Universidade do Minho
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Ivo Vaz Oliveira

Entomopathogenic fungi associated to *Prays oleae*: isolation, characterization and selection for biological control.

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Tese de Doutoramento em Ciências
Especialidade em Biologia

Trabalho realizado sob a orientação da
Prof. Doutora Teresa Lino-Neto
do
Prof. Doutor Albino Bento
e da
Prof. Doutora Paula Baptista

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Entomopathogenic fungi associated to *Prays oleae*: isolation, characterization and selection for biological control

Abstract

Entomopathogenic fungi (EF) are nowadays considered one of the most promising alternatives to chemical pesticides. The use of fungal species or strains recovered from and adapted to a specific environment and host will increase the chances of success of biocontrol measures. In this work, we focused on one of the major pests affecting the olive culture, the lepidopteran *Prays oleae* Bern. A revision of available knowledge on entomopathogenic fungi is presented, focusing on their ecology and environmental factors that affected their occurrence, abundance and diversity. The infection mechanism is also addressed, as well as the enzymes and toxins related to both insect infection and death. The review on the compatibility of EF with chemical pesticides and other biological methods of pest control shows that different outcomes of those interactions are to be expected. Finally we address the current data about the use of EF for the control of olive pests.

The first step was to ascertain which fungal entomopathogenic species were associated to *P. oleae* in an olive ecosystem. The undertaken survey, on larvae and pupae of the three annual generations (phyllophagous, antophagous and carpophagous) of *P. oleae* resulted in the identification of 43 species from 24 genera. Besides species best described as phytopathogenic, a large number of possible biocontrol agents, including antagonistic and entomopathogenic fungal species were found. *Beauveria bassiana* was the one found in higher abundance, especially in the phyllophagous generation.

The internal transcribed spacer (ITS) region has been adopted as a barcode region for fungi, although many doubts still persist about the reliability of this marker. This work intends to evaluate and compare the efficiency of different molecular barcode markers (ITS, β-tubulin and RPB2), by amplifying these regions of fungal isolates retrieved from mycosed larvae and pupae of *P. oleae*. Although results showed higher performance of the ITS region, use of multiple barcode regions for fungal identification should be considered, as the use of β-tubulin and RBP2 increased the number of identified sequences. This multi-locus approach allowed the identification of fungal species associated with the death of larvae and pupae of *P. oleae* that present distinct roles in the ecosystem.

The pathogenicity of four isolates of the major fungal taxa identified, *B. bassiana*, was evaluated on a lepidopteran pest of chestnut, *Cydia splendana*. The use of six concentrations of

each fungal isolates showed a time- and concentration-dependent mortality of larvae, with mortalities ranging from 40 to 100%. Variations of virulence parameters were detected between isolates, both for median lethal concentration (LC_{50}) and median lethal time (LT_{50}). The results showed a high susceptibility of *C. splendana* to *B. bassiana*, indicating that EF can be useful to control this pest.

After having determined the presence of EF in olive orchards, and their ability to infect insect pests, we decided to study some factors that can be responsible for the detected variation on their diversity and abundance in olive orchards. One of the studied factors was the impact that soil tillage has on EF. Larvae and pupae were sampled from olive orchards with or without soil tillage, and EF species associated to *P. oleae* were identified. A total of 120 isolates belonging to 8 EF species were found, being *B. bassiana* the most abundant one. Although no significant differences were observed between no-till and tilled orchards, higher occurrence, diversity and abundance was found in the former type of soil management. Four species were found exclusively on no-till orchards, indicating that the presence of natural vegetation creates more suitable conditions to EF, in olive orchards.

The effect of the olive plant organs (leaves, flowers and fruits) on the manipulation of the entomopathogens *B. bassiana* and *Paecilomyces formosa* was studied under *in vitro* conditions. Both fungi are influenced by olive tree organs, but in different ways: *Beauveria bassiana* was more negatively affected by olives, while *P. formosa* was more negatively affected by leaves. Both volatile and diffusible compounds, as well as their interaction, are responsible for the observed effects. These results suggested that olive plant organ may be involved in the recruitment or maintain of specific fungal species which could partly explained the differences on fungal occurrence between *P. oleae* generations.

Overall results showed that a pool of EF, for use as biocontrol agents, can be found in olive orchard, namely found in association to *P. oleae*, in high diversity and abundance. The most abundant species, *B. bassiana*, besides being isolated from *P. oleae*, also showed to be pathogenic to another lepidopteran pest, *C. splendana*. Of the factors that can interact with EF, different plant organs showed to have considerable influence on fungal behaviour. By other hand, different soil management practices didn't result in significant differences, although the presence of natural vegetation appears to be more suitable for EF. Combined information should give a significant thrust forward on research on EF for olive pest control, as more knowledge is needed, for the understanding of the interaction EF- *P. oleae*- olive tree.

Fungos entomopatogénicos associados a *Prays oleae*: isolamento, caracterização e selecção para controlo biológico

Resumo

Os fungos entomopatogénicos (FE) são, hoje em dia, uma das mais promissoras alternativas aos pesticidas químicos. O uso de espécies ou estirpes fúngicas isoladas e adaptadas a um determinado ecossistema e hospedeiro resultará num aumento das probabilidades de sucesso de métodos de luta biológica. Este trabalho focou-se numa das principais pragas que afecta a cultura da oliveira, o lepidóptero *Prays oleae* Bern. É apresentada uma revisão sobre o conhecimento disponível sobre os FE, incidindo sobre a sua ecologia, e factores que afectam a sua ocorrência, abundância e diversidade. O mecanismo de infecção destes fungos é também abordado, assim como enzimas e toxinas responsáveis pela infecção e morte do hospedeiro. A compatibilidade de EF com meios de controlo de pragas, quer químicos, quer de luta biológica é também apresentada. Finalmente, apresentam-se os dados disponíveis sobre o uso de FE contra pragas da oliveira.

O primeiro passo foi verificar as espécies de FE encontradas associadas a *P. oleae* no ecossistema olival. A pesquisa efectuada, em larvas e pupas das três gerações anuais (filófaga, antófaga e carpófaga) de *P. oleae* resultou na identificação de 43 espécies. Para além de espécies descritas como fitopatogénicas, foi encontrado um grande número de possíveis agentes de luta biológica, incluindo espécies de fungos antagonistas e entomopatogénicas. *Beauveria bassiana* foi a espécie encontrada em maior abundância, principalmente na geração filófaga.

A região espaçadora interna transcrita (internal transcribed spacer - ITS) foi recentemente definida como a região padrão -barcode- para os fungos, apesar de ainda subsistirem dúvidas sobre a sua confiabilidade. Este trabalho teve como objectivo avaliar e comparar a eficiência de diferentes regiões barcode (ITS, β-tubulin e RPB2), amplificando-os de isolados fúngicos obtidos de larvas e pupas de *P. oleae*. Apesar de os resultados mostrarem maior eficiência da região ITS, o uso de múltiplas regiões deve ser considerado, uma vez que aumenta o número de sequências identificadas. Esta abordagem multi-regiões permitiu a identificação de fungos associados *a P. oleae*, com distintas funções no ecossistema.

A patogenicidade de quatro isolados da principal espécie identificada, *B. bassiana*, foi avaliada em *Cydia splendana*, um lepidóptero praga da cultura da castanha. A utilização de seis concentrações de cada isolado mostrou uma mortalidade de larvas dependente do tempo e da concentração, com valores compreendidos entre 40 a 100% de mortalidade. Variações nos

parâmetros de virulência foram detectados entre isolados, quer na concentração letal média (LC_{50}), quer no tempo letal médio (LT_{50}). Os resultados mostraram uma alta susceptibilidade de *C. splendana* à infecção por *B. bassiana*, indicando que os FE podem ser úteis para o controlo desta praga.

Após ter sido determinada a presença de FE em olivais, e a sua capacidade de infectar insectos, foram estudados alguns factores que poderão influenciar a sua diversidade e abundância nos olivais. Um desses factores estudados foi o impacto da mobilização do solo nos FE. Foram recolhidas larvas e pupas da praga em olivais com e sem mobilização de solo, identificando-se as espécies isoladas. No total, 120 isolados, pertencentes a 8 espécies de FE foram identificados, sendo *B. bassiana* a mais abundante. Apesar de não terem sido detectadas diferenças significativas entre olivais com e sem mobilização solo, foi observada maior ocorrência, diversidade e abundância em solos sem mobilização. Quatro espécies de FE foram encontradas exclusivamente em olivais sem mobilização, indicando que a vegetação natural pode criar condições mais favoráveis aos FE.

O efeito dos órgãos de oliveira (folhas, flores e frutos) nos FE *B. bassiana* e *Paecilomyces formosa* foi estudado em condições *in vitro*. Ambas as espécies foram influenciadas pelos órgãos da oliveira, ainda que de forma distinta. *Beauveria bassiana* foi negativamente mais afectada pelos frutos, sendo que *P. formosa* foi negativamente mais afectada pelas folhas. Compostos voláteis, difusíveis, e a sua interacção são responsáveis pelos efeitos observados. Estes resultados sugerem que os órgãos da oliveira podem estar envolvidos no recrutamento ou manutenção de FE específicos, o que pode explicar, parcialmente, as diferenças na ocorrência de FE entre gerações de *P. oleae*.

Os resultados globais revelaram a existência de um conjunto de FE no olival, em associação com *P. oleae* e em grande abundância e diversidade, que podem ser explorados como agentes de luta biológica. A espécie mais abundante, *B. bassiana*, além de isolada de *P. oleae*, também se mostrou patogénica em outra praga, *C. splendana*. Entre os factores que podem interagir com FE, os diferentes órgãos da planta mostraram ter uma influência considerável sobre o comportamento dos fungos. Por outro lado, os diferentes sistemas de maneio do solo não causaram diferenças significativas, embora a presença de vegetação natural pareça beneficiar os FE. A informação resultante deste trabalho deverá dar um impulso significativo à pesquisa sobre FE para a luta contra pragas de oliveira, uma vez que mais informação é necessária, para a compreensão da interacção FE- *P. oleae*- oliveira.

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List of abbreviations

μL – Microliter
μM – Micromolar
A – Antagonistic
AG – Antophagous generation
ANOVA - Analysis of variance
ATP - Adenosine triphosphate
BLAST- Basic Local Alignment Search Tool
bp – Base pair
β-tub – β tubulin
CFU – Colony-forming unit,
CG – Carpophagous generation
COI - Cytochrome c-oxidase
D – Simpson diversity index
DNA - Deoxyribonucleic acid
dNTP - Deoxynucleotide triphosphates
DTX – Destruxins
E – Entomopathogenic
EDTA - Ethylenediaminetetraacetic acid
EF – Entomopathogenic fungi
Ef1-α - Elongation factor-1 alpha
FAO – Food and Agriculture Organization of the United Nations
H - Shannon-Wiener diversity index
HCl - Hydrochloric acid
ITS – Internal transcribed spacer
LC50 – Median lethal dose
LT50 - Median lethal time
MatK - megakaryocyte-associated tyrosine kinase.
MgCl - Magnesium chloride
NaCl – Sodium chloride
NaOAc - Sodium acetate
NCBI - National Center for Biotechnology Information
ng – Nanogram
P – Phytopathogenic
PCA - Principal component analysis
PCR - Polymerase chain reaction
PDA – Potato Dextrose Agar
PG – Phyllophagous generation
rbcL - Ribulose-bisphosphate carboxylase
RH – Relative humidity
RPB1 - RNA polymerase II large subunit
RPB2 - RNA polymerase II 2nd largest subunit
SDS - Sodium dodecyl sulfate
SPSS - Statistical Package for the Social Sciences
UV – Ultraviolet light

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Chapter 1

Entomopathogenic fungi: current knowledge and potential against olive pests

Entomopathogenic fungi: current knowledge and potential against olive pests.

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Manuscript in preparation.

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Abstract

Entomopathogenic fungi have been extensively studied and their use as biocontrol agents is now a reality. They have no adverse effects on human and the environment, and are able to infect their hosts by direct contact only, without the need to be ingested. Secondary metabolites of entomopathogenic fungi can also be used as control agents against several pests. The studies regarding their ecology show that they are mainly found in soil, but are also present in the phylloplane and as endophytes of several plant species. Identification of entomopathogenic fungi is possible using different methodologies, including molecular ones, and using several markers. Also, a number of factors are known to affect their abundance and diversity, such as soil-related and climatic-related factors. Their compatibility with other pest control agents has already been evaluated. In this review, all these different topics will be address, in order to elucidate entomopathogenic fungal characteristics, as well their applicability against olive pests, like *Bactrocera oleae*, *Prays oleae* and *Saissetia oleae*.

Keywords: Entomopathogenic fungi; Ecology; Secondary metabolites; Biocontrol; Olive pests

Entomopathogenic fungi are able to regulate the natural population of pests, like insects, ticks and mites (Butt et al., 2001). They comprise a large group of fungal pathogens that includes approximately 700 species from almost 85 genera (Charnley and Collins, 2007). Due to their large host range, entomopathogenic fungi could be potentially useful as control agents against different insect orders. The use of entomopathogenic fungi to control insect pests was proposed more than 130 years ago, by Metchnikoff (Lord, 2005). From that point on, research on that possibility increased dramatically. In those early years, the initial mass production and several field trials were implemented. However, with the discovery and widespread application of chemical agents, the use of entomopathogenic fungi as a biocontrol agent was set aside. Even more, an erroneous comparison standard was created, when fungi-based insecticides are compared to chemical insecticides, generating expectations of chemical-like efficacies (Vega et al., 2009).

In the last few years, the situation has been changing, as research and development of new mycoinsecticides are considerably increasing (Faria and Wright, 2007). This is mainly due to an increased pressure by the public, demanding healthier products, to the requirements imposed by importing countries concerning chemical residues levels, and also to the awareness of farmers and industrial corporations about the long term environmental damage caused by chemicals. Furthermore, the resistance that some insect pests have acquired to chemical products, and increasing restrictive laws regarding the application of those chemicals, have given an significant thrust towards the research, production and commercialization of biocontrol agents. Up to 2007, at least 171 fungal products have been developed for the control of insects and pests (Faria and Wright, 2007) (Table 1). Of those, 129 were commercialized, at the date of the study. Two fungal species represented the major part of the available mycoinsecticides, *Beauveria bassiana* and *Metarhizium anisopliae*, each one representing 33.9% of the total available products. These fungal-based products are applied for controlling a large variety of pests, which include species belonging to several insect orders and families (Faria and Wright, 2007). Accordingly, the insect pests controlled by entomopathogenic fungi have been described to comprise whiteflies, cockroaches, corn and coffee-berry borers, grasshoppers and locusts, among others (Butt et al., 2001).

Although some disadvantages can be pointed out to fungal-based insecticides (speed of kill and cost) in comparison to conventional insecticides (Lacey et al., 2001), they also present considerable advantages. The possible infection of beneficial insects may be overcome by using

entomopathogens. Indeed, the fungal isolates retrieved from a given insect species tend to be more virulent to that species and could present a narrower host range (Goettel, 1995).

Table 1 Entomopathogenic fungal species used for mycoinsecticides development (adapted from Faria and Wright, 2007).

Fungal species	Number of products
Aschersonia aleyrodis Webber	1 (0.6%)
Beauveria bassiana (Bals.) Vuill.	58 (33.9%)
Beauveria brongniartii (Sacc.) Petch	7 (4.1%)
Hirsutella thompsonii F.E.Fisher	3 (1.8%)
Isaria fumosorosea Wize	10 (5.8%)
Isaria sp.	1 (0.6%)
Lecanicillium longisporum (Petch) R.Zare & W.Gams	2 (1.2%)
L. muscarium (Petch) R.Zare & W.Gams	3 (1.8%)
Lecanicillium sp.	11 (6.4%)
Metarhizium anisopliae (Metschn.) Sorokın	58 (33.9%)
M. anisopliae var. *acridum* Driver & Milner	3 (1.8%)
Nomuraea rileyi (Farl.) Samson	1 (0.6%)
Sporothrix insectorum de Hoog & H.C.Evans	3 (1.8%)
Conidiobolus thromboides Drechsler	2 (1.2%)
Lagenidium giganteum Couch	1 (0.6%)
Mix of two or more species	7 (4.1%)
Total	171 (100%)

Further advantages include the lack of detrimental effect on mammals, high persistence in the environment, as well as the possibility to modify, through biotechnological research, production of enzymes and toxins responsible for infection and mortality (Wan, 2003). In addition, no reports of resistance acquisition by insect pests to the entomopathogenic fungi infection have been reported (Shelton *et al.*, 2007). Contrary to other insect control agents, the use of entomopathogenic fungi does not rely on ingestion by the insects to cause mycosis. The entomopathogenic fungi are able to infect insects by penetrating the insect cuticle. Their proliferation will then lead to the insect's death. However, according to Lacey et al. (2001), the use of entomopathogens should even be

enhanced by performing some improvements on their performance, production efficiency, formulation and virulence. Their environmental advantages should be also confirmed for increasing the growers and general public acceptance for these biological control agents.

Ecology of entomopathogenic fungi

Entomopathogenic fungi present a cosmopolitan distribution (Bidochka et al., 1998). They have been isolated from almost all regions of the world and belong to several genera of Ascomycota and Zygomycota divisions (Roy et al., 2006) (Table 2). The ecology of this kind of fungi has already been thoroughly reviewed (e.g. Vega et al., 2009; Roy et al., 2010) and will be only briefly addressed.

Table 2 – Classification of the most common entomopathogenic fungi (Roy et al., 2006).

Division	Class	Order	Family	Genus
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Beauveria*
				Cordyceps
				Cordycepioideus
				Lecanicillium
				Metarhizium
				Nomuraea
Zygomycota	Sordariomycetes	Entomophthorales	Entomophthoraceae	*Entomophaga*
				Entomophthora
				Erynia
				Eryniopsis
				Furia
				Massospora
				Strongwellsea
				Pandora
				Tarichium
				Zoophthora
			Neozygitaceae	*Neozygites*

The presence of entomopathogenic fungi in all habitats proves their successful evolution, and the ability to take advantage from the interaction with plants, insects and other nutritional sources (Cory and Ericsson, 2010), and their life cycle is complex (Figure 1). However, the soil is considered the natural reservoir of entomopathogens. The resting stages of entomopathogenic fungi are commonly found in soil of several field crops, orchards (Sun et al., 2008) and forests (Bidochka et al., 1998). The fungal entomopathogens most recurrently found in soil belong to *Beauveria*, *Isaria* and *Metarhizium* genera (Meyling and Eilenberg, 2007).

Life in the soil presents some opportunities for fungal entomopathogens. The soil provides a protective effect from U.V. radiation, as well as from extreme temperatures, and also offers an optimal humidity level (Inglis et al., 2001). In addition, a wide diversity of insects is present in the soil that may be potential hosts for the entomopathogenic fungi (Vega et al., 2009). Indeed, the continuous proximity between hosts and pathogens has been described as an important factor for the evolution of fungal entomopathogenecity (Humber, 2008). However, the competition caused by the presence of other microorganisms in the soil, as well as the presence of their antimicrobial metabolites, may have a negative effect on the ability of entomopathogens to infect their insect hosts (Vega et al., 2009). In addition, even after insect infection, the entomopathogenic fungi could have their nutritional resources, namely dead insects, infected by opportunistic microorganisms. To overcome this competition, the production of secondary metabolites by entomopathogens belonging to the Hypocreales order has already been described (Strasser et al. 2000a). These metabolites apparently have the function to increase the ability of the fungus to outcompete those opportunistic organisms. However, the possibility of retrieving other nutritional sources should not be discarded. Some fungi appear to be able to retrieve nutrients from plants, after depleting those offered by the insect host (Vega et al., 2009).

Entomopathogenic fungi can also become associated with plants, being found as endophytes in a large variety of plant species (Meyling and Eilenberg, 2007). At least twelve species of entomopathogenic fungi have been reported as endophytes in several plant species, in which they naturally occurred or were introduced using different techniques (Vega, 2008). For instance, *B. bassiana* has been found as an endophyte in several plant species, including cocoa seedlings and coffee (Posada and Vega, 2005), corn (Bing and Lewis, 1993), poppy (Quesada-Moraga *et al.*, 2006a), potato (Reddy *et al.*, 2009), *Carpinus caroliniana* (Bills and Polishook, 1990), *Phoenix dactylifera* (Gómez-Vidal *et al.*, 2006), among several other species (Vega, 2008). The presence of endophytic entomopathogenic fungi is believed to work as a plant adaptation for

the defence against herbivorous insects (Elliot *et al.,* 2000; White *et al.*, 2002). Indeed, plants appear to be able to "control" endophytic entomopathogens proliferation by adjusting their abundance, germination or contact rates (Elliot *et al.,* 2000). Besides the described endophytic activity of entomopathogenic fungi, their presence in the phylloplane of several plant species has already been described (Meyling and Eilenberg, 2006; Asensio *et al.*, 2007, Talwar, 2005). Most probably the fungal entomopathogens were deposited on the leaf surface by the action of the wind or rain. However, the endophytic behaviour of such fungi should not be neglected (Meyling and Eilenberg, 2006).

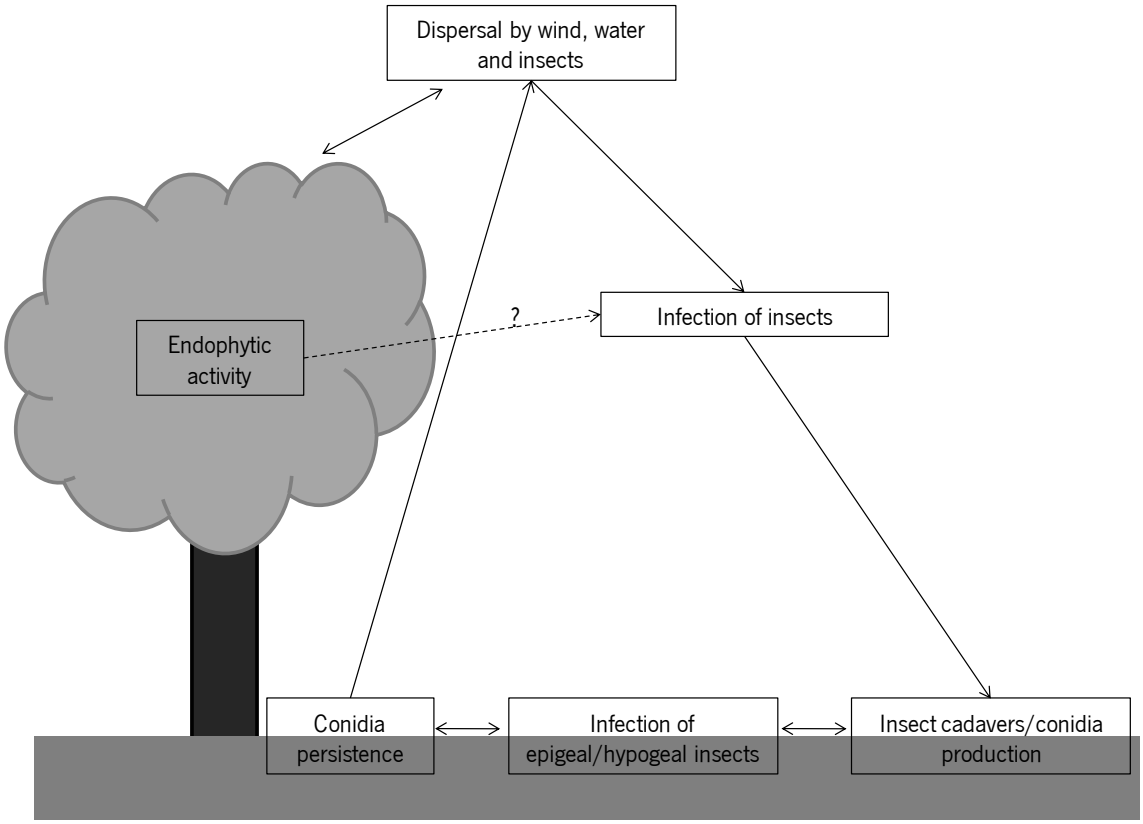

Figure 1 Suggestion of entomopathogenic fungi lifecycle, based on the current knowledge. Arrows represent the dispersal pathways; dotted arrow indicates a possible infection pathway. Dark grey area indicates soil environment, and white background indicates aboveground environment (adapted from Meyling and Eilenberg, 2007).

Molecular identification of entomopathogenic fungi

As occurring for other fungal species, the identification of entomopathogenic fungi was first determined by morphological traits. The advent of molecular studies led to the identification of previous cryptic entomopathogenic species, corresponding to those species classified as a single nominal species because they were morphologically indistinguishable (Bickford et al., 2006). Examples of such cryptic fungi include *B. bassiana* (Rehner and Buckley, 2005; Rehner et al.,

2006), *Metarhizium* (Bidochka et al., 2001, 2005), *Paecilomyces lilacinus* (Inglis and Tigano, 2005), *Nomuraea rileyi* (Neelapu et al., 2009) and *Ophiocordyceps unilateralis* (Kobmoo et al., 2012). Furthermore, the use of molecular tools for identification of entomopathogenic fungi has recently resulted in the description of new six species belonging to *Beauveria* genus (Rehner et al., 2011).

Different molecular markers have been described for the molecular identification of entomopathogens and for the phylogenetic studies of these fungi, as well as for the molecular characterization of fungal isolates. The number of works dealing with the molecular identification and characterization of *B. bassiana* population is a good example of such molecular markers diversity. Several loci are now currently used in such studies, like the internal transcribed spacer (ITS) (Rehner and Buckley, 2005; Ghikas et al., 2010; Sevim et al., 2010; Rehner et al., 2011; Johny et al., 2012; Meyling et al., 2012; Wang and Zheng, 2012), the elongation factor 1-alpha (EF1-α) (Rehner and Buckley, 2005; Sevim et al., 2010; Garrido-Jurado et al., 2011; Rehner et al., 2011; Meyling et al., 2012; Wang and Zheng, 2012; Johny et al., 2012), the RNA polymerase II largest subunit (RPB1) (Rehner et al., 2011) and the RNA polymerase II second largest subunit (RPB2) (Rehner et al., 2011). In the particular case of *B. bassiana*, specific primers have been also designed for the nuclear intergenic region Bloc (Rehner et al., 2006), which exhibits more variability than the ITS region (Meyling, 2008). Due to its informative potential, this locus has already been used in several studies that confirmed the ability of this marker to further resolve species within the genus (Rehner et al., 2011; Johny et al., 2012; Meyling et al., 2012). Other entomopathogenic fungi have also been subject to phylogenetic studies using distinct molecular markers, such as *M. anisopliae* (Bischoff et al., 2009; Freed et al., 2011), *Cordyceps sinensis* (Chen et al., 2004), *Nomuraea rileyi* (Han et al., 2002), *Paecilomyces fumosoroseus* (Tigano-Milani et al., 1995), among others. Multilocus analysis has also led to the report of new species of *Metarhizium*, including the type species, *M. anisopliae*, previously composed of four varieties (Bischoff et al, 2009).

The use of a standard DNA region, a DNA barcode, has been established for a given taxonomic group identification. Examples of DNA barcodes include, the portion of the mitochondrial gene *COI* for animals (Hebert et al., 2003) and regions of the plastid genes *matK* and *rbcL* for land plants (CBOL Plant Working Group, 2009). The most consensual DNA barcode for fungi has been the ITS region (Seifert, 2009, Begerow et al., 2010). However, the size of this region has been pointed out as a major limitation. Indeed, the amplification of a sequence of less

than 500 bp, in some taxonomic groups, could result in insufficient variation and lead to an ambiguous species identification (Seifert, 2009). Furthermore, intraspecific and even intra-individual variability of this region has been reported for entomopathogenic fungi (Kårén et al., 1997; Smith et al., 2007). The ITS region exhibited lower species resolution that elongation factor 1-alpha (EF1-a) region, for *Beauveria* isolates (Rehner and Buckley, 2005). As referred before, for this genus, the nuclear intergenic Bloc region (Rehner et al., 2006) presents more information than the ITS region (Meyling, 2008).

Species identification, especially of those fungi presenting cryptic species or small variations between species, should be carefully evaluated. Whenever possible more than a single locus should be studied. The use of reliable barcode databases (http://www.boldsystems.org; www.fungalbarcoding.org) will allow the comparison the obtained sequences with barcode sequences and provide the rapid identification of fungal species. Currently, these databases rely on a reduced number of available deposited sequences.

Environmental factors affecting entomopathogenic fungi

Fungal entomopathogens are affected by several factors that influence their abundance, diversity, viability and infection efficiency in a given ecosystem. Fungal entomopathogens are particularly affected by abiotic factors, like temperature, relative humidity and solar radiation (Inglis et al., 2001; Vidal and Fargues, 2007). The impact of these factors, regarding some of the most important entomopathogenic species, has been intensively reviewed (Zimmermann, 2007a, b; Zimmermann, 2008; Jaronski, 2010).

Temperature is one of the key factors affecting entomopathogen germination, growth, sporulation and survival. The influence of temperature is also noticed during host-pathogen interactions (Blanford and Thomas, 2000). Optimal germination and growth temperatures for entomopathogens range from 23°C to 28°C, decreasing with temperatures above 30°C, and being arrested for temperatures over 34°C (Jaronski, 2010). However, these values could present some variations. *B. bassiana*, for instance, has an optimal growth temperature of about 23-28°C, presenting a minimum of 5-10°C, but a maximum of 30-38°C (Zimmermann, 2007a). Indeed, this fungal species has been described as being able to adapt to different temperature regimes. Isolates of *B. bassiana* collected in Africa presents a reduced growth below 15°C and over 35°C, ranging the optimal temperature between 20 and 30°C (Tefera and Pringle, 2003) or

25 and 30°C (Ekesi et al. 1999). Isolates from sub-antarctic soils germinated at temperatures as low as 5°C (Roddam and Rath 1997).

When placed at different temperatures, variations on germination rates and mycelia growth have also been recorded for different isolates (Devi et al., 2005). For example, the important entomopathogenic fungus, *M. anisopliae*, presents an optimal germination temperature of 25-30°C (Zimmermann, 2007b), but different isolates can germinate at 35°C or even at 15°C (Dimbi et al., 2004). For this species, the germination of conidia at 40°C was also recorded, as well as at 45°C (Rangel et al. 2005).

Other entomopathogenic fungi appear to have a higher optimal growth temperature than *B. bassiana* or *M. anisopliae*. For example, *Isaria* sp. presented an improved growth at 30°, compared to the average 25°C optimal temperature for other fungi (Cabanillas and Jones, 2009). Also, *P. fumosoroseus* presents similar germination at 25°C and 35°C, decreasing slightly at 40°C and more remarkably at 45°C (Smits et al., 1996).

As temperature affects spore germination and growth of entomopathogenic fungi, the fungal virulence will be subsequently affected by this environmental factor. Indeed, several studies have described the influence of temperature on the infection of numerous pests. When the green peach aphid, *Myzus persicae*, was treated with different strains of entomopathogenic fungi, the median lethal time (LT_{50}) keeping constant the relative humidity, varied greatly, e.g. from 4.4 days at 20°C to 1.55 days at 30°C (Vu et al., 2007). When the malaria vector mosquito, *Anopheles arabiensis*, was infected with *B. bassiana* under two different temperatures, a difference in fungus-induced mortality rates was detected between temperature treatments, increasing considerably when temperature was changed from 21° to 25°C (Kikankie et al., 2010). *B. bassiana* also presented similar virulence to the red spider mite, Tetranychus urticae, at different temperatures (Bugeme et al., 2009). High mortality rates were also described by Vandenberg et al. (1998), when the diamondback moth (*Plutella xylostella*) was exposed to *B. bassiana* at temperatures of 25°C. In addition, the time required for causing mortality of infected pests, evaluated as the median lethal time (LT_{50}), depends on the temperature (Vestergaard et al., 1995). When analyzing the pathogenicity of *M. anisopliae* to thrips, LT_{50} values of about 4 days at temperatures of 18° and 20°C, were decreased to 3 days, at temperatures of 23° and 26°C (Vestergaard et al., 1995). The time needed to cause mortality of *Aphis fabae* and *Myzus persicae*, when infected by *B. bassiana*, *P. fumosoroseus* and *Verticillium lecanii* also illustrates the influence that temperature has on the ability of entomopathogenic fungi to infect and kill (Yeo

et al., 2003). For example, for a *B. bassiana* strain infecting *M. persicae*, the LT_{50} values decreased from 20.88 days, at 10°C, to 4.59 days, at 23°C. Also, when applied to the mite *Tetranychus evansi*, at different temperatures, the fungus *Neozygites floridana* requires high temperatures (29°C) to achieve low LT_{50} values (around 3 days) (Wekesa et al., 2010).

The relative humidity is another abiotic factor known to strongly influence the entomopathogenic fungi (Jaronski, 2010). Indeed, the relative humidity may be considered the decisive factor for the appearance of epizootic outbreaks, being required for germination, infection and sporulation of entomopathogenic fungal species (Hesketh et al., 2010). Most of the fungal species require at least 90% of relative humidity for germination (Inglis et al., 2001). For *B. bassiana*, reduction of germination was recorded at 99% of RH, and it is delayed at 94%, as growth is at 92% (Zimmermann, 2007a). A high humidity level is necessary for germination of *M. anisopliae* spores (Zimmerman 2007b). Luz and Fargues (1997) also reported that a minimum of 95.5% of relative humidity is required for germination of B. bassiana spores, at 25°C, below which no germination was detected.

The importance of humidity conditions on the infection ability of entomopathogenic fungi as been extensively reported. A higher humidity level than 96% is required for the effective infection of *Rhodnius prolixus* by *B. bassiana* (Luz and Fargues, 1999). The importance of humidity is also evident in this system by the LT_{50} values that significantly increased when relative humidity was changed cyclically to lower values (Fargues and Luz, 1999, 2000). Mortality of *Triatoma infestans* caused by *B. bassiana* and *M. anisopliae* was also significantly decreased with the reduction of relative humidity (Lazzarini et al., 2006). Relative humidity of 98%, promoted almost 100% of mortality, while humidity levels of 75% or 43% only promoted 23.7% of mortality or even lower. The humidity requirements could be even more extreme for other fungal species, like *Hirsutella thompsonii* and *Lecanicillium* spp. The germination of their spores requires a relative humidity of 80% to 95%, on the leaf surface, for several days (Jaronski, 2010).

Depending on the host-fungus system the humidity conditions could be more or less important. For example, *B. bassiana* is able to cause 98% mortality of the spruce bark beetle, *Ips typographus*, at relative humidity of 40%. Furthermore, experimental results with *B. bassiana*- and *Lecanicillium lecanii*-based formulations showed that environmental relative humidity may be a secondary factor affecting infection, in contrast to the target-insect habitat, such as leaf surface (Fargues et al., 2003)

The sunlight, particularly UV-B (290-330 nm) and UV-A (330-400nm) radiation, is another major factor affecting entomopathogenic fungi (Zimmermann, 2007a). Conidia are relatively short lived on leaves at the top of the canopy, compared to the ones deposited in the middle of the canopy (Inglis et al, 1993). Indeed, sunlight is considered the most important parameter, when considering the survival of conidia on epigeal habitats (Daoust and Pereira, 1986; Inglis et al., 1993). However, attention must be paid to inter- and intra- specific variation of resistance to sunlight. These variations were clearly showed by the work of Fargues et al. (1996). Their results showed an overall higher resistance of conidia from *Metarhizium flavoviride* to simulated sunlight, followed by *B. bassiana* and *M. anisopliae*. Conidia from *P. fumosoroseus* were the most susceptible, but presented intra-specific variations. These intra-specific variations were also detected for *M. anisopliae* (Braga et al., 2001; Rangel et al., 2004; Rangel et al., 2005) and *B. bassiana* (Leland, 2005; Fernandes et al., 2007; Huang and Feng, 2009) spores.

The radiation also affects the fungal infection ability. When exposed to increasing doses of UV-B radiation, a strong decline of mortality rates of aphid *Myzus persicae* were observed for *B. bassiana* and *M. anisopliae* (Yao et al., 2010). These results confirmed previous data, which showed that radiation affects the virulence of entomopathogenic fungi (Inglis et al., 1997; Rangel et al., 2008).

In agricultural ecosystems, the production systems and agricultural practices, as well as the soil management, could also affect the natural enemies of pests (Altieri, 1999; Hummel et al. 2002a, b). The effect of soil properties on the presence of entomopathogens has been reported (Ali-Shtayeh et al., 2002; Shapiro-Ilan et al., 2003; Quesada-Moraga et al., 2007; Oddsdottir et al., 2010; Imoulan et al., 2011; Medo and Cagáň, 2011), as well as the influence of the habitat type (Vänninen, 1995; Chandler et al., 1997; Bidochka et al., 1998; Meyling and Eilenberg, 2006; Sookar et al., 2008; Sun et al., 2008; Goble et al., 2010; Medo and Cagáň, 2011; Schneider et al., 2012; Wakil et al., 2013). In addition the soil management system, such as the tillage practice, may also influence the entomopathogenic fungi, as previously described (Sosa-Gómez et al., 2001; Hummel et al. 2002b; Jabbour and Barbercheck, 2009). However, distinct fungal species exhibit different responses to this particular soil management practice, depending also on the plant culture field. For example, while *B. bassiana* was equally found in no-tilled and conventionally tilled soils of soybean cultures (Sosa-Gómez et al., 2001), tillage increased the soil CFU number of *B. bassiana* in corn fields (Bing and Lewis, 1993). In contrast, *Metarhizium* and *Isaria* species were found to be more prevalent in no-tilled soils than in conventionally tilled soils

(Sosa-Gómez et al., 2001). Other factors linked to soil management, like weed density, crop residues and abundance and diversity of insect pest can also influence the levels of entomopathogenic fungi (Jabbour and Barbercheck, 2009). Although differences can be found in soils with different management practices, these variations may not be detected when analysing fungal abundance and diversity at the canopy level (Meyling et al., 2011; Sosa-Gómez et al., 2001; Oliveira et al., accepted publication).

