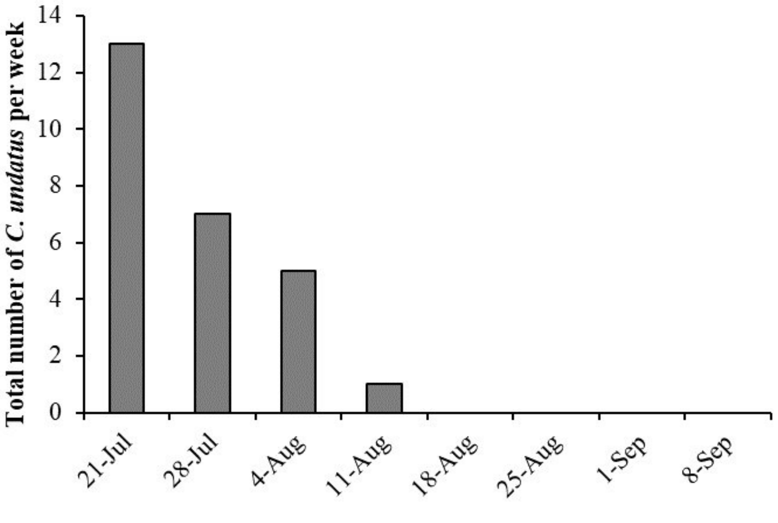

Figure 4 - Total number of *Coraebus undatus* adults caught weekly at 6 purple traps baited with green leaf volatiles (GLVs) ((E)-2-hexenal, (E)-2-hexenol, 1-hexanol, and (Z)-3-hexenyl acetate e n-hexyl acetate) in Calangianus (Sardinia) in 2022.

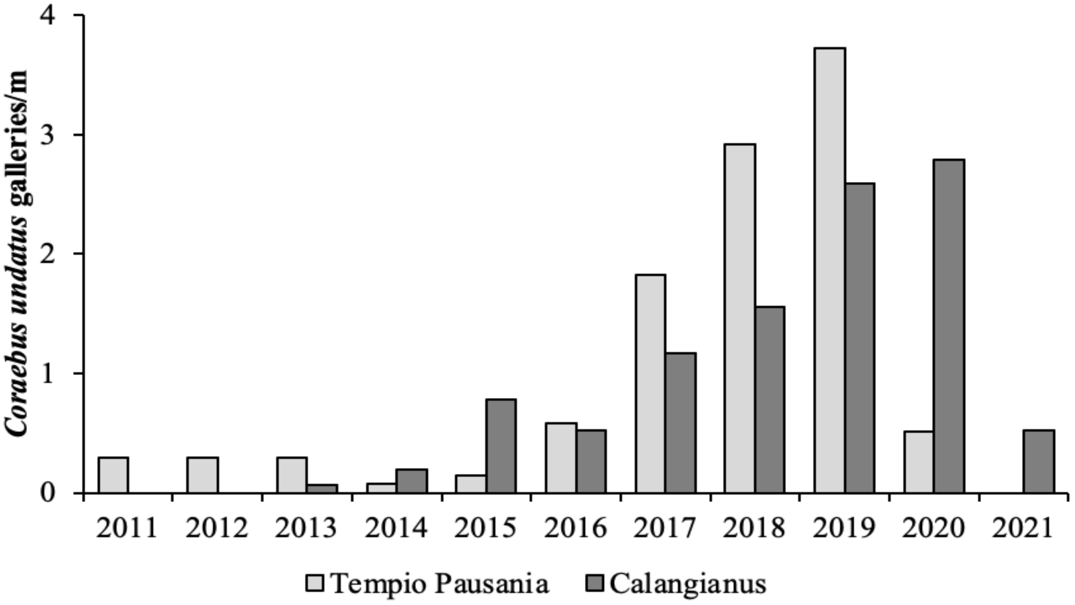

Figure 5 - Average number of *C. undatus* galleries per linear meter of cork strip in the "Cusseddu-Miali-Parapinta" Experimental Farm of AGRIS Sardegna (Tempio Pausania, Sardinia) in 2021 (light grey) and in the private "La Talga" cork oak forest (Calangianus, Sardinia) (dark grey). The galleries were separated according to the year of attack.

2.5. Conclusions

The findings of this preliminary study need to be confirmed by multi-year observations. It would be important to study the population dynamics of the insect over a long time, and to verify whether the damage observed in recent years is the result of a temporary outbreak or whether environmental conditions are favorable to its constant presence at damaging levels. For this reason, it is essential to ascertain whether the insect is native to Sardinia or possibly introduced with cork planks imported into Gallura from other known infested areas (e. g., Sicily, Tuscany, Iberian Peninsula). A genetic comparison between C. *undatus* samples from different geographic regions would elucidate the origin of the spread found in Sardinia.

2.6. Acknowledgement

The work was partially funded by Fondo di Ateneo per la ricerca 2019 (University of Sassari), and the Regione Autonoma della Sardegna, Assessorato della Difesa dell'Ambiente through a scientific-technical collaboration agreement.

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CHAPTER III - Potential of endophytic *Beauveria bassiana* for the management of *Coraebus* (Coleoptera: Buprestidae) species in cork oak forests

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3.1. Abstract

Oak borers in the genus *Coraebus*, including the bark- and the wood-boring beetles *C. florentinus* and *C. undatus*, are major pests of the cork oak tree *Quercus suber*, and under conditions of high population density, forest protection interventions are necessary. In the present study a novel strain of the entomopathogenic fungus *Beauveria bassiana* has been isolated from the forest ecosystem and its insecticidal potential against coleopterans was evaluated in laboratory bioassays involving the model species *Tenebrio molitor* and the two target *Coraebus* species. This strain (UNISS 22) proved to be effective against all insect stages with a concentration dependent efficacy.

In comparative experiments with other commercially available strains, *B. bassiana* UNISS22 was found to have distinctive abilities in the production of increased biomass and the ability to penetrate and spread within oak plants through the lymphatic system with characteristic endophytic behaviour. Finally, the whole genome of the new strain was sequenced and annotated, revealing its own gene profile with specific adaptations to insecticidal action and endophytism.

Keywords: *Quercus suber*, *Coraebus florentinus*, *Coraebus indatus*, *Beauveria bassiana*, endophytism

3.2. Introduction

Oak borers of the genus *Coraebus* (Coleoptera: Buprestidae), in particular the bark- and the wood-boring beetles *Coraebus florentinus* Herbst and *Coraebus undatus* Fabricius, are major pests of the cork oak tree *Quercus suber* L., with a significant impact on the economy of the cork industry and the health of forest ecosystems in different geographical areas (Tiberi et al., 2009; Furstenau et al., 2014). The activity of these species also contributes to the decline of forests in Europe (Gallardo et al., 2012), due to their boring activity and their association with microbial plant pathogens, for which they can serve as carriers (Pinna et al., 2019). Although these two species share an endophytic attitude, they have differentiated behaviours and are usually found in different ecological niches on the plant. More specifically, *C. florentinus* larvae typically feed by boring longitudinal tunnels under the bark of young, vigorous branches. As the larva grows this activity interrupts the sap flow with subsequent death of the corresponding branch, usually coinciding with the metamorphosis of the insect (Gallardo et al. 2018). On the other hand, young larvae of *C. undatus* feed in the phellogen layer by boring typical sinuous tunnels with an elliptical cross-section, which later become blackish. Older larvae pierce the cork to prepare the pupation chamber, after which the adults emerge, usually in late spring/early summer. All this damage to plant tissues impairs the regenerative ability of the tree and reduces its production of acorns, cork and wood (Romanyk et al., 1992; Aronson et al., 2009). In addition, it reduces the quality of the cork at hulling and its commercial value, and hinders the cork removal process (Evans et al., 2004). Consequently, *C. undatus* is the subject of special attention in terms of economic impact, which can be particularly important in conditions of significant infestation (Gallardo et al., 2012; Jiménez et al., 2012).

The endophytic behaviour and the still limited knowledge of the biology of *Coraebus* make it difficult to develop a timely and effective management strategy. The lack of commercially approved active substances that specifically target these pests and are compatible with the forest environment adds further issues (Solinas, 1974). Accordingly, there is an urgent need to develop new and alternative forest compatible approaches to the management of these pests. One frontier in this direction is the use of naturally occurring microbial control agents that are effective in their natural containment. These include entomopathogenic fungi such as *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales). This entomopathogen has been successfully used in agriculture and typically acts by contact through the adhesion of its aerial conidia to the surface of the target insect's body, where they germinate under favourable conditions. This triggers a mechanical and enzymatic action that results in the tegumental barrier being overcome by penetrating hyphae. The fungus reaches the internal tissues where the pathogenic process causes ultrastructural, biochemical and physiological changes that, together with a toxic action, lead to the insect’s death (Quesada-Moraga et al., 2020).

A key step in the pathogenic process is therefore the initial physical interaction of the fungus with the host, which is more difficult in the case of endophytic larvae. Nevertheless, the endophytic behaviour has been observed in several *B. bassiana* strains, opening the possibility of their use against insect borers (Quesada Moraga, 2020).

The main hypothesis of the present study is that an entomopathogenic fungus, naturally adapted to endophytic behaviour and capable of acting against *Coraebus* species, may be a promising tool for their management. Accordingly, a new strain of *B. bassiana*, isolated from a *Coraebus* larva during an infestation in an oak forest, was tested for its insecticidal potential, including a comparison with currently available commercial strains. In addition, observations were made on its endophytic behaviour. Finally, the whole genome of the new strain was sequenced and annotated, revealing a gene profile with specific adaptations to insecticidal action and endophytism.

3.3. Materials and methods

3.3.1. ***Beauveria bassiana strain isolation***

Beauveria bassiana strain UNISS22 was isolated from a naturally infected *C. florentinus* larva found inside a branch of an infested oak tree in Sardinia (Italy) during forest surveys in spring 2022. The endophytic larva was dead and completely covered with a compact white fungal mycelium. The fungus was grown on potato dextrose agar (PDA) plates for morphological observations and preliminary identification according to Humber (1997), followed by ITS and Tef-1α gene sequencing. The strain was cryo-preserved at -80 °C in cryovials containing a glycerol solution at the Microbial Collection of the University of Sassari.

Other *B. bassiana* strains as reference in this study were ATCC74040, GHA, and PPRI 5339, which represent the active substances of the commercial products Naturalis (Biogard), Botanigard (Certis Europe B.V.) and Velifer (BASF), respectively.

3.3.2. Fungal growth conditions and preparations

Beauveria bassiana was routinely grown on PDA plates at 25 °C for 10-15 days to allow good production of mycelium and aerial conidia, which were harvested by gently scraping the surface with a glass spatula and the help of a solution of water and 0.02% Tween 80. Conidial suspensions were quantified using a Neubauer chamber, and the concentration was adjusted as required for bioassays.

3.3.3. Comparative fungal growth observations

The growth of *B. bassiana* strain UNISS22 was monitored after inoculation on PDA in comparison with the reference strains ATCC74040, GHA, and PPRI 5339. To monitor horizontal growth on plates, a hole (8 mm diameter) was made in the centre of a Petri dish (9 cm diameter) and inoculated with 100 µL of a fresh fungal suspension (10^7 conidia/mL). Vertical growth was measured instead in 50 mL tubes containing PDA in which the medium surface was inoculated with 100 µL of the same fungal suspension. Cultures were then incubated in the dark at 25 °C and their horizontal and vertical growth was measured with a millimetre ruler daily during the following 12 days. At least three measurements were taken in different directions on each mycelial body in the plate to determine the average diameter, so that the shape of its surface was approximately circular. Measurement data were based on three replicates and all experiments were repeated four times. Measurements of horizontal and vertical growth were combined to calculate the volume of fungal biomass produced in culture (= base area x height of mycelial mass). Comparisons were made after two weeks of growth.

3.3.4. Insect rearing and maintenance

The coleoptera used in this study were the two target species, *Coraebus florentinus* and *C. undatus*, and the lab-reared model coleoptera, *Tenebrio molitor*.

Coraebus florentinus larvae were collected from cork oak branches in highly infested forests in Sardinia (Italy), in the area of Buddusò (North-West of the island), and directly used in bioassays, after a short acclimatization period in the laboratory at 25 °C. *Coraebus undatus* adults were collected in the cork oak forests of Tempio Pausania using emergence traps. This approach was chosen because of the difficulty in rearing these species through their entire life cycle in the laboratory.

Tenebrio adults, pupae and larvae used in the bioassays were routinely provided by the laboratory rearing facilities of the Department of Agricultural Sciences of the University of Sassari.

3.3.5. Insect bioassays

Bioassays were conducted by exposing different stages of *Coraebus florentinus*, *C. undatus*, and *Tenebrio molitor* to *B. bassiana* conidial suspensions in order to determine the lethal effects and the ability of the fungus to complete its pathogenic cycle. In all cases, insects (larvae, pupae, or adults) of the different coleopteran species were individually immersed in a suspension of conidia or in distilled water (control) for 30 seconds before being maintained together in a group of 10 individuals in a Petri dish (9 cm diameter). Before this treatment, each insect was surface sterilized with ethanol (70%) and sodium hypochlorite (0.05%)and then washed with sterile water. Petri dishes, which represented the experimental unit, were incubated in the dark at 25 °C for observations. Different conidia concentrations were tested on *T. molitor* (10^7, 10^6, and 10^5 conidia/mL), whereas *Coraebus* samples were exposed to a standardized concentration of 10^7 conidia/mL.

The experimental design involved four replicates, and each experiment was repeated 2-3 times overtime. When the number of available field specimens of *Coraebus* was limited, experiments were conducted using the same experimental design but reducing the number of individuals per plate to five. Plates were inspected daily to record mortality and the appearance of pathological symptoms over a period of 2 weeks.

3.3.6. Endophytic behaviour observation

The ability of *B. bassiana* strain UNISS22 to penetrate plant tissues and translocate within the plant was investigated by applying conidial suspensions at different concentrations to oak tree seedlings and then examining internal tissues for the presence of the fungus. For this purpose, the roots of 1-year-old (30 cm tall) cork oak tree seedlings were washed, surface sterilized with 70% ethanol and 0.05% sodium hypochlorite, then rinsed in distilled water and immersed in conidial suspensions or just water (control) for 30 minutes. Plants were then transplanted into pots with soil, kept in good condition in a room at 25°C with natural photoperiod (14:10). The experimental design involved four replicates and different treatments (10^7 and 10^6 conidia/mL). After three weeks, five small portions (3 mm diameter) of roots, stems, and leaves were excised from each plant and surface-sterilized before being placed on PDA plates supplemented with 0.05% streptomycin to allow growth of any fungal mycelium or propagules present in the internal plant tissues. Plates were incubated at 25°C and checked daily for detect the presence of *B. bassiana* white mycelium and then sampled to confirm identification.

3.3.7. Genomic analysis on B. bassiana UNISS22

For genomic analysis DNA from *B. bassiana* UNISS22 was extracted from a pure culture using the Fungi/Yeast Genomic DNA Isolation Kits (cat. 27300, Norgen Biotek Corp, CANADA) according to the manufacturers' instructions.

The DNA libraries were prepared using Illumina DNA prep kit (Illumina, Sandiego, USA) with unique dual indexes (UDIs) and sequenced on Illumina Novaseq 6000 platform at BMR Genomics (Padua, Italy) in 150PE format following the manufacturer's instructions. The quality of raw paired-end (PE) reads was checked using FASTQC v0.11.8 (Andrews, 2010).

MetaPhlan v4.0.1 (Blanco-Míguez et al., 2023) was employed for microbial profiling to assess sample content, including potential contaminants.

SPAdes v3.15.5 (Bankevich et al., 2012) was used for *de novo* assembly, whereas QUAST v5.2.0 (Gurevich et al., 2013) and BUSCO v5.4.3 (Manni et al., 2021) were used to evaluate assembly quality.

Gene prediction and annotation was done with eggnog-mapper version 2.1.9 on eggNOG DB database version 5.0.2 (Cantalapiedra et al., 2021).

The genome was then analyzed for the presence of genes potentially involved in entomopathogenic activity and related to the endophytic properties of the fungus. The sequence of a selection of these genes was aligned with the sequence of reference fungal strains to determine the degree of homology, using the BLAST tool of NCBI.

3.4. Statistical analysis

Data processing and statistical analyses were performed using R software, version 4.4.2 (R Core Team, 2022). One-way analysis of variance (ANOVA) was used to analyse fungal growth data, whereas two-way ANOVA was used to analyse insect mortality data. Levene's test was used to confirm the assumption of homogeneity of variance across groups. ANOVA was followed by Tukey post-hoc test (Keselman and Rogan, 1977).

Repeated measures ANOVA (proc. mixed) and LSMEANS comparison (adjust = Tukey) for means separation were used to analyse insect survival rate over time. Time to death means were compared between two different insect species exposed to *B. bassiana* using *t*-tests.

3.5. Results

3.5.1. Fungal growth behaviour

Growth indicators of the strain UNISS22 of *B. bassiana* measured two weeks after inoculation on PDA in comparison with other strains are shown in Table 1. While the strain ATCC74040 showed the greatest horizontal growth ($F_{3,12} = 6.22$; $P = 0.00856$), strain UNISS22 revealed the greatest ability to grow vertically ($F_{3,12} = 5.462$; $P = 0.0134$). As a result, the biomass produced by strains UNISS22 and ATCC74040 was comparable and about twice that of strains GHA and PPRI 5339 ($F_{3,12} = 33.95$; $P < 0.0001$).

Table 1 - Comparative production of *Beauveria bassiana* fungal biomass on PDA

***B. bassiana* strain**	**Horizontal growth (mm)**	**Vertical growth (mm)**	**Biomass volume (mm^3)**
ATCC74040	34.5 ± 1.1 a	5.8 ± 0.5 a	7074.7 ± 294.6 a
GHA	27.2 ± 5.4 b	4.8 ± 1.0 a	3487.0 ± 585.2 b
PPRI 5339	24.9 ± 3.9 b	6.0 ± 0.8 a	3666.5 ± 1124.3 b
UNISS22	26.2 ± 1.3 b	9.8 ± 3.5 b	6737.9 ± 247.2 a

3.5.2. Insect bioassays

Different stages of *T. molitor*, used as a model insect, exposed by contact to *B. bassiana* UNISS22 suspensions at an average concentration of 10^7 conidia/mL were significantly more susceptible than the untreated control ($F_{1,20} = 107.406$; $P < 0.0001$), reaching more than 70% death for larvae and pupae, and 100% death for adults five days after treatment (Figure 1).