The abundance, diversity and persistence of entomopathogenic fungi in a given ecosystem, could be also affected by factors occurring at the phylloplane, such as the leaf surface chemistry, the microflora, and the presence of pesticides residues (Jaronski, 2010). Furthermore, the plant species is known to exert considerable influence on entomopathogenic fungi, through chemical cues that have an effect on pest attraction, infection, fungal sporulation and viability (reviewed by Cory and Ericsson, 2010). Plant secondary compounds are responsible for direct effects on entomophagous fungi. The most studied secondary compounds of plants that are able to influence entomopathogens are volatile compounds. Other compounds produced by plants, like alkaloids and glycoalkaloids, phenolics, terpenoids, isothiocyanates or exudates, are also able to affect conidia of entomopathogenic fungi (Lopez-Llorca and Olivares-Bernabéu, 1997; Vega et al., 1997; Lacey and Mercadier, 1998; Inyang et al., 1999; Poprawski et al., 2000; Poprawski and Jones, 2000; Klingen et al., 2002; Jaronski, 2007; Cory and Ericsson, 2010).

Infection mechanism of entomopathogenic fungi

Entomopathogenic fungi are able to infect their hosts, either penetrating through the insect cuticle or through body openings (Tanada and Kaya, 1993), being able to deplete nutrients, and, afterwards, re-emerge from host, as hyphae (Fig. 2). This kind of fungi evolved in such manner that they present mechanisms to overcome the insect defences. The first defence relies on the insect physical barrier of the cuticle, formed by the epicuticle, the procuticle and the epidermis, from the exterior to the interior (Pedrini et al., 2007). The epicuticle is very thin (0.1 – 3 μm) but multi-layered. The first layer - lipid layer – is resistant to enzymatic degradation (Hadley, 1981) and, unless physically disrupted, can prevent the passage of cuticle degrading fungal enzymes (Pedrini et al., 2007).

Spores and conidia are the structures responsible for the infection, which begins with the adhesion of these structures to the insect cuticle. The adhesion process occurs in three distinct stages: adsorption of fungal propagules to the cuticle, adhesion or consolidation of the interface

between pre-germinant propagules and epicuticle, and fungal germination and development in the cuticle surface (Fargues, 1984). The adhesion of the spores is believed to be possible due to the existence of non-specific hydrophobic forces. Although some fungal species (for instance, *V. lecanii* and *Hirsutella thompsonii*) present mucilaginous coatings that facilitate the adhesion to the insect, *B. bassiana* spores rely on a layer of rodlets fascicles that exert those kinds of forces (Boucias *et al.,* 1988). Other single-cell propagules of *B. bassiana* (blastospores and submerged conidia) also present different physical and chemical properties, which differ from each other and from aerial conidia (Holder et al., 2007).

At the end of the adhesion process, the appressorium will be developed to start the penetration stage. If the ideal conditions are present (nutrients, water activity, temperature and pH), and in the absence of possible antifungal compounds produced by the host, the germination of spores and formation of the infective structures – appressorium – will occur (Samson *et al.*, 1988). The formation of this structure is dependent on several signals, like the presence of polar cuticle fraction from an appropriate host, the adequate nutrient levels or the presence of a hydrophobic surface (Wang and St. Leger, 2005). In addition, secondary intracellular messengers, like Ca^{2+} and cyclic AMP, are also involved (St Leger *et al.,* 1991). Once the infective structures are formed, the fungus initiates the penetration in the insect cuticle. In order to do so, the entomopathogenic fungi use a combination of mechanical (pressure exerted by appressorium) and enzymatic mechanisms. Chitinases, lipases, esterases and, at least, four different proteases have been described to be involved in fungal penetration (Wan, 2003).

After penetration, the fungus will develop inside the insect host. This fungal growth happens as yeast-like blastospores, hyphal bodies or protoplasts (Clarkson and Charnley, 1996). The death of the host arises as a result of a combination of factors, including nutrient depletion, physical obstruction or invasion of organs, and toxinosis (Wraight *et al.*, 2007). After death of the host, the entomopathogenic fungus emerges from the cadaver as hyphae, and, in most cases, sporulation occurs on the insect surface. However, oomycetes, many species of zygomycetes, and some ascomycetous fungi produce spores inside the host cadaver (Wraight *et al.*, 2007). Other entomopathogenic fungal species are able to modify the behaviour of hosts, in order to ensure an enhanced dispersal of their spores. The best known behavioural change is the so called “summit disease”, where the host, in the hours leading to its death, climb to an elevated position to die. Other behaviour modifications caused by entomopathogenic infection include the host attachment to substrate, either by fungal holdfasts or host structures (Roy *et al.*, 2006).

Other entomopathogenic species are also able to produce conidia and resting spores, while the host is still alive, without visible changes in its behaviour. The final objective of all these changes of behaviour, as well as the sporulation ability while the host is still alive, is to increase the probability of transmission of the fungus itself (Roy *et al.*, 2006).

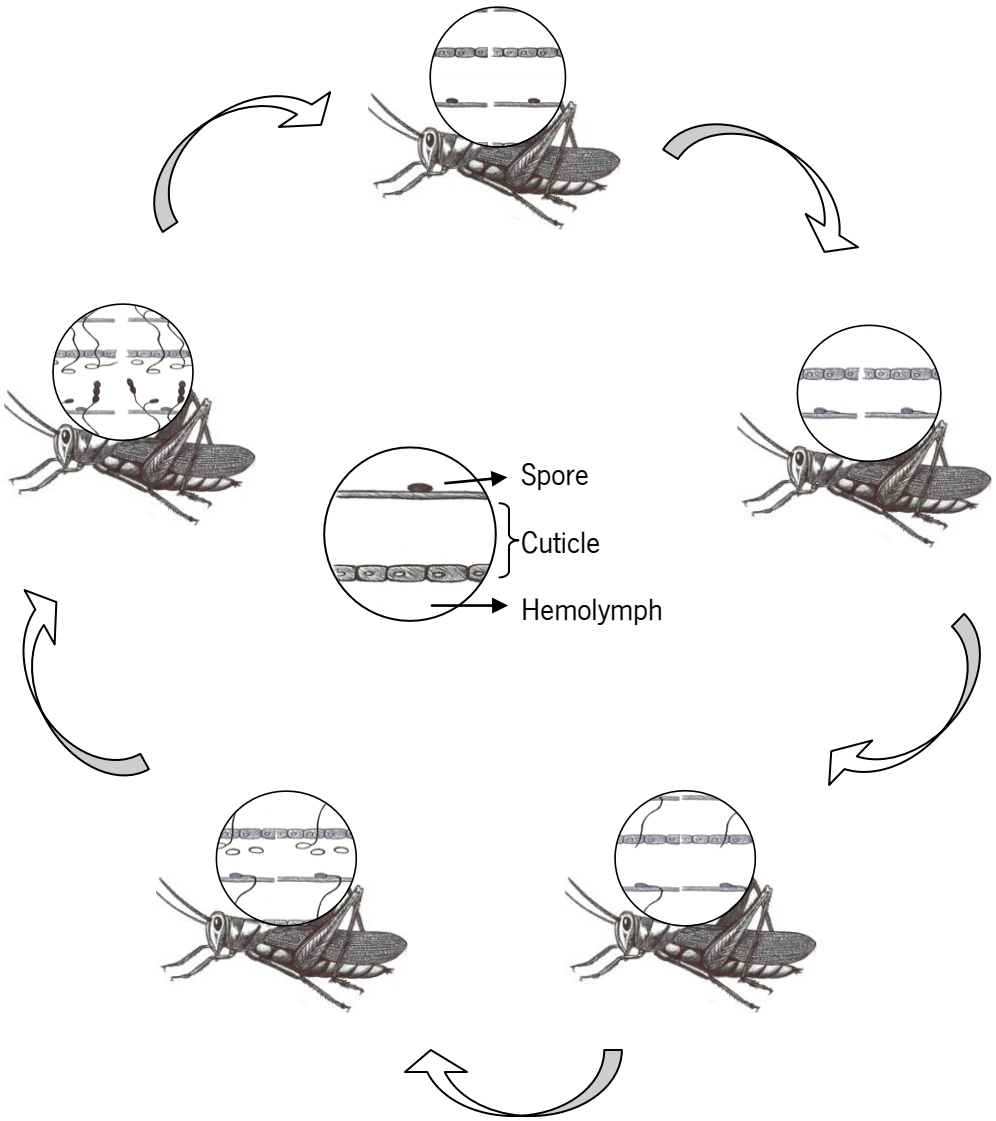

Figure 2 Fungal entomopathogen infection cycle. Starting on the top, and clockwise: spore adhesion to the host cuticle; germination and appressorium formation; penetration through host cuticle; fungal growth, mycelium proliferation and sporulation outside the host cadaver.

Fungal enzymes responsible for infection and pathogenesis

The insect cuticle consists of a thin outer epicuticle, containing lipid and proteins, and a thick procuticle, consisting of chitin and proteins (Fang et al., 2005). As referred, this first barrier against pathogens is degraded by the action of a number of extracellular enzymes produced by entomopathogenic fungi, which are generally grouped in proteases and peptidases, chitinases and lipases. Together, these enzymes are able to degrade the cuticle components, allowing the penetration of the formed appressoria into the insect haemolymph (Fan et al., 2007). The first enzymes to be produced are proteases and esterases (< 24h), while the activity of chitinases and lipases is only detected after 4 to 5 days of contact with the insect (St. Leger *et al.,* 1986). The biochemistry behind the fungal degradation process of insect epicuticle has been a matter for a review (Pedrini et al., 2007). Thorough reviews about cuticle degrading enzymes and toxins of entomopathogenic fungi are also available (Samuels and Paterson, 1995; Khachatourians and Qazi, 2008; Schrank and Vainstein, 2010). Besides cuticle degradation, proteolytic enzymes of entomopathogenic fungi play other roles in insect pathogenesis, like the activation of the prophenol oxidase in the insect haemolymph and virulence (Khachatourians and Qazi, 2008).

The Pr1 protease is the main enzyme produced by entomopathogenic fungi during infection (St. Leger et al. (1987). This enzyme is known to be directly linked to the fungal penetration of the cuticle, since its inhibition delays the penetration but does not affect the viability of spores or the formation of appressoria (St. Leger et al. 1987). Pr1 enzyme is thus an important virulence determinant, which is induced by the presence of the insect cuticle, derepressed in starvation conditions, and repressed in the presence of excess nutrients (Dhar and Kaur, 2010). This regulation is consistent with a multiple-control model (Bidochka and Khachatourians, 1988). Nitrogen and carbon sources are together implicated in the regulation by repressing the Pr1 synthesis, although considerable basal levels are found when carbon or nitrogen sources are applied alone (Gupta et al., 1992; Dias et al., 2008; Donatti et al., 2008). In addition, distinct carbon and nitrogen sources differentialy affect the production of proteases (Bidochka and Khachatourians, 1987; Campos et al., 2005; Donatti et al., 2008). The presence of glucose or alanine also represses the expression of Pr1 and supresses the formation of the appressorium (Clarkson and Charnley, 1996). When deprived from nutrients, Pr1 protease is up-regulated in the appressorium leading to the penetration of the insect cuticle (Goettel et al., 1989; St Leger et al., 1989). These results suggest that nutrient starvation may be one of the environmental signals leading to a change in the nutritional mode of fungi, from saprotrophic to

pathogenic (Clarkson and Charnley, 1996). In contrast to exponentially growing mycelia, the addition of carbon and nitrogen sources is not relevant during the initial interaction of conidia or during pre-germination phase (Qazi and Khachatourians, 2008). This result suggested that conidia and active growing mycelia should present different regulatory mechanism for the synthesis of Pr1 and Pr2 enzymes. In conidia, the carbon/nitrogen repression is only expected to occur in nutrient rich substrates, as could be the case of the insect haemolymph.

In addition to carbon and nitrogen levels, also the presence of insect cuticle promotes the production of proteases. When growing in a culture medium supplemented with insect cuticle, *B. bassiana* overproduces proteases. Indeed, the activity of Pr1 and Pr2 proteases was detected after *B. bassiana* growth in a culture medium containing cuticle of *Rhammatocerus schistocercoides* (Donatti et al., 2008). Both enzymes (Pr1 and Pr2) are thought to be complementary in the splitting of the peptidic bonds of the cuticle (Dhar and Kaur, 2010). However, their expression in *B. bassiana* does not seem to be coordinated, in contrast to results achieved using *Metarhizium* strains (Gillespie et., 1998), where Pr2 is produced earlier that Pr1.

Besides Pr1 and Pr2 proteases, at least two other proteases have been identified in *B. bassiana*: Bassiasin I (Kim et al., 1999) and *B. bassiana* protease (BBP) (Urtz and Rice, 2000). This later enzyme exhibits a comparable cuticle-degrading activity to Pr1. More recently, new proteases have been described in entomopathogenic fungi, as an alkaline protease from *Beauveria* sp. (BAP) (Shankar et al., 2011), and a subtilisin-like protease of *Cordyceps militaris* (Semenova et al., 2011).

Chitinases also play an important role in fungal infection. Chitin is the major component of insect cuticle, representing 25 to 40% of its mass (Hegedus and Khachatourians, 1995). Chemically, chitin is a polymer formed by β-linked N-acetylglucosamine units, embedded in a protein matrix (Samuels and Paterson, 1995) Chitinolytic enzymes of *B. bassiana* include chitinases with 33 kDa (Bbchit1, Fang et al., 2005), 45 and 110 kDa (Havukkala et al., 1993), chitosanase-like proteins (Bclp with 28 kDa, Fuguet et al., 2004) and chitinolytic proteins with 55 kDa (Murad et al., 2007). Extracellular chitinases have been considered as virulence determinant factors (Khachatourians, 1991, 1996; Charnley 1997). Accordingly, the chitinase production by *B. bassiana* has been linked to the virulence of this entomopathogenic fungus. The activity of several *B. bassiana* enzymes (chymoelastase, chymotrypsin, endochitinase, esterase and N-acetyl glucosaminidase) is related with the onset and rate of mortality of *Galleria mellonella* and *Trichoplusia ni* larvae (Gupta et al., 1994). The endochitinase activity showed an apparent

relation to early onset of mortality, whereas N-acetyl glucosaminidase was related to the rate of mortality of *G. mellonella* larvae. In addition, bioassays conducted using overproducing Bbchit1 *B. bassiana* transformants, enhanced the virulence of the fungus, when compared to the wild-type strain (Fang et al., 2005). More recently, Pelizza et al. (2012) also found a direct relationship between high chitinolytic activity and virulence of Argentinean fungal strains. The regulation of chitinases synthesis occurs through an inducer-repressor mechanism, by soluble monomers of chitin, glucosamine, N-acetylglucosamine and chitobiose (Bidochka and Khachatourians, 1993; Havukkala et al., 1993; St. Leger et al., 1993).

As the epicuticle of insects also contains lipoproteins, fats and waxy layers, the action of lipases and lipoxygenases is an important factor in fungal penetration into the host (Khachatourians, 1996). Also for other microbial pathogens, lipolytic enzymes have been described as virulence factors, presenting different roles in the infection process (Ali et al., 2010). In general, lipases are hydrolyzing enzymes that work in aqueous conditions (Zibaee et al., 2008), and have as natural substrates long-chain triacylglycerols (Gupta et al., 2004). Lipases secreted by *M. anisopliae* have been implicated in the recognition of a susceptible host, as well as in the production of the first nutrient molecules to support conidia germination (Silva et al., 2010). When lipase activity was inhibited by using ebelactone B inhibitor, spore germination was arrested in ticks, but not in culture media. This result indicates that, at least for *M. anisopliae*, lipases are an important part of the process of infection and pathogenesis (Silva et al., 2010).

Besides supporting spore germination, the adhesion to the host cuticle, due to hydrophobic interactions, is also promoted by the lipolytic activity that releases free fatty acids (Göttlich et al., 1995). Phospholipases can also lead to a membrane dysfunction and/or physical disruption, by hydrolysing membrane phospholipids.

In the light of this data, it is possible to conclude that the success of fungal infection is directly linked to the amount of secreted enzymes by entomopathogenic fungi (Khachatourians, 1996). In fact, entomopathogenic fungi present more sequenced genes encoding secreted enzymes, namely proteases and lipases, than other fungi (Gao et al., 2011). As referred, this large number of enzymes is probably involved in the transition of nutritional mode, from saprotroph to insect pathogenicity (Gao et al., 2011). The diversity of enzymes may also serve as a way to overcome the insect defences, which include protease inhibitors. In addition, different enzymes could play distinct roles in pathogenesis, increase fungal adaptability and host range, or even display different functions in survival habitats outside the host (Bagga, et al., 2004).

Fungal toxins produced by entomopathogenic fungi

Fungal toxins are a complement to enzymes, determining the end result of the interaction between entomopathogenic fungi and insect hosts (Khachatourians and Qazi, 2008). Several toxins of entomopathogenic fungi have been described (Table 3). Based on their structure, toxins can be separated into non-peptide and peptide groups.

Table 3 Toxin metabolites produced by entomopathogenic fungi (adapted from Khachatourians and Qazi, 2008)

Fungus	Toxin	References
Beauveria bassiana	Tenellin	Khachatourians (1991, 1996)
	Bassianin	Caragh-Moore et al. (1998)
	Oxalic acid	Bidochka and Khachatourians (1993), Alverson (2003), Kirkland et al. (2005)
	Beauvericin	Khachatourians (1991), Tang et al. (2005)
	10-kDa toxin	Khachatourians (1996)
	TF-1 and TF-2	Khachatourians (1996)
	Hirsutellin	Liu et al. (1996)
	Bassiacridin	Quesada-Moraga and Vey (2004)
B. brongniartii	Oosporin	Strasser et al. (2000a)
B. tenella	Oosporin	Michelitsch et al. (2004)
Cordyceps heteropoda	Cicapeptins	Krasnoff et al. (2005)
Hirsutella sp.	Hirsutide	Lang et al. (2005)
H. thompsonii var. thompsonii	Toxic metabolite	Vey et al. (1993)
	Hirsutellin	Liu et al. (1996)
Fusarium avanacium	Enniatin complex	Khachatourians (1991)
F. sambucinum	Enniatin complex	Khachatourians (1991)
Metarhizium spp.	Destruxins	Khachatourians (1991), Jegorov et al. (1998), Pedras et al. (2002)
Paecilomyces	Leucinostins	Krasnoff and Gupta (1991)
P. tenuipes	Paecilomycine	Kikuchi et al. (2004)
P. fumosoroseus	Beauvericin	Khachatourians (1991), Tang et al. (2005)
Tolypocladium cylindrosporum	Efrapeptins	Bandani et al. (2001), Bandani (2004)
T. geodes	Efrapeptins	Bandani et al. (2001), Bandani (2004)
	Tolypocin	Jegorov et al. (1993)
T. inflatum	Kojic acid	Alverson (2003)
	Tolypin	Khachatourians (1991, 1996)
T. niveum	Efrapeptins	Krasnoff and Gupta (1991)

Among the toxins produced by entomopathogenic fungi, some of the most studied are destruxins (DTXs), as reviewed by Schrank and Vainstein (2010) and Liu and Tzeng (2012). These compounds have insecticidal, anti-viral, phytotoxic, and are also being studied for their toxicity to

cancer cells (Schrank and Vainstein, 2010). Destruxins were first isolated from the fungal species, *Oospora destructor*, from which the toxin name comes from. This species was then renamed as *M. anisopliae*. Currently, it is known that these toxins are the most prevalent secondary metabolites from this fungal species (Khachatourians and Qazi, 2008).

There are as much as 39 destruxins or destruxins analogs, divided into six groups, each with an associated letter, from A to F (Pedras et al., 2002). In the last review concerning destruxins, four more DTXs are referred: [Phe3, N-MeVal5] destruxin-B, pseudo destruxin-C, [β-MePro] destruxin-E chlorohydrins and a regioisomer of destruxin-E chlorohydrins (Liu and Tzeng, 2012). The mechanism of action of destruxins has been reviewed (Pedras et al. 2002). Destruxins A and E are the most toxic and have the ability to depolarize lepidopteran muscle membrane, by activating calcium channels. They can also inhibit the functions of insect hemocytes (Bradfisch, 1990) and block the activity of H^+-ATPase (Muroi et al., 1994). The fact that these toxins are able to damage the muscular systems, besides reducing the ability of hosts to feed, also impedes the insect travel to places with higher temperature (Elliot et al., 2002). As a higher temperature inhibits the fungal infection, the reduced mobility caused by destruxins will reduce this defensive behaviour.

Other relevant toxic compounds are produced by entomopathogenic fungi, such as oosporin, efrapeptins, beauvericin, bassianolid, bassiacridin and beauveriolides. Oosporein is a red-pigmented dibenzoquinone, included in the group of non-peptide toxic metabolite. The properties and relevance of oosporein have been comprehensively reviewed (Strasser et al., 2000a; Vey et al., 2001; Seger et al., 2005). Besides being produced by species from *Beauveria* genus, this toxin is also produced by a large number of soil inhabiting fungi (Strasser et al., 2000b; Charnley, 2003; Khachatourians and Qazi, 2008; Molnár et al., 2010). This toxin exhibits antifungal, antibiotic and antiviral activities (Love et al., 2009), leading to enzyme malfunction due to their reaction with proteins and aminoacids (Wilson, 1971). This toxic metabolite appears to be produced only in the insect cuticle, rather than in the haemolymph (Charnley, 2003). Probably, due to the antimicrobial activity previously described, the production of this toxin will help the suppression of saprophytic microbiota present is the host surface. However, no correlation has been established between the virulence of a given isolate and the amount of oosporein in the insect cadaver (Charnley, 2003). This suggests that oosporein may not be a determinant factor in the pathogenesis.

Efrapeptins are a group of linear peptides, containing 15 aminoacids (Charnley, 2003). These toxins were isolated from the entomopathogenic *Tolypocladium* species (Strasser et al., 2000b; Charnley, 2003), but also from the non-entomopathogenic *Acremonium* sp. (Boot et al., 2006). For the efrapeptin-producing *Tolypocladium* species, there is inter- and intra-specific variation of production (Krasnoff and Gupta, 1992). These toxins have showed insecticidal activity against mites, potato beetle, tobacco budworm and diamondback moth, either by injection or contact (Matha et al., 1988; Krasnoff et al., 1991). Antifungal and antibacterial activity of these compounds was also detected, although limited. Other properties include antifeedant and growth inhibition effects (Bandani et al., 2000). These peptides have been described as potent inhibitors of ATPase from mitochondria, bacteria, and chloroplasts, disturbing the ion regulation (Gledhill and Walker, 2006). Efrapeptin binding to a vacuolar type ATPase, present in the midgut of the honeycomb moth (*Galleria mellonella*), has also been described (Bandani et al., 2001). After infection with *T. niveum*, these peptides were found in haemolymph and bodies of *G. mellonella*, but the amounts found were lower than those required to cause the death of the host (Bandani et al., 2000). This result suggests that efrapeptins may work together with other pathogenicity determinants.

Beauvericin is a hexadepsipeptide toxin, isolated from *Beauveria* and *Paecilomyces* entomopathogenic species, as well as from the plant-pathogenic *Fusarium spp*. and *Polyporus fumosoroseus*. Interestingly, the production of this toxin was not found in all isolates of *B. bassiana* (Frappier et al., 1975). Although insecticidal properties have been described for these compounds (Suzuki et al., 1977; Kanaoka et al., 1978; Champlin and Grula, 1979; Qadri et al., 1989; Zizka and Weiser, 1993; Gupta et al., 1995), some insect showed no susceptibility (Champlin and Grula, 1979). Beauvericin also exhibits antibiotic activity against several bacteria (Ovchinnikov et al., 1971). Two different forms (A and B) of this toxin were described by Gupta et al. (1995). These compounds are able to increase membrane permeability, by creating Na^+ and K^+ complexes (Ovchinnikov et al., 1971). As it acts as an ionophore, beauvericin is able to increase the cytoplasmic concentration of Ca^{2+} (Tang et al., 2005), which is followed by the ATP depletion and activation of calcium-sensitive cell apoptotic pathways (Jow et al., 2004; Chen et al., 2006). Comparative studies, using a *B. bassiana* strain without the gene encoding for beauvericin production revealed that this toxin is not indispensable for the virulence, although having a highly significant role (Xu et al., 2008).

Another toxic peptide, bassianolide, has been isolated from *B. bassiana* and *Lecanicillium* spp. (Xu et al., 2009a). Studies have revealed that bassianolide induces the atony of *Helicoverpa zea* larvae (Champlin and Grula, 1979), being also toxic to silkworm larvae (Suzuki et al., 1977). In cadavers of silkworm larvae, killed by *B. bassiana* infection, bassianolide was detected, indicating that this compound is compatible and coincident with the infection. This toxin has been described as an ionophore antibiotic (Suzuki et al., 1977), exhibiting anti-mycobacterial activity (Jirakkakul et al., 2008).

Bassiacridin is a toxic protein isolated from *B. bassiana* when infecting *Dociostaurus maroccanus* (Quesada-Moraga and Vey, 2004). The virulence potential of this protein was proved when nymphs of *Locusta migratoria* were injected with this toxin, at low dosage, and a mortality of near 50% was promoted (Quesada-Moraga and Vey, 2004). Although the mechanisms that are involved in these insecticidal effects are still unclear, the fungal production of bassiacridin and its involvement in pathogenesis seems to be clear.

Beauverolides, also referred to as beauveriolides (Molnár et al., 2010), are peptides with a similar structure to beauvericin and bassianolide (Namatame et al. 1999, 2004). Moderate insecticidal activity was observed against *Spodoptera litura* and *Callosobruchus chinensis* (Mochizuki et al., 1993). However, these compounds have an apparent high potential in human medicine, as they showed antiartherosclerotic effects (Namatame et al., 2004). Indeed, beauverolides are now considered as leader compounds for antiartherosclerotic agents. Another study has also revealed their potential for Alzheimer's disease treatment by reducing the formation of senile plaques in the brain (Witter et al., 2009).

This large amount of data shows that entomopathogenic fungi are able to produce a wide array of toxins that, together with fungal enzymes are responsible for virulence and pathogenicity. Furthermore, as these compounds can be over-expressed in modified strains of entomopathogenic fungi, an increase the performance can be achieved.

Compatibility between entomopathogenic fungi and chemical pesticides

As the unfavourable environmental conditions may affect the efficacy of entomopathogenic fungi under field conditions, one way to overcome this fact for the control of pests is to add low dosages of chemical pesticides. The combined application of mycoinsecticides and chemical pesticides may have synergistical effects, allowing the use of lower concentrations of chemicals and decreasing the likelihood of resistance to either agent (Boman, 1980). In recent years, some

research is being performed in the compatibility of different entomopathogenic fungi and insecticides. Different outcomes of the interaction with chemical pesticides have been observed. For instance, the combination of benzoylphenyl ureas with *B. bassiana* resulted in a reduced infectivity, up to 50% (Olson and Oetting, 1999; Irigaray et al., 2003). Other works recorded additive effects between *B. bassiana* and triflumuron (Anderson et al., 1989), diflubenzuron-B (Delgado et al., 1999) and azadirachtin (Hernández et al., 2012). Synergistic effects with this entomopathogenic fungus were also recorded when using diflubenzuron (Reuter et al., 1995) and flufenoxuron (Hernández et al., 2012). A study conducted to evaluate interactions between exposure of the longhorned beetle *Anoplophora glabripennis* to imidacloprid and *Metarhizium brunneum* also showed synergistic effect of the dual treatment (Russell et al., 2010). The association between deltamethrin and *M. anisopliae*, to control *Boophilus microplus* tick, resulted in higher larvae mortality rates than those obtained with non-associated formulations (Bahiense et al., 2006). In contrast, no synergistic effect was recorded when evaluating the effect of two insecticides (thiamethoxam and imidacloprid) with *M. anisopliae* in the second-instar larvae of *F. occidentalis* (Niassy et al., 2012). However, the combination of thiamethoxam and *M. anisopliae* conidia resulted in a shorter LT_{50} (6 days) than the single treatments of thiamethoxam (13 days) or *M. anisopliae* conidia (11 days) (Niassy et al., 2012).

These conflicting results could be due to the effect of insecticides on mycelial growth, sporulation, conidial germination and cuticle-degrading enzyme production of entomopathogenic fungi. A wide range of pesticides (11 active ingredients) caused a significant inhibition of mycelial growth and spore production of *M. anisopliae* (Akbar et al. 2012). Although conidial germination was also found to be affect, two active ingredients (spinosad and indoxicarb) were significantly compatible and considered to be safe to conidial germination and fungal growth (Akbar et al. 2012). The compatibility of this same fungus (*M. anisopliae*) with other agrochemicals was also studied (Niassy et al., 2012). The effect of insecticides (thiamethoxam, L-cyhalothrin, imidacloprid, chlorpyrifos, diazinon), acaricides (abamectin, spiromesifen) and fungicides (carbendazim, copper hydroxide, probineb) was accessed by evaluating the vegetative fungal growth, mycelial mass and conidial production, as well as the fungal ability to infect the second-instar larva of *Frankliniella occidentalis*. While some compounds (abamectin and imidacloprid) were highly compatible with the use of *M.anisopliae*, others negatively affected the fungal vegetative growth and sporulation (azadirachtin and L-cyahalothrin). The same occurred with the acaricides, being abamectin highly compatible and spiromesifen moderately toxic to the fungus.

As expected, all three fungicides showed to be very toxic to the *M. anisopliae*. Anhalt et al. (2010) also tested the compatibility of *M. anisopliae* with chemical insecticides. While a compatibility with tebufenozide was detected, three other compounds (chlorpyrifos-ethyl, methidathion and fenitrotion) showed to be highly toxic to *M. anisopliae*. The *in vitro* effects of different concentrations of fipronil, permethrin, imidacloprid, NeemAzal and amitraz were also investigated in *M. anisopliae* (Schumacher and Poehling, 2012). All pesticides were compatible with the two tested strains, but higher concentrations were able to cause inhibition of conidial germination, colony size and sporulation.

Similar results were obtained with other entomopathogenic species, in which a variation of effects was detected from product to product. *Lecanicillium muscarium* showed to have its germination reduced by Majestik, Spray Oil, Agri-50E, Savona, Oberon, buprofezin, teflubenzuron, nicotine and imidacloprid chemical insecticides, all of them used for the control of *Bemisia tabaci* on poinsettia (Cuthbertson et al., 2009), tomato and verbena plant (Cuthbertson et al., 2005). Interestingly, the infection rates of *L. muscarium* on foliage, which still presents dry residues of the insecticides, were similar to rates on residue-free leaves. *Isaria fumosorosea* conidial germination, radial growth and sporulation are also significantly affected by chemical insecticides (chlorfenapyr, chloranthraniliprole, indoxacarb, hubendamide + avermectin, chlorpyrifos, imidacloprid) (Ali et al., 2012). Furthermore, the production of cuticle-degrading enzymes (chitinases, subtilisin-like activity (Pr1), trypsin activity (Pr2) and lipase) was also affected by all these chemical insecticides.

All these results reveal a great variability of entomopathogenic fungi tolerance to chemical pesticides. This variability is linked to each particular fungal strain, as well as to the type of chemical and concentration. To use entomopathogenic fungi simultaneously with chemical pesticides, sequential applications of insecticides and entomopathogenic fungi should be preferred, instead of combined applications.

Compatibility between entomopathogenic fungi and other biological control agents

For using entomopathogenic fungi to control pests in agroecosystems, the impact that these fungi may have on beneficial insects, like predators and parasitoids, should be taken in consideration. Intraguild interactions commonly occur when using biological control agents, likely affecting the outcomes of the biological control measures (Polis and Holt, 1992; Rosenheim et al., 1995). Different results from these relations could be expected, from deleterious effects to

synergism. Therefore, the study of such interactions is of major importance and essential to devise biocontrol programs without the detrimental effects on beneficial organisms. Some reviews about the most studied entomopathogenic fungal species (*Beauveria* species, *M. anisopliae* and *Isaria* species, formerly named as *Paecilomyces,* address this subject (Zimmermann, 2007a, b; Zimmermann, 2008).

Regarding *Beauveria*, the available data indicate that these species can be used without disadvantageous effects on non-target organisms (Zimmermann, 2007a). Accordingly, beneficial insects were not significantly affected by the entomopathogenic *Beauveria* fungi. The *B. bassiana*-based product BotaniGard® was found to be compatible with *Dicyphus hesperus*, a predator of the greenhouse whitefly *Trialeurodes vaporariorum* (Labbé et al., 2009). Also, the *B. bassiana*-based mycopesticide, Naturalis-L, had no detrimental effect on *Amblyseius cucumeris*, a predatory mite of western flower thrips, *Frankliniella occidentalis* (Jacobson et al., 2001). Similar results were observed for a strain of *B. bassiana* applied to control *Frankliniella occidentalis*, which presented a minor risk to the predator *Orius sauteri* nymphs and adults (Gao et al., 2012). *B. bassiana* also proved to be compatible with other predators, like *Chrysoperla externa*, only affecting the third instar larvae when applied at high concentrations (Pessoa et al., 2005). Compatibility between *B. bassiana* and predators was also recorded for *Harmonia axyridis* and *Chrysoperla carnea* (Zhu and Kim, 2012), *Podisus nigrispinus* (França et al., 2006), *Euborellia annulipes* (Oliveira et al., 2011). However, harmful effects of *B. bassiana* have been also described for a number of predators. This entomopathogenic fungus adversely affected the longevity and fecundity of the predatory mite *Phytoseiulus persimilis* (Pozzebon and Duso, 2009; Seiedy et al., 2012), *Teretriosoma nigrescens* and the predator of *Prostephanus truncates* (Bourassa et al., 2001). Regarding parasitoids, the available studies describe different effects of *B. bassiana*. While this species has been described to have no harmful effects in parasitoids (Rashki et al., 2009; Dean et al., 2012), other studies reveal a negative impact of *B. bassiana* on parasitism (Furlong, 2004; Castillo et al., 2009).

The available information about *M. anisopliae* interactions with parasitoids and predators is also contradictory. Some results indicate that this entomopathogenic fungi is safe to parasitoids and predators (Husberg and Hokkanen, 2001; Stolz et al., 2002; Tounou et al., 2003; Ekesi et al., 2005; Nielsen et al., 2005; Polanczyk et al., 2010; Oliveira et al., 2011). However, reports of a negative effect of this fungus on those natural enemies are also available (Bourassa et al., 2001; Broglio-Micheletti et al., 2006; França et al., 2006; Potrich et al., 2009).

The variation of entomopathogens effects on beneficial insects is also noticed for *Isaria* (formerly *Paecilomyces*) species. Compatibility was detected between *Isaria* species and predators and parasitoids (Mesquita and Lacey, 2001; Tounou et al., 2003; Avery et al., 2008; Zhou et al., 2010; Hamdi et al., 2011; Pick et al., 2012), but *Isaria* species are also described as possessing harmful effects on other biological control agents (Pell and Vandenberg, 2002). In contrast, other fungal species have been described as safe to parasitoids and predators, like *Verticillium lecanii*, *Pandora neoaphidis*, *Lecanicillium* spp. or *Zoophthora radicans* (Furlong and Pell, 1996; Kim et al., 2005; Fatiha et al., 2008; Baverstock et al., 2009; Xu et al., 2009b; Aiuchi et al., 2012).

The safety of entomopathogenic fungi to beneficial insects in a given ecosystem should be carefully evaluated. The effects of entomopathogenic fungi are known to be linked to the physiological (range of insect species that can be infected in the laboratory) and ecological host range (insects can be infected in nature or under field conditions) (Zimmermann, 2007), and beneficial insects infected under laboratory assays, may not necessarily be infected in field conditions. As previously referred, their effects are also dependent on fungal persistence, dispersion and production of metabolites (Vestergaard et al., 2003). Although not presenting a direct negative effect on predators and parasitoids, the fungal entomopathogens could still be considered as harmful, by reduction of the host population, which will affect predators and parasitoids that rely on such host (Goettel and Hajek, 2001). Laboratory results are not useful when predicting risks under field conditions (Jaronski et al, 2003). In addition, the effects of entomopathogenic fungi on beneficial insects should take into account the host plant, the application on soil or canopy, field or glasshouses conditions.

Other interesting results show that some predators are able to detect and avoid fungal entomopathogens (Meyling and Pell, 2006). This fact reveals that insects are able to prevent detrimental effects caused by entomopathogenic fungi. Therefore, the adverse effects of these fungi on predators and parasitoids may be reduced, not due to the lack of infection ability by the fungi, but by the referred avoidance of contact by the insect.

Entomopathogenic fungi in olive pests

The key olive tree pests in the Mediterranean region include olive fruit fly (*Bactrocera oleae* Gmelin), olive moth (*Prays oleae* Bern.) and olive black scale (*Saissetia oleae* Bern.) (Haniotakis, 2003). Few studies are available, on the presence of entomopathogenic fungi in olive orchards, as well as their ability to control these pests. Their presence in olive orchards has already been

described by Marannino et al. (2006), Quesada-Moraga et al. (2006b) and Oliveira et al. (2012). However, the application of entomopathogenic fungi against olive pests is scarce, and only a few publications address this subject.