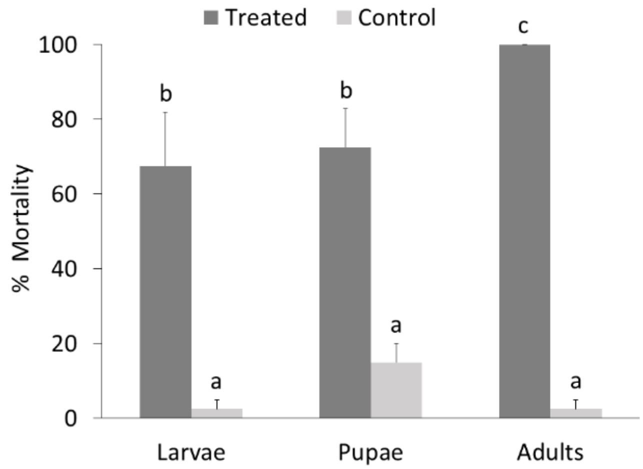

Figure 1 - Mortality (mean ± s.e.) of different *Tenebrio molitor* stages exposed to *Beauveria bassiana* UNISS22

Survival rate was significantly affected by treatment in *T. molitor* adults ($F_{2,45} = 9.49$; $P = 0.0003$) and larvae ($F_{2,105} = 30.89$; $P < 0.0001$), with a significant reduction in survival rate when insects were exposed to a higher fungal concentration (10^7 conidia/mL) (Figures 2 and 3).

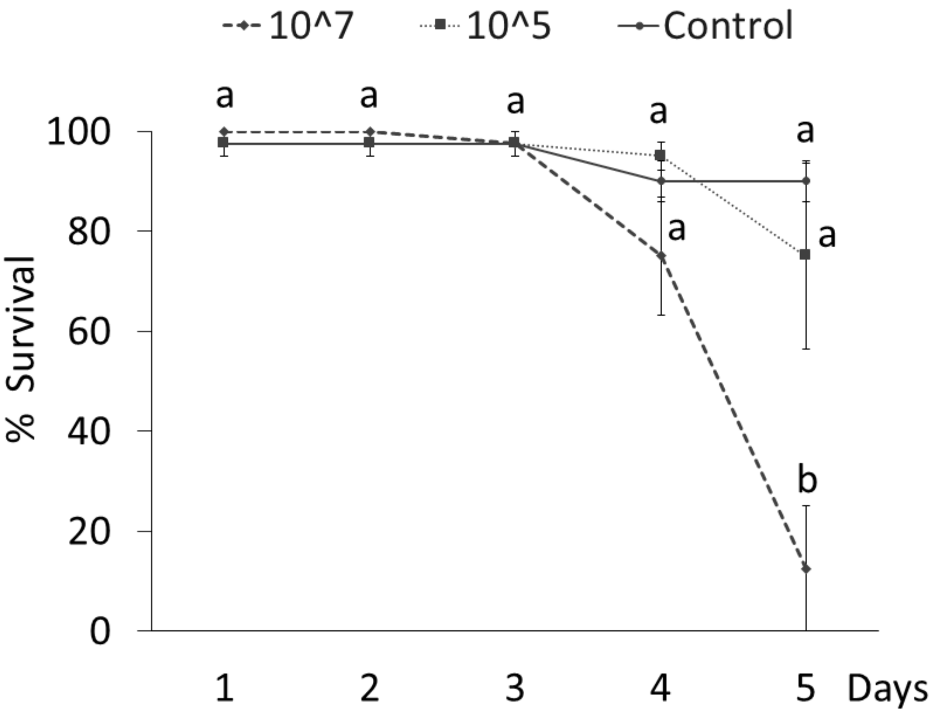

Figure 2 - Over time survival (mean ± s.e.) of different *Tenebrio molitor* adults exposed to different concentrations of *Beauveria bassiana* UNISS22

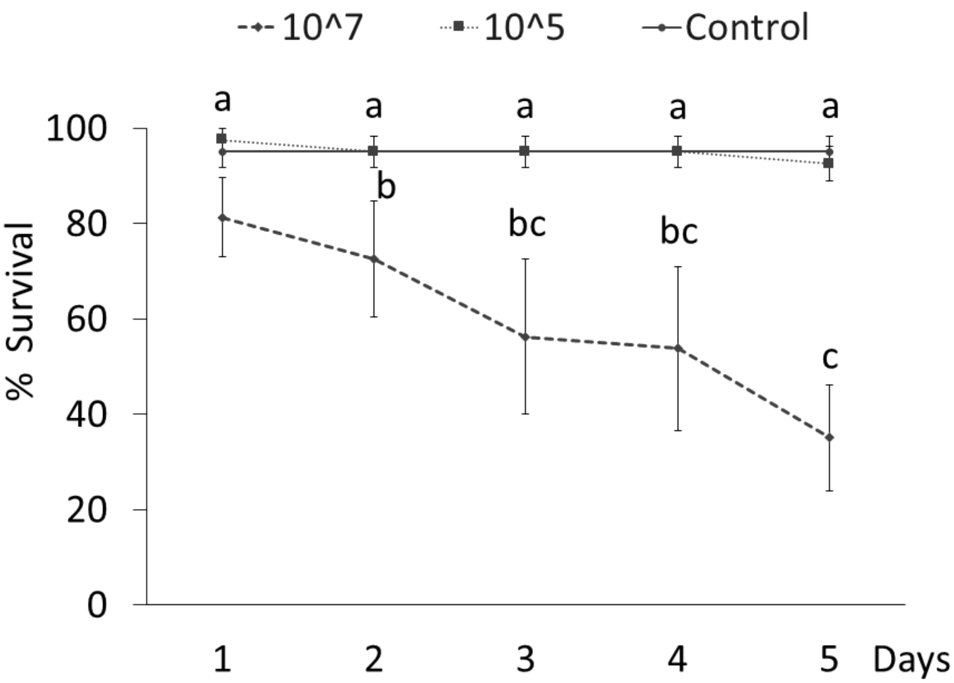

Figure 3 - Over time survival (mean ± s.e.) of different *Tenebrio molitor* larvae exposed to different concentrations of *Beauveria bassiana* UNISS22

Coraebus samples were also highly susceptible to *B. bassiana* UNISS22 ($F_{1,27} = 319.63$; $P < 0.0001$), with 100% mortality achieved in 5 days after treatment for both *C. florentinus* larvae and *C. undatus* adults (Figure 4). Mortality results were comparable to those observed in *T. molitor* larvae and adults, also taking into account a slightly higher larval mortality in the *Coraebus* control under laboratory conditions.

The average time to death (mean ± s.e.) was also slightly faster for *Coraebus* (2.58 ± 0.57 days) compared to *Tenebrio* (3.46 ± 0.39 days) larvae, although these differences were not significant (*t* = 2.0930, df = 19, P =0.2188).

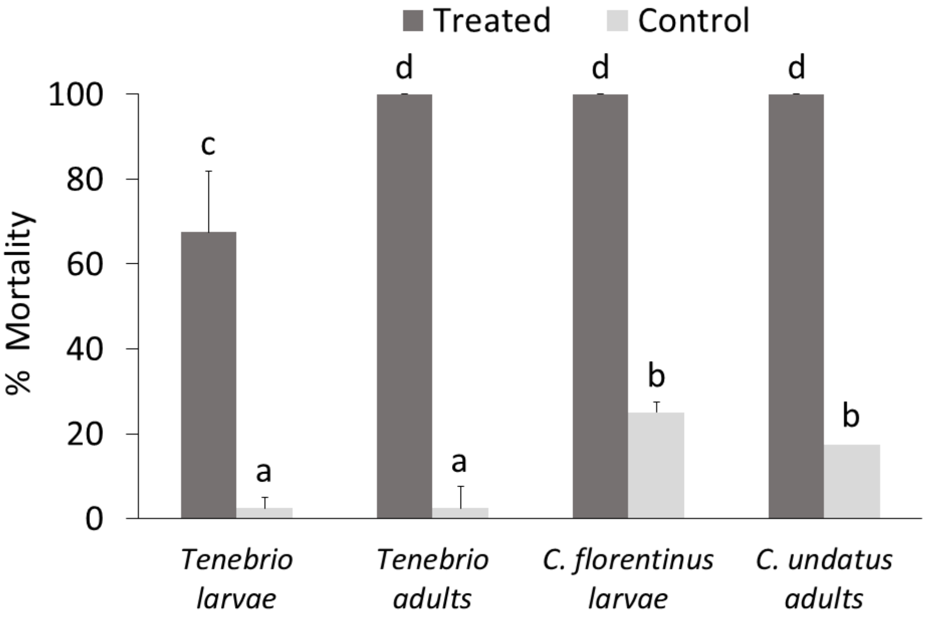

Figure 4 - Mortality (mean ± s.e.) of different *Coraebus* species and stages exposed to *Beauveria bassiana* UNISS22 in comparison with *Tenebrio molitor*.

3.5.3. Endophytic behaviour

Beauveria bassiana strain UNISS22 was found inside all *Quercus suber* plants whose roots had been treated by immersion in a conidial suspension. The fungus was found most frequently in tissue samples taken from the roots, followed by the stem and leaves. A higher percentage of occurrence was associated with a higher concentration of root treatment

(Figure 5). Significant differences were therefore influenced by treatment concentration (F2,31 = 5.229; P = 0.011) and the portion of the plant sampled (F2,31 = 3.548; P = 0.041).

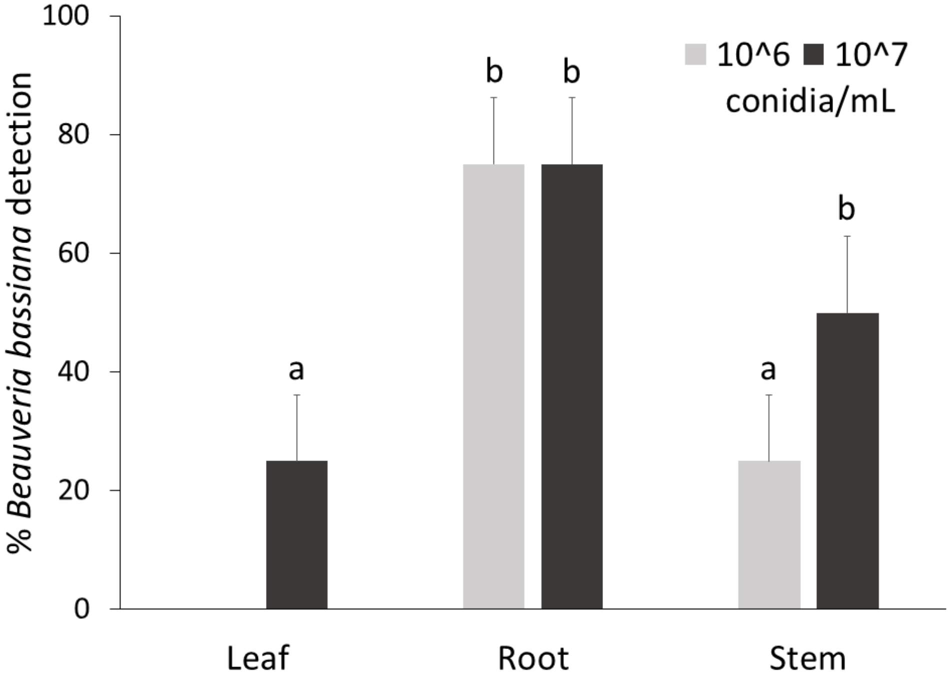

Figure 5 - Percentages (mean ± s.e.) of *Beauveria bassiana* detection in different tissues (leaf, root, stem) of *Quercus suber* plants treated with conidia of the fungus by root immersion.

3.5.4. Genomic insights on B. bassiana UNISS22

Whole-genome sequencing resulted in a total number of 61,409,652 paired-end reads and the assembled genome consisted of 137 contigs (≥1000 bp), with twelve L50 contigs and an N50 value of 968,729 bp. The guanine + cytosine (GC) content was 50.90 % and the total estimated size of the genome was 34,177,820. Functional genome annotation resulted in the distribution of the Clusters of Orthologous Groups (COGs) shown in Figure 6. Among the identified protein-coding sequences, several genes implicated in the entomopathogenic (e.g. chitinases, proteases, and hydrophobins) or in the endophytic (e.g.

cellulases, glucanases) properties of strain UNSS22 were identified. Alignment of these gene sequences with known sequences of other entomopathogenic fungal strains, including *Beauveria bassiana* ARSEF 2860, *Cordyceps militaris* CM01, and *Cordyceps (=Isaria) fumosorosea* ARSEF 2679, revealed a significant uniqueness of the strain compared to others (Table 2).

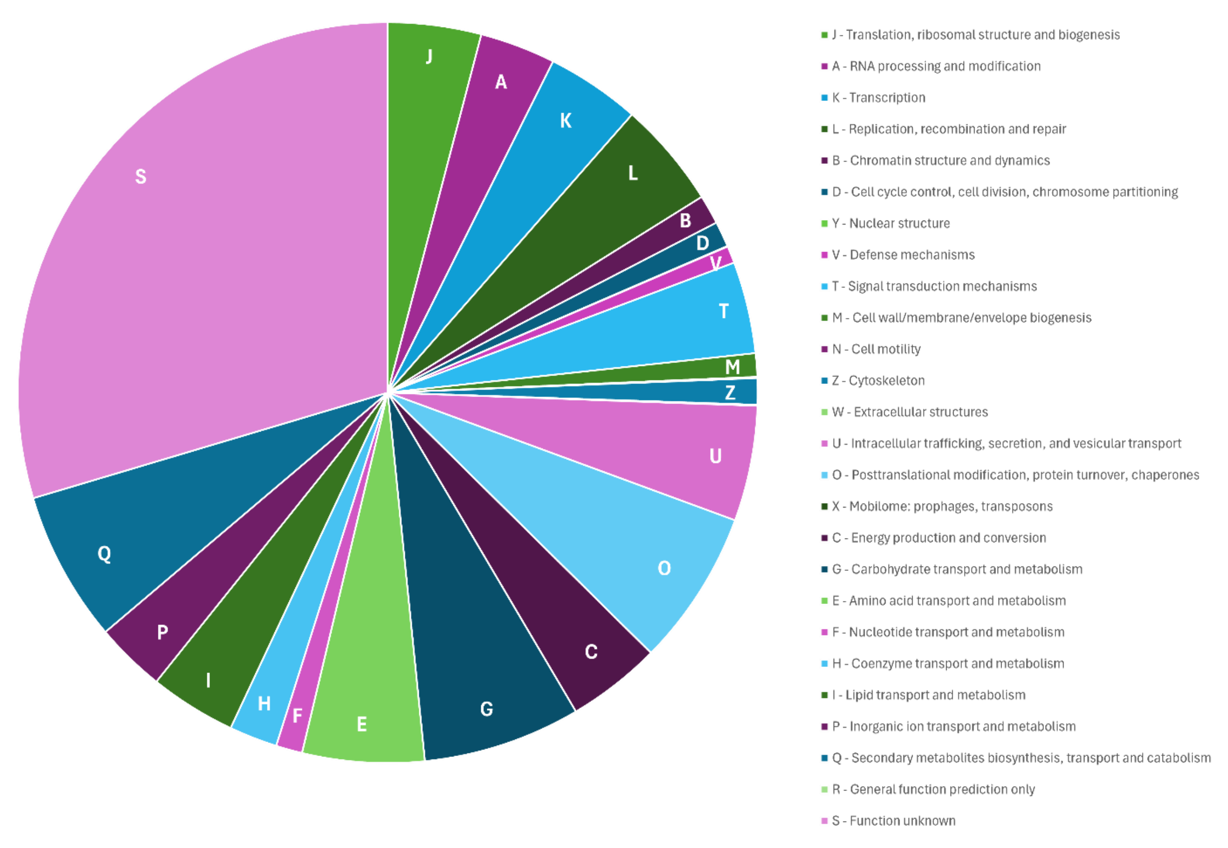

Figure 6 – Pie chart of COG functional category classification result for *B. bassiana* UNISS22 genome

Table 2 - Homology of *Beauveria bassiana* UNISS22 gene selection involved in pathogenesis and endophytism.