The susceptibility of *B. oleae* to entomopathogenic fungi has already been described. Konstantopoulou and Mazomenos (2005) tested two species of *Beauveria*, *B. bassiana* (isolated from pupae of *B. oleae*) and *B. brongniartii* (isolated from *Melolontha* sp.), against adults of olive fruit fly. After 21 days of fungal contact, a mortality percentage of 66.8% and 38.6% were obtained for *B. bassiana* and *B. brongniartii*, respectively. Similar results were obtained when insects were fed with a diet containing fungal spores. After 21 days, the insect mortality attained 62.2% and 36.5% for *B. bassiana* and *B. brongniartii*, respectively. The median lethal time (LT_{50}) corresponded to 17 days for the oral bioassay and less than 14 days for contact bioassays. When a product based on *B. bassiana* was tested in *B. oleae*, a higher virulence was found in oral bioassays (80%) than in contact bioassays (60.8%), after 20 days (Mahmoud, 2009a). The time-mortality response was also different for contact and oral bioassays. While a LT_{50} of 14.7 days was found for oral bioassays, in contact assays a value of 16.6 days was detected. In addition, combinations of *B. bassiana* and two other entomopathogenic fungi, *Metarhizum anisopilae* and *Lecanicillium lecanii*, were also tested for controlling *B. oleae* (Mahmoud, 2009a). The combination of *B. bassiana* and *M. anisopilae* provided a synergistic response, increasing mortality to 100%. In contrast, the combination of *B. bassiana* and *L. lecanii* gave an antagonistic response, reducing the mortality of *B. oleae* to 72%. Altogether, these studies gave an indication of the susceptibility *B. oleae* to entomopathogenic fungi, namely *B. bassiana*, which should then be further studied as an alternative to chemical insecticides.

Besides *B. bassiana*, other entomapathogens could control *B. oleae*. The insecticidal activity of metabolites from a strain of *Mucor hiemalis* presents a strong toxic effect against *B. oleae* adults (Konstantopoulou et al., 2006). The toxicity symptoms included lethargy after 1-2 hours after treatment, with 82 to 97% death arising in 24 hours.

In addition to *B. oleae*, several *Bactrocera* species are similarly susceptible to entomopathogenic fungi. Indeed, five entomopathogenic fungal species (*B. bassiana*, *Isaria tenuipes*, *Metarhizium flavoviride*, *M. anisopliae* and *Paecilomyces lilacinus*) are effective against *Bactrocera spp*. pupa (Mar and Lumyong, 2012); the soil inoculation with *M. anisopliae* was reported to suppress *Bactrocera invadens* (Ekesi et al., 2011); *Bactrocera zonata* was described to be susceptible to *B. bassiana*, *M. anisopliae* and *Lecanicillium muscarium* (Mahmoud,

2009b); and *M. anisopliae*, *B. bassiana* and *P. fumosoroseus* exhibit pathogenicity towards *Bactrocera zonata* and *Bactrocera cucurbitae* adults (Sookar et al., 2008)

There are no available studies on the use of entomopathogenic fungi to control the other two major pests in olive orchards, *Prays oleae* (from Lepidoptera order) and *Saissetia oleae* (from Hemiptera order). However, promising results obtained with insects belonging to the same taxonomical groups may indicate the possibility of biocontrol using entomopathogens. The susceptibility of different species of lepidopterans to *B. bassiana* has been demonstrated by Wraight *et al.,* 2010. Several species of lepidopterans, including *Plutella xylostella*, *Ostrinia nubilalis*, *Helicoverpa zea*, *Spodoptera frugiperda, Agrotis ipsilon*, *Pieris rapae* and *Trichoplusia ni*, were susceptible to 43 different isolates of *B. bassiana*. In most cases, this fungus caused high mortality rates that attained up to 98%. Other reports also describe *B. bassiana* susceptibility of different several lepidopteran species, including *Argyresthia conjugella* (Vänninen and Hokkanen, 1997), *Ostrinia nubilalis* (Safavi *et al*., 2010), *Chilo partellus* (Tefera and Pringle, 2003), *Diatraea saccharalis* (Alves *et al*., 2002), *Strymon megarus* (Inclán *et al*., 2008) and *Busseola fusca* (Maniania *et al*., 2011). There is also one study that is focused on the capacity of *B. bassiana* to infect insect species from the same family as the olive moth, Yponomeutidae, namely *Plutella xylostella* (Godonou *et al*., 2009). This work tested six different isolates of *B. bassiana*, which presented different results. All isolates where able to infect, kill and grow externally on larvae of *Plutella xylostella*, after 3 days of treatment, but with differences in the percentage of cumulative dead larvae. Only one of the isolates reached a value of 94% mortality. Interestingly, when using this *B. bassiana* isolate to manage the pest in cabbage plots, the yields of production were approximately three-fold higher than in plots treated with insecticide or in untreated plots.

For scale insects, as *Saissetia oleae*, data regarding infection by *B. bassiana* is almost inexistent. Only one study is available that evaluates the virulence of *B. bassiana* on *Saissetia coffeae* (Nahla et al., 2008). In laboratory assays, *B. bassiana* was able to infect nymphs and adults of *Saissetia coffeae*, with higher pathogenicity to nymphs than to adults. In field trials, where *B. bassiana* was applied to *Cycas revoluta* plants infected with *Saissetia coffeae*, the population of nymphs and adults was reduced in 74.1% and 69.7%, respectively, after 30 days from treatment. The efficacy of entomopathogenic fungi, other than *B. bassiana*, to control scale insects has been reported. When analysing the role of parasitoids and entomopathogenic fungi in the mortality of two scale insects (*Ceroplastes destructor* and *C. sinensis*), two fungal species

(*Verticillium lecanii* and *Fusarium* spp) were identified as the main death promoters of these scale insects (Lo and Chapman, 1998). *Alternaria infectoria* is also able to infect *Ceroplastes rusci*, causing disease on a large percentage of eggs (91%), 74.5% of which will die and decreasing by 39.6% the hatching of the remaining eggs (Shabana and Ragab, 1997).

The combined information, regarding lepidopterans and scale insects, suggests the likelihood of the application of entomopathogenic fungi, including *B. bassiana*, to control these two major pests in olive orchards, the olive moth and the olive black scale.

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Objectives and outline of the thesis

Biological control of insect pest, using beneficial organisms, has been increasing, to minimize the harmful effect known to chemical pesticides. One of the approaches to control insect pests is the use of entomopathogenic fungi (EF). These fungi, studied for their natural occurrence in several habitats, characteristics of infection, production of secondary metabolites, have already been tested as biocontrol agents against insect pests, and commercial products are already available on the market. However, different factors, both host-related and environmental-related are known to influence the pathogenicity and virulence of EF. Several works point out the fact that a fungal strain, retrieved from a given ecosystem, and found infecting a particular host, will have increased pathogenicity and virulence for that host, while minimizing possible effect on non-target organisms.

One of the most important cultures in Portugal is the olive tree, grown in large part of the country. Several pests affect this culture, being one of them *Prays oleae* Bern., responsible for high losses of production. This pest is mainly controlled using chemical insecticides. The detrimental effects caused by this type of compounds have resulted in an increase of the search for alternative measures to control insect pest, which should include entomopathogenic fungi.

Thus, the main objectives of this work were to evaluate the diversity of fungal entomopathogens associated to one of the key pests of olive, *Prays oleae*, assessing their pathogenicity and virulence, as well as appraising the effect of selected factors in fungal behaviour and occurrence. The fungal diversity associated to the three annual generations of *P. oleae* was studied, in an olive ecosystem (Chapter 2). The applicability of different molecular markers (ITS, β tubulin and RPB2) in the identification of the fungal isolates obtained was further analysed (Chapter 3). The evaluation of fungal pathogenicity and virulence of the native fungal strains is one of the key steps of the design of a pest control program based on EF. These important fungal traits were determined on the most abundant EF isolated, *Beauveria bassiana*, against a lepidopteran pest, *Cydia splendana* (Chapter 4). The biological control efficiency of entomopathogenic fungi depends greatly of several biotic and abiotic factors, including agricultural ones. One of those is soil tillage, performed recurrently in olive orchards. The impact that soil management practices, with or without soil tillage, on EF fungi was also studied in this work (Chapter 5). Plants can also affect fungal entomopathogens. As *P. oleae* attacks different olive tree organs (leaves, flowers and fruits), in each of its annual generations (phyllophagous,

antophagous and carpophagous), the effect of these different plant organs on two native fungal entomopathogens was evaluated (Chapter 6).

Altogether, the results of this work are expected to provide an insight of the entomopathogenic fungal community associated to *P. oleae*, and their characteristics, in order, to ultimately, help the creation of a biocontrol program using EF against *P. oleae*.

Chapter 2

Fungal diversity associated to the olive moth, *Prays oleae* Bernard: a survey for potential entomopathogenic fungi

Fungal diversity associated to the olive moth, *Prays oleae* Bernard: a survey for potential entomopathogenic fungi..

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ABSTRACT

Olive production is one of the main agricultural activities in Portugal. In the region of Trás-os-Montes this crop has been considerably affected by *Prays oleae*. In order to evaluate the diversity of fungi on *P. oleae* population of Trás-os-Montes olive orchards, larvae and pupae of the three annual generations (phyllophagous, antophagous and carpophagous) were collected and evaluated for fungal growth on their surface. From the 3828 larvae and pupae, a high percentage of individuals exhibited growth of a fungal agent (40.6%), particularly those from the phyllophagous generation. From all the moth generations, a total of 43 species from 24 genera were identified, but the diversity and abundance of fungal species differed between the three generations. Higher diversity was found in the carpophagous generation, followed by the antophagous and phyllophagous generations. The presence of fungi displaying entomopathogenic features was highest in the phyllophagous larvae and pupae, being *B. bassiana* the most abundant taxa. The first report of *B. bassiana* presence on *P. oleae* could open new strategies for the biocontrol of this major pest in olive groves, since the use of an already adapted species increases the guarantee of success of a biocontrol approach. The identification of antagonistic fungi able to control agents that cause major olive diseases, such as *Verticillium dahliae*, will benefit future biological control approaches for limiting this increasingly spreading pathogen.

Keywords: Olive tree; *Prays oleae*; Fungal diversity; Moth life cycle generation

INTRODUCTION

The olive tree is an important crop for Mediterranean basin countries including Portugal. Extensive areas occupied by olive groves in Trás-os-Montes region (Northeast of Portugal), not only have a significant economic impact, but also exhibit a social, environmental and landscape significance. The olive moth, *Prays oleae* Bern., is one of the major pests on these olive orchards, being responsible for high losses in the olive yield as much as 40% (Ramos et al., 1998). This lepidopteran presents three generations per year that damage the olive tree in different organs. The antophagous generation, occurring from April to June, causes damages to the olive tree flowers; the carpophagous generation, which usually appears from July to September, attacks the fruits leading to their premature fall; and the phyllophagous generation, present from October to March of the following year, damages the leaves (Ramos et al., 1998).

Due to the growing awareness of detrimental effects of pesticides to the ecosystems (Cuthbertson and Walters, 2005) agricultural practices in these orchards have been changing to biological agriculture. Therefore, the search for methods to control pests and diseases has acquired a new motivation. One of the promising methods to control pests can be the use of entomopathogenic fungi, which are known by their ability to infect and kill several insect species (Meyling and Eilenberg, 2007).

Entomopathogenic fungi comprise a large group of pathogens that includes approximately 700 species in almost 85 genera (Charnley and Collins, 2007). Due to their large host range, entomopathogenic fungi could be potentially useful as control agents against different insect orders. When compared to conventional chemical pesticides, the use of insect pathogens presents many advantages, such as the safety for humans and other non-target organisms, environmental reduction of pesticides residues and a smaller effect on the natural biodiversity (Lacey et al., 2001). However, pest management using entomopathogenic fungi has been difficult to achieve. The ecological and environmental variations within agro-ecosystems turn the formulation and application of this kind of biocontrol agent difficult to manage (Vega et al., 2009). The isolation of native fungi could provide a collection of isolates, for the development of potential control agents already adapted and suited to a particular habitat. In this work, the fungal diversity encountered on *P. oleae* population was evaluated in olive groves from Trás-os-Montes and will be discussed taking into account the olive moth generation where it appears. From this survey, potential entomopathogenic fungi for future biocontrol strategies will be selected.

METHODS

Study area

Larvae (mostly in the fourth and fifth instars of development) and pupae of *Prays oleae* were collected in six olive groves located in Mirandela – Bragança region, Northeast of Portugal (GPS coordinates: 41°34'03.77''N; 7°05'39.21''W; 41°33'53.29N, 7°05'40.23''W; 41°33'52.51''N, 7°05'30.59W; 41°33'33.11N, 7°05'35.62W; 41°33'08.02''N, 7°07'24.87''W; 41°32'35.20''N, 7°07'26.27''W). This region comprises an olive tree growing area around 65.000 ha, corresponding to 22% of the olive tree covered area in Portugal. It contains 20% of Portuguese olive trees, which altogether are responsible for 39% of national olive oil production (Pereira et al., 2004). The topography of this region is mountainous, with altitudes ranging between 300 and 500 m. The prevailing climate is Mediterranean, with cold and rainy winters and long, hot and dry summers. The average annual rainfall ranged from 600 to 800 mm, occurring mainly between October and February, and the annual mean temperature ranged from 9 to 20°C. For this study, the selected orchards have been managed through organic (European Union, 207) or integrated production guidelines (Malavolta et al., 2002) and display high levels of olive moth infestations, as observed by delta traps baited with pheromone. Although two different management systems are referred, the studied olive groves are very similar, with low-external inputs. The groves area ranged from 0.4 to 1.5 ha. Trees are of medium size with a planting density of 7 x 7 m. Pruning was made every 2-3 years. No irrigation was done, and only copper based products were used in the beginning of autumn for fungal diseases control and no other pesticides were used in the last five years. The differences between the two management systems are related with the products allowed in soil fertilization. The most important cultivars were Cobrançosa and Verdeal Transmontana.

Experimental design and collection of plant material

The collection of larvae and pupae was performed in 15 randomly chosen trees in each of the selected orchards for the three annual generations of *P. oleae*. Sampling dates were variable according to the life stages of the pest. For the phyllophagous generation the collection was conducted between 17th and 27th March of 2009, for the antophagous generation between 24th May and 5th June and for the carpophagous generation between 1st and 3rd of September of the same year. For the first two generations, sampling of leaves (phyllophagous generation) and flower clusters (antophagous generation), in which larvae and pupae were present, was

performed. For the carpophagous generation, the collection of whole fruits was randomly performed, due to the presence of larvae and pupae within olives. The plant material (leaves and flower clusters) and olives were collected individually to sterile bags, and processed within a few hours after sampling.

Isolation of fungi from mycosed larvae and pupae of *P. oleae*

In the laboratory, the plant material was further examined for the presence of olive moth larvae and pupae. Since carpophagous larvae develop inside the olive stone, the stone was smoothly broken to expose larvae. Whenever present, the larvae and pupae were individually placed into sterile tubes containing a food source (leaves for the phyllophagous generation, flower buds for the antophagous generation, and olive stones for the carpophagous generation). The tubes were sealed and maintained at 25 ± 2°C, under 16/8 hours light/dark regime, for an average period of 10 days. Larvae and pupae were daily observed, in order to detect their death or evaluate the insect emergence. Each time a fungal agent was growing on the surface of dead larvae or pupae, the fungal specimen was isolated by inoculating Potato Dextrose Agar (PDA) medium, supplemented with 0.01% (w/v) chloramphenicol (Oxoid). Pure cultures of each isolate were deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

Extraction of fungal DNA

Fungal isolates were inoculated onto PDA medium and maintained at 25 ± 2°C in the dark for 1-2 weeks. The colony morphology, spore size and shape were used for the first identification and to group strains. Spores were collected from each isolate and used for DNA extraction. Isolation of genomic DNA was performed by transferring spores into a microtube containing 500µL of Lysis buffer (200 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM EDTA pH 8.0 and 0.5% SDS) and sterile glass spheres. Tubes were vortexed for 5 minutes to disrupt cells. After addition of 250 µL of cold 3M NaOAc pH 5.5, the mixture was gently homogenized by inversion and incubated for 10 minutes at -20°C. Following centrifugation at 10.500 rpm (4°C) for 10 minutes, the supernatant was collected to another microtube and one volume of isopropanol (-20°C) was added. This mixture was slowly homogenized and incubated at -20°C for one hour. The DNA precipitate was collected by centrifugation at 10.500 rpm (4°C) for 10 minutes and the pellet washed with cold 70% ethanol. The DNA pellet was air-dried for 20 min at room temperature and re-suspended in 50 µL of ultra pure water. DNA was stored at -20°C until use.

Molecular identification of fungal isolates

Molecular identification was achieved by amplification of the internal transcribed spacer region (ITS), using the universal primers *ITS1* and *ITS4* (White et al., 1990). PCR reactions (50 µl) comprised 50 ng of genomic DNA, 0.2 µM of each primer (*ITS1* and *ITS4*), 1x GoTaq® Flexi buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.2 µM dNTP Mix (Fermentas) and 1 U GoTaq® DNA polymerase (Promega). Amplifications were carried out in the thermocycler Biometra UNO II (Thermoblock, Biotron) using a temperature gradient protocol as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 55°C, 1 min at 72°C, and a final 10 min extension at 72°C. PCR amplification products were analysed by electrophoresis and those reactions that amplified a single PCR product were selected for purification using the JETquick PCR product purification kit (Genomed). Amplified fragments were sequenced using both *ITS1* and *ITS4* primers at the sequencing services of STAB Vida (Oeiras, Portugal). DNA sequences were analysed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database (http://www.ncbi.nlm.nih.gov) and BLAST algorithm.

The ecological classification of identified species was only based on the impact they can have on olive orchards regarding their potential role on pests and diseases control. Even though many of the identified fungi are also considered general saprophytes, they were only classified into three ecological roles: phytopathogenic, antagonistic and entomopathogenic. The classification given to each fungal species was based on previously described characteristics.

Data Analysis

For each *P. oleae* generation, the species richness, *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes, total and relative abundances were estimated. Calculations of *Simpson* and *Shannon-Wiener* diversity indexes were done using the software *Species Diversity and Richness* (v. 3.0). Total abundance (N) was estimated as the number of isolates per fungal taxa, whereas the proportion of isolates from each fungal taxa in relation to the total number of fungal isolates was considered as the relative abundance of a certain taxa. Principal component analysis (PCA) was applied to access the relationship between fungal taxa and the three *P. oleae* generations. PCA was performed using the SPSS software, version 17.0 (SPSS, Inc.). It was applied as an unsupervised approach for reducing the number of variables (43, corresponding to the number of identified fungal species) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information. This analysis will define which fungal

species are correlated with each olive moth generation. PCA analysis also allowed the recognition of patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). The aim of the PCA is to produce components suitable to be used as predictors or response variables in subsequent analysis. The number of factors to keep in data treatment was evaluated by the Scree plot, taking into account the eigenvalues and the internal consistency by means of αCronbach's value (Maroco, 2003; Rencher, 1995).

RESULTS

Diversity and abundance of fungi encountered in *P. oleae*

From the 3828 larvae and pupae (2552 larvae and 1276 pupae), collected in all generations of *P. oleae*, an insect emergence percentage of 38.6% was observed (Table 1).

Table 1 Larvae and pupae collected from each generation of *P. oleae*. The insect emergence percentage was determined for each moth generation. The percentage of cadavers exhibiting fungal growth on their surface was determined in relation to the total of dead larvae and pupae.

Generation	N° of collected larvae and pupae	Insect emergence	Cadavers presenting surface fungal growth
Phyllophagous	1246	30.1%	61.5%
Antophagous	1745	53.9%	32.7%
Carpophagous	837	19.5%	23.0%
Total	3828	38.6%	40.6%

Of the total number of dead larvae and pupae (1477), 40.6% (599) exhibited the growth of a fungal agent on their surface (Table 1). From those, the isolation of fungi allowed the identification of 43 species, belonging to 24 genera and 14 families (Table 2). The families comprising more diversity were Pleosporaceae and Quambalariaceae (8 species each) and Mucoraceae and Nectriaceae (5 species each). These families accounted for as much as 62% of the total identified species. The greatest number of taxa belonged to the genera *Penicillium* (7), *Arthrinium*, *Mucor*, *Fusarium* and *Alternaria* (all with 4 taxa). Concerning abundance, 166 different isolates were obtained in this work (Table 2). The most common taxa were *Beauveria bassiana* (N=50) and *Fusarium oxysporum* (N=25), representing together 45.2% of the total identified isolates. The number of fungal taxa identified in the present study varied between the three generations of *P. oleae* (Table 2).

In the phyllophagous generation, where a total of 70 fungal isolates were obtained, 16 different species were identified, belonging to 13 genera and 9 families. The most representative family was Quambalariaceae, comprising 25% of the identified species in this generation, being the genus *Penicillium* the most represented (3 species).

Table 2 Total abundance (N) and relative percentage (%) of each fungal taxa isolated from dead P. oleae larvae and pupae in relation to the total number of identified fungi. Values are presented for all the three moth generations. The ecological role (ER) of each fungus is also presented (P – phytopathogenic, A – antagonistic, E – entomopathogenic).

Family, genera and species	ER	Phyllophagous		Antophagous		Carpophagous		Total	
		N	%	N	%	N	%	N	%
Amphisphaeriaceae									
Truncatella									
T. angustata (Pers.) S. Hughes	P	0	0.0	0	0.0	1	2.3	1	0.6
Apiosporaceae									
Arthrinium									
A. phaeospermum (Corda) M.B. Ellis	A	1	1.4	0	0.0	0	0.0	1	0.6
Arthrinium sp1	A	0	0.0	1	1.9	0	0.0	1	0.6
Arthrinium sp2	A	0	0.0	1	1.9	0	0.0	1	0.6
Arthrinium sp3	A	0	0.0	1	1.9	0	0.0	1	0.6
Bionectriaceae									
Bionectria									
B.ochroleuca (Schwein.) Schroers & Samuels	P, A	2	2.9	0	0.0	0	0.0	2	1.2
Botryosphaeriaceae									
Botryosphaeria									
B. dothidea (Moug.) Ces. & De Not.	P	0	0.0	1	1.9	0	0.0	1	0.6

Microdiplodia									
M. hawaiiensis Crous	P, A	0	0.0	0	0.0	1	2.3	1	0.6
Chaetomiaceae									
Chaetomium									
C. funicola Cooke	P, A	0	0.0	0	0.0	1	2.3	1	0.6
Cordycipitaceae									
Beauveria									
B. bassiana (Bals.-Criv.) Vuill	A, E	49	70.0	0	0.0	1	2.3	50	30.1
Cordyceps									
C. sinensis (Berk.) Sacc.	E	1	1.4	0	0.0	0	0.0	1	0.6
Davidiellaceae									
Cladosporium									
C. cladosporioides (Fresen.) G.A. de Vries	P, A, E	1	1.4	0	0.0	1	2.3	2	1.2
Hypocreaceae									
Trichoderma									
T. gamsii Samuels & Druzhin.	A	0	0.0	8	15.4	0	0.0	8	4.8
Incertae sedis									
Septogloeum									
S. mori (Lév.) Briosi & Cavara	P	0	0.0	0	0.0	1	2.3	1	0.6

Trichothecium									
T. roseum (Pers.) Link	A	1	1.4	2	3.8	0	0.0	3	1.8
Mucoraceae									
Lichtheimia									
L. ramosa (Zopf) Vuill.	-	0	0.0	0	0.0	1	2.3	1	0.6
Mucor									
M. circinelloides Tiegh.	P, A, E	0	0.0	0	0.0	3	6.8	3	1.8
M. fragilis Bainier	P	1	1.4	0	0.0	0	0.0	1	0.6
M. hiemalis Wehmer	P, E	0	0.0	1	1.9	0	0.0	1	0.6
M. racemosus Bull.	P	1	1.4	0	0.0	0	0.0	1	0.6
Nectriaceae									
Fusarium									
F. equiseti (Corda) Sacc.	P, A,	0	0.0	8	15.4	0	0.0	8	4.8
F. oxysporum Schltdl.	P, A, E	2	2.9	23	44.2	0	0.0	25	15.1
F. solani (Mart.) Sacc.	P, E	0	0.0	1	1.9	0	0.0	1	0.6
Fusarium sp. 1	P, A, E	0	0.0	1	1.9	0	0.0	1	0.6
Gibberella									
Gibberella sp. 1	P	1	1.4	0	0.0	0	0.0	1	0.6
Pleosporaceae									

Alternaria									
Alternaria arborescens E. G. Simmons	P	0	0.0	0	0.0	1	2.3	1	0.6
Alternaria sp.1	P, A	3	4.3	3	5.8	6	13.6	12	7.2
Alternaria sp.2	P, A	0	0.0	0	0.0	2	4.5	2	1.2
Alternaria tenuissima (Kunze) Wiltshire	P	0	0.0	0	0.0	5	11.4	5	3.0
Embellisia									
Embellisia sp.	P	0	0.0	0	0.0	1	2.3	1	0.6
Epicoccum									
E. nigrum Link	P, A	1	1.4	0	0.0	0	0.0	1	0.6
Stemphylium									
S. solani G. F. Weber	P	0	0.0	0	0.0	1	2.3	1	0.6
S. vesicarium (Wallr.) E.G. Simmons	P	0	0.0	0	0.0	1	2.3	1	0.6
Quambalariaceae									
Quambalaria									
*Q. cyanescens (*de Hoog & G.A. de Vries) Z.W. Beer, Begerow & R. Bauer	P	3	4.3	0	0.0	0	0.0	3	1.8
Penicillium									
P. biourgeianum K.M. Zalessky	P	1	1.4	0	0.0	0	0.0	1	0.6
P. commune Thom	P	1	1.4	0	0.0	0	0.0	1	0.6

P. echinulatum Fassat.	P	1	1.4	0	0.0	0	0.0	1	0.6
P. italicum Wehmer	P	0	0.0	0	0.0	1	2.3	1	0.6
P. pinophilum Thom	A	0	0.0	1	1.9	7	15.9	8	4.8
Penicillium sp. 1	P, A, E	0	0.0	0	0.0	1	2.3	1	0.6
Penicillium sp. 2	P, A, E	0	0.0	0	0.0	1	2.3	1	0.6
Trichocomaceae									
Aspergillus									
A. ustus (Bainier) Thom & Church	P, A, E	0	0.0	0	0.0	6	13.6	6	3.6
Talaromyces									
T. flavus (Klöcker) Stolk & Samson	A	0	0.0	0	0.0	1	2.3	1	0.6
Total		70	100.0	52	100.0	44	100.0	166	100.0

In the antophagous generation, 52 isolates were obtained belonging to 13 species, 8 genera and 8 families. The families Nectriaceae and Apiosporaceae include the majority of the identified taxa (54%). The genus that included more taxa (4) was *Fusarium* (Nectriaceae), which contained 31% of the fungal species surveyed in this generation. Concerning the carpophagous generation, 44 fungal isolates were obtained being identified 21 species, belonging to 14 genera and 10 families. Pleosporaceae was the family representing more species (33%), and the genera *Alternaria* (Pleosporaceae) and *Penicillium* (Quambalariaceae), both with 4 species each, comprised 38% of the total fungal taxa found in this generation of *P. oleae*.

The most abundant species also differed between generations. For the phyllophagous generation, the most abundant one was *B. bassiana* (N=49), which represented 70% of the relative abundance in this generation. For the antophagous generation, *F. oxysporum* was the most abundant (N=23), corresponding to a relative abundance of 44.2%. In the carpophagous generation, several taxa presented comparable relative abundances, being *P. pinophilum* the most frequent (N=7, corresponding to a relative abundance of 15.9%), followed by *Alternaria sp.1* and *A. ustus*, both with N=6 corresponding to 13.6% of relative abundance.

In order to provide more information about fungal community composition in each *P. oleae* generation, the *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes were determined (Table 3). In the present work, both diversity indexes differed between the three *P. oleae* generations. As verified for species richness, the highest fungal diversity was verified in the carpophagous generation (*D*=0.09 and *H*=2.70). In fact, when using larvae or pupae from this generation, the highest number of unique species (17) was obtained.

Table 3 Simpson (*D*) and Shannon-Wiener (*H*) diversity indexes of fungal taxa identified in the three generations of *P. oleae*.

Generation	Diversity indexes	
	D	*H*
Phyllophagous	0.50	1.39
Antophagous	0.25	1.68
Carpophagous	0.09	2.70

The number of exclusive species identified from phyllophagous and antophagous generations was only 11 and 9, respectively. The lowest value of species diversity (*D*=0.5 and *H*=1.39) was detected in the phyllophagous generation.When performing a principal component analysis (PCA) using the total fungal taxa, according to the moth generation from which they were isolated, it was possible to separate the samples in eight groups (Fig 1). The phyllophagous generation is clearly related to groups 4 and 7, the antophagous generation is linked to groups 3 and 6, and the carpophagous generation is associated to groups 1 and 2. The two remaining groups (5 and 8) do not appear associated to a specific generation. Group 5 is mainly correlated to the antophagous generation, although presenting some relation to the phyllophagous generation. Group 8 is equally correlated to both phyllophagous and carpophagous generations.

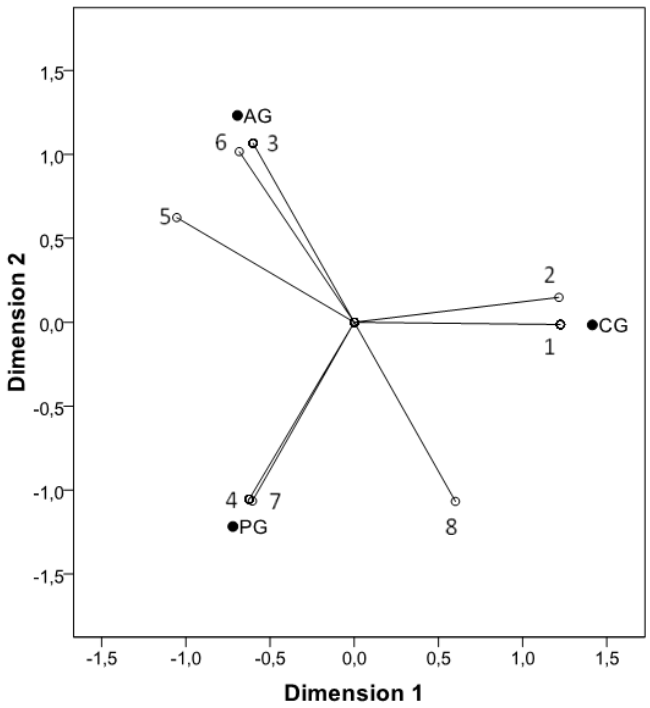

Figure 1 Principal component analysis (PCA) of the identified fungal taxa, according to the moth generation from which they were isolated (represented as closed circles; AG - antophagous generation; PG - phyllophagous generation; CG - carpophagous generation). Each number corresponds to a fungal group defined by the coordinates generated by SPSS. Fungal groups are defined as follows: 1 - *C. funicola*, *S. solani*, *P. italicum*, *S. vesicarium*, *A. tenuissima*, *T. flavus*, *Alternaria sp.1*, *Penicillium sp.1*, *M. hawaiiensis, A. ustus*, *Embellisia sp.*, *A. arborescens, S. mori*, *M. circinelloides*, *T. angustata*, *L. ramosa*, *Penicillium sp.2*, *Alternaria sp.2*; 2 - *P. pinophilum*. 3 - *Arthrinium sp.1*, *Fusarium sp.1*, *T. gamsii*, *F. solani, Arthrinium sp2*, *M. hiemalis*, *B. dothidea*, *F. equiseti*, *Arthrinium. sp3*; 4 - *M. racemosus*, *P. biourgeianum*, *A. phaeospermum, P. commune*, *P. echinulatum*, *Gibberella sp.*, E. nigrum, *C. sinensis*, *B. ochroleuca*, *Q. cyanescens*, *M. fragilis*; 5 - *T. roseum*; 6 - *F. oxysporum*; 7 - *B. bassiana*. 8 - *C. cladosporioides*.

Fungal ecological roles

Taking into account the potential use for the biological control of pests and diseases, 45% of the isolates found in this work have been described in the literature as presenting the useful features of antagonism and/or entomopathogenecity (Fig 2A).

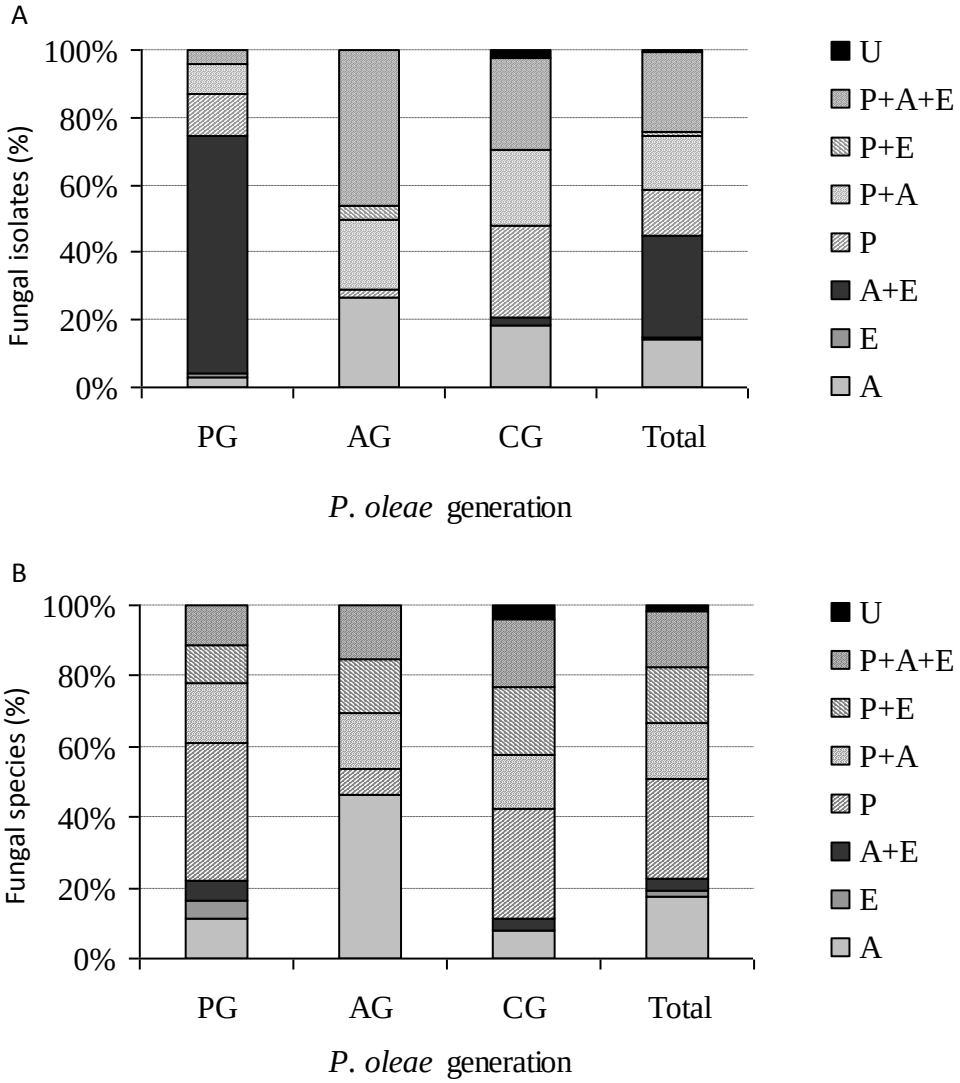

Figure 2 Percentage of fungal isolates (a) and fungal species (b) presenting different ecological roles. Isolates were obtained from dead larvae and pupae of the three generations of *P. oleae* (PG – phyllophagous generation; AG – antophagous generation; CG – carpophagous generation). After molecular identification, fungi were grouped according to their described ecological role (A – antagonistic, E – entomopathogenic, P – phytopathogenic, U – unknown).

The remaining 55% of the isolates presented phytopathogenic features, displaying also in large extent (41%) antagonistic and/or entomopathogenic characteristics. When comparing the number of identified species, the majority (75%) has been described as phytopathogenic fungi (Fig 2B). While the mentioned attribute was present alone in 28% of the identified species, the remaining 47% also displayed antagonistic and/or entomopathogenic characteristics. From those species described as non-phytopathogenic (23%), the majority are defined as antagonistic (17%).

The ecological roles of the identified fungi differed between generations. In the phyllophagous generation, the majority (87%) of the fungal isolates have been described as antagonistic and entomopathogenic (Fig 2A). Included in the phytopathogenic fungi (23%), 13% of fungal isolates also display antagonistic and/or entomopathogenic characteristics. This relation is reversed when considering the number of identified fungal species. The number of phytopathogenic taxa was higher (77%) than those displaying only antagonistic and/or entomopathogenic features (23%) (Fig 2B).

In the antophagous generation, a large fraction of isolates (73%) has been described as displaying phytopathogenic characteristics, of which 71% also displays antagonistic and/or entomopathogenic features (Fig 2A). The remaining 27% has been described as antagonistic fungi. Concerning the carpophagous generation, the higher fraction of fungal isolates displays phytopathogenic characteristics (77%) (Fig 2A). Included in these, 50% of fungal isolates also exhibits antagonistic and/or entomopathogenic features. Fungal isolates displaying only antagonistic and/or entomopathogenic features were also found but in a lower proportion (20%). When considering the number of identified fungal taxa, the same trend was observed for the antophagous and carpophagous generations. In both, the phytopathogenic fungi comprised the majority of identified taxa, but most of them also present antagonistic and/or entomopathogenic features. It was in the carpophagous generation that the lowest amount of non-phytopathogenic species with antagonistic and/or entomopathogenic properties were found (12%). This was the only generation where a fungal taxa (*Lichtheimia ramosa*) with no described ecological role was isolated.