Gene	Sequence Coverage	Sequence Identity	Acc. Number	Homologous fungal strain
Class V chitinase Chi100	99%	93.76%	XM_006669963.1	*Cordyceps militaris* CM01
	99%	88.79%	XM_018843980.1	*Isaria fumosorosea* ARSEF 2679
	86%	95.05%	XM_008604503.1	*Beauveria bassiana* ARSEF 2860
Chitinase 18-3	100%	84.39%	XM_008602996.1	*Beauveria bassiana* ARSEF 2860
Chitinase 18-4 (Chi-6)	84%	96.24%	XM_008603039.1	*Beauveria bassiana* ARSEF 2860
Subtilase-like protein	96%	91.36%	XM_008600400.1	*Beauveria bassiana* ARSEF 2860
Alkaline serine protease AorO	99%	93.00%	XM_008603888.1	*Beauveria bassiana* ARSEF 2860
Peptidase S8 family	99%	83.60%	XM_056202738.1	*Akanthomyces muscarius*
	98%	78.98%	XM_018846784.1	*Isaria fumosorosea* ARSEF 2679
	100%	92.81%	XM_008604367.1	*Beauveria bassiana* ARSEF 2860
	98%	82.48%	XM_056192465.1	*Akanthomyces muscarius*
	96%	76.89%	XM_006669501.1	*Cordyceps militaris* CM01
Hydrophobin	95%	86.73%	XM_008601696.1	*Beauveria bassiana* ARSEF 2860
Hydrophobin 2	58%	88.85%	XM_008595627.1	*Beauveria bassiana* ARSEF 2860
Adhesin protein Mad1	79%	90.56%	XM_008597516.1	*Beauveria bassiana* ARSEF 2860
Aspartokinase-like protein	100%	88.84%	XM_008603841.1	*Beauveria bassiana* ARSEF 2860
Cytochrome P450	100%	92.94	XM_008600044.1	*Beauveria bassiana* ARSEF 2860
Glycosyl hydrolase 5 (cellulase A)	100%	91.14%	XM_008599623.1	*Beauveria bassiana* ARSEF 2860
	96%	81.62%	XM_006671857.1	*Cordyceps militaris* CM01
	96%	80.62%	XM_018848077.1	*Isaria fumosorosea* ARSEF 2679
Cellulase putative	99%	93.26%	XM_008600575.1	*Beauveria bassiana* ARSEF 2860
	99%	82.00%	XM_006669699.1	*Cordyceps militaris* CM01
	99%	81.57%	XM_056200721.1	*Akanthomyces muscarius*
	99%	78.86%	XM_018846030.1	*Isaria fumosorosea* ARSEF 2679
Glycosyl hydrolase 12 (cellulase H)	100%	95.92%	XM_008595400.1	*Beauveria bassiana* ARSEF 2860
	100%	87.07%	XM_056203914.1	*Akanthomyces muscarius*
	88%	84.48%	XM_006670591.1	*Cordyceps militaris* CM01
Cellulase (glycosyl hydrolase family 5)	80%	95.20%	XM_008600714.1	*Beauveria bassiana* ARSEF 2860
	80%	85.40%	XM_056202372.1	*Akanthomyces muscarius*
	80%	84.36%	XM_018850046.1	*Isaria fumosorosea* ARSEF 2679
Beta-1,6-glucanase precursor	100%	94.39%	XM_008603898.1	*Beauveria bassiana* ARSEF 2860
	96%	88.58%	XM_006665929.1	*Cordyceps militaris* CM01
	100%	86.83%	XM_056193425.1	*Akanthomyces muscarius*
	100%	85.16%	XM_018849630.1	*Isaria fumosorosea* ARSEF 2679
	100%	79.63%	XM_014687521.1	*Metarhizium brunneum*
	100%	79.54%	XM_066952036.1	*Metarhizium anisopliae*
	100%	79.05%	XM_007818996.1	*Metarhizium robertsii* ARSEF 23
	99%	78.74%	XM_007817346.2	*Metarhizium acridum*
Glucan 1,3-beta-glucosidase	100%	89.61%	XM_008596840.1	*Beauveria bassiana* ARSEF 2860

3.6. Discussion

Beauveria bassiana strain UNISS22, isolated from a naturally infected *Coraebus florentinus* larva found inside an infested oak branch, proved to be an effective entomopathogen of coleopterans as determined in laboratory bioassays using the model species *Tenebrio molitor*. These experiments have also documented a concentration-dependent insecticidal effect and the ability of the fungus to attack all stages (larvae, pupae, adults) of the insect and successfully complete the pathogenic process. Similarly, *B. bassiana* strain UNISS22 was able to kill *Coraebus* larvae and adults by carrying out its entire biological cycle on the host, up to the external production of mycelium and conidiophores bearing new aerial conidia for dispersal (Mascarin and Jaronski, 2016). These findings corroborate several studies reporting the pathogenicity of *B. bassiana* administered at comparable concentrations to different beetle species, including the spruce bark beetle *Ips typographus* L. (Coleoptera: Curculionidae) (Kreutz et al., 2004), the elm bark beetle *Scolytus scolytus* (Coleoptera: Scolitidae) (Doberski, 1981), the red turpentine beetle (RTB) *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae), and *C. florentinus* (El-Khoury and Tarasco, 2020). However, many promising results have come from laboratory studies in which the target insects are exposed by direct contact with the fungus, whereas real field conditions require the fungus to be able to reach the larvae within the tunnels dug in the wood or bark (McKinnon et al., 2017). Accordingly, we conducted observations on the growth behaviour of the fungus and its ability to penetrate plant tissues. Comparative experiments with other commercially available strains revealed that *B. bassiana* strain UNISS22 has pronounced biomass-producing capabilities, which may be related to greater pathogenicity and diffusion capacity across environmental surfaces such as soil and plant material, thereby enhancing fungal persistence. Equally important is the ability of the fungus to penetrate plant tissues in perspective of its use in the management of endophytic pest species (Quesada Moraga et al., 2020). *B. bassiana* UNISS22 showed significant endophytic properties, being able to penetrate tissues and spread within the plant through the lymphatic system (Quesada Moraga et al., 2014). Consistently, the fungus was found not only in the roots, but also in the stem and leaves of oak seedlings treated by root immersion.

Analysis of the genome of *B. bassiana* strain UNISS22 revealed an arsenal of genes encoding for proteins related to the insecticidal potential, such as hydrophobins normally involved in the adhesion to the host cuticle as well as proteases and chitinases involved in the penetration inside the host (Quesada-Moraga et al., 2020). Interestingly, the sequences of these genes showed significant variations with respect to homologous sequences in the reference genome of *B. bassiana* strain ARSEF 2860 and in other entomopathogenic species like *Cordyceps militaris* and *Cordyceps (=Isaria) fumosorosea*. These findings raise the possibility that strain UNISS22 may have its own characteristics and potential with respect to *Coraebus*, from which it was originally isolated. Moreover, the presence of several genes encoding for enzymes involved in the interaction with the plant, such as cellulases, supports an adaptation of the fungus to the forest environment from which it originates. Although the general genetic profile of this strain is consistent with an expected broad spectrum of activity, the variability of the sequences found outlines its own uniqueness, which deserves further investigation (Ortiz-Urquiza and Keyhani, 2016).

According to the results of this study, *B. bassiana*, and in particular strain UNISS22, appears to be a well-adapted fungus to the forest environment, with significant potential for the management of *Coraebus* borers populations, given the endophytic habits of the larvae. The observed susceptibility of adults may provide further opportunities for the management of these oak pests, envisioning methods in which adults could be infected in the field by inoculation devices, and then subsequently act as carriers of the fungus to other hosts or to the plants where immatures develop (Dowd and Vega, 2003; Gálvez et al., 2023).

However, successful field use will require the development of appropriate application techniques to reach the damaging larvae so as to achieve the required efficacy (Hajek and Bauer 2007).

The use of a selected strain of *B. bassiana* could complement other control methods that are also suitable for further implementation, such as the use of natural enemies, the use of adult traps, and the removal of infested branches or whole plants. The results of the present study therefore contribute to the development of eco-friendly management strategies to protect forests from *Coraebus* beetles.

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CHAPTER IV - Safety of the entomopathogenic fungus *Beauveria bassiana* for wild and laboratory-reared *Chrysoperla lucasina* strains

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4.1. Simple summary

Natural predators such as the chrysopid *Chrysoperla lucasina* occur in agroecosystems, where they naturally contribute to containing pest populations. Their biological properties can be leveraged by mass rearing in the laboratory and releasing them in the field, where they can express their predatory potential against pest species. To ensure the preservation of these beneficial species, plant protection products should be harmless to them. Accordingly, the use of biopesticides based on microbial entomopathogens, such as the fungus *Beauveria bassiana* strain ATCC 74040, is promoted as they are generally regarded as safe to non-target species. However, such safety should be demonstrated case by case. In addition, safety should be ensured for both the wild species and those released for biological control. This study involved experiments including laboratory-reared and wild *C. lucasina* larvae and determined only slight or no effects of *B. bassiana* on their survival, immature development, adult emergence, and reproductive potential. These findings highlight the compatibility of the predator and this strain of *B. bassiana*, emphasising the opportunity for their combined use in environmentally friendly pest management strategies.

4.2. Abstract

The need to reduce the impact of plant protection products on agroecosystems fosters the use of augmentative biological control involving the release of beneficial species into the field, the employment of entomopathogenic microbials, and the protection of naturally occurring biocontrol agents. This study aimed to investigate the compatibility of the entomopathogenic fungus *Beauveria bassiana* with the generalist insect predator *Chrysoperla lucasina*, in comparative experiments involving a laboratory-reared and a wild chrysopid strain. The larvae of the predators were exposed to different concentrations of fungal conidia up to a concentration of 10^7 conidia/mL by contact and ingestion. The treated insects showed only slight differences in terms of survival and immature development time compared to the untreated control insects. A significant decrease in the proportion of the male adults of *C. lucasina* that emerged from the laboratory-reared larvae that were exposed to higher concentrations of the fungus suggested a potentially different susceptibility between the sexes. A slightly lower adult emergence rate was observed in the wild strain, while no significant differences were recorded in the adult reproductive performance. These findings indicate that the *B. bassiana* strain ATCC 74040, at concentrations commonly used in the field, did not pose a significant risk to *C. lucasina* and can be safely used in combination with this predator for sustainable pest management.

Keywords: biological control; predator; pest management; microbials; compatibility; IPM.

4.3. Introduction

The need to protect agricultural production systems from the deleterious action of pests while minimizing the impact on the environment fosters the use of bio-based solutions, among which biological control agents (BCAs) like predators and parasitoids are often employed successfully. This can be achieved with different techniques, including the release of beneficial insects or the enhancement of naturally occurring antagonists through ecosystem manipulation (Baker et al., 2020). Accordingly, several chrysopid species occur on a variety of crops, where they act as generalist predators regulating the action of various pests, including aphids, thrips, and whiteflies. *Chrysoperla lucasina* (Lacroix) (Neuroptera: Chrysopidae), is a member of the *Chrysoperla carnea* (Stephens) group, commercialized as a BCA for inundative biocontrol programs (Pappas et al., 2011). This insect is therefore present both in the wild form and as a product of artificial breeding. Consequently, ecosystem management interventions should be compatible with this useful entomofauna (Genyz et al., 2010). Another bio-based approach for pest management is the use of microbials like entomopathogenic bacteria, viruses, fungi, and nematodes (EPNs), which cause diseases that specifically target pests. Among these, the fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. is widely used for its efficacy and broad spectrum of action against a variety of crop pests across different orders, including Lepidoptera, Coleoptera, Heteroptera, Homoptera, Diptera, and Hymenoptera (Zimmerman et al., 2007; Mascarin et al, 2016). The action of *B. bassiana* typically relies on the adhesion of aerial conidia to the hydrophobic surface of a host’s cuticle, followed by germination and penetration through the cuticle to reach the rich haemocoel environment where the fungus can produce further propagules and hyphae that infect diverse host organs and tissues, and release metabolites to intoxicate the host and overcome its immune system, thus leading to the insect's death. If environmental conditions are favourable, the fungus can produce conidiophore-carrying aerial conidia on the surface of the dead host, which will ensure its dissemination (Ortiz-Urquiza et al., 2010; Quesada-Moraga et al., 2023). Several studies have demonstrated the successful use of *B. bassiana* in various agroecosystems with little ecological impact. The compatibility of this fungus with most of its natural enemies has been reported (Koller et al., 2023), including Coleoptera, Hemiptera, Collembola (Thungrabeab et al., 2007), and Hymenoptera (Aguila et al., 2021; Al-mazra'awi et al., 2007). On the other hand, this fungus was reported to interfere with the recognition mechanisms of honeybee nestmates (Cappa et al., 2019). This highlights the importance of conducting extensive studies to assess the potential impact on beneficial species not only in terms of acute toxicity, but also in terms of the behavioural or developmental effects that could occur even with sublethal exposure.

The purpose of this study was to investigate the possible impact of *B. bassiana* on wild and laboratory-reared populations of the predatory lacewing *C. lucasina* when exposed to different concentrations of the entomopathogenic fungus *B. bassiana*. Developmental and life-table parameters in treated and untreated insects were also compared.

4.4. Materials and methods

4.4.1 Fungal preparations

The entomopathogenic fungus *Beauveria bassiana* strain ATCC 74040, commercially available as the active substance of the product Naturalis® (CBC Europe S.r.l., Nova

Milanese, Italy), was used in this study (Atzeni et al., 2020). Microbial cultures were routinely grown at 25 ± 1 ◦C on potato dextrose agar (PDA) (Liofilchem, Teramo, Italy) to ensure continuous availability during the experiments (Atzeni et al., 2020). The aerial conidia that were used in bioassays were scraped from fresh PDA plates into a 0.02% Tween 80 solution. The resulting conidia suspensions were checked under a phase microscope (Zeiss, Novara, Italy) for purity and quantified in a Neubauer chamber (Blaubrand, Wertheim, Germany). Distilled water was used to adjust the concentration of the suspension as needed for bioassays. The conidia suspensions were used in bioassays immediately after their preparation to ensure the highest viability (>90%).

4.4.2. Insect rearing

During this study, two colonies of *C. lucasina* were maintained in the laboratory, of which the first was normally used in augmentative biocontrol programs (laboratory strain) and the second (wild strain) was initiated using wild-caught specimens from the countryside of Olmedo (Sardinia, Italy). The insects were reared at 25 ˚C with a photoperiod of L16:D8 in compliance with previously described methods (Pasqualini, 1975) with necessary modifications (Loru et al., 2013).

Larvae (mealworms) of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), used in bioassays or as lacewing prey, were provided by the insect rearing facility of the Department of Agricultural Sciences of the University of Sassari (Italy) (Ruiu et al., 2020).

4.4.3. Insect bioassays

4.4.3.1. Survival bioassays

The potential lethal effects of *B. bassiana* were evaluated by exposing chrysopid larvae to fructose drops containing fungal conidia, so as to encourage their contact and ingestion. To set up a reliable experimental model, preliminary observations were conducted on larvae that were exposed for 24 h to 20% fructose drops (4 μL) with a red vegetable-based food dye (Ehrenberg et al., 2019). During this period, the larvae were regularly observed making contact with the droplets and feeding on them. Thereafter, the larvae were observed under a phase microscope to detect the presence of the dye in their gut, which confirmed the ingestion of the coloured liquid and thus also the fungal conidia when present (Figure 1).

Figure 1 - *Chrysoperla lucasina* larva fed with 20% fructose drops containing a food dye with evidence of red coloration showing through the gut (arrows).

According to this method, first instar larvae of *C. lucasina* were individually placed in transparent plastic pots (2 cm diameter × 3 cm high) with a drop (4 μL) of 20% fructose suspension containing a specific conidia concentration (10^7, 10^6, or 10^5/mL) or without conidia (control). Treated and control larvae were incubated at 25 °C and maintained in the following period on a diet consisting of two mealworms per individual, provided every other day. Each treatment involved four replicates (10 individuals each), and the insects were inspected daily, assessing mortality after 7 days. Individual maintenance of larvae was necessary to avoid cannibalism.

To assess the virulence of the conidia suspension stocks that were used in the experiments with chrysopids, additional bioassays were conducted in parallel on *T. molitor* larvae. For this purpose, groups of 10 mealworms were immersed for 30 s in *B. bassiana* suspensions at different concentrations (10^7, 10^6, or 10^5 conidia/mL) and incubated at 25° C on sterile plates with filter paper for 96 h, finally assessing larval mortality. The experimental design involved 4 replicates for each treatment and for the untreated control group (larvae immersed in sterile water).

All of the experiments were repeated over time with at least three different cohorts of insects and *B. bassiana* batches from different laboratory preparations.

4.4.3.2. Bioassays on Life-Table Parameters

According to the experimental design previously described, individually reared chrysopid larvae that were exposed to different concentrations of fungal conidia (10^7, 10^6, or 10^5 conidia/mL) or left untreated (control), were maintained individually in pots that were inspected daily and provided with two mealworms per individual on alternate days, to follow their preimaginal development until pupation and adult emergence. After recording insect survival and the developmental stage duration, the emerging adults were transferred into new cages (10 cm diameter and 10 cm high) at a 1:1 sex ratio, allowed to mate, and were fed ad libitum with pollen and water. Each cage included a removable paper sheet in the top side where eggs were regularly oviposited by females (Loru et al., 2013). The paper sheet was replaced daily to allow continuous egg counts over the following three-week period. The insect mortality in each cage was also recorded (Ruiu et al., 2020).

Four groups (replicates) of 10 eggs from each cage (treated and control) were periodically analysed to assess their viability. For this purpose, eggs were placed individually in a separate pot and monitored over a week until hatching. These analyses were performed 4 times during the oviposition period.

4.5. Data analysis and statistics

Data were analysed using R software, version 4.1.2 (R Core Team, 2022). Two-way ANOVA (factors: treatment and *C. lucasina* strain) was used to analyse the *C. lucasina* data on survival, larval and pupal development time, adult emergence, sex ratio, oviposition, and egg hatching. The percentage data were arcsin-squareroot-transformed before analysis (Ahrens et al., 1990). The assumption of homogeneity of variance across the groups was confirmed by Levene’s test. One-way ANOVA was used to analyse the mortality data for *Tenebrio molitor* larvae. A post-hoc comparison based on the Tukey test (Keselman et al., 1977) was used when significant main effects were detected.

4.6. Results

4.6.1. Survival bioassays

4.6.1.1. Bioassays with Chrysoperla lucasina

The survival of newly hatched *C. lucasina* larvae that were exposed by contact and fed on fructose drops containing different concentrations of *B. bassiana* conidia appeared to be consistently above 90% after 7 days and without significant differences compared with the untreated control group ($F_{3,24} = 0.99$; $p = 0.4100$). Furthermore, no differences were detected in the comparison between laboratory and wild strains ($F_{1,24} = 1.94$; $p = 0.1750$), nor in the interaction between treatments and chrysopid strains ($F_{3,24} = 1.71$; $p = 0.1920$) (Figure 2).

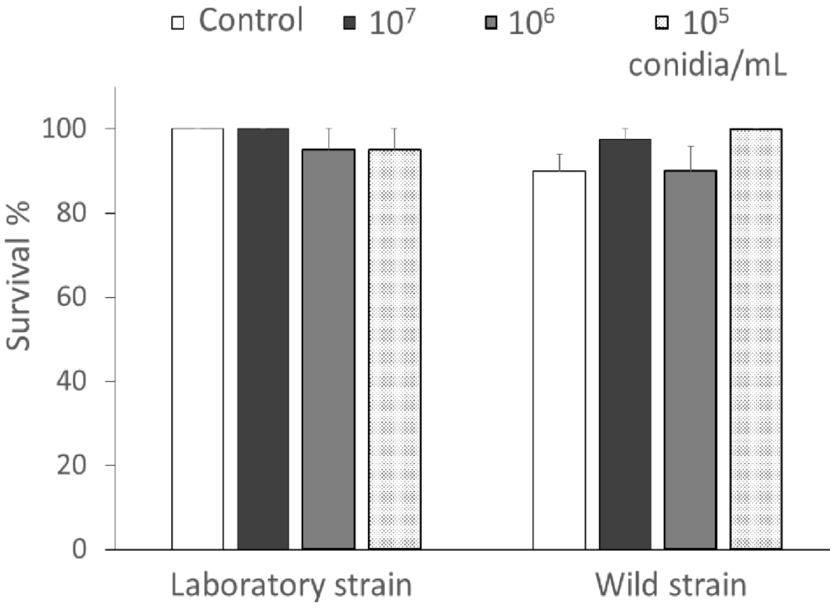

Figure 2 - Survival percentage (mean ± SE) of *Chrysoperla lucasina* larvae exposed to different concentrations (10^7, 10^6, or 10^5 conidia/mL) of *Beauveria bassiana* conidia. Means were not significantly different (two-way ANOVA on transformed data, $p > 0.05$).