DISCUSSION

In the present work, the diversity of fungi isolated from dead larvae and pupae of a major olive pest (*P. oleae*) was evaluated for the first time. Following molecular identification, it was possible to identify 43 fungal species, belonging to 24 genera and 14 families. The identification of such a high number of fungal taxa described as presenting antagonistic, entomopathogenic or phytopathogenic features was only possible because the studied olive groves were maintained under organic or integrated production guidelines. If sampling had been performed on a conventional orchard the expected fungal diversity would have been lower. Organic and integrated production management creates a healthier and safer environment with higher biological diversity (Mander et al., 1999). Similar diversity levels (46 fungal species and 27 genera) were obtained when studying insect-associated fungi isolated from soil samples of different field crops (wheat/maize, corn and soybean) and orchards (peach and apple) (Sun et al., 2008).

Fungal diversity and abundance

Concerning genera diversity, three main patterns were detected: (i) species-rich and highly abundant genera, e.g. *Fusarium*, *Alternaria* and *Penicillium* (about 35% of the taxonomic diversity and 42% of the total of isolates), (ii) species-rich genera, but displaying low abundance, e.g. *Mucor* and *Arthrinium* (about 19% of the taxonomic diversity and 5% of the total of isolates); and (iii) species-poor but highly abundant genera, e.g. *Beauveria* and *Trichoderma* (about 5% of the taxonomic diversity and 35% of the total of isolates). Among the 43 species recorded during the present study, the genera *Alternaria, Arthrinium, Fusarium, Mucor* and *Penicillium* were the most represented in terms of taxa number. These genera include some of the most ubiquitous fungal species in nature, which have been found in soils, plants and agricultural communities. The wide presence of such microorganisms has been related to the broad ecological roles they play, either as saprophyts, phytopathogens or biocontrol agents (De Lucca, 2007).

The genera that contributed most to species diversity depended on which *P. oleae* generation was used for fungal isolation. While in phyllophagous generation *Penicillium* represented the genus with highest taxonomic diversity (18%), in antophagous generation that genus was *Fusarium* (31%) and in carpophagous generation were *Alternaria* and *Penicillium* (representing together 39%). The composition of fungal community was also inferred by the *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes, which offer valuable information about

rarity and frequency of species in a community. Both indexes provide more information than simply species richness, since they also take the relative abundances of different species into account. While the carpophagous generation presented the highest fungal diversity (displaying the lowest *D* and highest *H* values), the phyllophagous generation displayed the lowest diversity (presenting the highest *D* and lowest *H* values). This difference between moth generations could be related to two main factors: climatic conditions during larvae and pupae collection and the moth life cycle.

Climate conditions, in particular relative humidity and temperature, are known to affect both conidia dispersion and germination (Topbaş et al., 2006). The collection of larvae and pupae from phyllophagous generation (March) matched with the time of year where the temperature is low, decreasing the ability of spore germination and thus reducing fungal diversity. Furthermore, the moth life cycle also seems to strongly affect the fungal diversity observed in this generation, as a large part of phyllophagous moth development occurs in the leaves as miner larvae. Therefore, the reduced fungal diversity observed in this generation can be associated to the low chances of larvae contact with fungal spores. The high occurrence of the entomopathogenic *B. bassiana* in the phyllophagous generation (70% of the isolates) could be related with an epizootic outbreak that might have occurred during the time of larvae and pupae sampling. These epizootics are described as being dependent on host population dynamics, the number and viability of infective stages in the pathogen population, infection efficiency and development, in addition to a complex set of environmental factors and timing (Ordentlich and Nachmias, 1990). Although dispersal of *B. bassiana* conidia by larvae could have been limited, due to their small displacement in trees, the wind and rain may have caused the spread of those infectious structures. Also, the high number of infected *P. oleae* individuals could have functioned as sources of infective conidia, thus contributing for the abundance of *B. bassiana* in this generation.

In antophagous and carpophagous generations, the most abundant taxa were *Fusarium oxysporum* (44% of the total isolates) and *Penicillium pinophilum* (15.9% of the total isolates), respectively. As referred, these two taxa are very frequent in nature, being present in almost all environments. Furthermore, the amount of spores of *Fusarium* species is known to increase from April to July (Topbaş et al., 2006) and those from *Penicillium* species rise between August and October (Medrela-Kuder, 2010). These periods are coincident with the sampling dates of larvae and pupae from antophagous (May-June) and carpophagous (September) generations, explaining

in some degree the high abundance of *Fusarium* and *Penicillium* genera in antophagous and carpophagous generations, respectively.

The association of climate and life cycle negatively affect the fungal diversity when using antophagous larvae and pupae. In the region where the study was conducted, temperature rapidly increases during May and June, leading to an extremely fast larvae development. Therefore, larvae and pupae stay a short period exposed to the surrounding environment, reducing the possibility of fungal infection. In contrast, carpophagous larvae are exposed to fungal spores for a longer period. After ecloding from the egg, larvae must travel from the oviposition site in the fruits to the petiole and bore down into the stone, where they usually feed for several weeks. After completing their development, larvae re-emerge from the fruit. The chance of infection is still enhanced by the extension of carpophagous generation which, together with the favourable climatic conditions, allows the development of more fungal taxa. Accordingly, it was in this moth generation that higher species diversity was found.

Fungal ecological roles

The relation between the amount of dead larvae and pupae displaying fungal growth and the ecological role of the identified fungal taxa provides interesting data. The higher amount of infected dead larvae and pupae was observed in the phyllophagous generation (61.5%). It was also from this generation that the most isolates exhibiting entomopathogenic features (76%) were obtained. As the presence of fungal growth on dead larvae and pupae decreases (from 61.5% to 32.7% and finally 23.0%, in the phyllophagous, antophagous and carpophagous generations, respectively), the abundance of fungi exhibiting entomopathogenic characteristics also decreases (76%, 50% and 30%, respectively). These results seem to suggest that the infection with entomopathogenic fungi could have led to larvae and pupae death. Most of the fungal isolates (86%) and species (70%) identified in this work has been described as antagonistic and/or entomopathogenic, although some of them have also been considered as displaying phytopathogenic features. Because only the non-phytopathogenic fungi displaying antagonistic and/or entomopathogenic features could be explored for limiting fungal diseases and/or pests, the percentage of identified fungal species that might have a future application as biocontrol agents is reduced to 23%.

From identified taxa in this work, *B. bassiana* seems to be the most conspicuous among the entomopathogenic/antagonistic species. The natural occurrence of this fungus in over 700

insect hosts from almost all taxonomic orders is well documented (Meyling and Eilenberg, 2007). However, there are still some reservations about the host range of this fungus. Some authors claim that *B. bassiana* is a "species complex", referring that different isolates have a restricted host, while others point out that this fungus has no host specificity (Pathan et al., 2007). Accordingly, several pests are susceptible to the entomopathogenic aptitude of this fungus, like *Alphitobius diaperinus* (Santoro et al., 2008), whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* (Quesada-Moraga et al., 2006), *Capnodis tenebrionis* (Marannino et al., 2006), *Lutzomyia longipalpis* (Amóra et al., 2009), *Callosobruchus maculates* (Murad et al., 2007), and *Tetranychus urticae* (Eken and Hayat, 2009). Some studies also indicate the presence of this fungus associated to several lepidopterans (Maurer et al., 1997; Fuguet and Vey, 2004; Aquino de Muro et al., 2005; Dalzoto et al., 2006; Quesada-Moraga et al., 2006; Santoro et al., 2008; Amóra et al., 2009). In addition, natural occurrence of mycoses caused by *B. bassiana* is reported in lepidopteran pests such as *Helicoverpa armigera* (Hübner) and *Spodoptera litura* (Fab.) (Devi et al., 2005), *Argyresthia conjugella* (Vänninen and Hokkanen, 1997) and *Plutella xylostella* (Silva et al., 2003). As far as we know this is the first report of *B. bassiana* related to *P. oleae*, where a large amount of isolates were obtained from larvae and pupae of the phyllophagous generation. The presence of this fungus in olive grove soils has already been reported (Marannino et al., 2006; Quesada-Moraga et al., 2006), as well as its ability to control pests in this crop, such as *Bactrocera oleae* (Konstantopoulou and Mazomenos, 2005; Mahmoud, 2009). All these evidences may suggest that this fungus could be effective in controlling *P. oleae* in olive orchards, although experiments to evaluate its infecting ability towards *P. oleae* have to be performed. The natural occurrence of this species in the studied olive groves guarantees an already adapted and suited strain to be used as a control agent in this particular ecosystem.

Another potential entomopathogen identified in the present study that could be able to control *P. oleae* larvae is *Cordyceps sinensis*. Although no literature is available for its ability to infect *P. oleae*, the capacity of infecting other lepidopterans larvae was already described, such as those of *Hepialus armoricanus* (Paterson, 2008). Nevertheless, these fungal taxa described as entomopathogenic cannot be definitely linked to the cause of death of *P. oleae* larvae and pupae. Assays confirming the infection ability and virulence of such fungi must be performed, in order to confirm their entomopathogenic potential.

The identification of fungi that could limit the growth of other infectious fungi by their antagonistic properties may also be important for designing future biocontrol strategies for restricting fungal diseases in olive groves. The most abundant taxa with antagonistic characteristics identified in the present study were *Penicillium pinophilum* and *Trichoderma gamsii. P. pinophilum* is one of the most important antagonists of *Rhizoctonia solani*, a fungal pathogen of tobacco (Alagesaboopathi, 1994). Although scarce information is available about *T. gamsii*, antagonistic properties of *Trichoderma* species against a great number of fungal species have already been described, including *Verticillium dahliae* that causes one of the most severe diseases affecting olive (Verticillium wilt) (Ordentlich and Nachmias, 1990; Verma et al., 2007). The identification of several isolates with antagonistic features (44%), some of them against fungi that cause olive diseases, opens up the possibility of further research on those antagonistic species for limiting the occurrence of such phytopathogenic fungi in olive orchards.

In this work, besides the identification of potential biocontrol agents for limiting pests and fungal diseases in olive groves, many other fungi were identified that could play a role in olive grove ecosystems. One of the most common genus found in this work was *Alternaria* that comprises species that have already been reported to cause spoilage of olives (Roussos et al., 2006) and cause a disease on olive shoots grown under greenhouse conditions (Bourbos et al., 1999). The most frequent *Alternaria* species isolated in the present work was *A. tenuissima*, which has been associated to late blight of pistachio and black point of small-grain cereals (Logrieco et al., 2003), among other crops. One of the most abundant fungal taxa identified in this study was *Fusarium oxysporum* that exhibits antagonistic, entomopathogenic and phytopathogenic properties. This species has been mainly described as phytopathogenic, causing vascular wilts or rot and crown rots in a large number of crops, including tree crops (Fravel et al., 2002). This species also presents antagonistic features against *Colletotrichum gloeosporioides* and *Pestalotia psidii* (Pandey et al., 1993), and most important, against *Verticillium dahliae* (Mercado-Blanco et al., 2004). Furthermore, *F. oxysporum* has also been described as an opportunistic insect-pathogen (Sun et al., 2008). Accordingly, it has been isolated from several insect hosts, such as those from Homoptera and Coleoptera orders, being able to parasite the greenhouse whitefly, *Trialeurodes vaporariorum* (Torres-Barragán et al., 2004).

However, the identification of entomopathogenic fungi from dead *P. oleae* larvae and pupae cannot be unequivocally associated to their ability to infect this lepidopteran, since dead larvae and pupae could just become increasingly susceptible to fungi.

CONCLUSION

As far as we know, the present work describes for the first time the assessment of fungal diversity directly obtained from mycosed dead *P. oleae* larvae and pupae.

The strategy used for obtaining fungal isolates (collection of larvae and pupae from the field and isolation of fungi from cadavers) allowed the identification of 43 fungal species, displaying several ecological roles. The diversity and abundance of fungal species differed when using larvae or pupae from different moth generation. Higher fungal diversity was found in the carpophagous generation, followed by antophagous and phyllophagous generations. Although the identified taxa could not be unequivocally associated with the cause of moth death, almost 37% of identified taxa presented entomopathogenic properties.

The identification of entomopathogenic and antagonist fungi in olive orchards provided a pool of biocontrol agents that could be used in the future for controlling pests and fungal diseases. The first report of *Beauveria bassiana* presence on *P. oleae* could open new strategies for the biocontrol of this major pest in olive groves. This fungus was isolated with high incidence from phyllophagous generation larvae and pupae. Future studies will be directed to screening the entomopathogens isolates for virulence to adult *P. oleae*. As the environmental conditions influence the performance of a given strain of fungus, the use of already adapted species to a particular ecosystem increases the guarantee of success of a biocontrol approach. The occurrence of antagonistic fungi able to control one of the major fungus attacking olive tree (*Verticillium dahliae*) may as well be investigated, in order to control this pathogen that is spreading throughout olive orchards.

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Chapter 3

Use of different barcoding regions to identify fungi associated to *Prays oleae*

Use of different barcoding regions to identify fungi associated to *Prays oleae.*

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ABSTRACT

Species identification using molecular barcodes is now definitely established for animals and for plants. Although the internal transcribed spacer (ITS) region has been adopted as a barcode region for fungi, many doubts still persist about the reliability of this marker. For identifying the fungal community associated to *Prays oleae*, this work intends to evaluate and compare the efficiency of different molecular barcode markers (ITS, β-tubulin and RPB2). For this purpose, fungal isolates retrieved from mycosed larvae and pupae of *P. oleae* were amplified using universal primers for these regions. The genomic DNA from the 43 obtained isolates was amplified using the ITS primers (100% of DNA samples), β-tubulin primers (62.8%) and RPB2 primers (83.7%). A total of 30 fungal isolates were identified. The ITS barcode marker allowed the identification of 22 isolates, β-tubulin identified 8 isolates and RPB2 identified the genera/species of 16 isolates. Most identifications (19) relied in only one region, specially ITS (13 identifications). The results of this work show that the use of multiple barcode regions for fungal identification should be considered. Although the characteristics of the ITS region turns this barcode region the most reliable to perform fungal identification, the use of β-tubulin and RBP2 increased the number of identified sequences. This multi-locus approach allowed the identification of fungal species associated with the death of larvae and pupae of *P. oleae* that present distinct roles in the ecosystem.

Keywords: DNA barcoding; ITS; β-tubulin; RPB2; Fungi

INTRODUCTION

The molecular identification of fungal species is essential for many research efforts on fungal ecology and is giving new insights into the diversity and ecology of scarcely known fungal species. The use of molecular techniques has been largely applied for the identification of fungal species (Bruns et al., 1991), but the benefits of a DNA barcoding in the context of taxa identification was only recently recognized (Hollingsworth, 2007). The main difference between molecular identification tools and the "DNA barcode" approach is that the latter involves the use of a standard DNA region, specific for a taxonomic group. For animals and plants, those regions have already been established, corresponding to a segment of the mitochondrial DNA cytochrome c oxidase gene (*COI*) for animals (Hebert et al., 2003) and regions of the plastid genes *matK* and *rbcL* for land plants (CBOL Plant Working Group, 2009). In contrast, the definition of an appropriate fungal DNA barcoding is still a matter of debate.

The first step for barcoding fungi, even though without this purpose, began with the work of White et al. (1990). This work described universal primers used for amplifying three main components of the fungal ribosomal operon: the large subunit (LSU), the small subunit (SSU) and the internal transcribed spacer (ITS) region. The described primers have been largely used by the scientific community, as they are very robust and able to function in the large majority of fungi (Seifert, 2009). The ITS region comprises two sections (ITS1 and ITS2) that flank the conserved 5.8S region. This region has been considered as the preferred DNA barcoding marker region for fungi, either for the identification of single taxa (*e.g.* Kelly et al., 2011) or to mixed environmental samples (*e.g.* Buée et al., 2009). Accordingly, the ITS region was recognized as the barcode needed for fungal identification by the official website for barcode (http://www.boldsystems.org) (Ratnasingham and Hebert, 2007). The recognition of ITS as a fungal barcode is largely due to the fact that this sequence is the most sequenced region of fungi, being routinely used for systematic, phylogeny and identification (Begerow et al., 2010). However, this advantage is to some extent hampered by misidentifications or sequencing errors that have been deposited in public DNA repositories (Nilsson et al., 2006). In addition, some taxonomic groups present an ITS region with less than 500 pb (Seifert, 2009). In order to provide a sufficient amount of variability within this region that allows unmistakable identification of species, a ITS length of 500 pb was established as the optimal lower limit for an effective DNA barcode (Seifert, 2009). Also, the intraspecific variation detected in this sequence could compromise the use of ITS as a barcode. Intraspecific and even intra-individual variability of this region has been described

(Kårén et al., 1997; Smith et al., 2007). A thorough study of ITS sequences in the International Nucleotide Sequence Database revealed that this region is not equally variable in all groups of fungi (Nilsson et al., 2008). Some genera have been described as being difficult to be classified using ITS barcode. Some of these include *Alternaria*, *Embelisia* (Seifert, 2009; Pryor and Michailides (2002), *Cladosporium* (Schubert et al. 2007), *Penicillium* (Skouboe et al. 1999) and *Fusarium* (O'Donnell & Cigelnik, 1997).

The problems associated with using ITS as the unique DNA barcode marker, as well as the recent approaches of selecting a small number of markers for botanical molecular identification (CBOL Plant Working Group, 2009), have led to the application of a multilocus DNA barcoding strategy to fungi (Dupuis et al., 2012). Several works have used this multi-locus approach for fungal identification, with the recurrent use of ITS, β-tubulin, EF-1α and RPB2 genes, such as those regarding *Neonectria* (Zhao et al., 2011a), an, in addition to those markers, also including actin, calmodulin and LSU genes, for genus *Mycosphaerella* (Quaedvlieg et al., 2012), and RPB1 and mitochondrial ATP6, for *Cordyceps* genus (Sung et al., 2007) However, the need of a second barcoding region could be only required in the case of specific studies, depending if a general survey or a more particular group identification is pretended (Schoch et al., 2012). Secondary markers should be used to accurately report genetic diversity in those taxa that present low ITS interspecific variability (Gazis et al., 2011). Besides ribosomal regions, various gene sequences have been used for the identification of fungi, such as partial β-tubulin (Samson et al., 2004), partial elongation factor 1-alpha (EF-1α) (Geiser et al., 2004), COI (Seifert et al., 2001) and the second largest subunit of RNA polymerase II (RPB2) gene sequences (Liu et al., 1999; Froslev, 2005). However, the use of these sequences as barcode markers could be associated with potential problems, such as the difficulty with amplification, the presence of multiple/mobile introns of differing lengths, the presence of multiple gene copies and poor resolution in some taxa.

In this study, the efficacy of three molecular markers (ITS, RPB2 and β-tubulin) was evaluated for the identification of those fungal species associated to pupae and larvae of *Prays oleae*. The fungal community associated to *P. oleae*, one of the major pests on olive orchards, have been described to play several roles in the ecosystem. Studies of the fungal community associated with olive moth have been performed in order to develop potential control agents (Oliveira et al., 2012). The use of a reliable method and barcode regions for fungi identification is required to speed up the characterization and survey of this fungal community.

METHODS

Fungal isolates

Fungal isolates were obtained from mycosed larvae and pupae of *Prays oleae* Bern. Larvae were collected from randomly chosen trees in selected orchards, managed through organic or integrated production guidelines. Sampling of larvae and pupae was performed in the three annual generations of *P. oleae* (phyllophagous, antophagous and carpophagous). Larvae and pupae were placed in sterile tubes, sealed and maintained at 25 ± 2°C, under 16/8 hours light/dark regime, for an average period of 10 days. Larvae and pupae were daily observed, in order to detect fungal growth. When present, fungi were isolated by inoculating Potato Dextrose Agar (PDA) medium, supplemented with 0.01% (w/v) chloramphenicol (Oxoid). Pure cultures of each isolate were deposited in the culture collection of the Polytechnic Institute of Bragança (School of Agriculture).

Molecular methods

Genomic DNA of each isolate was extracted from fungal spores, which were obtained by incubating the fungus in PDA medium, at 25 ± 2°C in the dark, during an average of 15 days. Spores were collected with a sterile scalpel, placed in a sterile microtube and then broken with the help of sterile glass spheres. DNA purification proceeded by performing a DNA precipitation with isopropanol (Oliveira et al., 2012).

Three different gene regions of each fungal isolate were amplified: the non-coding ITS (comprising ITS1 + 5.8S + ITS2 sequences), partial β-tubulin gene sequence (from exon 1 to exon 4, including 3 introns), and partial RPB2 gene sequence (spanning the conserved regions 6 and 7 and including a single intron). The primers used to amplify the described regions are listed in Table 1.

Table 1 Primers and PCR conditions used for amplifying each DNA barcode marker.

Marker	Direction	Sequence (5'-3')	References	Amplification conditions	
					94°C - 3 min
ITS					94°C – 30 s
				35 x	55°C – 30 s
ITS1	Forward	TCCGTAGGTGAACCTGCGG	White et al., 1990		72°C – 1 min
ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al., 1990		72°C - 10 min
					95°C - 5 min
β Tubulin					95°C – 45 s
				35 x	52°C – 1.5 min
T1	Forward	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik 1997		72°C – 1.5 min
Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson 1995		72°C - 10 min
					95°C - 1 min
RPB2					95°C – 45 s
				35 x	52°C – 40 s
					72°C – 2 min
RPB2-980F	Forward	TGYCCIGCIGARACICCHGARGG	Reeb et al., 2004		95°C – 45 s
				15 x	52°C – 40 s
					72°C – 2 min
fRPB2-7Cr	Reverse	CCCATRGCTTGYTTRCCCAT	Liu et al., 1999		72°C – 10 min

Amplification reactions (50 µL) were prepared using 50 ng of genomic DNA, 0.2 µM of each primer, 1x GoTaq® Flexi buffer (Promega), 0.2 µM dNTP Mix (Fermentas) and 1 U GoTaq® DNA polymerase (Promega). For ITS amplification a concentration of 2 mM $MgCl_2$ (Promega) was used, but for β-tubulin and RPB2 amplifications a higher concentration (2.5 mM) was employed. All amplifications were carried out in a MyCycler (BioRad) thermocycler, using the conditions detailed in Table 1.

PCR amplification products were analysed by electrophoresis, in a 1.2% agarose (Fluka) gel containing 0.5 µL GelRed™ (Biotium) nucleic acid gel stain. Following electrophoresis (in 1x TAE buffer), gel was analyzed under UV light using EagleEye II image capture system (Stratagene). Those reactions that amplified a single PCR product were selected for purification using the JETquick PCR product purification kit (Genomed). Amplified products were sequenced at the STABVida sequencing services (Oeiras, Portugal), using both forward and reverse primers for amplification.

Data analysis

The obtained DNA sequences were assembled in contigs using SeqMan software (DNASTAR Lasergene). Initial assessment of sequence identity was performed via BLASTN searches (Altschul et al., 1997) of GenBank (Benson et al., 2010). Those top-hit species (and closely related species) were retrieved for further analysis. Sequence aligments were performed using ClustalW method in MegAlign software (DNASTAR Lasergene). Before submitting sequences for further fungal identification, the trimming of low quality data at the start and end of sequences was performed for obtaining highly similar anchor regions, as close to the primer binding sites as possible. A successful identification was considered when the maximal identity (Max Ident) reached 90% (with 99–100% of coverage). The three top-hits obtained were taken into consideration for determining the probable correct identification.

RESULTS AND DISCUSSION

The precise identification of unknown fungal species should and must be the foundation of any given biodiversity assessment study. Misidentifications could have a major impact in such biodiversity surveys and ecological studies, leading to erroneous management decisions (Bortolus, 2008). Aiming the evaluation of fungal biodiversity associated with the death of *Prays oleae*, we collected larvae and pupae from the field and maintained them under aseptic conditions. For the precise identification of fungi that infected larvae and pupae, three different DNA barcode markers were selected (ITS, β-tubulin and RPB2).

The amplification success was different for each DNA barcode marker (Table 2). The ITS primers (ITS1 and ITS4) allowed the amplification of all tested samples, the primers T1 and Bt2b (β-tubulin) only amplified 62.8% of DNA samples, while RPB2-980F and fRPB2-7Cr primers (RPB2) resulted in 83.7% of successful amplifications.

Table 2 Results obtained after using ITS, β-tubulin and RPB2 as barcode markers. Success of amplification and positive identifications are presented, as well as amplicon length.

	ITS	**β-tubulin**	**RPB2**
% of successful amplification	100 (43/43)	62.8 (27/43)	83.7 (36/43)
Amplicon length (range)	500-653	495-793	657-834
% of successful amplification, identification (Max Ident≥98%; Query Cov≥99%)*	20.9 (9/43)	11.1 (3/27)	5.6 (2/36)
% of successful amplification, identification (Max Ident≥98%; Query Cov≥90%)*	62.8 (27/43)	29.6 (8/27)	44.4 (16/36)

* Results for percentage of successful amplification and probable correct identification were calculated using BLAST hits with more than 98% of maximum identity (Max Ident) and query coverage higher than 99%. In these results, samples obtaining BLAST hits of "uncultured fungus"," fungal sp." or "No significant similarity found" are also included.

The amplification success using ITS primers was already recognized (Zhao et al., 2011b; Quaedvlieg et al., 2012; Schoch et al., 2012) and has been attributed to the multi-copy nature of this marker that facilitates its amplification from low quantity or moderate quality DNA (Gardes and Bruns, 1993; Nilsson et al., 2006; Nilsson et al., 2008). In a wide-scale study, in which distinct laboratories used different routine methods for ITS amplification, there were not major differences on ITS performance (Schoch et al., 2012). Most experiments (80%) did not report any

problems with PCR or sequencing, and most of the times (90%) the PCR product presented high-quality. The higher performance of ITS primers in relation to RPB2 and β-tubulin markers has also been reported in other studies (Pino-Bodas et al., 2013; Quaedvlieg et al., 2012). Low values of PCR success using either universal or specific primers of RPB2 was reported for lichen-forming fungal genus *Cladonia* (Pino-Bodas et al., 2013). As this protein-coding gene is single copy (Liu and Hall, 2004) more efforts would be expected for its amplification. In spite of being a multiple copy gene, β-tubulin has been also described as being difficult to amplify (Kobmoo et al., 2012). Indeed, although the protein-encoding markers have been described as presenting more resolving power at species level (Schoch et al., 2012), the PCR and sequencing problems jeopardize their use as an all-fungi barcode region (Schoch et al., 2012).

Taking into consideration the used primer pairs, the amplicon length of the three tested markers was as expected (Table 2). With exception of a single β-tubulin amplified product, all amplicons exhibited more than 500 bp, which has been considered as the minimum limit for a DNA barcode (Seifert, 2009). The median length of ITS amplicons was 546 bp, with only two outliers above the 1.5-fold of the upper quantile (Fig. 1).

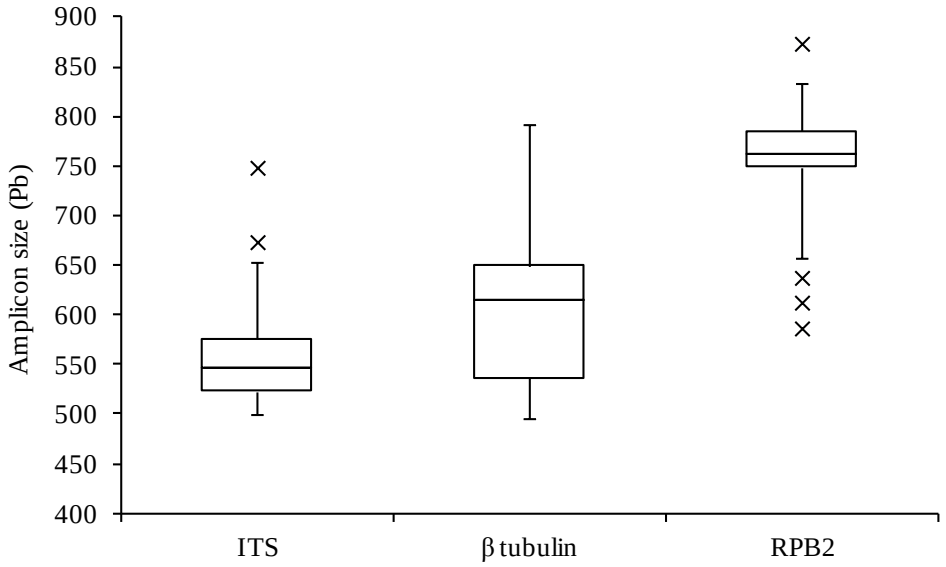

Figure 1 Amplicon length of each DNA barcode region. Primers for each barcode marker (ITS, β-tubulin and RPB2) were used for amplification of 43 fungal DNA samples. Only successful amplifications were considered (43 for ITS; 27 for β-tubulin; 36 for RPB2). A box-and-whisker plot is shown for each DNA barcode marker. Median is represented by a bold line; lower/upper quantiles are represented by a box; outliers that lie more than 1.5-fold of the interquartile range (the difference between upper and lower quantiles) are indicated by x.

While β-tubulin amplifications resulted in amplicons with a proximate median size (614 bp), RPB2 amplifications yielded amplicons with a higher median size (762 bp). The higher variation on the amplicon size between isolates was observed for gene-coding sequences. When using the partial β-tubulin gene as barcode marker, a difference of 298 bp was detected between the largest and smallest amplicon. As the amplified sequence spans three introns, the variation of their size would largely affect the amplicon size. Variation of β-tubulin introns length has already been reported for fungi (Schroeder et al., 2002; Msiska and Morton, 2009). RPB2 amplicons, which exhibits a variation of 177 bp, comprises a single intron that could also present length variations, as already referred for some fungal taxa (Malkus et al., 2006). The ITS amplicons exhibited the slightest variation between samples (153 bp difference).

For comparing the barcoding efficiency of the three markers, all the amplicons were aligned and trimmed to obtain similar regions. The trimmed sequences were then used for BLASTN searches (Altschul et al., 1997). The three top-hits for each fungal DNA sample, using the three barcode markers, will be considered (Supplementary Table 1). In a first analysis, only those sequences that presented more than 98% of maximum identity (with query coverage of more than 99%) were considered as probable correct identifications (PCI_{99}). Using these conditions, only few fungal isolates were positively identified (Supplementary Table, dark gray hits). About 21% of the ITS amplicons successfully identified the fungal isolates, but the identification using β-tubulin and RPB2 barcode markers was less than 12% and 6% of the amplified sequences, respectively (Table 2). As the query coverage is strongly dependent on sequence length variation, the same analysis was performed considering as probable correct identifications those sequences that still present more than 98% of maximum identity, but present a query coverage of more than 90% (PCI_{90}). Taking these criteria into consideration, the number of positively identified fungal isolates increased for all the three barcode markers. However, a higher rise was obtained in the case of RPB2 (8-fold more) than with β-tubulin (2.7-fold more) or ITS (3-fold more) markers. These results are related with the size differences between the amplicons and reference samples, as evaluated by the query coverage value. The majority of queries using β-tubulin resulted in low query coverage (13 samples), in which the query sequence was larger than the reference sequence. Indeed, the widely used primers for β-tubulin are Bt2a and Bt2b (Hubka and Kolarik, 2012) that result in an amplicon smaller than 500 bp (e.g. Aghayeva et al., 2004; Slippers et al., 2005; Visagie et al., 2009; Lu et al., 2009). When considering just this region for analysis, higher values of query coverage would have been

obtained and would eventually lead to new identifications. In contrast to β-tubulin, the obtained RPB2 amplicon (spanning the conserved regions 6 and 7 and including a single intron) was smaller than the reference samples. The most widely used primers for RPB2 amplify the regions 5 to 7 of the gene (Houbraken and Samson, 2011), leading to a much larger amplicon (Matheny et al., 2007; Reeb et al., 2004), being more difficult to amplify (about 1100 bp product). However, the region 6-7 has been suggested as phylogenetically useful for discriminating between closely related taxa (Liu et al., 1999).

A total of 32 fungal isolates (out of 43) were highly similar (PCI_{90}) to sequences already deposited in public DNA repositories (Table 3). However, the taxa identification was not always possible, since frequently the most similar sequences were annotated as "uncultured organism" or "Ascomycota sp."

Indeed, five ITS amplicons were highly similar to deposited sequences, but as they were not annotated to specific taxa, their identification was not possible. At the end, ITS barcode marker allowed the identification of 22 isolates (51.2% of total fungal isolates), β-tubulin identified 8 isolates (18.6%) and RPB2 identified the genera/species of 16 isolates (31.3%). The high number of positive identifications when using the ITS barcode marker is related to the number of reference sequences deposited on the public DNA repertories. The higher is the number of reference amplicons available for each barcode region, the higher are the chances of a positive identification. When compared to ITS sequences, the number of β-tubulin and RPB2 sequences is comparatively low in NCBI database. A quick search on this database (performed at April 2013) retrieved 32,224 nucleotide sequences for β-tubulin, 14,672 sequences for RPB2 and 288,640 ITS sequences. The disparity in the number of available sequences had already been recognized by Begerow et al. (2010).

Table 3 Probable correct identification of the studied fungal isolates by BLAST analysis, using as criteria ≥ 98% of maximum identity and ≥ 90% of query coverage. The species presenting the highest scores are presented. In brackets, the numbers refer to maximum identity/query coverage. Sequences annotated as "uncultured organism" or "Ascomycota sp." are presented in italics. NA – no amplified product, NI – no successful identification (maximum identity less than 98% and/or query coverage less than 90%, as well as no significant similarity found).

Sample ID	ITS	β-tubulin	RPB2
A1GC09	NI	NA	NA
A7_10GC10	NI	NI	NI
A11GF10	NI	NA	NI
A19GF10	Penicillium spinulosum (99/99) Penicillium glabrum (99/99)	NI	Penicillium glabrum (98/97)
A35GC09	NI	NI	NI
A39GF09	NI	NI	Beauveria sp. (98/99)
A49GC09	NI	NA	NI
A65GC09	Acremonium cellulolyticus (99/99) Penicillium pinophilum (99/99)	NI	NI
A69GC09	Alternaria sp. (99/97)	NI	Lewia eureka (99/100)
A108GF09	Fusarium oxysporum (98/99)	NI	NI
A181GC09	*Uncultured organism (99/93)*	NI	NI
AC1_8GC10	Penicillium sp. (99/99)	NI	NI
AC9_2GC10	Aspergillus niger (99/96)	NI	NI
AC95GF09	NI	Arthrinium phaeospermum (99/100) Arthrinium serenense (99/100)	NI
AC112GF10	Penicillium spinulosum (99/98) Penicillium thomii (99/98)	NI	NI
AC173GF10	NI	NA	NI
AC187GF10	NI	Passalora fulva (98/99)	NI
AL5GC09	Mucor circinelloides (99/98)	NA	NI
AL8_4GC10	NI	NI	NA
AL11GC09	Mucor circinelloides (100/98)	NA	NI
AL23GC09	Uncultured Alternaria (100/98)	NA	Alternaria tenuissima (99/93) Alternaria alternata (98/94)
AL31GF10	Valsaria ceratoniae (99/90)	NI	NI

AL49GF10	NI	NI	NI
AL83GC	Uncultured organism (99/98)	NA	Alternaria tenuissima (100/94)
AL83GF10	Cladosporium sp. (99/92)	NA	NA
AL97GF	NI	NA	Alternaria arborescens (99/95)
LC9_4GA10	Lecanicillium psalliotae (99/98)	NA	NA
LC10_10GC10	Cladosporium sp. (99/99)	NI	NI
LC25GA09	Fusarium chlamydosporum (99/95) Fusarium oxysporum (99/95)	Fusarium sp. (99/99) Fusarium equiseti (98/98) Fusarium incarnatum (98/98)	Fusarium equiseti (98/97)
LC25GC	Alternaria arborescens (99/95) Alternaria brassicae (99/95)	Alternaria arborescens (100/93) Alternaria tenuissima (99/93)	Alternaria arborescens (100/93)
LC53GC	*Uncultured organism (100/99)*	NA	Alternaria tenuissima (99/93) Alternaria alternata (99/93)
LC58GF	NI	Fusarium sp. (100/95) Fusarium subglutinans (99/95)	Fusarium subglutinans (99/90)
LC61GF	NI	Cylindrocarpon destructans (99/97) Fusarium solani (98/94)	Fusarium sp. (99/94)
LC83GC	Lewia infectoria (98/98)	NA	NI
PA19GF	NI	NI	Fusarium oxysporum (99/95)
PA167GF	Penicillium glabrum (99/99)	NI	Penicillium glabrum (98/98)
PNM4GF	Alternaria sp. (99/99)	NI	Alternaria tenuissima (99/93) Alternaria alternata (99/95)
PNM19GF	Alternaria sp. (100/97)	Alternaria tenuissima (100/97)	Alternaria tenuissima (100/94) Alternaria alternata (100/94)
PNM31GC	NI	NA	NI
PNM37GF	*Ascomycota sp. (99/99)*	NI	NI
PNM47GF	Alternaria arborescens (100/92) Alternaria brassicae (100/92)	Alternaria arborescens (99/97) Alternaria tenuissima (99/97)	Alternaria arborescens (100/95)
PNM53GF	Ulocladium chartarum (99/92)	NA	NA
PNM75GF10	*Uncultured organism (99/97)*	NA	NA

Using all the barcode markers, 30 fungal isolates (70%) were successfully identified. Although using different barcode markers, most fungal identifications (19 out of 30) were achieved using only a single barcode. Most of these were possible by using ITS barcode (13), but β-tubulin and RPB2 marker also resulted in exclusive identifications (2 and 4, respectively). In contrast, four fungal isolates (LC25GA09, LC25GC, PNM19GF and PNM47GF) were identified by the three barcode markers as species from *Alternaria* (3 isolates) or *Fusarium* (1 isolate) genus. The remaining fungal isolates were identified by ITS and RPB2 (5 isolates) or by β-tubulin and RPB2 (2 isolates). Most of these identifications resulted in species from the same genus: *Penicillium* (2 isolates), *Alternaria* (2 isolates) and *Fusarium* (1 isolate). However, different results were obtained for isolate A69GC09 (using ITS and RPB2 barcodes) and isolate LC61GF (using β-tubulin and RPB2). This divergence could be a result of reduced representation of specific barcode sequences in public DNA databases that lead to the identification of phylogenetically related species. However, in most of the cases, the discrepancy is due to the presence of erroneous identifications deposited on databases. According to this, only 62.7% of fungal isolates presented the same identification result in the three top hits (Supplementary Table). In addition, different genera were equally identified for the same fungal isolate (A65GC09), using the same barcode marker (ITS). A considerable amount of misidentified sequences in public DNA repositories like NCBI have been detected, which can represent over 20% of the database (Bridge et al., 2003; Nilsson et al., 2006). Due to these database inconsistencies, several species had been previously wrongly identified (Bruns and Shefferson 2004; Clapp et al., 2002; Schüßler et al., 2003; Deckert et al., 2002). This fact is of major concern, as identification errors will propagate, and some recommendation to reduce those inaccuracies was already suggested (Crous, 2002). Although some attempts to reduce such errors have been made, the results were described as unsuccessful (Pennisi, 2008). Curated fungal sequence databases already exist, but they are usually devoted to a single genus, like *Fusarium*, *Phaeoacremonium* or *Trichoderma*, as well as to mycorrhizal fungi (Balajee et al., 2009). These curated databases have the inconvenience of the limited number of available sequences, often overlooking the most significant species (Balajee et al., 2009).