4.6.1.2. *Bioassays with Tenebrio molitor*

In virulence bioassays with *T. molitor*, aimed at assessing the entomopathogenic potential of *B. bassiana* against insects, conidia that were brought into contact with the mealworms' integument caused an average mortality exceeding 80% at 96 h after treatment at all concentrations assayed (10^7, 10^6, and 10^5 conidia/mL), while low mortality (<4%) was observed in the untreated control group (F3,11 = 18.64; P < 0.001) (Figure 3).

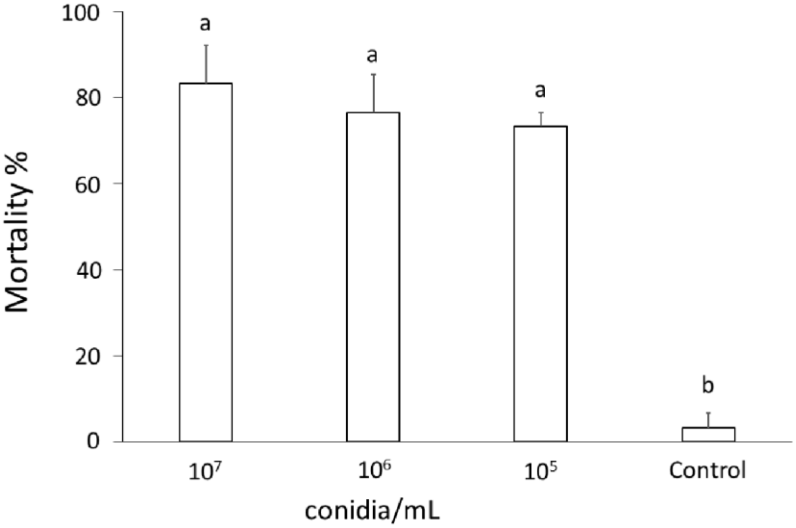

Figure 3 - Mortality percentage (mean ± SE) of *Tenebrio molitor* larvae exposed to different concentrations (10^7, 10^6, or 10^5 conidia/mL) of *Beauveria bassiana* conidia. Different letters above bars indicate significantly different means (one-way ANOVA on transform

4.6.2. Bioassays on life-table parameters

The average larval and pupal development time and adult emergence rate of *C. lucasina* individuals that were exposed to different concentrations of *B. bassiana* conidia at the first larval instar are reported in Table 1. The development time was not significantly different for the treated compared to the control larvae (F3,24 = 0.53; p = 0.6652), and for the wild compared to the laboratory strain (F3,24 = 3.47; p = 0.0746), and no differences between treatments (F3,24 = 0.01; p = 0.9988) and strains (F3,24 = 0.16; p = 0.9203) were observed in the duration of the pupal stage.

Table 1 - Means (± SE) of larval and pupal development time, percentage of adult emergence, and sex ratio of two *Chrysoperla lucasina* strains (laboratory and wild) exposed to *Beauveria bassiana* conidia at different concentrations.

Beauveria bassiana **Concentration (Conidia/mL)**	**Development Time (Days)**		**Adult Emergence**[c] **%**	**Sex Ratio (Male) %**
	Larva[a]	**Pupae**[b]		
Laboratory strain				
10^7	16.3 ± 1.26 a [d]	11.5 ± 0.93 a	92.5 ± 2.50 ab	73.1 ± 5.33 b
10^6	16.6 ± 1.62 a	11.4 ± 1.19 a	95.0 ± 2.89 a	55.0 ± 5.49 a
10^5	16.8 ± 1.42 a	11.1 ± 1.01 a	92.5 ± 2.50 ab	59.4 ± 5.24 a
Control	15.8 ± 1.37 a	11.5 ± 1.03 a	97.5 ± 2.50 a	49.4 ± 12.48 a
Wild strain				
10^7	17.5 ± 1.29 a	11.71 ± 0.99 a	80.0 ± 7.07 b	51.6 ± 10.70 a
10^6	19.0 ± 1.98 a	11.83 ± 1.02 a	82.5 ± 8.54 b	37.5 ± 11.09 a
10^5	17.8 ± 1.90 a	12.0 ± 0.89 a	85.0 ± 6.45 b	31.5 ± 12.78 a
Control	17.1 ±1.20 a	11.7 ± 0.91 a	85.0 ± 6.45 b	54.6 ± 6.61 a

[a] calculated from egg hatching to pupation. [b] calculated from pupation to adult emergence. [c] calculated on the initial number of larvae. d Different letters in a column indicate significantly different means (two-way ANOVA on transformed data in the case of percentages, followed by the Tukey test, P < 0.05).

A slightly lower, though not significant, adult emergence rate was observed in the treated compared with the control groups of both the laboratory and wild strains of *C. lucasina* ($F_{3,24}$ = 0.40; *p* = 0.7514). Adult emergence was significantly higher in the lab-reared in comparison with the wild chrysopids ($F_{3,24}$ = 5.94; *p* = 0.0217).

The sex ratio was similar in the untreated laboratory-reared and untreated wild *C. lucasina*. The laboratory strain had an adult sex ratio of 73% male in the highest inoculum treatment that differed significantly from that of the control group or other inoculum concentrations ($F_{3,24} = 5.35$; p = 0.0269). Other variations in differently treated groups were not significant ($F_{3,24} = 1.38$; p = 0.2860).

The average number of eggs per female that were laid by individuals that emerged from the larvae that were exposed to *B. bassiana* conidia at different concentrations was not affected by treatment ($F_{3,24} = 0.23$; p = 0.8694) or the C. lucasina strain ($F_{3,24} = 3.96$; p = 0.0580). Similarly, no differences were observed in the egg-hatching rate between treatments ($F_{3,24} = 0.26$; p = 0.8510) or strains ($F_{3,24} = 1.60$; p = 0.2160) (Table 2).

Table 2 - Means (± SE) of eggs/female and egg hatching rate of two *Chrysoperla lucasina* strains (laboratory and wild) exposed to *Beauveria bassiana* conidia at different concentrations.

Beauveria bassiana Concentrations (Conidia/mL)	N [a]	Eggs/Female	Eggs Hatching [b] %
Laboratory strain			
10^7	11	288.4 ± 46.03 [c]	55.0 ± 8.66
10^6	17	355.0 ± 54.94	60.0 ± 4.08
10^5	16	234.8 ± 63.38	62.0 ± 6.29
Control	18	294.5 ± 64.42	70.0 ± 4.08
Wild strain			
10^7	13	211.9 ± 54.27	72.5 ± 11.09
10^6	16	182.7 ± 31.75	77.5 ± 7.50
10^5	16	237.6 ± 14.44	67.5 ± 8.53
Control	14	254.1 ± 57.32	55.0 ± 6.45

[a] Number of ovipositing females. [b] Egg hatching was determined on four occasions during the experiment. Mean values are reported. [c] Means in each column are not significantly different (two-way ANOVA on transformed data in the case of percentages, P > 0.05).

4.7. Discussion

The need to limit the use of broad-spectrum chemical insecticides for pest management in agroecosystems is well recognized for the preservation of entomophagous species. The mass breeding of beneficial species for field release is also required for augmentative biological control (Collier et al., 2004).

However, the quality of laboratory-reared insects is frequently lower, in terms of their biological characteristics, biotic potential, and predator or parasitoid performance, due to their reduced genetic diversity and the artificial rearing conditions (Van Lenteren et al., 2004). Though this aspect is the subject of continuous studies aimed at improving the quality of these insects, it follows that a significant difference in their ability to adapt to different environmental conditions may exist when comparing between laboratory-reared and wild strains (Gilkeson et al., 2019).

Another aspect that influences the quality of the biocontrol activity that is carried out in the field by the beneficial species, and which could highlight differences between lab-reared and wild strains, is their susceptibility to biotic factors, including the entomopathogenic agents that are used as biopesticides (Zimmermann et al., 2007; Grenier et al., 2003).

Among the latter is the fungus *Beauveria bassiana*, whose entomopathogenic action against insects in different orders begins with the adhesion of aerial conidia to the host's integument, followed by mechanical penetration through the cuticle by means of special structures that are aided by specific enzymes (chitinases and proteases), which allows the fungus to reach the insect's haemocoel where it finds nutrients that promote its development and further spread in the insect's body. The pathogenic process ends with the production of conidiophore branches carrying new aerial conidia that will promote spreading in the environment (Quesada-Moraga et al., 2020). The action of the fungus is therefore nonspecific and requires favourable environmental conditions for its accomplishment. It therefore becomes essential to verify its selectivity and compatibility towards beneficial insects (Zimmermann et al., 2007).