The discrimination between different species from the same genus was difficult to be achieved when using ITS as barcode marker (isolates from *Alternaria* (2), *Penicillium* (2) and *Fusarium* (1) genera), β-tubulin (isolates from *Alternaria* (2), *Fusarium* (2) and *Arthrinium* (1) or RPB2 (isolates from *Alternaria* (4) genera). The lack of resolution within *Alternaria* (Seifert, 2009;

Pryor and Michailides (2002), *Penicillium* (Skouboe et al. 1999) and *Fusarium* (O'Donnell & Cigelnik, 1997) genera was already reported when using the ITS barcode regions. However, the use of protein-encoding markers has been describing as presenting more resolving power than ITS, at least at species level (Schoch et al., 2012). Indeed, a smaller region of β-tubulin amplicon (only comprising the third exon) was described to discriminate between fungal samples from cheese (Panelli et al., 2012).

The use of ITS region as a barcode region has been considered as presenting some limitations: much of the deposited sequences lack the formal barcode standards (Nilsson et al., 2006), the lack of resolution at species level is often recognized, the length of ITS region is less than the lower limit of a barcode for some taxonomic groups (Seifert, 2009) and intraspecific and intra-individual variability have been reported (Smith et al., 2007; Simon and Weiß, 2008). However, this barcode region is still the most reliable region for fungal identification. Although lacking some barcode criterion, the large number of available sequences still allows a large basis for sequence comparison of obtained sequences. Therefore, a careful analysis of query results could give a strong indication of its identification. Indeed, the thorough work of Schoch et al. (2012) has suggested that fungal ITS barcode region has a similar probability of correct species identification than the two-marker plant barcode system based on plastid *matK* and *rbcL* genes.

The fact that ITS lacks resolution for some genera like *Alternaria*, *Embelisia* (Seifert, 2009; Pryor and Michailides (2002), *Cladosporium* (Schubert et al. 2007), *Penicillium* (Skouboe et al. 1999) and *Fusarium* (O'Donnell & Cigelnik, 1997), was the hint to create a two-marker barcode, as in plants. However, Schoch et al. (2012) showed that the increase on the probability of correct identification using only ITS or the combination of this region with other markers is small, and that such more-than-one marker approach should be dependent on the type of study.
In the present work, the use of β-tubulin and RBP2 (in addition to ITS barcode sequence) increased the number of identified sequences. However, due to the less amplification efforts and number of probable identifications achieved, the ITS region was the most successful barcode region tested. Therefore, the ITS region is still the most reliable barcode region to perform fungal identification, especially when the identity of samples is completely unknown. The use of additional barcode markers could be advantageous for overcoming the fail in identification with ITS. Using the multi-locus approach presented in this work the identification of fungal species associated with the death of larvae and pupae of *P. oleae* that present distinct roles in the ecosystem was possible. This multi-locus approach should be taken in consideration whenever

possible for this kind of fungal surveys, although the ITS region proved to be the most efficient barcode. The additional use of other markers could help in the identification efforts.

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Supplementary Table 1 Amplicon size and BLAST top hits of query sequence, size, accession number and identification of deposited sequence

	ITS					β-Tubulin					RPB2				
Sample ID	Amplicon size (pb)	Most similar accessions	Size (pb)	Max ident (%)/ Query coverage	E value	Amplicon size (pb)	Most similar accessions	Size (pb)	Max ident (%)/ Query coverage	E value	Amplicon size (pb)	Most similar accessions	Size (pb)	Max ident (%)/ Query coverage)	E value
A1GC09	568	HE653031.1 *Aspergillus* sp.	1093	98/85	0.0		N.A.					N.A.			
		HE653030.1 *Aspergillus* sp.	1093	98/85	0.0										
		HE615091.1 *Aspergillus insuetus*	1085	98/85	0.0										
A7_10GC10	564	JQ272372.1 *Penicillium spinulosum*	614	97/99	0.0	643	AB566106.1 *Penicillium adametzii*	473	94/73	0.0	752	JN406535.1 *Penicillium crocicola*	912	97/86	0.0
		GU566252.1 *Penicillium spinulosum*	615	97/99	0.0		EU128574.1 *Penicillium glabrum*	469	93/72	0.0		FJ004469.1 *Penicillium glabrum*	808	91/98	0.0
		GU566248.1 *Penicillium spinulosum*	615	97/99	0.0		GQ367517.1 *Penicillium thomii*	421	96/65	0.0		FJ004468.1 *Penicillium glabrum*	808	91/98	0.0
A11GF10	526	KC119203.1 *Fusarium oxysporum*	1641	100/87	0.0		N.A.				829	EF470149.1 *Gibberella intermedia*	1806	99/85	0.0
		KC119197.1 *Fusarium* sp.	1646	100/87	0.0							FR870306.1 *Fusarium lactis*	1945	96/95	0.0
		JN624910.1 *Gibberella moniliformes*	1072	100/87	0.0							FR870303.1 *Fusarium lactis*	1944	96/95	0.0
A19GF10	524	**HQ680956.1 *Penicillium spinulosum***	**580**	**99/99**	**0.0**	640	AB566106.1 *Penicillium adametzii*	473	98/74	0.0	759	**FJ004469.1 *Penicillium glabrum***	**808**	**98/97**	**0.0**
		HQ680955.1 *Penicillium glabrum*	**541**	**99/99**	**0.0**		FJ004410.1 *Penicillium glabrum*	452	98/71	0.0		FJ004468.1 *Penicillium glabrum*	808	98/97	0.0
		HM469402.1 *Penicillium glabrum*	1131	99/99	0.0		FJ004409.1 *Penicillium glabrum*	452	98/71	0.0		EF198601.1 *Penicillium glabrum*	1011	99/87	0.0
A35GC09	507	JN098084.1 Fungal sp.	522	97/96	0.0	699	AY753379.1 *Penicillium pinophilum*	465	97/65	0.0	749	EU021620.1 *Talaromyces flavus*	1138	94/86	0.0

		JN098097.1 Fungal sp.	520	97/96	0.0		AY753373.1 *Talaromyces macrosporus*	465	93/64	0.0		XM_002153105.1 *Penicillium marneffei*	4153	91/88	0.0
		JN098086.1 Fungal sp.	521	97/96	0.0		AY766252.1 *Talaromyces flavus*	457	92/65	0.0		JF417426.1 *Talaromyces flavus*	976	94/74	0.0
A39GF09	585	GQ302680.1 *Beauveria bassiana*	569	99/79	0.0	638	EU604131.1 *Isaria tenuipes*	338	98/6	$7e^{10}$	801	**HQ880941.1 *Beauveria* sp.**	**2163**	**98/99**	**0.0**
		HQ115700.1 *Cordyceps bassiana*	569	99/79	0.0		EU604141.1 *Cordyceps militaris* cf. Var. *sphaerochephala*	334	98/6	$3e^{9}$		HQ880943.1 *Beauveria* sp.	2163	98/99	0.0
		GU109336.1 *Beauveria bassiana*	569	99/79	0.0		EU604139.1 *Isaria tenuipes*	332	98/6	$3e^{9}$		HQ880940.1 *Beauveria* sp.	2163	98/99	0.0
A49GC09	571	AY373874.1 *Aspergillus ustus*	594	97/97	0.0		N.A.				586	No significant similarity found			
		FN397275.1 Uncultured fungus.	608	97/97	0.0										
		FJ878628.1 *Aspergillus insuetus*	594	97/97	0.0										
A65GC09	**535**	**AB474749.2 *Acremonium cellulolyticus***	**637**	**99/99**	**0.0**	666	JF910279.1 *Penicillium pinophilum*	433	99/65	0.0	749	EU021620.1 *Talaromyces flavus*	1138	92/89	0.0
		JN624915.1 *Penicillium pinophilum*	**538**	**99/99**	**0.0**		AY525431.1 *Talaromyces flavus* var. *flavus*	489	95/71	0.0		FJ004498.1 *Penicillium verruculosum*	808	91/92	0.0
		JQ776546.1 *Penicillium pinophilum*	789	99/99	0.0		AY525426.1 *Talaromyces flavus* var. *flavus*	484	95/71	0.0		XM_002153105.1 *Penicillium marneffei*	4153	91/89	0.0
A69GC09	**585**	**FN868462.1 *Alternaria* sp.**	**611**	**99/97**	**0.0**	514	JQ672054.1 *Alternaria calendulae*	347	93/66	$1e^{140}$	**724**	**DQ677938.1 *Lewia eureka***	**1509**	**99/100**	**0.0**
		FR799468.1 *Alternaria* sp.	532	99/91	0.0		JQ671958.1 *Crivellia papaveracea*	347	93/66	$1e^{140}$		JF331615.1 *Alternaria vanuatuensis*	733	93/95	0.0
		AF212307.1 *Embellisia* sp.	571	97/97	0.0		JQ672055.1 *Alternaria ricini*	347	93/66	$6e^{139}$		JF331614.1 *Alternaria vanuatuensis*	733	93/95	0.0
A108GF09	**524**	**JF776163.1 *Fusarium oxysporum***	**545**	**98/99**	**0.0**	511	EF101456.1 *Cladosporium velox*	552	94/83	0.0	750	GU371782.1 *Mycosphaerella zeae-maydis*	1153	82/96	$7e^{175}$
		JF429684.1 *Fusarium* sp.	543	98/99	0.0		JN712647.1 *Coniothyrium nitidae*	536	90/95	$7e^{178}$		EU874849.1 *Phoma koolunga*	994	82/99	$4e^{172}$

		EF495230.1 *Fusarium oxysporum*	547	99/79	0.0		FJ427107.1 *Epicoccum nigrum*	343	99/67	$1e^{-176}$		EU874850.1 *Didymella exigua*	995	81/99	$5e^{-171}$
A181GC09	**519**	**JN660485.1 Uncultured organism.**	**553**	**99/93**	**0.0**	566	EF101455.1 *Cladosporium oxysporum*	489	94/86	0.0	762	DQ677971.1 *Davidiella tassiana*	904	83/94	$2e^{-179}$
		JN660484.1 Uncultured organism.	553	99/93	0.0		EF101454.1 *Cladosporium oxysporum*	478	93/84	0.0		DQ677945.1 *Cladosporium* sp.	1505	88/41	$1e^{-98}$
		KC33977.1.1 *Cladosporium* sp.	547	99/93	0.0		JQ217373.1 *Cladosporium* sp.	380	93/66	$1e^{-156}$		AF107799.1 *Aureobasidium pullulans*	2808	74/84	$8e^{-60}$
AC1_8GC10	540	**EU330619.1 *Penicillium* sp.**	**577**	**99/99**	**0.0**	600	JQ973898.1 *Penicillium* sp.	459	98/74	0.0	750	JX965298.1 *Talaromyces amestolkiae*	1038	99/89	0.0
		JQ912017.1 *Penicillium* sp.	604	99/99	0.0		JQ973897.1 *Penicillium* sp.	459	98/74	0.0		JX965292.1 *Talaromyces amestolkiae*	1038	99/89	0.0
		JQ912016.1 *Penicillium* sp.	604	99/99	0.0		FM991734.1 *Penicillium* sp.	506	95/84	0.0		JX965291.1 *Talaromyces amestolkiae*	1038	99/89	0.0
AC9_2GC10	569	**AJ280010.1 *Aspergillus* sp.**	**604**	**99/95**	**0.0**	783	FR775312.1 *Aspergillus brasiliensis*	1996	97/91	0.0	758	EU021641.1 *Aspergillus brasiliensis*	1014	93/82	0.0
		JQ316521.1 *Aspergillus brasiliensis*	599	99/94	0.0		AM270161.1 *Aspergillus niger*	348198	93/97	0.0		EF661064.1 *Aspergillus brasiliensis*	1014	93/82	0.0
		Kc119204.1 *Aspergillus niger*	1279	99/96	0.0		FR775313.1 *Arpergillus tubingensis*	790	93/88	0.0		EF661063.1 *Aspergillus brasiliensis*	1014	93/82	0.0
AC95GF09	673	KC139506.1 *Arthrinium* sp.	604	99/73	0.0	657	**AB220314.1 *Arthrinium phaeospermum***	**1633**	**99/100**	**0.0**	779	DQ368650.1 *Apiospora setosa*	1062	91/88	0.0
		GU055649.1 Uncultured *Arthrinium*	1193	98/73	0.0		**AB220297.1 *Arthrinium serenense***	**1633**	**99/100**	**0.0**		DQ810234.1 *Apiospora setosa*	1034	91/88	0.0
		AJ279456.1 *Arthrinium phaeospermum*	640	98/73	0.0		AB220287.1 *Arthrinium serenense*	1633	99/100	0.0		DQ368656.1 *Seiridium eucalypti*	1067	77/88	$6e^{-106}$
AC112GF10	**547**	**FR670336.1 *Penicillium spinulosum***	**576**	**99/98**	0.0	619	AB566106.1 *Penicillium adametzii*	473	91/76	$3e^{-178}$	761	JN406535.1 *Penicillium crocicola*	912	92/84	0.0
		DQ132828.1 *Penicillium spinulosum*	**565**	**99/98**	0.0		EU128574.1 *Penicillium glabrum*	469	91/76	$3e^{-177}$		JN406621.1 *Penicillium subericola*	912	89/84	0.0
		DQ132826.1 *Penicillium thomii*	566	99/98	0.0		FJ491776.1 *Penicillium glabrum*	479	91/74	$9e^{-173}$		JN406593.1 *Penicillium patens*	912	88/85	0.0

AC173GF10	651	DQ118998.1 *Amylomyces rouxii*	638	97/98	0.0		N. A.				657	JN993501.1 *Mucor circinelloides* f. *lusitanicus*	2796	85/95	0.0
		DQ118987.1 *Mucor circinelloides*	639	97/98	0.0							EF014398.1 *Mucor hiemalis*	2652	83/91	$1e^{-146}$
		JN561250.1 *Mucor circinelloides*	641	97/98	0.0							HM488820.1 *Lepiota roseolivida*	689	92/12	$2e^{-21}$
AC187GF10	549	JX241671.1 *Trichothecium roseum*	590	100/82	0.0	614	**EF432762.1 *Passalora fulva***	**1676**	**98/99**	**0.0**	811	DQ676599.1 *Trichothecium roseum*	654	100/80	0.0
		JX217818.1 *Trichothecium roseum*	589	100/82	0.0		JQ434527.1 *Trichothecium roseum*	357	100/57	0.0		DQ676586.1 *Trichothecium roseum*	654	100/80	0.0
		JQ434580.1 *Trichothecium roseum*	639	100/82	0.0		JQ434526.1 *Trichothecium roseum*	357	100/57	0.0		DQ676583.1 *Stachybotrys kampalensisi*	640	78/32	$2e^{-36}$
AL5GC09	653	**DQ118990.1 *Mucor circinelloides***	**638**	**99/98**	**0.0**		N. A.				673	JN993501.1 *Mucor circinelloides* f. *lusitanicus*	2796	88/99	0.0
		AM745433.1 *Mucor circinelloides*	643	99/98	0.0							EF014398.1 *Mucor hiemalis*	2652	86/98	0.0
		DQ118989.1 *Mucor circinelloides*	638	99/98	0.0							DQ302787.1 *Umbelopsis ramanniana*	2317	76/94	$2e^{-89}$
AL8_4GC10	569	GU973744.1 *Penicillium* sp.	564	97/96	0.0	642	JF910277.1 *Penicillium minioluteum*	433	92/68	$3e^{-168}$		N.A.			
		GU973797.1 *Penicillium* sp.	558	96/96	0.0		EU597716.1 *Penicillium pinophilum*	359	91/56	$2e^{-134}$					
		GU566240.1 *Penicillium minioluteum*	634	94/99	0.0		AY753371.1 *Talaromyces trachyspermus*	479	84/72	$1e^{-116}$					
AL11GC09	580	**JX241658.1 *Mucor circinelloides***	**615**	**100/98**	**0.0**		N. A.				695	JN993501.1 *Mucor circinelloides* f. *lusitanicus*	2796	87/93	0.0
		JQ683248.1 *Mucor circinelloides* f. *circinelloides*	634	100/98	0.0							EF014398.1 *Mucor hiemalis*	2652	85/94	0.0
		JQ085484.1 *Mucor circinelloides* f. *circinelloides*	634	100/98	0.0							JN993687.1 *Leucoagaricus orientiflavus*	722	76/30	$3e^{-19}$

AL23GC09	576	JN660464.1 Uncultured organism	572	100/98	0.0		N.A.				785	**JQ811955.1** ***Alternaria tenuissima***	**733**	**99/93**	**0.0**
		JN660443.1 Uncultured organism	569	100/98	0.0							DQ677980.1 *Alternaria alternata*	1822	98/94	0.0
		KC206496.1 **Uncultured Alternaria**	**620**	**100/98**	**0.0**							JQ811961.1 *Alternaria tenuissima*	733	98/93	0.0
AL31GF10	505	**EU040213.1** ***Valsaria ceratoniae***	**1923**	**99/90**	**0.0**	673	JX241677.1 *Gibberella intermedia*	850	89/25	$5e^{-51}$	873	GU456353.1 *Valsaria insitiva*	923	87/62	$8e^{-175}$
		JQ694114.1 *Valsaria insitiva*	604	99/88	0.0		JQ412112.1 *Gibberella intermedia*	1294	89/25	$5e^{-51}$		FN868688.1 *Hypomyces odoratus*	1609	95/9	$1e^{-24}$
		AB454268.1 *Guignardia bidwellii*	1211	85/77	$2e^{-98}$		AB725614.1 *Gibberella intermedia*	870	89/25	$5e^{-51}$		FN868689.1 *Hypomyces odoratus*	1609	95/9	$1e^{-24}$
AL49GF10	557	AF310978.1 *Fusarium lateritium*	1456	97/97	0.0	666	FJ427160.1 *Phoma pimprina*	343	84/19	$2e^{-25}$	834	HQ728166.1 *Fusarium heterosporum*	939	92/79	0.0
		AF310979.1 *Fusarium lateritium*	1456	97/97	0.0		EU541422.1 *Phoma exigua* var. *exigua*	308	75/41	$2e^{-25}$		JX171594.1 *Fusarium heterosporum*	1862	92/77	0.0
		EU520062.1 *Fusarium oxysporum*	562	95/97	0.0		EU541423.1 *Phoma exigua* var. *linicola*	307	75/41	$7e^{-25}$		JX171593.1 *Fusarium heterosporum*	1862	92/77	0.0
AL83GC	586	**JN660559.1** **Uncultured organism**	**568**	**99/98**	**0.0**		N.A.				762	**JQ811956.1** ***Alternaria tenuissima***	**733**	**100/94**	**0.0**
		JN660550.1 Uncultured organism	583	99/98	0.0							JQ811954.1 *Alternaria tenuissima*	733	100/94	0.0
		JN660460.1 Uncultured organism	586	99/98	0.0							JQ811953.1 *Alternaria alternata*	733	100/94	0.0
AL83GF10	514	**GU395509.1** ***Cladosporium* sp.**	**525**	**99/92**	**0.0**		N.A.					N.A.			
		KC007185.1 *Cladosporium* sp	1055	99/92	0.0										
		AB746922.1 *Cladosporium* sp	1063	99/92	0.0										
AL97GF	530	KC145172.1 *Alternaria* sp.	569	99/85	0.0		N.A.				637	**JQ811964.1** ***Alternaria arborescens***	**733**	**99/95**	**0.0**

		JX159646.1 Uncultured *Alternaria* clone	607	99/85	0.0						JQ811963.1 *Alternaria arborescens*	733	99/95	0.0
		JX159633.1 Uncultured *Alternaria* clone	607	99/85	0.0						JQ811962.1 *Alternaria arborescens*	733	99/95	0.0
LC9_4GA10	587	**AB083034.1 *Lecanicillium psalliotae***	**2295**	**99/98**	**0.0**		N.A.				N.A.			
		AB160994.1 *Lecanicillium psalliotae*	2299	98/98	0.0									
		AF455489.1 *Aphanocladium aranearum*	612	98/97	0.0									
LC10_10GC10	537	**KC339770.1 *Cladosporium* sp.**	**545**	**99/99**	**0.0**	793	FR775312.1 *Aspergillus brasiliensis*	1996	97/89	0.0	768	EU021641.1 *Aspergillus brasiliensis*	1014	97/85	0.0
		FR799495.1 *Cladosporium* sp	557	99/99	0.0		AM270165.1 *Aspergillus niger*	348198	93/89	0.0		EF661064.1 *Aspergillus brasiliensis*	1014	97/85	0.0
		HE977532.1 Uncultured *Cladosporium*	574	99/99	0.0		FR775313.1 *Aspergillus tubingensis*	790	93/86	0.0		EF661063.1 *Aspergillus brasiliensis*	1014	97/85	0.0
LC25GA09	514	JX914477.11 *Fusarium* sp.	572	99/95	0.0	**574**	**GQ915447.1 *Fusarium* sp.**	**1308**	**99/99**	**0.0**	830	**GQ915491.1 *Fusarium equiseti***	**2028**	**98/97**	**0.0**
		JX867235.1 *Fusarium chlamydosporum*	**513**	**99/95**	**0.0**		**JX241676.1 *Fusarium equiseti***	**850**	**98/98**	**0.0**		GQ915497.1 *Fusarium* sp.	2028	98/97	0.0
		JN400714.1 *Fusarium oxysporum*	**546**	**99/95**	**0.0**		**GQ915444.1 *Fusarium incarnatum***	**1309**	**98/98**	**0.0**		GU250674.1 *Fusarium* sp.	882	100/89	0.0
LC25GC	516	**KC415806.1 *Alternaria arborescens***	**518**	**99/95**	**0.0**	500	**JQ811950.1 *Alternaria arborescens***	**544**	**100/93**	**0.0**	775	**JQ811964.1 *Alternaria arborescens***	**733**	**100/93**	**0.0**
		JX857165.1 *Alternaria brassicae*	**571**	**99/95**	**0.0**		**JQ811948.1 *Alternaria arborescens***	**544**	**100/93**	**0.0**		JQ811963.1 *Alternaria arborescens*	733	100/93	0.0
		JX564139.1 Uncultured *Alternaria*	571	99/95	0.0		JQ811946.1 *Alternaria tenuissima*	544	99/93	0.0		JQ811962.1 *Alternaria arborescens*	733	99/93	0.0
LC53GC	576	**JN660559.1 Uncultured organism**	**586**	**100/99**	**0.0**		N.A.				775	**JQ811956.1 *Alternaria tenuissima***	**733**	**99/93**	**0.0**
		JN660550.1 Uncultured organism	583	100/99	0.0							**JQ811954.1 *Alternaria tenuissima***	**733**	**99/93**	**0.0**

		JN660460.1 Uncultured organism	586	100/99	0.0						JQ811953.1 *Alternaria alternata*	733	99/93	0.0
LC58GF	542	JX511973.1 *Gibberella moniliformis*	524	98/88	0.0	553	**AF160317.1 *Fusarium* sp.**	**569**	**100/95**	**0.0**	814	JX171599.1 *Fusarium subglutinans*	1862	99/90	0.0
		JF499676.1 *Gibberella moniliformis*	548	98/88	0.0		**U34417.1 *Fusarium subglutinans***	**537**	**99/95**	**0.0**		HM347216.1 *Fusarium subglutinans*	1839	99/90	0.0
		GU982311.1 *Gibberella moniliformis*	550	98/88	0.0		GU737302.1 *Fusarium* sp.	530	99/95	0.0		JX171618.1 *Fusarium quttiforme*	1862	97/90	0.0
LC61GF	748	FM177683.1 Uncultured compost fungus	1071	97/99	0.0	505	**AM419086.1 *Cylindrocarpon destructans* var. *destructans***	**550**	**99/97**	**0.0**	793	**GU170588.1 *Fusarium* sp.**	**938**	**99/94**	**0.0**
		HQ285687.1 *Lichtheimia ramosa*	859	97/99	0.0		JX241678.1 *Fusarium solani*	949	96/99	0.0		GU170591.1 *Fusarium* sp.	938	99/94	0.0
		FJ174686.1 *Mycocladus corymbiferus*	855	97/99	0.0		**GQ121902.1 *Fusarium solani***	**481**	**98/94**	**0.0**		GU170589.1 *Fusarium* sp.	929	99/93	0.0
LC83GC	537	**KC254057.1 *Lewia infectoria***	**599**	**98/98**	**0.0**		N.A.				768	DQ677938.1 *Lewia infectoria*	1509	91/97	0.0
		JF449874.1 Uncultured *Lewia*	1155	98/98	0.0							DQ677980.1 *Alternaria alternata*	1822	89/97	0.0
		GQ999401.1 Uncultured fungus	620	98/98	0.0							JF331616.1 *Alternaria ascaloniae*	733	91/88	0.0
PA19GF	649	JQ316442.1 Fungal endophyte	912	93/100	0.0	540	No significant similarity found.				814	**JX885464.1 *Fusarium oxysporum***	**1152**	**99/95**	**0.0**
		JQ316438.1 Fungal endophyte	877	94/100	0.0							DQ790583.1 *Fusarium oxysporum*	1920	99/93	0.0
		JQ316434.1 Fungal endophyte	870	94/99	0.0							DQ790581.1 *Fusarium oxysporum*	1920	99/93	0.0
PA167GF	549	**JX421718.1 *Penicillium glabrum***	**560**	**99/99**	**0.0**	634	AB566106.1 *Penicillium adametzii*	473	94/72	0.0	754	**FJ004468.1 *Penicillium glabrum***	**808**	**98/98**	**0.0**
		JX421729.1 *Penicillium glabrum*	564	99/99	0.0		EU128561.1 *Penicillium glabrum*	449	94/70	0.0		FJ004469.1 *Penicillium glabrum*	808	98/98	0.0
		JX421727.1 *Penicillium glabrum*	576	99/99	0.0		EU128585.1 *Penicillium glabrum*	461	93/72	0.0		EF198601.1 *Penicillium glabrum*	1011	99/87	0.0

PNM4GF	523	**KC139510.1 *Alternaria* sp.**	**568**	**99/99**	**0.0**	534	JQ811947.1 *Alternaria tenuissima*	544	93/66	$6e^{-144}$	778	**JQ811955.1 *Alternaria tenuissima***	**733**	**99/93**	**0.0**
		KC139505.1 *Alternaria* sp.	557	99/99	0.0		JQ811941.1 *Alternaria tenuissima*	544	93/66	$6e^{-144}$		**DQ677980.1 *Alternaria alternata***	**1822**	**99/95**	**0.0**
		KC139494.1 Alternaria sp.	560	99/99	0.0		JQ811950.1 *Alternaria arborescens*	544	93/66	$3e^{-144}$		JQ811961.1 *Alternaria tenuissima*	733	99/93	0.0
PNM19GF	546	**JQ346879.1 Uncultured *Alternaria***	**940**	**100/97**	**0.0**	495	**JQ811947.1 *Alternaria tenuissima***	**544**	**100/97**	**0.0**	762	**JQ811956.1 *Alternaria tenuissima***	**733**	**100/94**	**0.0**
		KC145172.1 *Alternaria* sp.	569	99/90	0.0		JQ811941.1 *Alternaria tenuissima*	544	100/97	0.0		JQ811945.1 *Alternaria tenuissima*	733	100/94	0.0
		JX159646.1 Uncultured *Alternaria*	607	100/97	0.0		JQ811946.1 *Alternaria tenuissima*	544	99/97	0.0		JQ811953.1 *Alternaria alternata*	733	100/94	0.0
PNM31GC	523	GU934566.1 *Stemphylium globuliferum*	580	95/92	0.0		N.A.				723	AF107804.1 *Pleospora herbarum*	2826	95/100	0.0
		EU859960.1 *Stemphylium globuliferum*	506	95/92	0.0							DQ247794.1 *Pleospoara herbarum*	1574	94/100	0.0
		EF104157.1 *Stemphylium solani*	522	95/91	0.0							DQ470924.1 *Dendryphiella arenaria*	1796	94/100	0.0
PNM37GF	528	**GU566233.1 Ascomycota sp.**	**607**	**99/99**	**0.0**	591	AB539436.1 *Corynespora smithii*	953	80/76	$1e^{-81}$	612	No significant similarity found			
		GQ153104.1 Dothideomycetes sp.	1098	99/86	0.0		AB539201.1 *Corynespora cassiicola*	954	79/76	2^{e-79}					
		GQ153158.1 Dothideomycetes sp	512	99/96	0.0		AB539171.1 *Corynespora cassiicola*	954	79/76	2^{e-79}					
PNM47GF	510	**KC415806.1 *Alternaria arborescens***	**518**	**100/92**	**0.0**	516	**JQ811950.1 *Alternaria arborescens***	**544**	**99/97**	**0.0**	752	**JQ811964.1 *Alternaria arborescens***	**733**	**100/95**	**0.0**
		JX857165.11 *Alternaria brassicae*	**571**	**100/92**	**0.0**		JQ811948.1 *Alternaria arborescens*	544	99/97	0.0		JQ811963.1 *Alternaria arborescens*	733	100/95	0.0
		JX564139.1 Uncultured *Alternaria*	571	100/92	0.0		**JQ811946.1 *Alternaria tenuissima***	**544**	**99/97**	**0.0**		JQ811962.1 *Alternaria arborescens*	733	99/95	0.0
PNM53GF	522	**JQ565884.1 *Ulocladium chartarum***	**531**	**99/92**	**0.0**		N.A.					N.A.			

PNM75GF10	500	JQ585683.1 *Ulocladium chartarum*	531	99/92	0.0	N.A.	N.A.
		HQ829119.1 *Ulocladium* sp.	573	99/92	0.0		
		JN660485.1 **Uncultured organism**	**553**	**99/97**	**0.0**		
		JN660484.1 **Uncultured organism**	**553**	**99/97**	**0.0**		
		KC339771.1 *Cladosporium* sp.	547	99/97	0.0		

Chapter 4

Pathogenicity evaluation of different *Beauveria bassiana* strains against *Cydia splendana* Hübner

Pathogenicity evaluation of different *Beauveria bassiana* strains against *Cydia splendana* Hübner

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ABSTRACT

Four native strains of *Beauveria bassiana* isolated from Lepidoptera were tested against the chestnut tortrix, *Cydia splendana* (Hübner) under laboratorial conditions. Conidial suspensions of *B. bassiana* isolates were applied to *C. splendana* larvae, at six different concentrations (from 10^5 to 10^8 conidia/mL). All isolates showed high pathogenicity against *C. splendana*, with mortality ranging from 40 to 100%. Concentration and time-dependent mortalities were also observed in all assays. A marked variation of virulence between the tested isolates was observed. Among the isolates tested, three gave rise to an earlier onset of disease (< 5 days) and mortality (over 70%) when applied at low concentrations (LC_{50} ranging from < 10^5 to 1.43 x 105). Results showed that *C. splendana* is susceptible to this entomopathogenic fungus, which open up the possibility of its use as biocontrol agent of this pest.

Keywords: *Beauveria bassiana*, *Cydia splendana*, Biological control, Bioassay, Virulence

INTRODUCTION

Chestnut fruit (*Castanea sativa* Mill.) is one of the oldest edible fruits cultivated in Portugal (Ferreira-Cardoso et al., 1999). This crop is affected by several pests, being the most important the chestnut tortrix, *Cydia splendana* Hübner (Lepidoprera: Torticidae) (Bento et al., 2007). The damages caused by this lepidopteran are the consumption of the fruit, creating small tunnels, leaving behind excrements. *Cydia splendana* it able to cause several economic losses, as infested fruits lose all commercial value, and infestation can reach as much as 80% of potential production (Bento et al., 2007). Control of this pest is not easy neither effective, and involves cultural and biotechnical measures, in alternative to the only chemical product approved in Portugal, for such purpose (Aguin-Pombo et al., 2009). All of these control measures presenting important drawbacks, leading to the need of an alternative, feasible and effective method to reduce this pest. One of the alternatives that should arise is the use of entomopathogenic fungi.

Although no data is available about the presence of this kind of fungi associated to *C. splendana*, previous works indicate that insects of the same family (Tortricidae) are susceptible to fungal entomopathogens (Anhalt et al., 2010; Goble et al., 2010; Goble et al., 2011; Ihara et al., 2009). One the most studied entomopathogenic fungal species is *Beauveria bassiana* (Bals.-Criv.) Vuill.. This entomopathogen has been isolated from several insect species, tested as biocontrol agent of numerous pests and commercially exploited as microbial pesticide (Shah and Pell, 2003; Faria and Wright, 2007; Zimmermann, 2007). *Beauveria bassiana* has been already described has caused of disease in a large number of lepidopteran species (Alves et al., 2002; Inclán et al., 2008; Maniania et al., 2011; Tefera and Pringle, 2003; Safavi et al., 2010; Vänninen and Hokkanen, 1997; Wraight et al., 2010).

Hence, the aims of this work were: i) to access the ability of B. bassiana to act as a pathogen of *C. splendana* and ii) to compare the pathogenicity of four native *B. bassiana* isolates obtained from naturally infected *Prays oleae* Bern. (Lepidoptera, Plutellidae) against *C. splendana*.

METHODS

Insects

Cydia splendana larvae were hand-picked from a local chestnut processing factory. All larvae were collected at the fourth larval stage. Larvae were placed in sterile plastic bags, immediately taken to the laboratory, and used in the bioassays within a few hours.

Fungal isolates and preparation of conidial suspension

Four isolates of *B. bassiana* (A67GF09; A39GF09; PA95GF10 and LC39GF10) were retrieved from the culture collection of the School of Agriculture of the Polytechnic Institute of Bragança. These isolates were obtained in a previous work (Oliveira et al., 2012), from infected larvae and pupae of *Prays oleae* Bern, and were identified by amplification and sequencing of the Internal Transcribed Spacer (ITS) region. These isolates were maintained in an aqueous glycerol solution (30%, v/v) at -80°C.

An aliquot of spore suspension was taken from the culture collection, placed in Potato Dextrose Agar (PDA) medium, and incubated in the dark at 25°C for 10-15 days for spore production. Fungal conidia were then collected by scrapping the surface of 10-15 days-old cultures with a sterile scalpel blade and suspended in 10 mL of 0.02% (v/v) Tween 80 sterile solution. The number of conidia per mL was counted in a Thoma counting chamber, followed by serial dilution in sterile aqueous Tween 80 (0.02%, v/v) and further used as inoculum in the bioassays.

Bioassay procedure

Cydia splendana larvae were randomly assigned to one of the different assays. The treatments consisted on six conidia concentrations (1×10^5; 1×10^6; 5×10^6; 1×10^7; 5×10^7; 1×10^8 conidia/mL), and control was performed with sterile 0.02% (v/v) Tween 80 solution. A sterile filter paper (Whatman n° 4) was placed in round plastic containers (8 cm of diameter and 5 cm of height), and one mL of conidia suspension was evenly placed over the paper, by carefully pipetting using sterile micropipette tips. Ten larvae were placed on top of the filter paper, and the plastic containers were placed in a room at 24±2 °C, 55-65% relative humidity and photoperiod 16:8 (light:dark). Each treatment consisted in five replicates, with a total of 50 larvae per conidial concentration. Mortality of the larvae was monitored daily, for 25 days, and dead larvae were collected and further used to indicate fungus-induced mortality. For this, dead larvae were surface sterilized by sequential immersion in 70% (v/v) aqueous ethanol for 1 min, 3-5% (v/v)

sodium hypochlorite for 3 min, re-immersed in 70% (v/v) aqueous ethanol for 1 min, followed by three consecutive washes of 1 min in sterile distillate water. After removing the excess of water with a sterile filter paper, the larvae's was transferred to Petri dishes (9 cm diameter) containing PDA medium and incubated in the dark at 25°C to facilitate fungal development. Fungal infection was further confirmed and the identity of the fungus was determined by morphological characteristics.