The hypothesis in the present study was that larvae of the predator *C. lucasina,* that normally move about the plant in search of prey, come into contact with droplets of the plant protection product containing *B. bassiana* conidia. Under our experimental conditions, it has been shown that these larvae, in addition to coming into contact with the fungus, can also ingest it, adding to the above-described action by contact also an action by ingestion (Mannino et al., 2019).

However, at the concentrations assayed (equal to and less than 10^7 conidia/mL), which reflect those that the predator might encounter in the field in accordance with the application doses indicated in the biopesticide product label, no effect on the survival of either the laboratory or wild strains was observed. This result aligns with several studies in which different chrysopid species were exposed to other strains of *B. bassiana* (Mingotti Dias et al., 2019; Leyva et al., 2011).

Greater variability was observed in the sex ratio of adults that emerged from early age larvae that were exposed to the fungus. On this, there were no substantial differences between the two strains of *C. lucasina*, although a significant reduction in the proportion of female individuals was observed as an effect of treatment at the higher concentration, which supports sex-specific differences in their susceptibility to infection.

Alongside a wide individual variability, the fecundity of *C. lucasina* females that emerged from the treated larvae was not affected by the treatments and no differences between the lab and wild strains emerged, which corroborates several other studies with chrysopids reporting slight to non-significant effects of fungi and other entomopathogens on reproductive parameters (Ruiu et al., 2020; Hamze et al., 2022). This aligns with a generally good ecotoxicological profile being recognized for *B. bassiana* that is compatible not only with chrysopids, but with a variety of other predators such as coccinellids and hemipterans, and with soil insects such as collembolans (Thungrabeab et al., 2007; Broza et al., 2001; Dromph et al., 2002).

According to our study, no specific risks resulted from the exposure of *C. lucasina* larvae to conidia of *B. bassiana* strain ATCC 74040, which supports the simultaneous use of these two agents for biological control (predator and fungus) and highlights the safety of this *B. bassiana* strain for wild chrysopid populations. In contrast, there are also studies that report a deleterious effect of *B. bassiana* on different chrysopid species (Amorim et al., 2005; Portilla et al., 2017). In addition to a variable degree of susceptibility of different host species, different studies have employed diverse strains of the fungus, which can be associated with variable virulence against different targets (Zhang et al., 2020; Usman et al., 2021).

Studies on the compatibility of a *B. bassiana* strain with chrysopids merit further exploration into the combined use of entomopathogens and predators, as demonstrated in studies with the predators *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and *C. carnea* that were inoculated with *B. bassiana* conidia to disseminate the fungus in the environment (Zhu et al., 2012). On the other hand, every specific use should be accurately studied, especially in field conditions where the complexity of the relationships among species and the presence of other stress factors may lead to new or unexpected effects (Donegan et al., 2003).

4.8. Conclusions

This investigation contributes to defining the safety and a good ecotoxicological profile of a commercially available *B. bassiana* strain that is largely employed as a valuable biosolution for pest management, according to the principles of eco-sustainability. The evidence for the compatibility of this *B. bassiana* strain with *C. lucasina* arising from this study also provides practical information for the combined employment of these pest management agents in crop systems.

4.9. Authors contributions

Conceptualization, W.M. and L.R.; methodology, W.M., M.T.N. and L.R.; data analysis, W.M. and L.R.; manuscript preparation and editing, W.M. and L.R.; funding acquisition, L.R. All authors have read and agreed to the published version of the manuscript.

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5. DISCUSSION

The Mediterranean region is facing increasing difficulties in managing insect populations that threaten *Quercus* species, with serious implications for the cork industry. *Coraebus undatus* (Fabricius), a member of the Buprestidae family, is a major pest that poses a significant risk to cork production. *C. undatus* larvae attack the phellogen layer in cork oak (*Quercus suber*), excavating extensive galleries that eventually integrate into the cork layer as it grows, affecting the physical and mechanical properties of the cork and dramatically reducing its quality (Jiménez et al., 2012). In Sardinia*, C. undatus* infestations have mostly been intermittent. However, since 2016, the number of damaged cork planks has increased significantly, notably in the northeastern parts of the island. This pest behaviour has led to increased monitoring efforts to determine its real impact and to study the dynamics of *C. undatus* infection during various harvesting periods (2011-2021) (Tiberi et al., 2016).

Our field research focused on the spread of *C. undatus* infestations and found a strong relationship between infestation intensity and proximity to industrial cork storage facilities. Infestation rates were highest in forests close to storage areas and decreased dramatically within a 2-km northwest and 10-km southeast radius of these spots. These findings show that the proximity of the cork industry can influence *C. undatus* populations, creating favourable conditions for the pest spread. The prevalence of this pest infestation was very low between 2011 and 2015 but increased exponentially from 2016 onwards. Cork layer analysis was used to track temporal dynamics, with feeding galleries dated based on their position between annual cork rings (Cárdenas et al., 2021).

In addition to regional and temporal dispersion, phenological investigations of *C. undatus* have revealed important details about the life cycle of this pest. Adult emergence was reported to begin in early July, peak in mid-July and decline progressively until the flight period ended in late August. Observations from subsequent years reflect this seasonal pattern, but further multi-year research is needed to confirm these phenological trends and refine management plans (Cárdenas et al., 2012).

Recently, there has been renewed interest in the use of biological control agents to manage the *Coraebus* population as part of integrated pest management (IPM) strategies. *Beauveria bassiana*, an entomopathogenic fungus known to infect a wide range of insect pests, is one of the most promising biological control agents. It infects insects by adhering to their cuticle, germinating, and entering their bodies, ultimately killing them through toxicological effects, nutrient loss and mechanical disruption. Unlike conventional pesticides, *B. bassiana* has low environmental impact, making it a promising alternative for sustainable pest management in vulnerable environments such as Mediterranean oak forests (Koller et al., 2023).

Our results showed that *B. bassiana* is highly effective against *Coraebus* species at all life stages, with considerable mortality rates in larvae, pupae, and adult beetles. These findings are consistent with previous research demonstrating the broad-spectrum efficacy of *B. bassiana* against various beetle species (Liu et al., 2008). The ability of *B. bassiana* to affect *Coraebus* at different life stages is an added value, in the prospect of a more complete control throughout the life cycle of the pest, thus reducing the possibility of resurgence. On the other hand, one of the main concerns about the use of biological control agents, such as entomopathogens, is the potential impact on non-target species. Maintaining biodiversity and ecological balance is critical in natural environments because many beneficial insects, such as pollinators and predators, play important roles in ecosystem functions. Accordingly, the widespread use of biological control agents may affect these non-target organisms, resulting in unforeseen ecological consequences.

Our study investigated this compatibility by focusing on *Chrysoperla lucasina*, a generalist predator whose larvae prey on a variety of pests under field conditions. Wild and laboratory-reared *C. lucasina* strains were exposed in the laboratory to increasing concentrations of *B. bassiana* conidia by contact and ingestion, up to a maximum of 10^7 conidia/mL. Slight changes in survival rates and immature development time were observed in the treated groups compared to the control groups, indicating that *B. bassiana* has no substantial impact on *C. lucasina*. These findings align with the work of Thungrabeab et al. (2007), who also reported minimal effects of entomopathogenic fungi on non-target beneficial insects.

Although exposure to higher fungal concentrations (up to 10^7 conidia/mL) led to a slight reduction in the emergence rate of adult males in the laboratory-reared *C. lucasina* strain, this difference was not observed in the wild strain. Furthermore, there were no significant differences in the reproductive performance of adults that emerged from the treated larvae, indicating that *B. bassiana* poses a low risk to *C. lucasina*. These results, as suggested by Quesada-Moraga et al. (2021), support the use of *B. bassiana* in integrated pest management (IPM) programs that protect predator populations and result in a more balanced approach to pest control.

The compatibility of *B. bassiana* with *C. lucasina* is an important step towards the development of effective and sustainable IPM techniques for oak ecosystems, particularly in Sardinia, where *Coraebus* species infestations are increasing. This study demonstrates that *B. bassiana*, and in particular the newly isolated strain UNISS22, can efficiently control insect populations. Given the need to reduce reliance on chemical pesticides, *B. bassiana* provides an environmentally friendly option that adheres to agroecological principles by targeting pests without altering the overall ecosystem (Ruiu et al., 2020).

6. CONCLUSION

The management of *Coraebus* species in Sardinian oak forests is a serious concern due to the increasing frequency of infestations and the ecological relevance of the trees affected. Our findings indicate that the *B. bassiana* strain UNISS22 has potential as an effective biological control agent for managing *Coraebus* species, with strong efficacy throughout the pest life cycle. The biological properties of this strain, including its increased biomass production, adaptation to the forest environment and endophytic behaviour, as a result of its genetic uniqueness, outline a considerable potential against these pests. Based on the current knowledge and our additional study on the safety of *B. bassiana* to non-target species, the introduction of strain UNISS22 as a pest management tool is very promising. However, further experiments are needed to assess its efficacy under field conditions and to implement appropriate techniques to effectively apply this endophytic *B. bassiana* strain to infested oak trees.

More generally, integrating *B. bassiana* into IPM programs for forest pest management seems to be a promising strategy for the long-term protection of *Quercus* species in

Sardinia and the Mediterranean region. Biocontrol measures need to be continuously monitored, researched, and refined to ensure their long-term effectiveness and environmental safety.

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