Data analysis

The cumulative mortality data was recorded every five days. In order to achieve the virulence of *B. bassiana* strains both LC_{50} and LT_{50} were calculated. The LC_{50} (expressed as conidia/mL) is the estimated concentration required to kill 50% of the test larvae whereas the LT_{50} is the estimated time to kill 50% of the larvae's, in days. The LC_{50} was calculated by probit analysis using SPSS (Statistical Package for Social Sciences) software, version 19.0 (IBM Corporation, New York, U.S.A.). To calculate LT_{50} values, probit mortality was regressed against the number of days after treatment.

RESULTS

Results show that the tested isolates of *B. bassiana* are able to infect and kill larvae of *C. splendana*, at all tested conidia concentrations. Mortality of larvae in the control assays was recorded only after 25 days of exposure to sterile aqueous Tween 80 solution. A concentration-dependent mortality of larvae was observed, although with variations between the tested isolates. In fact, one of the isolates (LC39GF10) proved high pathogenicity to *C. splendana* larvae even at the low concentrations tested (Fig. 1A). For this isolate, recorded mortality using the two low concentrations of conidia (1 x 10^5; 1 x 10^6 conidia/mL) stabilized after 15 days of exposure, at levels of 50% and 90% of dead larvae. At high conidial concentrations (5 x 10^6; 1 x 10^7; 5 x 10^7; 1 x 10^8 conidia/mL) this isolate caused the mortality of 100% of the larvae, at five days post-inoculation. For the isolate PA95GF10 (Fig. 1B), 100% of mortality was recorded when using the concentrations of 1 x 10^7, 5 x 10^7; 1 x 10^8 conidia/mL, after 10 days of exposure.

The other two isolates (A67GF09 and A39GF09) tested against *C. splendana* larvae showed a completely different behaviour. Stabilization of the recorded mortality of larvae didn´t occur (isolate A39GF09; Fig. 1D) or only took place at conidial concentrations higher than 5 x 10^7conidia/mL (isolate A67GF09; Fig. 1C). Isolate A67GF09 provided 100% mortality of larvae, at the highest concentration tested (1 x 10^8 conidia/mL) after 10 days of exposure (Fig. 1C), while isolate A39GF10 achieved a mortality of 100%, in the two high concentration tested, only after 25 days post-inoculation.

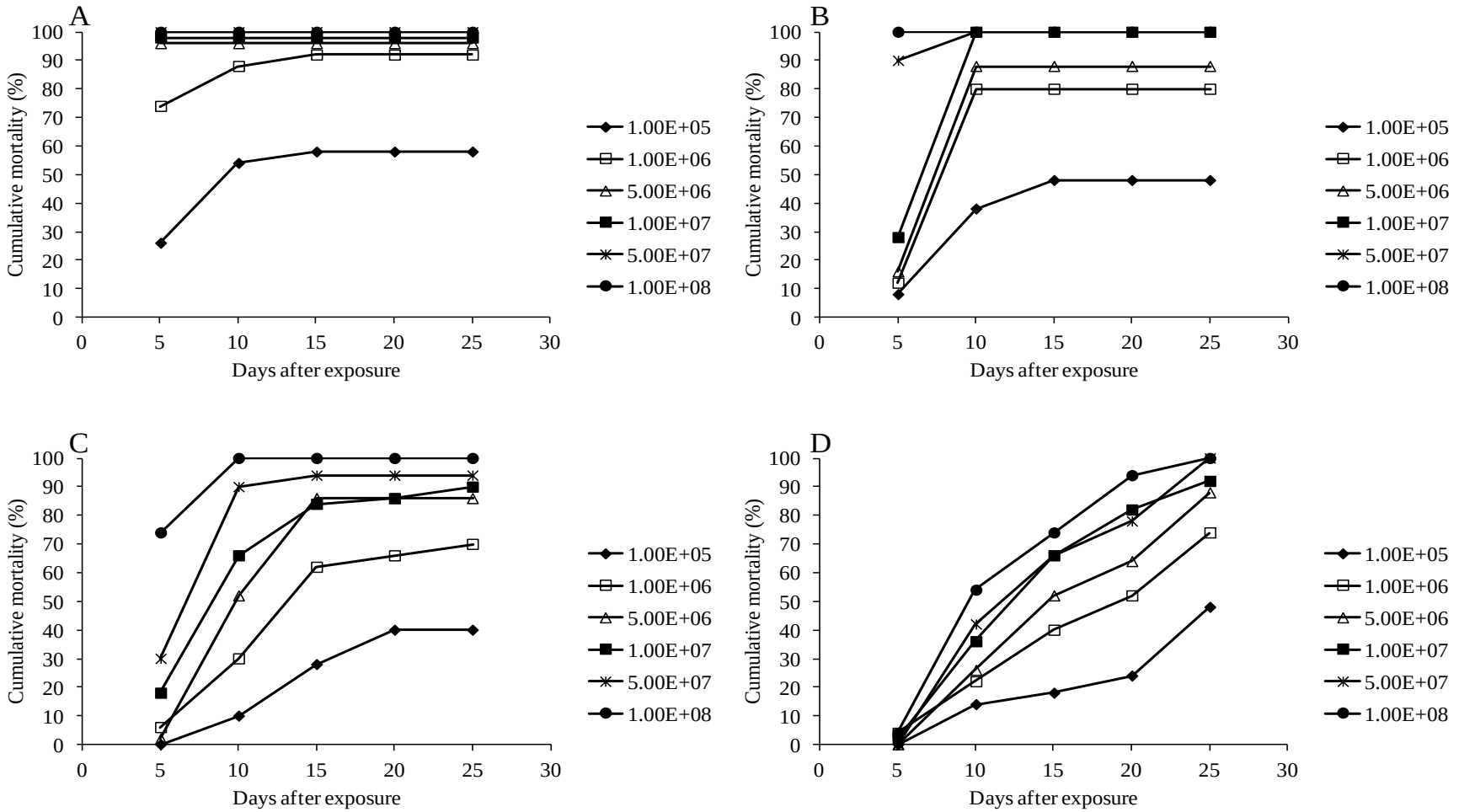

Figure 1 Cumulative mortality (%) of larvae of *C. splendana* (n = 50) in assays with strains of *B. bassiana* (A – LC39GF10; B – PA95GF10; C – A67GF09; D – A39GF09) at different concentrations (conidia/mL)

The determination of LC_{50} values showed similarly considerable differences between *B. bassiana* isolates (Table 1). Low values were observed for isolate LC39GF10 (< 1 x 10^5 conidia/mL, 5.76 x 10^4 as calculated by probit), followed by the isolates PA95GF10 (1.28 x 10^5 conidia/mL) and A67GF09 (1.43 x 10^5 conidia/mL). Isolate A39GF09 presented the poorer results, with the high LC_{50} value (2.13 x 10^5), which is almost four times higher than the value recorded with LC39GF10 isolate.

Table 1 Virulence of *B. bassiana* isolates to *Cydia splendana* larvae and LT_{50} values (days) of *B. bassiana* infecting the chestnut tortrix. Values between parentheses indicate minimum and maximum values, with 95% fiducial limits

B. bassiana isolates	LC_{50} (conidia/ml)	LT_{50} (10^5 conidia/mL)	LT_{50} (10^8 conidia/mL)
A39GF09	**2.13 x 10^5** (8.23 x 10^4 - 4.13 x 10^5)	**> 25**	**10.2 (9.2 – 11.2)**
A67GF09	**1.43 x 10^5** (5.31 x 10^4 – 2.81 x 10^5)	**> 25**	**< 5**
LC39GF10	**< 1 x 10^5** (1.67 x 10^4 – 1.21 x 10^5)	**12.6 (8.4 – 17.9)**	**< 5**
PA95GF10	**1.28 x 10^5** (5.25 x 10^4 – 2.36 x 10^5)	**20.5 (12.4 – 28.3)**	**< 5**

The median lethal time (LT_{50}) values of the four *B. bassiana* isolates were also determined, for the low and high concentrations of conidia applied to *C. splendana* larvae (Table 1). Results confirm the higher pathogenicity of LC39GF10 isolate in comparison to the other isolates. At the low concentration (1 x 10^5 conidia/mL), LC39GF10 showed a LT_{50} of 12.6 days. Highest LT_{50} was found for PA95GF10 isolate (20.5 days) and both isolates A67GF09 and A39GF09 didn't cause 50% of mortality of *C. splendana* larvae, during the duration of the assay (25 days). At the high concentration (1 x 108 conidia/ml), three of *the B. bassiana* isolates tested (A67GF09, PA95GF10 and LC39GF10) presented LT_{50} values lower than 5 days. Only the A39GF09 isolate presented LT_{50} higher than 5 days (10.2 days).

DISCUSSION

Laboratory experiments were conducted to evaluate the infectivity of *B. bassiana* entomopathogenic fungi in larvae of *C. splendana*. All the isolates of *B. bassiana* tested were pathogenic to *C. splendana* larvae, although with variations between isolates. Although all isolates caused 100% of larvae cumulative mortality, LC39GF10 and PA95GF10 isolates at the highest concentration killed 100% at 5 days post-inoculation, whereas the others isolates cause a similar percentage of mortality after 10 (isolate A67GF09) and 25 (isolate A39GF09) days of inoculation.

These pathogenicity differences between entomopathogenic fungal isolates have already been reported for numerous insect hosts, including lepidopterans (Goble et al., 2011; Godonou et al., 2009; Safavi et al., 2010; Wraight et al., 2010). Furthermore, variations have already been described for members of Tortricidae family, which showed different susceptibility to *B. bassiana* (between 21% to 93% of mycoses on pupae of *Thaumatotibia leucotreta*) depending on the tested isolate (Goble et al., 2011), fact than emphasises the need of strain selection and host-susceptibility tests. These variations may be due to different levels of enzyme production of the isolates of *B. bassiana*, has already been reported (Kaur and Padmaja, 2009; Dhar and Kaur, 2010; Murad et al., 2009), as well as relationships between enzyme production and virulence (Bidochka and Khachatourians, 1990; Fang et al., 2005; Gupta et al., 1994; Kaur and Padmaja, 2009; Montesinos-Matías et al., 2011). Other possible explanation for pathogenicity differences between *B. bassiana* isolates could be related with variability's in conidial size. Some studies have found positive correlation between infectivity of entomopathogenic fungi and the length of their conidia (Altre et al. 1999; Liu et al. 2003) or, by contrast, a negative correlation between conidia size and virulence (Samuels et al., 1989).

Even so, previous works regarding the virulence of *B. bassiana* against Tortricidae insects presented similar values of LC_{50} to the ones observed in the present work. Ihara et al. (2009) reported a value of LC_{50} of 3.5 x 10^5 conidia/mL for *B. bassiana* infecting *Cydia kurokoi* (Amsel) (Lepidoptera: Tortricidae), similar to the results observed for three of the tested isolates (A39GF09, A67GF09 and PA95GF10), with LC_{50} values, ranging from 1.28 to 2.13 x 10^5 conidia/mL. However, one of the tested isolates (LC39GF10) presented a considerable lower value of LC_{50} (< 1 x 10^5 conidia/mL). Other works with lepidopterans showed the susceptibility of these insects to *B. bassiana*, also detecting differences on the virulence of entomopathogenic strains (Cherry et al., 2004; Devi et al., 2001; Er et al., 2007; Talaei-Hassanloui et al., 2006; Safavi et al., 2010). Even though previous reports on *B. bassiana* infection of lepidopterans are

available, comparison of results should always keep in mind that each insect species have different susceptibilities to fungal entomopathogens. In fact, susceptibility of insects, even of the same taxonomic group, to the same fungal strain is a known reality, as detected by Wraight et al. (2010), when evaluating the ability of *B. bassiana* to infect several lepitopterans. We also detected variation of the LT_{50} between isolates. Related to these variations, besides the pathogenicity of each isolates, can also be the speed of germination. This specific parameters, while not evaluated in the present work, has been correlated to virulence (Altre et al, 1999; Yeo et al., 2003), and leads to faster infection of the host (Varela and Morales, 1996), resulting in low LT_{50}.

To date, no previous report of the presence of *B. bassiana* in chestnut orchards or associated to *C. splendana* are available, but the present work shows that this entomopathogenic species may be useful in the control of this pest. Although the isolates used in this work were not retrieved from infected *C. splendana* individuals, low values of LC_{50} and LT_{50} were observed. The fact that isolates retrieved from a given insect species tend to be more virulent to that species (Goettel, 1995), may indicate that lower LC_{50} and LT_{50} are to be expected.

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Chapter 5

Effect of soil tillage on natural occurrence of fungal entomopathogens associated to *Prays oleae* Bern.

Effect of soil tillage on natural occurrence of fungal entomopathogens associated to *Prays oleae* Bern.

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ABSTRACT

The olive tree is one of the most important crops in Portugal, where tillage is still a normal practice performed by the growers. This work intends to evaluate the effect of tillage practices on the diversity and abundance of entomopathogenic fungi associated to *Prays oleae* Bern. population in the olive grove ecosystem. In order to achieve this, three tilled and three non-tilled groves were sampled for *P. oleae* larvae and pupae, during two consecutive years. The fungi were isolated from diseased larvae and pupae and identified by rDNA sequencing. 120 isolates of fungi described as entomopathogenic were found, belonging to 8 different species. The most abundant species were *Beauveria bassiana* (60%), *Cladosporium cladosporioides* (18%) and *Cladosporium oxysporum* (14%). Olive groves under no-till system showed higher occurrence (2.7%), diversity (7 species) and abundance (65 isolates) of entomopathogenic fungi than tilled system (2.3%, 4 species and 55 isolates). Although these differences were found to be not significant, the results suggested that no-tilled olive groves with natural vegetation presented the most suitable conditions to increase the probability of *P. oleae* infection by entomopathogenic fungi. The highest number of exclusively species found in non-tilled groves (4 species) when compared to tilled ones (1 species) also reinforce this fact and indicated that vegetation cover may act as a reservoir for fungal species.

Keywords: Olive tree, *Prays oleae*, Entomopathogenic fungi, Tillage, Diversity.

INTRODUCTION

The natural biological control of insect pests is a safe phenomenon that can reduce the use of chemical insecticides. The entomopathogenic fungi (EF) are, among the natural enemies of insects' pests, considered as one of the most important groups in many cropping systems (Roy and Cottrell, 2008). The most common include *Beauveria bassiana* (Bals.) Vuill., *B. brongniartii* (Sacc.) Petch, *Metarhizium anisopliae* (Metschn.) Sorokin, *Isaria farinosa* Holms. Fr. and *I. fumosorosea* Wize (Zimmermann, 2008), all of which have a wide range of insect hosts (Toledo et al., 2008; Zimmermann, 2008). These fungi are especially common in the soils, where they often cause epizootics of disease in their hosts (Quesada-Moraga et al., 2007; Sun et al., 2008), and also occur as endophytes in a variety of host plants, including both agronomic and weedy species (Vega, 2008).

Consequences of agricultural practices on the occurrence and distribution of entomopathogenic fungi have been reported (Hummel et al., 2002; Quesada-Moraga et al., 2007; Jabbour and Barbercheck, 2009). Some works regarding the effects of tillage practices on the entomopathogenic fungal community showed contradictory results. Conventional tillage has been shown to increase the abundance and diversity of entomopathogens in soils (Jabbour and Barbercheck, 2009). However, no-tillage practice was found to increase the number of colony forming units (CFU) of soil entomopathogens when compared to tilled soils (Sosa-Gómez et al., 2001). Nevertheless, in other cases, no differences were observed (Sosa-Gómez et al., 2001; Jabbour and Barbercheck, 2009). Although these studies revealed an impact of tillage on soil fungal entomopathogens, they did not reflect the effect of this agriculture practice on the efficacy of entomopathogenic fungi under natural biotic and abiotic conditions. Only Bing and Lewis (1993) have reported that the number of infections caused by *B. bassiana* on *Ostrinia nubilalis* was the same between no-tilled and plow-tilled soils, but was twice in both soil managements that in chisel-plow soils.

In a given ecosystem, fungal entomopathogens establish a complex range of interactions with insects, plants and other microorganisms, which have important implications on the dissemination of entomopathogenic fungi, and their efficacy as a biocontrol agent (Baverstock et al., 2009; Meyling and Hajek, 2010). These interactions, as referred by Baverstock et al. (2010), are often overlooked on the large majority of laboratory bioassays that exclude biotic and abiotic conditions present in the natural habitat. Thus, in a biological control perspective, the assessment of the direct effect of tillage on the mortality of pests caused by entomopathogenic

fungi under natural habitats will provide a better understanding of this agricultural practice on the natural occurrence, infectivity and population dynamics of entomopathogenic fungi. This knowledge will also be very useful in improving the efficacy of these fungi as biological control agents, as well as in implementing conservation biological strategies.

The olive tree is one of the most important crops in the north-eastern Portuguese region of Trás-os-Montes (Pereira et al., 2004). Tillage is a traditional agricultural practice performed by a large number of the olive growers of this region. In this context, this work intends to evaluate the effect of tillage practices on the entomopathogenic fungal community present in the insect population of *Prays oleae* Bern. in the olive agroecosystem, during two consecutive years. This lepidopteran is a major pest of the olive trees in the region, able to cause up to 80% of losses of the potential production (Bento et al., 2001), and the natural occurrence of entomopathogenic fungi associated to this pest has been recently observed (Oliveira et al., 2012).

METHODS

Site description

This study was conducted in six olive groves 15 to 60 years old, located in Mirandela - Bragança region (41°28'51.85''N; 7°10'43.01''W). All the groves areas ranged from 0.4 to 1.5 ha and they present similar ecological conditions (exposition, slope and type of vegetation). Olive trees were of medium size with a planting density of 7x7 m, mainly comprising three different cultivars: Cobrançosa, Verdeal Transmontana and Madural. Pruning was made every two-three years and no irrigation was done. The olive groves were managed through the integrated production guidelines (Malavolta and Perdikis, 2012). Only copper based products were used in the beginning of autumn for fungal diseases control and no other pesticides were used in the last five years. Three olive groves are in a system using conventional tillage (using a disk plow) and the remaining three are no-tilled. Tillage was performed three times per year, the first at the end of March, the second three months later and the third at the end of September. The no-tilled olive groves have been kept under no-till management for, at least, three years. In these groves the vegetation was mainly dominated by *Andryala integrifolia* L., *Crepis vesicaria* L., *Anthemis arvensis* L., *Coleostephus myconis* (L.) Rchb.f. grasses. These weeds were cut using a chipper, usually in the beginning of summer, being the plant residues left in the grove.

The prevailing climate of this region is Mediterranean, with cold and rainy winters and long, hot and dry summers. The average annual rainfall varies from 600 to 800 mm, occurring mainly from October to February. The annual rainfall in the years of study was 800 mm in 2009, and 1400 mm in 2010. The annual mean temperature ranged from 9 to 20°C. Climatic data was retrieved using a field meteorological station. The predominant soils are dystric regosols (European Soil Bureau Network European Commission, 2005). These soils are characterized for being weakly developed, deep, well-drained, medium textured mineral soils, with limited horizons formed, with the exception of an ochric horizon (FAO, 2001).

Plant sampling

Fifteen olive trees were randomly chosen in each grove for collection of leaves, flower clusters and fruits to assess the presence of olive moth (*Prays oleae*) larvae and pupae. This moth develops three generations per year, attacking successively the leaves (phyllophagous), flower clusters (antophagous) and the fruits (carpophagous). The plant material collections were conducted in two consecutive years, 2009 and 2010, being the sampling dates dependent on

the life stages of the pest, which were monitored by regular field observations. Therefore, in 2009 the samplings were performed in March, May and September; and, in 2010 the samplings occurred in April, June and September according to the insect generations (phyllophagous, antophagous and carpophagous). After collection, the plant material was individually placed into sterile bags, and processed within a few hours after sampling.

Fungal isolation

The plant material was thoroughly examined for the presence of olive moth larvae and pupae. Whenever present, the larvae and pupae were placed individually in sterile glass tubes (25 mL) containing specific food sources. For larvae and pupae collected in March and April, leaves were placed in the tubes. Flower clusters served as food for larvae sampled in May and June, and olive stones were placed as food for larvae collected in September. Tubes were sealed with parafilm and kept under controlled temperature (25 ± 2°C), with a 16-8 hours light-dark regime, during an average of 10 days. Larvae and pupae were daily observed until the development of mycosis. The fungal agent growing on the surface of dead larvae or pupae was isolated in Potato Dextrose Agar (PDA), supplemented with 0.01% (w/v) chloramphenicol (Oxoid Ltd, Basingstoke, Hampshire, UK). Sub-culturing the initial mycelium in PDA medium allowed the establishment of pure cultures. Preliminary identification of the isolates was conducted by analysis of hyphal and spore morphology. The obtained fungal strains are currently maintained in the culture collection of the School of Agriculture of the Polytechnic Institute of Bragança (Oliveira et al., 2012).

Molecular identification of fungal isolates

The obtained fungal isolates were identified by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). The DNA of each isolate was extracted from spores, which were obtained by incubating the fungus in PDA medium, at 25 ± 2°C in the dark, during an average of 15 days. Spores were collected with a sterile scalpel, placed in a sterile microtube and DNA was extracted as previously described (Oliveira et al., 2012). ITS region amplification was carried out using the universal *ITS1* and *ITS4* primers (White et al., 1990) in a PCR protocol formerly described (Oliveira et al., 2012). Amplified products were sequenced using the STABVida services (Oeiras, Portugal). The obtained DNA sequences were analysed with DNASTAR v.2.58 software, and fungal identification was performed using the NCBI database

(http://www.ncbi.nlm.nih.gov) and the BLAST algorithm. All identified species are previously described as possessing entomopathogenic features.

Data Analysis

The fungal species richness, *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes, total abundance (N) and relative abundance of each identified fungal species were estimated. Calculations of *Simpson* and *Shannon-Wiener* diversity indexes were done using the software Species Diversity and Richness (v. 3.0). Total abundance was estimated as the number of isolates per fungal species; and the relative abundance is the proportion of isolates from each fungal species in relation to the total number of fungal isolates. The frequency of occurrence (FO) is given as the proportion of the number of isolates to the total number of sampled larvae and pupae, for each soil management. Similarity index (SI, %) between treatments (tillage *vs.* non-tillage) were calculated as described by Sørensen (1948) using the formula: SI = 2C/(A+B), where A is the total number of species in sample A, B is the total species number in sample B and C is the total number of species common to both samples (A and B). The effect of soil management (tillage and no-tillage) on entomopathogenic fungi diversity and abundance were evaluated by ANOVA. Once significant effects with a potency value of one were established, significant differences among groups were assessed using the Tukey test at $p<0.05$.

RESULTS

Fungal species richness, abundance and occurrence

During the course of this study, a total of 120 isolates, belonging to 8 fungal species, described as entomopathogenic, were recovered from larvae and pupae of *P. oleae* (Table 1). These 120 isolates were retrieved from a total of 4793 larvae and pupae of *P. oleae*, correspond to a frequency of occurrence of 2.5%.

Table 1 Total abundance (N) and frequency of occurrence (FO) of entomopathogenic fungal species collected during all the surveyed period and in each soil management systems, tillage and no-tillage.

	Soil management				Total		
	Tillage		No tillage		Abundance	Relative abundance (%)	FO (%)
Species	N	FO (%)	N	FO (%)			
Beauveria bassiana	35	1.47	37	1.54	72	60	1.5
Cladosporium cladosporioides	9	0.38	13	0.54	22	18	0.46
Cladosporium oxysporum	10	0.42	7	0.29	17	14	0.35
Cladosporium sp.	0	0	5	0.21	5	4	0.1
Isaria farinosa	1	0.04	0	0	1	1	0.02
Lecanicillium psalliotae	0	0	1	0.04	1	1	0.02
Lecanicillium muscarium	0	0	1	0.04	1	1	0.02
Paecilomyces formosus	0	0	1	0.04	1	1	0.02
Total	55	2.3	65	2.7	120	100	2.5

Over the two-year study, the fungal species that were most frequently found were *Beauveria bassiana* (72 isolates), *Cladosporium cladosporioides* (22 isolates) and *C. oxysporum* (17 isolates), with relative abundances of 60%, 18% and 14%, respectively. These species were found in 1.50% (*B. bassiana*), 0.46% (*C. cladosporioides*) and 0.35% (*C. oxysporum*) of the total sampled larvae and pupae. Four fungal species (*Isaria farinosa*, *Lecanicillium psalliotae*, *Lecanicillium muscarium*, and *Paecilomyces formosus*) were only detected once, which corresponds to a frequency of occurrence of 0.02 for each species.

Fungal species richness and abundance differed between years. The higher number of species (eight species) and fungal isolates (79 isolates) were found during 2010 whereas in 2009 only two species and 41 isolates were obtained. The occurrence of entomopathogenic fungi in the agroecosystem studied showed a bimodal pattern each year, with a peak in March - April (spring season) and another in September (autumn season) (Figure 1).

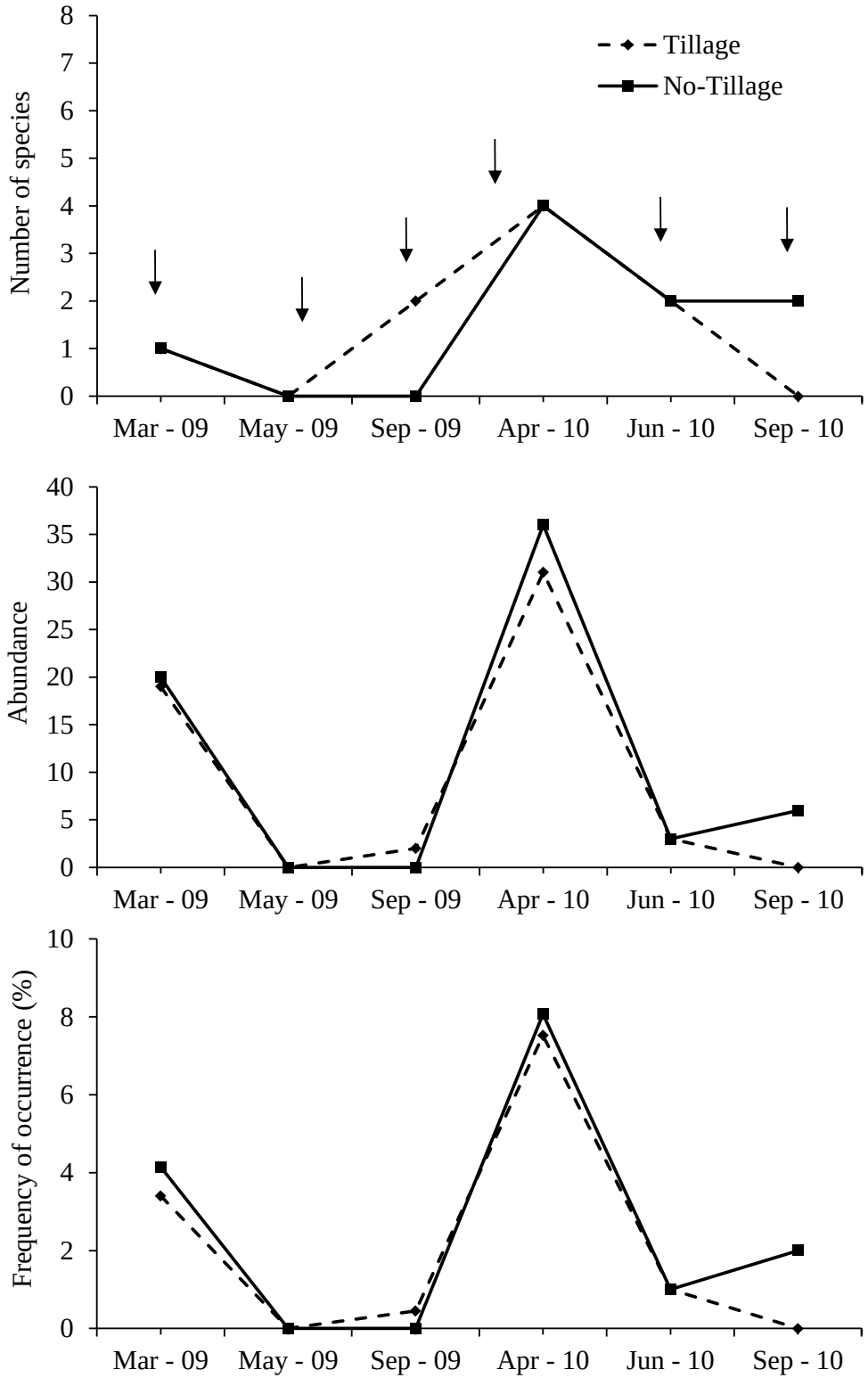

Figure 1 Number of species, abundance and frequency of occurrence of the entomopathogenic fungal species collected from tilled and non-tilled olive groves. Arrows indicate dates when tillage was performed.

The number of fungal isolates and species retrieved in March-April were higher than in September during the entire study period, but especially in 2010. The occurrence of the most abundant species also varied within and between years. The *B. bassiana* (Figure 2A) and *C. cladosporioides* (Figure 2B) were isolated from larvae and pupae collected in both spring and autumn seasons, being the highest number of isolates obtained in the first one. Note, however, that this last species was not always occurred in both spring and autumn of the same year. By contrast, the *C. oxysporum* (Figure 2C) was only occurred during the spring season and in a single year (2010).

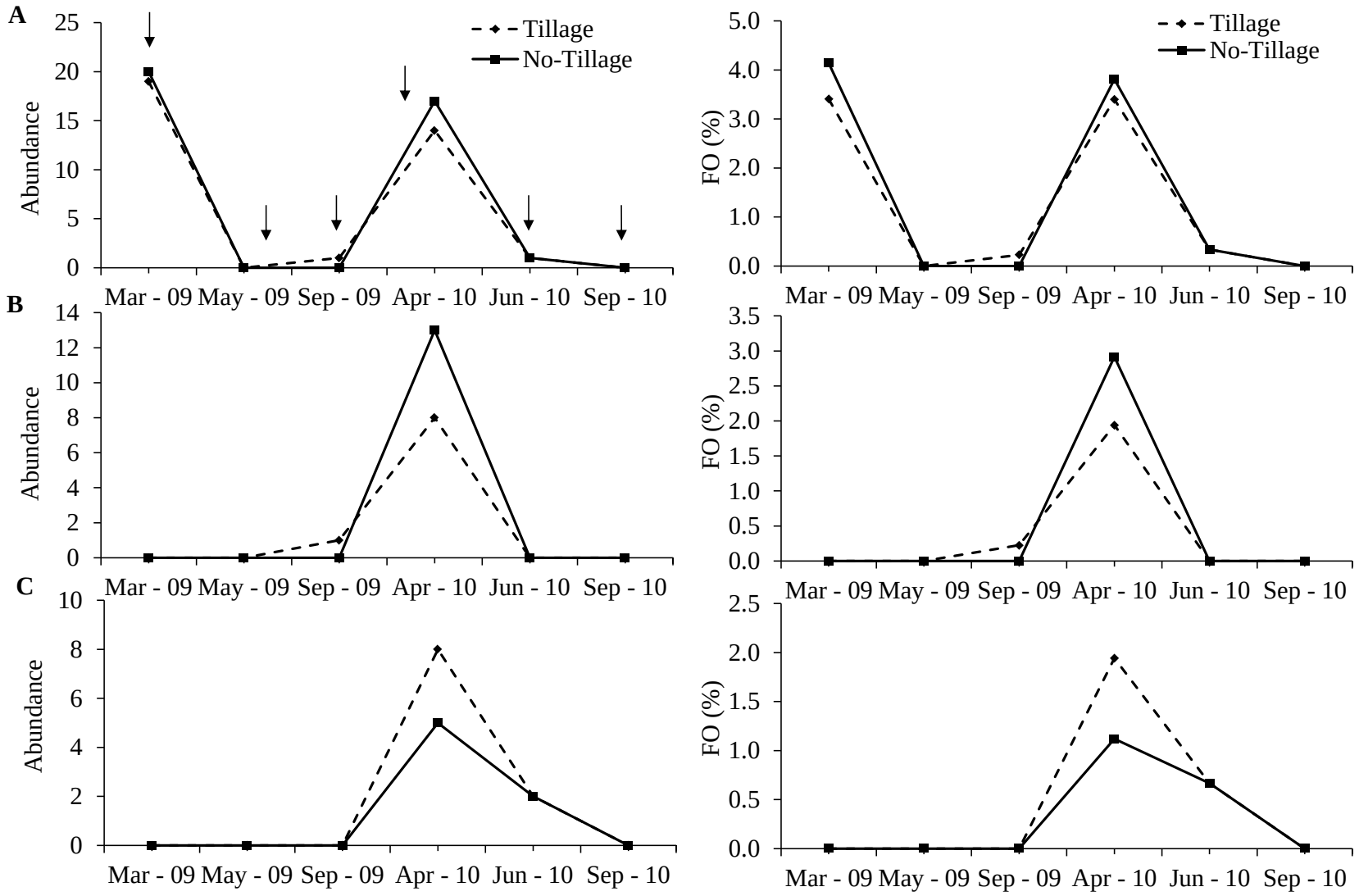

Figure 2 Effect of soil management practices on the total abundance and frequency of occurrence (FO) of the entomopathogenic fungal species: *Beauveria bassiana* (A), *Cladosporium cladosporioides* (B) and *C. oxysporum* (C). Arrows indicate dates when tillage was performed.

Effect of soil tillage on fungal community

Tillage practice was observed to be a factor that had slight influence on entomopathogenic fungal diversity and abundance. Although in the no tillage groves, the total number of entomopathogenic fungi species (7 species) and isolates (65) were higher than in tillage groves (4 species and 55 isolates) (Table 1), the differences were not statistically significant ($F_{1,5}$=6.25, P = 0.066; $F_{1,5}$=0.116, P = 0.750, respectively for the number of species and abundance). This result was corroborated by the no significantly different *Simpson* and *Shannon-Wiener* diversity indexes found between tilled and no-tilled groves (Table 2).

Table 2 *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes of fungal species collected per soil management system. Results are presented as mean ± SD, with n=3. Different superscripts indicate significant differences, $p < 0.05$.

	Soil management	
Diversity index	Tillage	No tillage
Simpson (D)	0.40 ± 0.04[a]	0.36 ± 0.15[a]
Shannon-Wiener (H)	1.09 ± 0.13[a]	1.26 ± 0.35[a]

The species that had occurred exclusively in no-tilled groves were *Cladosporium sp.*, *L. psalliotae*, *L. muscarium* and *P. formosus*, whereas in tilled ones only one exclusive species was found (*I. farinosa*) (Table 1). The species with occurrence in both groves types were *B. bassiana*, *C. cladosporioides* and *C. oxysporum*. The presence of only 3 common fungal species between the different types of soil management was also confirmed by the low *Sørensen* similarity index (0.54).

Although the frequency of occurrence of entomopathogenic fungi was slightly higher in no-tilled groves (2.7%) compared to tilled ones (2.3%) (Table 1), the differences was not statistically different ($F_{1,5}$=0.083, P = 0.775). *B. bassiana* was the most frequent fungus in both differently managed groves with an occurrence of 1.47% (of 2388 larvae and pupae collected) and 1.54% (of 2405 larvae and pupae), in tilled and no-tilled groves, respectively. *Cladosporium cladosporioides* was the second most frequent fungus found in no tilled groves, whereas *C. oxysporum* was the second most detected in tilled ones.

The frequency of occurrence, and the number of species and fungal isolates obtained during the surveyed period, both in tilled and no-tilled groves, is shown in Figure 1. In the spring sampling

dates (March, April and June), no variations on the occurrence of entomopathogenic fungal was found between tilled and no-tilled groves. The abundance as well as the number of species found in both tilled and no-tilled groves was very similar. By contrast, in the samplings performed in September some differences between the two types of soils management were observed. In 2009, the occurrence of entomopathogens was only noticed in tilled groves, while, in contrast, in 2010 they were only retrieved from no-tilled ones. However, for each of these sampling dates no significant differences were found on fungal abundance ($F_{1,5}$=2.286, P = 0.205) and richness ($F_{1,5}$=0.800, P= 0.422), between tilled and no-tilled groves.

A comparison of the frequency of occurrence of the most abundant entomopathogenic fungi species between tilled and no-tilled olive groves, for all the years of the study, showed different results according to the fungal species (Figure 2). The occurrence of *B. bassiana* in tilled and no-tilled olive groves was found to be very similar throughout of the study (Figure 2A). However, in the samplings performed in September was verified that this fungus had occurred only in tilled groves and in a single year (2009). By contrast, the frequency of occurrence of *C. cladosporioides* was higher in the no-tilled groves (Figure 2B), whereas of *C. oxysporum* was in the tilled ones (Figure 2C). However, these differences were not statistically different ($F_{1,5}$=0.325, P= 0.599, for *C. cladosporioides* and $F_{1,5}$=0.237, P= 0.652, for *C. oxysporum*) for both species. It is also important to note that the species *C. cladosporioides* was found, in one sampling date (September of 2009), exclusively in tilled groves

DISCUSSION

The present study constituted the first systematically monitoring of entomopathogenic fungi present in the natural *P. oleae* population in the olive grove agroecosystem. A total of eight entomopathogenic fungi species were identified from 120 isolates, retrieved from naturally infected *P. oleae* larvae and pupae. The species most frequently found were *B. bassiana* (60% of the total isolates), followed by species belonging to the *Cladosporium* genus (37% of the total isolates). Single occurrences of *I. farinosa*, *L. psalliotae*, *L. muscarium* and *P. formosus* were also observed during this study. Previously works have similarly found that *B. bassiana* was the most abundant entomopathogenic fungi in soils of olive groves (Quesada-Moraga et al., 2007). Curiously, in that study the second most abundant species was *Metarhizium anisopliae*; however its presence on *P. oleae* has not detected over the course of our work. Furthermore, and as far as we know, all the other species, excluding *B. bassiana*, are described in this work for the first time as being present in the olive grove agroecosystem.

The presence of entomopathogenic fungi in natural population of *P. oleae* was specifically noticed in three periods: March or April, June and September. It was in the first period that we have found the greatest number of species and isolates, followed by September and June. These differences on fungal occurrence, diversity and abundance throughout the year can be justified by two factors: the moth's life cycle and the climatic conditions. This particular lepidopteran feeds and develops in the leaves of the olive tree (during March and April), in the flower clusters (during May and June) and within the olive fruit (in September). It is known that fungal entomopathogens spend a fraction of their cycle as resting structures in different parts of plants, including in the leaf surface (Pell et al., 2010). Therefore, the number of isolates of entomopathogens is more likely to be higher in March-April, rather than in the other months (May, June and September), where *P. oleae* develops in the flower clusters and in the fruits. The insect development during the antophagous generation occurs very quickly which could explain the reduce infection of larvae and pupae by entomopathogenic fungi. On the other hand, during the development of the carpophagous generation, the larval stages of the insect develops inside of the fruits and therefore larvae are not so exposed to the surrounding environment, reducing the possibility of fungal infection. Climatic conditions, as temperature and humidity, are also known to affect entomopathogenic fungi (Jaronski, 2010). In the region where sampling was performed, the rainfall was considerable higher in March-April (30 mm in 2009; 150 mm in 2010) than in May-June (25 mm in 2009; 60 mm in 2010) or September (0 mm in 2009;

30 mm in 2010). Since the relative humidity has been shown to affect the efficiency of entomopathogenic fungi (Luz and Fargues, 1999; Luz et al., 2004), an increase of natural control by fungi during March-April would be expected. However, in these two months not only the high humidity was prevalent during prolonged periods, but also the mild temperatures were more favourable for fungal germination and growth. Although the average temperatures during March-April were 10°C (2009) and 12°C (2010), the average of maximum temperatures were 19°C (2009) and 22°C (2010). These conditions were more suitable for entomopathogenic fungal germination and growth than temperatures above 30°C (Jaronski, 2010). Thus, the combination of these two factors (humidity and temperature) could have resulted in the retrieval of more isolates in the March-April months.

The results obtained also indicated that among the total entomopathogenic fungi isolated, *B. bassiana* is the unique species that had occurred in all the three periods (March-April, June and September). *Cladosporium cladosporioides* and *C. oxysporum* have only occurred in two periods, in April and September, and in April and June, respectively. As mentioned previously, these differences on fungal species occurrence can be linked to climatic conditions which are known to affect entomopathogenic fungal activity, fitness and persistence, within and outside host (Hussein et al., 2010; Jaronski, 2010).

The tillage of olive groves soils shown to have minor influence on the diversity and abundance of entomopathogenic fungi associated to *P. oleae*. In fact, although the total number of entomopathogenic species and isolates were higher in no-tilled groves than in tilled ones the differences were not statistically significant. Also, according to *Simpson* and *Shannon-Wiener* diversity indexes, no significant difference was found between entomopathogens retrieved from groves with or without tillage practice. It is known that tillage disturbs the fungal propagules of entomopathogenic fungi present in the soil (Pell et al., 2010), and this action can have beneficial or detrimental effects. The disturbance of soil may positively affect fungal infection, by moving the propagules closer to the host, or by facilitating conidia dispersal by wind (Shimazu et al., 2002) or rain (Bruck and Lewis, 2002). Nevertheless, the same action may hinder the contact between fungi and insect, or expose conidia to adverse environmental conditions (UV light, temperature, humidity), that may reduce the ability of the fungi to survive (Pell et al., 2010). By contrast, on untilled soils the vegetation cover can increase the population of insects (Reeleder et al., 2006; Rodríguez et al., 2006), hence providing alternative hosts for certain fungal entomopathogens and contributing for their enhanced dispersion. Furthermore, some entomopathogenic fungi can

live inside of plant tissues (including of weedy species) as endophytes (Vega et al., 2008), which could provide a delivery system for the biological control of insects pests.

The lack of significant differences on entomopathogenic fungi diversity and abundance found between tilled and no-tilled olive groves may be attributed to the fact that the isolation of fungi was performed from infected larvae and pupae of *P. oleae* collected at the canopy level. In this habitat, the conditions for fungal growth are likely to be very similar between the different soil management practices (tillage and no-tillage). Therefore, even if tillage caused variations of the abundance and diversity of entomopathogenic fungi at the soil level (Bing and Lewis, 1993; Sosa-Gómez and Moscardi, 1994; Sosa-Gómez et al., 2001, Hummel et al., 2002, Wang et al., 2010), in the canopy these variations weren't noticeable. Similarly, Sosa-Gómez et al. (2001) have not found differences on entomopathogenic fungal density on leaves of soybean between the no-tillage and tillage systems. These authors suggested that the aerial part of the plants have similar microclimatic conditions that would be responsible for the lack of fungal community differences between the no-till and tillage systems. The factors that affect entomopathogenic fungi present in the canopy include sunlight, rainfall, temperature and humidity (Jaronski, 2010). However, some works have been showed that tillage doesn't cause significant variations of those factors at the canopy level (Cantonwine et al., 2007; Gucci et al., 2012). In such way, the variations in environmental conditions caused by tillage at the soil level (which may cause differences in the abundance and diversity of entomopathogenic fungi) are not reflected in the canopy of trees. Similarly, in our study was found that the practice of tillage in olive groves did not have an effect on the abundance and diversity of entomopathogenic fungi associated to *P. oleae* collected in olive tree canopy.

From the total of the identified entomopathogenic fungal species only *B. bassiana*, *C. cladosporioides* and *C. oxysporum*, have occurred in both tilled and no-tilled olive groves and were the most abundant. This result suggested that, these species seemed to have more capacity to infect *P. oleae* present in the olive tree canopy and that this capacity is not affected by the type of soil management (tillage and no-tillage). By contrast, four species (*Cladosporium sp.*, *L. psalliotae*, *L. muscarium* and *P. formosus*) have occurred exclusively in no-tilled, and in tilled groves only one exclusive species was found (*I. farinosa*). The presence of exclusive species on no-tilled groves is an indication that vegetation cover could be a reservoir for certain fungus, more appropriated to growth and produce spores on soil environmental conditions. The increased population of insects that a vegetation cover provides (Reeleder et al., 2006; Rodríguez et al.,

2006) may serve as hosts for these fungal species, such as *Lecanicillium* and *Paecilomyces*, known to have a wide host range (Goettel et al., 2008; Sandhu et al., 2012). As infected insects, as well as their predators, are able to disperse infective inoculum (Meyling et al., 2006) of entomopathogenic fungi, this fact may account for the detection of those species in no-tilled groves. Furthermore, the presence of fungal entomopathogens with endophytic capacity may also account for the presence of those species in the no-tilled groves. Indeed, some species belonging to the genera *Lecanicillium* (Gómez-Vidal et al., 2006), *Paecilomyces* (Cao et al., 2002; Tian et al., 2004) and *Cladosporium* (Ananda and Sridhar, 2002; Vega et al., 2008) are described as being endophytic to several plants. Although their presence as endophytic fungi in the plants present in the vegetable cover of olive groves as not yet been studied, it cannot be ruled out. The presence of fungal entomopathogens with endophytic capacity increases their ability to infect suitable hosts, therefore making their detection more likely to occur.

CONCLUSIONS

This work is the first report concerning the tillage effect on diversity and abundance of entomopathogenic fungi associated to *P. oleae*. The results obtained shown a minor effect of soil tillage on diversity and abundance of entomopathogens retrieved from *P. oleae* collected in the canopy of olive trees. Therefore, the probabilities of phylloplane inhabiting *P. oleae* insects becoming infected by entomopathogenic fungi seems to be the same, regardless of the tillage system. However, the exclusive occurrence of four entomopathogenic fungal species in no-tilled olive groves, suggested that the vegetable cover may serve as reservoir of entomopathogenic fungi, either by providing alternative hosts, or by providing a different way of prevalence in the environment. Although no significant differences were observed between the two soil management practices, the fact that a higher number of species, four of them exclusively to no-tilled groves, as well as a higher abundance, suggests that this type of soil management presents the most suitable condition to increase the probability of infection toward *P. oleae*. Seasonal climatic variations, namely of temperature and humidity, have been also shown to interfere with the fungal spores' dispersion, their germination, fungal growth and insect infection process. As fungal entomopathogens are engaged in a complex and yet fully undetermined array of interactions, with both plants and hosts, these results provide new insights about the population dynamics of entomopathogenic fungi in olive groves, and their relation with environment and agricultural factors. The findings described in this work will be important for devising cultural practices (specifically of tillage practices) aimed at augmenting the natural occurrence of entomopathogenic fungi on *P. oleae* population and, consequently, increasing the contribution of natural pest mortality in olive agroecosystems.

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Chapter 6

Plant-mediated effects on entomopathogenic fungi: how the olive tree influences fungal enemies of *Prays oleae*

Plant-mediated effects on entomopathogenic fungi: how the olive tree influences fungal enemies of *Prays oleae*

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ABSTRACT

Studies focusing the community of entomopathogenic fungi associated to one of a major pest of olive tree, *Prays oleae* Bern, revealed species-specificity to one of the three generations of this insect. For instance, the entomopathogens *Beauveria bassiana* and *Paecilomyces formosa* occurred almost exclusively in the phyllophagous (leaf) and carpophagous (fruit) generations, respectively. The main aim of this work was to evaluate the potential of plant-mediate effects on these entomopathogens, with an attempt to explain these differences on fungal occurrence. The nature (volatile or diffusible) of the compounds responsible for the detected effects was also assessed. For this, both fungi were challenged in divided or undivided Petri dishes by olive tree leaves, flowers or fruits and the outcome of interaction were assessed. Volatile composition of leaves and olives was evaluated and the major compounds were tested for their effects on both fungi. All the plant organs had a significant influence on both fungi. Olives were the most inhibitory to *B. bassiana* by reducing the germination (80%), growth (82%) and sporulation (88%), while for the *P. formosa* the most inhibitory effect was exert by leaves (reducing sporulation and viability in 172 and 37%, respectively). The olive effects were mostly result from the release of both volatile and diffusible compounds, whereas leaves effects were mainly caused by volatile compounds. The two volatiles produced by leaves and olives, (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate, were showed to affect both fungi being, however, the results no correlated with leaves and olives assays. This suggested that other compounds, together with those tested, may account for the observed effect. These results show an intricate relationship between plant organ, the compounds released by them and the entomopathogenic fungal species, broadening the available knowledge about fungal-plant interactions.

Keywords: Fungal entomopathogens, Olive, Fungal behaviour, Plant-mediated effects, Volatiles, Diffusible compounds

INTRODUCTION

It is known that plants can affect performance of natural enemies of herbivore insects, either positively or negatively, by modifying their features such as chemistry (volatiles, secondary compounds), resources (nectar, pollen) and/or morphology (trichomes, domatia) (Tkaczuk et al., 2007). However, the majority of the work focusing this issue has been mainly devoted to the study of the interactions between plants and predators/parasitoids of insect pests (Baverstock et al., 2005). By contrast, there has been little work done on the interactions between plants and other groups of insect natural enemies, such as entomopathogenic fungi. Cory and Ericsson (2010) provide an excellent background of plant-mediated effects on fungal entomopathogens and exhort researchers to examine the intricate interactions between entomopathogenic fungi, plants and insects. The understanding of such tritrophic interactions as well as of their effects may improve the efficacy of entomopathogenic fungi as biological control agents. According to the same authors, the plants could affect fungal entomopathogens either directly, by affecting conidia performance, contact rates or conidia persistence to the host insect, or indirectly, by altering insect condition and facilitating infection and contact rate with entomopathogenic fungi.

Good examples of direct effects are the allelochemicals compounds emitted by plant that exert some influence on entomophagous fungi. Some of the most studied are volatile compounds that have been shown to exert contradictory effects on fungal entomopathogens. For example, green leaf volatiles released from tobacco plants and volatiles released as a result of infestation with the tobacco aphid *Myzus nicotianae* have shown to inhibited conidial germination of the entomopathogen *Pandora neoaphidis* (Brown et al., 1995). By contrast, in a different system was verified that herbivore-induced plant volatiles increase conidiation of the entomopathogenic fungi *Neozygites tanajoae* (Hountondji et al., 2005). Other studies have reported no effects of plant volatile compounds either on the conidiation or on the *P. neoaphidis* fungus growth (Baverstock et al., 2005).

Other plant secondary metabolites can also affect germination, growth or infectivity of entomopathogenic fungi (Vega et al., 1997). Some compounds, most notably alkaloids (Lacey and Mercadier, 1998), phenolics (Lopez-Llorca and Olivares-Bernabéu, 1997; Lacey and Mercadier, 1998), glycoalkaloids (Poprawski et al., 2000), terpenoids (Poprawski and Jones, 2000) or isothiocyanates (Inyang et al., 1999a; Klingen et al., 2002) have shown generally to reduced spore germination, fungal growth, adhesion to host, and of their mortality and speed of kill. Other factors besides allelochemicals have been described as behaviour-modifiers of

entomopathogenic fungi. These include leaf surface topography or roughness (Inyang et al., 1998; Meekes et al., 2000), the degree and type of epicutilar wax formation (Inyang et al., 1998; Inyang et al., 1999b; Duetting et al., 2003), and the presence and type of exudates release by plants (Vega et al., 1997; Jaronski, 2007; Cory and Ericsson, 2010). These factors could directly affect spore retention, their survival and germination, or indirectly alter insect behaviour, which in turn will change rates of fungal acquisition (Inyang et al., 1998).

When studying the diversity of entomopathogenic fungi associated to one of a major pest of olive orchards, *Prays oleae* Bern, we have noticed that the entomopathogens *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Paecilomyces formosa* Sakag., May. Inoue & Tada ex Houbraken & Samson occurred almost exclusively in one of the three generations of this insect (Oliveira et al., 2012; Oliveira et al., accepted for publication). The first species occurred almost exclusively in the phyllophagous or leaf generation of *P. oleae* (1° flight), whereas the second one had occurred mainly in the carpophagous or fruit generation (3° flight). This lepidopteran presents an additional generation, the antophagous or bud and blooming generation (2° flight) that damage the olive tree flowers. One possible explanation to the exclusively occurrence of these entomopathogenic fungal species in a particular *P. oleae* generation could be related with the allelochemicals compounds produced by olive plant organs especially that of the leaves, flowers and fruits. Indeed, plants are thought to influence the behaviour of entomopathogens and use them as bodyguards against herbivores (Elliot et al., 2000). Such an interaction between the plant and the entomopathogenic fungus could either be numerical (altered population size of the fungus) or functional (enhanced efficacy of the fungus) (Elliot et al., 2000).

Therefore, the present work intends to evaluate the direct effect of olive plant organs (leaves, flowers and fruits) on *B. bassiana* and *P. formosa* growth, conidial germination (potential functional interaction) and sporulation (numerical interaction), and elucidate the nature of the plant organ compound (volatile and/or diffusible) that affects the behaviour of these entomopathogenic fungal species.

METHODS

Plant material

Olive plant organs (leaf, flower cluster, olive) were sampled from an orchard located in Mirandela, Bragança region (41°32'35.72''N; 7°07'27.17''W). The olives were about 50 years old, with a planting density of 7x7 m, of the Cobrançosa cultivar. Pruning was made every two-three years and no irrigation was done. The olive groves were managed through the integrated production guidelines (Malavolta and Perdikis, 2012). Leaves were hand-picked from the olive tree, and inspected for signs of disease or herbivore-caused damaged. Only fully healthy leaves were used for the assays. Olive flower buds were hand-picked when the first flowers where appearing - Code 60: First flowers open, of the BBCH (Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie) phenological scale (Sanz-Cortés et al., 2002). As for leaves, only completely healthy flower buds were selected for subsequent assays. Olives were collected from trees at a maturity index between 1 and 2, as defined by the method described by Hermoso et al. (1991).

Fungal cultures

Fungal strains of *Beauveria bassiana* (A39GF09) and *Paecilomyces formosa* (LC4.3GC10) used in this work were retrieved from the culture collection of the School of Agriculture of the Polytechnic Institute of Bragança (Oliveira et al., 2012).

Working cultures of fungi were prepared from frozen stock by transferring spores, with a bacteriological loop, from the aqueous glycerol solution previously thawed onto Petri dishes with Potato Dextrose Agar (PDA) medium. The dishes were incubated in the dark at 25°C for at least 7 days until colonies with multiple spores were produced. Spores were then collected by flooding fungal cultures with 2 mL of 0.02% (v/v) Tween 80 sterile solution. The number of spore per mL in the obtained spore suspension was counted in a Thoma counting chamber and further used as inoculum in the present study.

Experimental design

In order to assess the influence of the olive plant organ on *B. bassiana* and *P. formosa*, 5 µL of a spore suspension (10^6 spores per mL) of the tested fungus was placed on the surface of Petri dish (9 cm diameter) containing PDA medium. Three centimetres apart from the inoculum was placed the plant organ in question previously sterilized. The plant material was processed in the same day of its collection. After thoroughly washed in distillate water, the leaves, flowers and

fruits were surface sterilized throw sequential immersion in 70% (v/v) ethanol during 2 min, in 3-5% (v/v) sodium hypochlorite during 3 min, in 70% (v/v) ethanol during 1 min and three consecutive washes of 1 min in sterile distillate water. After removing the excess water, an incision in each plant organ was performed by using a scalpel, to mimic the damage caused by larvae of *P. oleae*. The plant material was then immediately transferred to Petri dishes. Controls were performed without the presence of the plant organ. In order to evaluate the chemical nature of the compounds (volatile or diffusible) involved in the interaction plant organ-fungus, the assay was performed in Petri dishes both undivided and divided into two sections. In this last case, the plant organ was placed in one section of the Petri dishes and the fungus in the other section. The *in vitro* effect of specific volatile compounds on *B. bassiana* and *P. formosa* was assess using a similar procedure, by using undivided plates. The volatiles tested were (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate, because of their high presence on the olive leaves. Both chemicals, (Z)-3-hexen-1-ol (>98%) and (Z)-3-hexenyl acetate (>98%), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Standards were diluted in polyethylene glycol (PEG) at two different concentrations: $1x10^{-3}$ (v/v) and 1ppm (v/v). Controls were performed with PEG. 5µL of both standards and PEG were directly placed onto the surface of the culture medium. Ten replicate of each combination (plant organ – fungi or volatile compound – fungi) were performed. The Petri dishes were sealed with parafilm and incubated at 25 ± 1°C in the dark. During interaction the germination, growth, sporulation and viability of both fungi were assessed.

Evaluated parameters

Germination – Spore germination was quantified 12 hours after inoculation of the PDA medium with the spore suspension (10^6 spores per mL). The percentage of germination was evaluated microscopically by counting the number of germinated and non-germinated spores, from a total of 300 spores per Petri dish. Only the spores with germ tubes longer than their width were considered to have germinated.

Radial Growth – Fungus growth assessment was achieved by measuring the four radial distances between the centre and the border of the colony, after 20 days of incubation.

Sporulation - The spores produced by each fungal strain, in each assay, was evaluated after 20 days of incubation. For this, a spore suspension was retrieved from fungus culture to 1 mL of an aqueous solution of Tween 80 (0.02%, v/v). The number of conidia was counted in a Thoma counting chamber. Results were expressed in spore per ml.

Spore viability – Twenty days after incubation, the viability of the spores produced by fungal strains in each assay was measured by quantifying the percentage of germination. For that purpose, 5µl of a spore suspension (10^6 spores per mL) retrieved from the spore suspension used to quantified sporulation was spread in a Petri dish, containing agar medium (15 g/L agar-agar). Five replicates of each assay were performed. After incubation, at 25±1°C in the dark for 12 h, the percentage of germination was evaluated by counting the number of germinated and non-germinated spores, from a total of 300 spores per Petri dish.

Volatile characterization of olives and leaves

Headspace-solid phase microextraction (HS-SPME) - For the characterization of volatile compounds, leaves and olive fruits were inspected for any possible cuts and wounds. Only complete healthy leaves and fruits were characterized. Samples were processed, at the most, 48 hours after being retrieved. They were stored at 4°C during transport and until processing. The HS-SPME was performed using a fiber coated with divinylbenzene/ carbonex/polydimethylsiloxane (DVB/CAR/PDMS), 50/30 µm, in 50 mL vials sealed with a polypropylene cap with PTFE/silicon septum. Volatiles from leaves (5 per analysis; n = 4) and fruits (1 fruit per analysis; n = 4) were exhaled by a ultrasound treatment at 40 °C (30 min and 1 h, for leaves and fruits respectively), followed by 1h of fiber adsorption at 40 °C.

Gas chromatography-mass spectrometry (GC-MS) conditions - Chromatographic analysis was performed using an Agilent 6890 series GC (Agilent, Avondale, PA, USA) coupled to a MS detector (Agilent 5973). Volatiles were separated using a 5% phenyl-methyl silicone (HP-5) bounded phase fused-silica capillary column (Hewlett-Packard, Pablo Alto, CA, USA, 33 m x 250 µm i.d., film thickness 0.25 µm), operating at 80 kPa column heads pressure, resulting in a flow of 1 mL min^{-1} at 40 °C. The oven temperature program was isothermal for 5 min at 40 °C, raised to 220 °C at a rate of 3 °C min^{-1} and maintained at 220 °C for 2 min. The transfer line to the mass spectrometer was maintained at 250 °C. Mass spectra were obtained by electronic impact at 70 eV, with a multiplier voltage of 2056 V, collecting data at a rate of 1 scan s^{-1} over the range 30 – 500. The constituents were identified by comparing the experimental spectra with spectra from NIST 98 data bank (NIST/EPA/NISH Mass Spectral Library, version 1.6, U.S.A.), and also by comparison of their GC Kovats index. For quantification purposes, the chromatographic peak areas were determined by a reconstructed full-scan chromatogram in the Single Ion Monitoring (SIM) using for each compound the correspondent base ion (m/z 100% intensity). The injection

port was in the splitless mode at 280 °C. Each identified compound is presented as peak areas and results expressed as relative abundance.

Data Analysis

All the data regarding radial growth, spore germination, number of spores and of their viability are express as percentage of variation against the control assay, and are present as the mean of ten independent experiments displaying the respective SE bars. The effect of each organ on fungal behaviour was evaluated by ANOVA. Once significant effects with a potency value of one were established, significant differences among groups were assessed using the Tukey test at $p<0.05$.

RESULTS

Interaction between plant - *Beauveria bassiana*

The results obtained showed that all the plant organs tested had a significant influence on *B. bassiana* (Fig. 1).

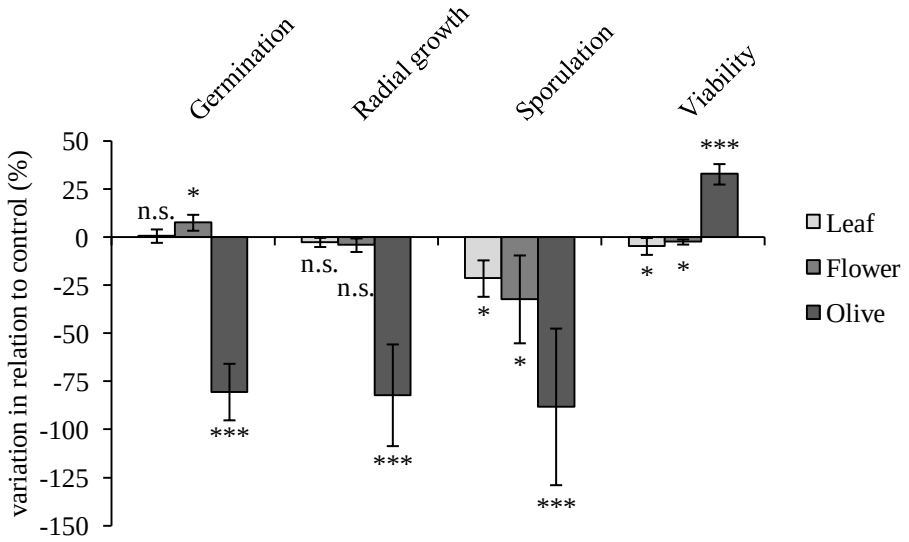

Figure 1 Variation relative to controls of conidial germination, radial growth, sporulation and viability of *Beauveria bassiana*, in the presence of different olive plant organs. Each value is expressed as mean ± SE (n=10). Asterisks indicates values that differ significantly from controls at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.s. – not significant.

The fruits showed to exert a greater influence comparatively to the other plant organs by inhibiting significantly the germination (80.4%), growth (82.0%) and sporulation (88.1%) of *B. bassiana*, when compared to the control. These percentages of inhibition were found substantially higher in undivided plates than in divided plates (Fig. 2C) which suggested that the association of both types of compounds (volatile and diffusible) is probably more responsible for this effect than volatiles compounds alone. The viability of spores on the presence of olives was, by contrast, significantly higher (32.9%) than in control (Fig. 1). This response was probably resulted from the combined effects of both volatile and diffusible compounds produced by olives, since the highest value of fungus viability was found in undivided plates (Fig. 2C).

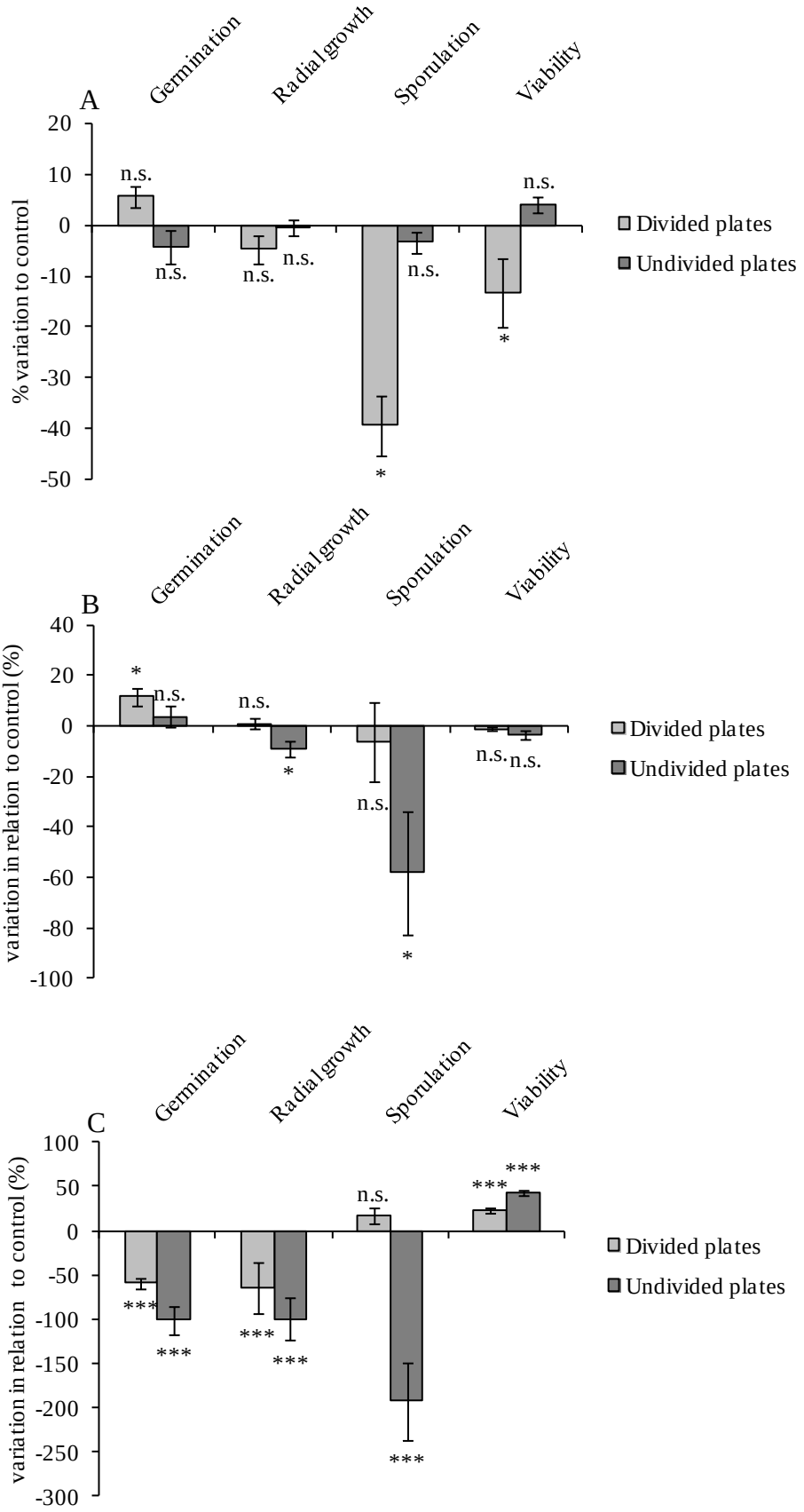

Figure 2 Variation of the evaluated parameters of *B. bassiana*, in medium in the presence of leaves (A), flowers (B) and olives (C) when inoculated in divided or undivided plates. Each value is expressed as mean ± SE (n=10). Asterisks indicates values that differ significantly from controls at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.s. – not significant.

The flowers inhibited significantly the sporulation (32.2%) and the viability of *B. bassiana* (2.3%) when compared to control (Fig. 1). These inhibitory effects are related to the diffusible compounds as indicated by the lowest values of fungus sporulation and viability of undivided plate assays (Fig. 2B). Regarding germination, there was a significant increase (7.7%) in relation to control (Fig. 1), caused by the release of volatiles from flowers, as it was in divided plates that a higher increase (11.6%) of this parameter occurred (Fig 2B). Although radial growth was not significantly influenced by flowers (Fig. 1), the detected decrease was likely caused by diffusible compounds (8.9%) (Fig. 2B).

Leaves only inhibited significantly the sporulation (21.4%) and the viability of fungi (4.6%) when compared to control (Fig. 1). In both cases, leaves appeared to suppress the sporulation and viability of fungus mainly via the volatile compounds (Fig. 2A), that caused 39.4% and 13.4% of reduction, for each parameter, respectively. Germination of *B. bassiana* was not significantly influenced by leaves (Fig. 1), but the slight increase (0.7%) detected in relation to control was caused by volatile compounds (5.7% of increase), while the combination with diffusible compounds inhibited this parameter (4.3%) (Fig. 2A).

Taken together the results indicate that the combination of volatile and diffusible metabolites have shown bigger reducing effect than volatile compounds alone, in the olives and flowers, whereas in the leaves it was the volatile compounds with the greatest inhibitory effect.

Interaction between plant - *Paecilomyces formosa*

The results of the interaction between *P. formosa* and plant showed that all organs are able to influence *P. formosa* (Fig. 3). Leaves exerted considerable more influence than flowers and olives, causing significant decrease of sporulation (172.0%) and of fungus viability (36.9%) when compared to control.

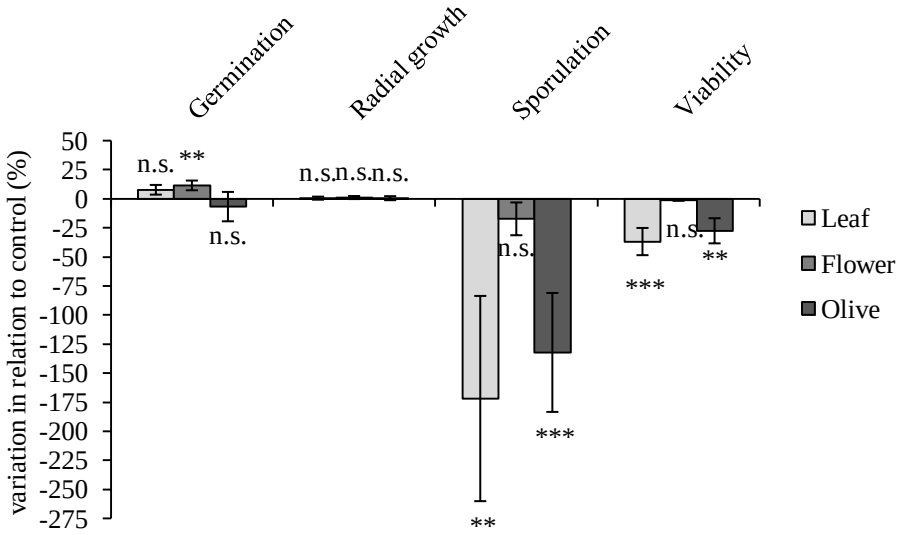

Figure 3 Variation relative to controls of conidial germination, radial growth, sporulation and viability of *P. formosa*, in the presence of different olive plant organs. Each value is expressed as mean ± SE (n=10). Asterisks indicates values that differ significantly from controls at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.s. – not significant.

The reduction in sporulation by leaves is mainly due to the release of diffusible compounds. In fact, only in undivided plates the leaves were showed to significantly inhibit fungus sporulation (347.9%) when compared to control (Fig. 4A). The viability of spores was affected by leaves differently, being volatiles compounds (divided plates) able to cause higher decrease (59.8%) than diffusible compounds (undivided plates) (14.1%), leading to the assumption that they are able to counteract, partially the inhibitory effects of volatiles. Leaves also caused an increase of germination (7.6 %) and radial growth (0.4 %) when compared to control, although variations were not significant (Fig. 3). Those variations were probably mainly caused by the combination of volatile and diffusible compounds, as increase of germination and radial growth was higher on undivided (14.9%) than on divided (0.2%) plates (Fig. 4A).

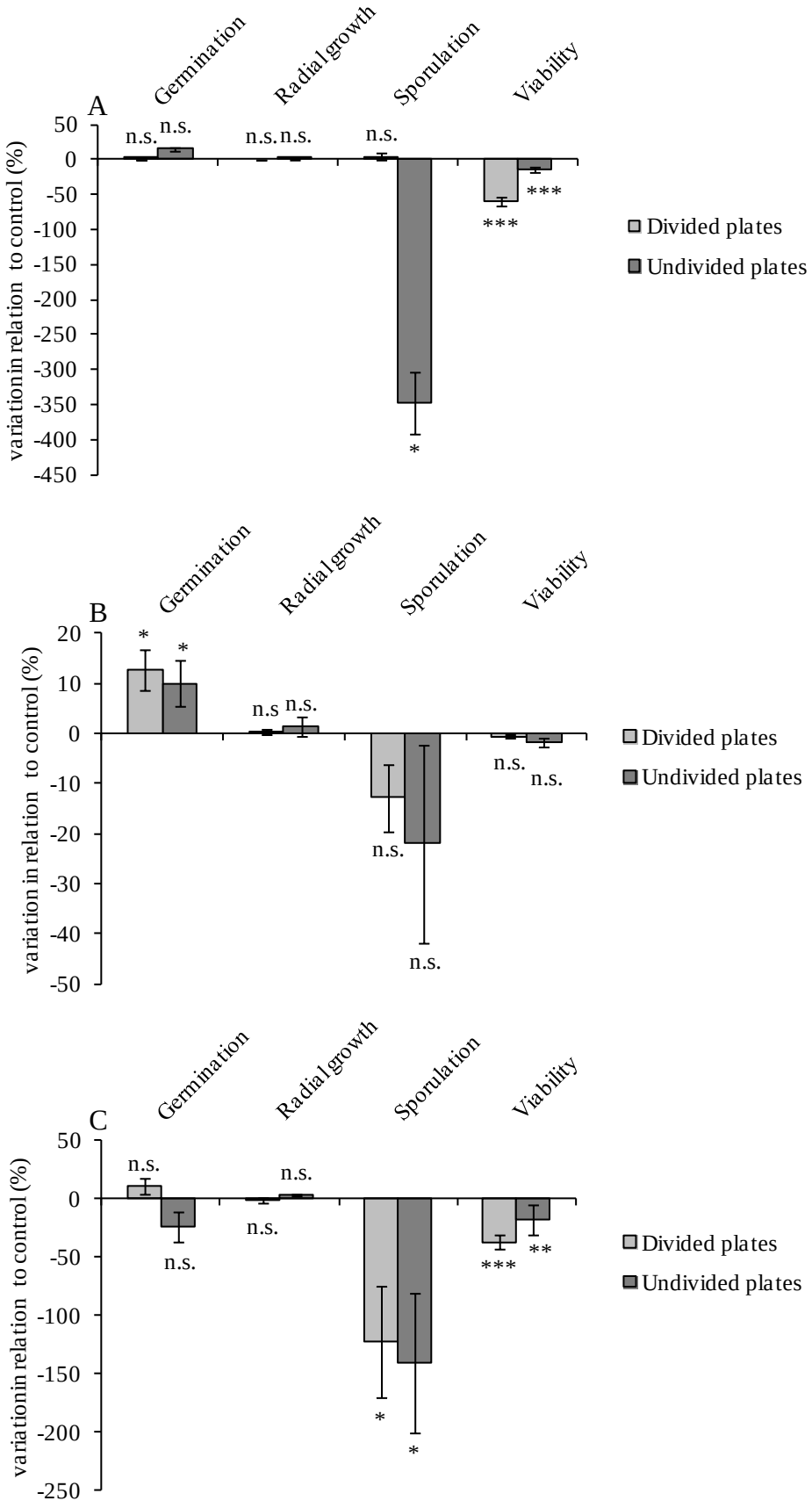

Figure 4 Variation of the evaluated parameters of *P. formosa,* in medium in the presence of leaves (A), flowers (B) and olives (C) when inoculated in divided or undivided plates. Each value is expressed as mean ± SE (n=10). Asterisks indicates values that differ significantly from controls at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.s. – not significant.

Olives led to significant variations in sporulation and viability, with a decrease of both parameters in 132.3% and 24.7%, respectively, when compared to control (Fig. 3). The reduction of the sporulation in the presence of olives was probably caused by the interaction of both volatiles and diffusible compounds, as similar inhibitory values were observed in divided (123.1%) and undivided (141.6%) plates (Fig. 4C). The decrease of fungus viability by olives seemed to be more influenced by volatile compounds (divided plates), which caused a more significant decrease of this parameter (37.4%) than diffusible compounds (undivided plates) (17.9%), that appear to partially neutralize the inhibitory effects of volatiles (Fig. 4C). No significant differences in radial growth and germination of *P. formosa* were observed between plates with olives and control (Fig. 3). However, the slightly differences found on those parameters were noticed to be higher on undivided than divided plates suggesting a greater involvement of diffusible compounds than volatile compounds in the response of fungi to olives (Fig. 4C).

The flowers showed to exert less influence on *P. formosa* than leaves and olives. The flowers were only found to influence significantly the germination, by increasing it in 11.4% when compared to control (Fig. 3). The stimulatory compounds released by flowers were most likely volatile than diffusible as indicated by the higher increase of germination (12.7%) in divided than in undivided plates (10.0%) (Fig. 4B). The presence of flowers also resulted in an increase of radial growth (0.9%) and in a decrease of sporulation (17.4%) and viability (1.2%) of *P. formosa* when compared to control (Fig. 3). Although the differences are not statistically significant, these effects were probably caused by the release of volatile but mostly due to the interaction of those compounds with diffusible compounds from flowers. In fact, variations of these fungal parameters in relation to the control were observed to be higher in undivided than in divided plates (Fig. 4B).

Volatile composition of leaves and olives

The results from *in vitro* studies indicated that volatiles compounds of leaves and olives were inhibitory to *B. bassiana* and *P. formosa*. In order to identify which volatile compound was probably responsible for this effect, the volatile composition of both leaves and olives of the Cobrançosa cultivar was analysed. The volatile profile from the leaves and olives revealed high variability both in the qualitative and quantitative fractions. The leaves were showed greater amounts (53 times more) and variability (in total 22 compounds) on volatile compounds than fruits, where only 12 compounds were detected. In total, five classes of chemical compounds

were detected, being the most important ones esters and alcohols, in leaves, and aldehydes and terpenes in olives. Sesquiterpenes were also detected in both plant organs.

In leaves, and regarding alcohols, four different compounds were detected (Fig. 5A), being (Z)-3-hexen-1-ol the most important (94.5%), and in lower amounts 3-methyl-1-butanol, 1-hexanol and 1-octanol. Regarding esters (Fig. 5B), nine compounds were detected, with (Z)-3-hexenyl acetate representing the major part of this class of compounds (65.9%). Other important compounds included (Z)-3-hexenoic acid methyl ester (18.9%), butanoic acid methyl ester (3.9%) and 3 methyl butanoic acid methyl ester (3.4%).

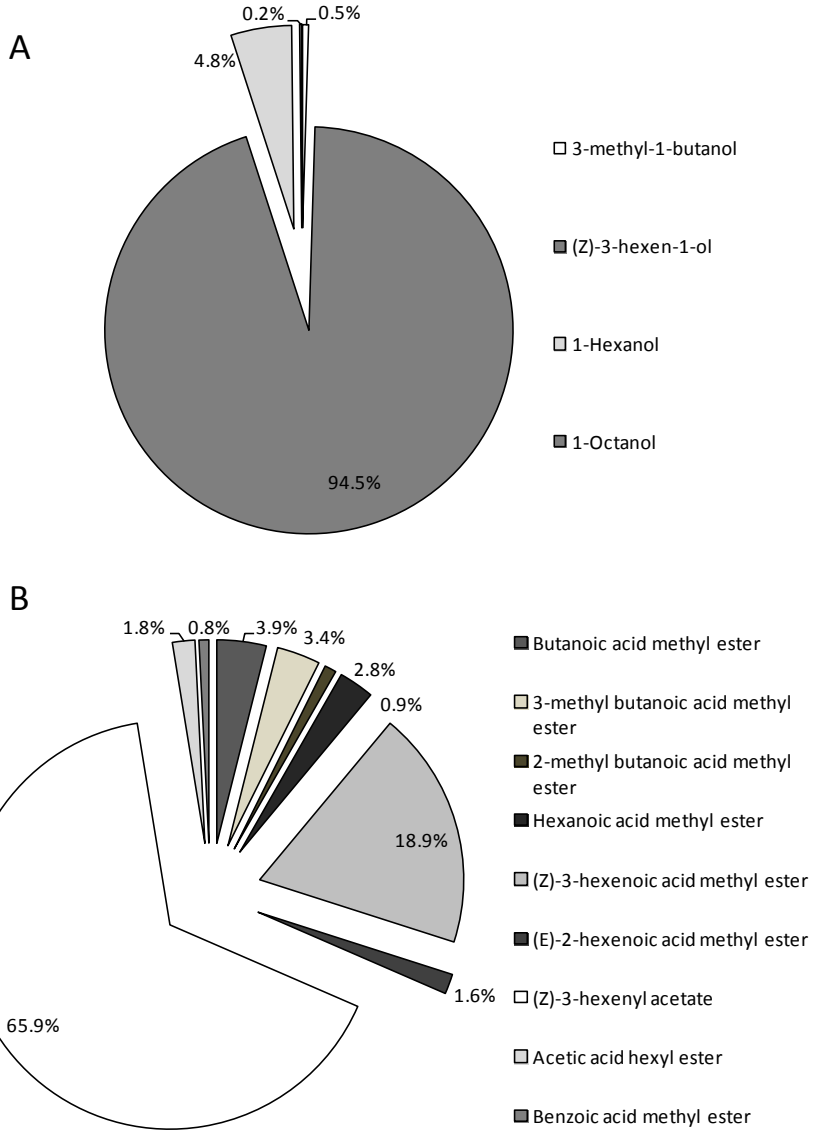

Figure 5 Alcohols (A) and esters (B) identified in leaves of Cv. Cobrançosa. Values are presented as relative abundance.

In fruits, major compounds detected were aldehydes, namely nonanal (36.8%) and octanal (20.0%) (Fig. 6A); and terpenes, especially limonene (78.2%) (Fig. 6B). Furthermore, the alcohol (Z)-3-hexen-1-ol was also present is considerable amounts (4.7% of the total).

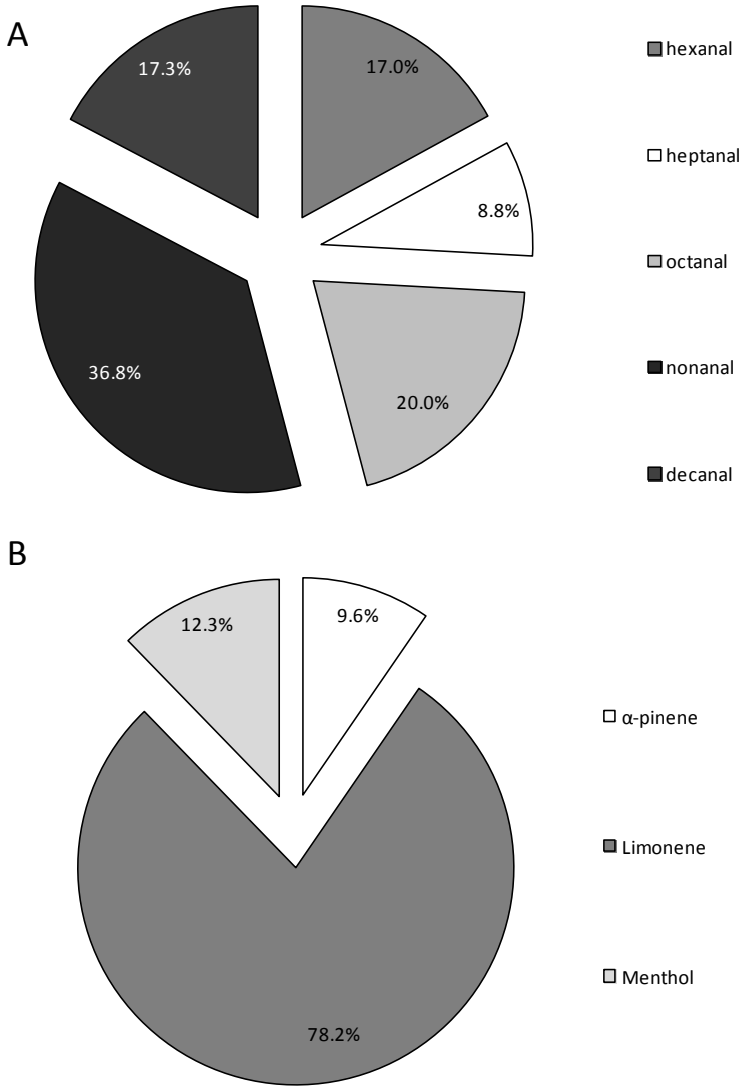

Figure 6 Aldehydes (A) and terpenes (B) identified in leaves of Cv. Cobrançosa. Values are presented as relative abundance.

Effect of selected volatiles on fungal behaviour

As we detected, in the assessment of volatile composition of leaves and fruits, that the former produced much higher amount of these compounds, being the most representative (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate, we used standards of both compounds to evaluate their influence on *B. bassiana* and *P. formosa*.

Overall results showed that the volatile compounds tested influence significantly both fungal species, regarding fungus sporulation ($F_{2,59}$ 27.347; $p< 0.001$), viability ($F_{2,59}$ 4.315; $p<0.05$) and radial growth ($F_{2,59}$ 4.310; $p< 0.05$). By contrast, the interaction between volatile compounds and its concentration, didn't result in significant influence on entomopathogenic fungal behaviour ($p>0.05$).

Regarding *B. bassiana*, a significant effect of the compound used was detected on radial growth ($F_{2,29}$ 3.496; $p< 0.05$), sporulation ($F_{2,29}$ 19.031; $p< 0.001$) and viability ($F_{2,29}$ 50.805; $p< 0.001$), while no significant effect was detected on germination ($p> 0.05$). The concentration factor alone didn't result in significant differences, and the interaction between compound and concentration only showed significant influence on radial growth ($F_{2,29}$ 3.914; $p< 0.05$). The overall results also indicated that the effect of both volatiles on *B. bassiana* was not directly proportional to the concentration used of these compounds. Both (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate used alone reduce significantly fungus growth (in average 5.1% and 5.9%, respectively; Fig. 7B), and increase significantly germination (in average 13.9% and 24.6%, respectively; Fig. 7A) and viability (in average 11.45 and 10.3%, respectively; Fig. 7D) in relation to the control, at any of the concentrations tested. When used alone, both volatiles compounds were also showed to reduce *B. bassiana* sporulation when compared to control, but without significant differences (Fig. 7C). The combined used of volatile compounds produced significant increases on *B. bassiana* germination (17.0%, Fig. 7A) and growth (2.9%; Fig. 7B) relative to controls only at the lowest concentration tested. By contrast, spore's viability was increase significantly compared to controls in the presence of both volatile compounds at any of the concentrations tested (29.5% for 1 x 10^{-3} and 22.8% for 1ppm; Fig. 7D).

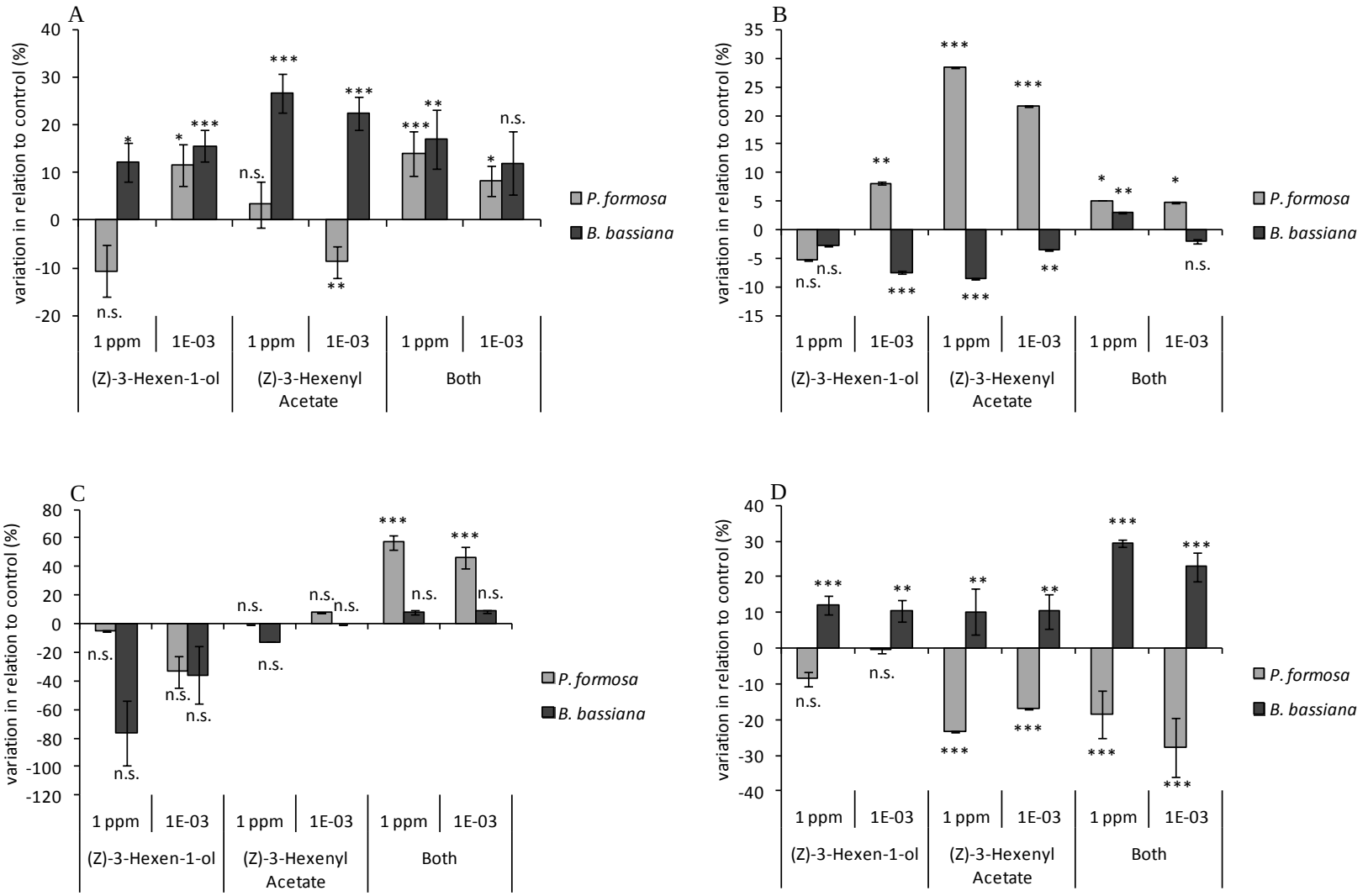

Figure 7 Variation relative to controls of conidial germination (A), radial growth (B), sporulation (C) and viability (D) of *B. bassiana* and *P. formosa*, in the presence of (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate either alone or in combination. Each value is expressed as mean ± SE (n=10). Asterisks indicates values that differ significantly from controls at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n.s. – not significant

Regarding *P. formosa*, a significant effect of the compound used was detected on all evaluated parameters, namely germination ($F_{2,29}$ 10.941, p< 0.001), radial growth ($F_{2,29}$ 65.172, p< 0.001), sporulation($F_{2,29}$ 71.705, p< 0.001) and viability ($F_{2,29}$ 5.583, p< 0.05). The concentration factor alone resulted in significant effect at sporulation level ($F_{2,29}$ 5.004, p< 0.05), and the interaction between compound and concentration showed to have significant influence on germination ($F_{2,29}$ 14.916, p< 0.001) and radial growth ($F_{2,29}$ 12.224, p< 0.001). The overall results also indicated that the effect of both volatile compounds on *P. formosa* was not concentration dependent. The (Z)-3-hexen-1-ol and (Z)-3-hexenyl used alone or combined reduced the viability (in average, 4.5%, 20.2% and 23.1%, respectively) when compared to control in either of the tested concentrations (Fig. 7D). Germination and radial growth of *P. formosa* were affected in different ways, by each of the compounds. (Z)-3-hexen-1-ol, at the lowest concentration, caused a decrease of both parameters (10.6% and 5.2%, respectively), and at higher concentration increase both parameters (11.6% and 8.1%, respectively) in comparison to control (Figs. 7A and 7B). (Z)-3-hexenyl increase significantly *P. formosa* germination (in average 3.3%, Fig. 7A) and growth (in average 25.1 %; Fig. 7B) in relation to control at any of the concentrations tested, with the exception of germination in the presence of 1ppm of (Z)-3-hexenyl, that resulted in a significant decrease (8.7%) (Fig. 7A). The combined used of (Z)-3-hexen-1-ol and (Z)-3-hexenyl resulted in a significant increase of *P. formosa* germination (in average 11.1 %; Fig. 7A) and radial growth (in average 4.9 %; Fig. 7B) in relation to control at any of the concentrations tested, being however the highest concentrations presenting smaller values than lowest concentration.

The sporulation of *P. formosa* colonies (Fig. 7C) was effected differently by (Z)-3-hexen-1-ol and (Z)-3-hexenyl. When compared to control, the former caused a reduction of sporulation in either concentration tested (5.3% and 33.3%, respectively for 1ppm and $1x10^{-3}$), while the latter caused an increase of 8.1%, at the highest concentration. When used in combination, the volatile compounds increased significantly the sporulation of *P. formosa*, in either concentration (56.9% and 46.3%, respectively for 1ppm and $1x10^{-3}$, in relation to control).

DISCUSSION

The olive moth, *P. oleae*, is one of the most serious pests of olives in the Mediterranean basin (Ramos et al., 1998). This species has three generations per year, attacking different organs in the olive trees, namely leaves (first generation), flower buds (second generation) and olives (third generation). Previous studies undertaken by us under field conditions have been shown that *P. oleae* larvae and pupae of the second (carpophagous) and third (phyllophagous) generations were most exclusively infected by the entomopathogenic fungi *P. formosa* and *B. bassiana*, respectively (Oliveira et al., 2012; Oliveira et al., accepted for publication). The present study intends to assess if these variations on entomopathogenic fungi occurrence through *P. oleae* generations may somehow be mediated by compounds (volatile or diffusible) released by the different olive plant organs (olives, flowers and fruits). Therefore, *P. formosa* and *B. bassiana* were challenged in divided and undivided Petri dishes by leaves, flowers or fruits of olive tree and during interaction the germination, growth, sporulation and viability of fungi were assessed.

Effect of leaves on fungi

The presence of leaves showed to reduce significantly sporulation and viability of *B. bassiana* and *P. formosa* in relation to control. By contrast, germination and growth of both fungi in the presence of leaves were not statistically different from control. Loss of conidia viability has already been reported for *B. bassiana* exposed to different extracts of neem (*Azadirachta indica*) (Depieri et al., 2005). These authors showed significant inhibition caused by emulsible oil and aqueous extracts of neem leaves, on the viability of *B. bassiana*. Results also showed that *P. formosa* was more negatively affected by leaves than *B. bassiana*. In fact, the reduction on sporulation and viability by leaves in comparison to control were, in average, 172% and 37%, respectively for *P. formosa* and only 21% and 5%, respectively, for *B. bassiana*. Therefore, although there was statistically negative effects of leaves on sporulation and viability of *B. bassiana*, this result indicate that the release of compounds from leaves allow a greater development of this fungus species in detriment of others species like *P. formosa*. This could partially explain the high occurrence of *B. bassiana* in the phyllophagous generation observed in our previous study (Oliveira et al., 2012).

The results from the divided and undivided plates indicate that reduction of *P. formosa* sporulation and viability by leaves was mainly via the release of diffusible and volatiles compounds, respectively; whereas in *B. bassiana* the reduction on both parameters were mainly

via the release of volatile compounds from leaves. Previous studies have also revealed the ability of plant volatiles compounds to inhibit entomopathogenic fungi sporulation (Hountondji et al. 2005). However, some of these studies reported contradictory effects by showing either inhibitory or no effect of plant volatiles on fungal sporulation. The effect of plant volatiles was showed to be isolate-dependent which could in part explain these contradictory results (Hountondji et al., 2005; 2006). For instances, the production of conidia of an isolate of the entomopathogenic fungus *Neozygites tanajoae* was found to be consistently higher (37%) when exposed to methyl salicylate than when not exposed to it, while, in another isolate of the same species, no significant differences were observed, between the presence or absence of methyl salicylate (Hountondji et al., 2006). In a different study, Baverstock et al. (2005) verified that *in vivo* sporulation of *Pandora neoaphidis* on *Acyrthosiphon pisum* was not affected by volatiles emitted by damaged plant of *Vicia faba*, when compared with volatiles of undamaged plants.

The volatile composition of the olive tree leaves reveals high amounts of esters and alcohols, which could account for the observed inhibition of fungal sporulation and viability. We detected high levels of six-carbon alcohols [(Z)-3-hexen-1-ol and 1-hexanol] and six carbon esters [e.g. (Z)-3-hexenyl acetate and (Z)-3-hexenoic acid methyl ester], that are known to be green leaf volatiles (GLVs) (Matsui et al., 2012). These compounds are described to interact with fungi (Brown et al., 1995; Arimura et al., 2001, Hountondji et al., 2009) and have already been linked to a decrease of fungal sporulation (Hountondji et al. (2005). These authors demonstrated that a range of C6 compounds alcohols present in high levels in clean cassava plants, were responsible for reduction in sporulation of *Neozygites tanajoae*.

The *in vitro* assays using the volatile standards (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate, the key volatiles component of olive tree leaves identified in the present study, revealed contradictory results from those obtained in the *in vitro* assays using leaves. The comparison of both assays indicated that only radial growth and sporulation of *B. bassiana*, and viability of *P. formosa* present a similar trend. In both assays these fungal parameters were reduced in relation to the respective controls. This suggests that (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate were not the main cause of the leaves effects on *B. bassiana* and *P. formosa*. Although the tested volatiles are the one found in higher abundance in leaves, it appears to be other compounds that, together with those tested, result in the observed effect from leaves. In fact, we detected 22 different volatiles in olives from Cv. Cobrançosa, and some of them should also be involved in this interaction. That could be the case of terpenes, which were found in low quantity (0.2%), but

that are showed to have activity as plant-signalling compounds (Halitschke et al. 2008) and, some of them, antifungal properties (Shai et al., 2008). Furthermore, the presence of several compounds emitted by leaves can result in several effects, as synergistic (Tasin et al., 2007), redundant or antagonistic (Linn et al., 2005). This is corroborate by our results since contradictory effects of (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate on *B. bassiana* and *P. formosa* were observed when these volatiles were used alone or combined. For instances, both (Z)-3-hexen-1-ol and (Z)-3-hexenyl acetate used alone have showed to reduce significantly *B. bassiana* growth, but their combined used increase significantly it, in relation to control. The absence of correlation between assays using volatile standards and leaves may have been a result of either the volatile concentration of the compounds tested not being the same as occurs naturally in the leaves. Another noteworthy fact is that the response of the fungus was not concentration dependent. Although it could be expected an increase of inhibition with increasing concentration, previous works also present similar results. Individual volatiles, tested at different concentrations, presented similar effects on the growth of *Aspergillus parasiticus*, when tested at high or low concentrations (Zeringue and Bhatnagar, 1994).

The inhibition of *P. formosa* sporulation caused by the release of diffusible compounds from leaves may be related to their composition on phenolics and fatty acids. Chemical analysis of olive leaves from Cv. Cobrançosa revealed high amounts on total phenolic compounds (about 36051 mg/kg of olive leaf lyophilized extract) (Pereira et al., 2007) and the presence of fatty acids as already been described (Bianchi et al., 1992a). Both compounds have already been described as possessing the ability to reduce the production of fungal spores (Russo and Pappelis, 1993; Boguś et al., 2012; Dambolena et al., 2012).

Cuticular waxes of leaves may additionally take part on the reduction of *P. formosa* sporulation. The main components of epicuticular waxes of olive leaves are triterpenic acids (oleanoic and betulinic), as well as sitosterol (Bianchi et al., 1992a). Oleanoic acid has already been described as having antifungal activity, when isolated from *Calendula officinalis* (Favel et al., 1994). Betulinic acid has been already tested as an antifungal compound, with results showing low minimum inhibitory concentration (< 47 µg/mL) against several fungi (Chaudhuri et al., 2004; Kuiate et al., 2007; Shai et al., 2008).

Effect of flowers on fungi

The presence of flowers significantly inhibited both sporulation and viability of *B. bassiana*, and increase significantly *P. formosa* germination, when compared to the respective control. The results from the divided plate's assays indicated that the effects observed on *B. bassiana* are most probably caused by diffusible compounds released by flowers. Although chemical composition of olive flowers is not available, other reports, regarding plants of the same family (Oleaceae), show that it includes phenolic compounds (Kunhachan et al., 2012). One of the phenolic compounds present in olive flowers is oleuropein (Malik and Bradford, 2006) which had shown antifungal activity (Korukluoglu et al., 2008). The significant increase of *P. formosa* germination was most likely caused by volatiles released from flowers. Although, and as referred before, volatiles are known to inhibit germination, an increase of germination of entomopathogenic fungal species exposed to volatiles has already been reported (Baverstock et al., 2005). Furthermore, germination of *Beauveria bassiana* increased slightly after exposure to *Impatiens wallerana* leaf disks, although significant differences were not detected (Ugine et al., 2007).

Effect of olives on fungi

Significant inhibitory effects of olives were detected on germination, growth and sporulation of *B. bassiana*, and on sporulation and viability of *P. formosa*, when compared to control. The olives have showed a greater inhibitory effect against *B. bassiana* than to *P. formosa*. This result may in part explain the lower abundance of *B. bassiana* on the carpophagous generation of *P. oleae*, which feeds on olives, detected in a previous study (Oliveira et al., 2012).

The results of the undivided and divided plates assays suggested that this inhibitory response was probably resulted from the combined effects of both volatile and diffusible compounds produced by olives. An exception was the decrease of *P. formosa* viability by olives which seemed to be more influenced by volatile compounds. Although producing considerable less volatile compounds than leaves, the major components of volatile fraction of fruits are aldehydes. These compounds, especially short-chain aldehydes, are used by plant as defence towards infection of plant-pathogenic fungi (Vaughn and Gardener, 1993; Shukla et al., 2009), which can in part explain the reduction of germination detected in divided plates with olives. Other works showed similarly inhibition of germination (Vega et al., 1997; Brown et al., 1995;

Inyang et al., 1999a; Klingen et al., 2002) and sporulation (Hountondji et al. 2005) by volatile compounds in several entomopathogenic fungi.

The inhibitory effects of olives against both *B. bassiana* and *P. formosa* could also be caused by diffusible compounds release by those organs. Phenolic could be one of those compounds. Present in olives (Vinha et al., 2005), phenolic compounds have been referred as inhibitors of entomopathogenic fungal germination (Vega et al., 1997), sporulation (Russo & Pappelis, 1993; Dambolena et al., 2012) and growth (Lopez-Llorca & Olivares-Bernabéu, 1997; Lacey and Mercadier; 1998). For instance, tannic acid was showed to reduced germination of *Isaria fumosorosea* (= *Paecilomyces fumosoroseus*) blastospores to 46% and 7%, at concentrations of 500 ppm and 1000 ppm, respectively (Vega et al., 1997). The inhibitory effect of this phenolic compound was further confirmed by Lacey and Mercadier (1998) that recorded a decrease of about 50% of the germination of aerial conidia and blastospores of *I. fumosorosea.* Catechol is another phenolic compound present in olive oils (Brenes et al., 2004), and therefore in olives, that has been described as a potent inhibitor of germination of *I. fumosorosea* blastospores (reduced the germination to 55% at 500 ppm, and to 7% of germinated blastospores at 1000 ppm) (Vega et al., 1997). Other phenolics present in olive have been described as inhibitors of conidial germination of several fungi, especially plant-pathogenic species. Quercetin 3-methyl ether and its glucosides (4'-O-glucoside and 7-O-glucoside) were able to completely reduce the germination of *Neurospora crassa* (Parvez et al., 2004). Gallic acid reduced the germination of the phytopathogenic fungi *Magnaporthe grisea*, from 97.3% to 67%, and, in a more considerable way, the formation of appressoria to 5.3% (Ahn et al., 2005).

Cuticular waxes of olives may also be responsible for the inhibition of the germination of both *B. bassiana* and *P. formosa*. The major components of olive fruit surface wax are oleanoic acid and triacylglycerols (Bianchi et al., 1992b). The first compound is described to be inhibitory to fungal growth (Favel et al., 1994). Although nothing is reported about the antifungal activity of triacylglcerols, the individual fatty acids that compose them have been described to displayed antifungal characteristics (Pohl et al., 2011). In fact, short chain fatty acids are able to prevent conidia germination by blocking the uptake of phosphate and thiamine (Kerwin, 1987). Some fatty acids with proved ability to reduce germination include caprylic and capric acid (Barnes and Moore, 1997) or (Z)-9-heptadecenoic acid (Carballeira, 2008).

Another interesting fact was noticed in the assay with olives: higher reduction of sporulation was detected in undivided plates, where the detected inhibitory effect of olives on

viability was less marked. In fact, an increase of viability would be expected from conidia collected from low-sporulating colonies, which indeed occurred in undivided plates with olives. This is due to the fact that some compounds, known as self-inhibitors, which are produced during sporulation (Macko, 1981), have the ability to reduce the germination of fungi, to ensure that conidia germinate under favorable conditions (Hegde and Kolattukudy, 1997). Hence, in the assays where sporulation was higher, an increased production of those compounds would occur, leading to a decrease on the viability of conidia.

CONCLUSION

As far as we know, this is the first work assessing the effect of olive tree organs on entomopathogenic fungi, exploring at the same time the nature of the compounds responsible for the detected effects. Results showed that all organs (leaves, flowers and olive) influence fungal germination, growth, sporulation and viability in different ways. In addition, both volatile compounds, as well as diffusible compounds are responsible for the detected effects on entomopathogenic fungi making these chemicals unlike to be involved in the recruitment or maintain of specific fungal species. The findings reported in this work show that entomopathogenic fungi are strongly influenced by the host plant, and, in particular, by different plant organs. Overall results indicated that olives were the more inhibitory to *B. bassiana* and for the *P. formosa* the same effect was noticed in the presence of leaves. This fact is helpful to explain occurrence of entomopathogenic fungi in the olive orchard ecosystems, increasing the knowledge of fungal-plant interaction. The understanding of such plant-mediated interactions would be very helpful when planning or assessing a biocontrol program.

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Chapter 7

Final remarks and future perspectives

FINAL REMARKS AND FUTURE PERSPECTIVES

The increasing pressure of the public, demanding healthier products, the awareness of both farmers and industry about deleterious effects of chemical pesticides, combined with restrictive laws about application of such products, some of them causers of insect resistance, led to a rise on research alternative control measures, namely entomopathogenic fungi. Although research has provided a large amount of data about this type of fungi, much is still unknown. Fungal behaviour is affected by numerous factors, both biotic and abiotic, and their presence, abundance and performance in a given ecosystem should be evaluated, before any control measures based in this type of fungi is taken. Furthermore, as fungal virulence characteristics are strain-specific, a large number of isolates should be characterized, in order to achieve maximum performance against pests. With all this into account, this work intended to identify fungal entomopathogens associated to one of the key pest of olive, *Prays oleae* Bern., evaluating the effect of selected factors on their abundance and behaviour. Furthermore, the virulence of four of the isolates was screened against another lepidopteran, *Cydia splendana* Hübner.

The first known survey of fungi associated to *P. oleae*, in larvae collected in the three generations that this lepidopteran presents throughout a year, showed a great number of species associated to this pest. As much as 43 fungal species, belonging to 24 genera were identified, showing that the methodology used for the collection of fungal isolates is appropriate. The same can be said about the use of the ITS region for fungal identification. The use of multi-locus approach for fungal identification should be taken in consideration whenever possible for this type of surveys. However, due to reduced amplification efforts and number of probable identifications achieved, the ITS region is still the most reliable barcode region to perform fungal identification, especially when the samples are completely unknown.

The identification of fungal species showed different values of abundance and diversity, depending on which generation the sampling of larvae and pupae was performed. Two main factors may be responsible for these results: i) the climatic conditions, known to affect fungi; and ii) the moth life cycle, attacking different olive tree organs in each of the generations, that leads to different exposure to fungal propagules and to different types of compounds emitted by those plant organs. Although the majority of the identified species have been described as phytopathogenic, this is the first report of entomopathogenic fungi associated to *P. oleae*, with special relevance to *Beauveria bassiana*.

As the most abundant EF found was *B. bassiana*, preliminarily tests were performed to evaluate their virulence to another lepidopteran, *Cydia splendana*. This first report of the infection of this chestnut pest by entomopathogenic fungi allowed two major observations: by one hand, *C. splendana* showed to be very susceptible to this entomopathogenic fungus; by other hand, although concentration-dependent mortalities were observed in four tested strains, results showed variation of virulence between the tested isolates. The good results achieved with these fungal isolates show that they possess considerable virulence towards lepidopteran species. Furthermore, in order to achieve improved results when using entomopathogenic fungi, the detected variations of virulence suggest that several strains should be tested, in order to select the most suitable one.

Since several environmental factors affect the occurrence and distribution of insect pathogenic fungi, the effect of soil management (tillage or no-tillage) was evaluated and correlated with the occurrence and distribution of entomopathogenic fungi associated to *P. oleae* in an olive grove ecosystem. Tillage is still a normal practice performed by the olive growers, which is known to affect entomopathogenic fungi. However, the majority of the studies focusing this issue reported only the effect of tillage practice on the entomopathogenic fungal community of the soil. The evaluation of the effect of soil tillage on entomopathogenic fungi, at canopy level, will provide a more accurate measurement of how this cultural practice influences fungal infection of host insects. The observed results showed that, although differences were observed between tillage and no-tillage, no significant differences were observed. Therefore, insects inhabiting olive tree canopy of tilled and no-tilled orchards will have the same probability to be infected by entomopathogenic fungi. However, results showed that some entomopathogenic species were only present in orchards where soil was kept undisturbed. This could be either due to the presence of alternative hosts in the vegetable cover or by providing a different way of prevalence in the environment. Furthermore, climatic condition could have also influenced the results, as it is known that they are able to influence fungal spores' dispersion, their germination, fungal growth and insect infection process

Increasing evidence suggests that plant-mediate effects can also have impact on the entomopathogenic fungal community. In the present study, the observed variations on entomopathogenic fungal diversity and occurrence throughout the three *P. oleae* generations suggested that these fungi can be manipulated by the olive tree. In fact, the entomopathogens *B. bassiana* and *Paecilomyces formosa* were occurred almost exclusively in the phyllophagous (leaf)

and carpophagous (fruit) generations of *P. oleae*, respectively. Therefore, in order to evaluate how the different olive tree organs (leaves, flowers and fruits) affect entomopathogenic fungi, both *B. bassiana* and *P. formosa* were grown in the presence of leaves, flowers or olives, in either divided or undivided Petri dishes. All plant organs have shown to influence both fungal species. *Beauveria bassiana* was more inhibited by olives while *P. formosa* was more inhibited by leaves. These inhibitory effects were mostly resulted from the release of both volatile and diffusible compounds from leaves and fruits, being however found a more significative effect of volatiles in the case of the leaves. These results showed that olive organs influence fungal entomopathogens, in a complex way, which depends on the organ, fungal species ant type of compounds emitted by the organ. The fact that fungal entomopathogens are affected in such dissimilar ways by olive organs may help explaining the variation detected on the abundance and diversity of these fungi, in the different sampled generations of *P. oleae*.

The overall results achieved in this work showed that entomopathogenic fungi are present, in high abundance and diversity in olive orchards, associated to one of the key pests, *P. oleae* Bern. The isolates retrieved showed high pathogenicity to a lepidopteran pest, although showing variations between the tested isolates. Some factors were found to influence fungal entomopathogens: the olive organs (leaves, flowers and fruits) and their volatile and non-volatile compounds, as well as soil tillage, that, although not influencing abundance or mycosis detection, appears to influence entomopathogenic fungal community in olive orchards.

An intricate web of relations was found, that should be the leading course on future research on this field. The continuous monitoring of the influence of biotic and abiotic factors on entomopathogens, in field context, should be pursued, allowing a better understanding of their dynamics, which is of vital importance for future control programs that rely on these fungi. Interactions with olive trees, aiming to the determination of cultivar influence, for instance, or how other agricultural practices, like fertilization, impact on entomopathogens are key aspects to be studied. Another important aspect that should be studied is the effect of entomopathogens on beneficial insects of the olive culture. Although most works show that entomopathogens are safe to beneficial insects, and that fungi are isolated from one species, they are more virulent to insects of that species, this evaluation has to be done, regarding the specific ecosystem where entomopathogens are to be used. More importantly, the virulence of all the isolates obtained has to be confirmed against olive pests, namely *P. oleae*, as well as their characterization, regarding the production of secondary metabolites of interest. This virulence screening will allow the

selection of the most suitable isolates, which will be used for field trials, which should provide data for the creation of biological control programs using entomopathogenic fungi. Ultimately, the goal must be the formulation of a commercial product based on the isolated fungal strains in this work.