FRANCISCO ÁNGEL BUENO PALLERO

ENTOMOPATHOGENIC FUNGI FROM ALGARVE REGION (PORTUGAL): STUDIES ON THEIR NATURAL OCCURRENCE IN THE SOIL AND MULTITROPHIC INTERACTIONS THAT SHAPE THEIR BIOCONTROL POTENTIAL.

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ENTOMOPATHOGENIC FUNGI FROM ALGARVE REGION (PORTUGAL): STUDIES ON THEIR NATURAL OCCURRENCE IN THE SOIL AND MULTITROPHIC INTERACTIONS THAT SHAPE THEIR BIOCONTROL POTENTIAL.

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Trabalho efectuado sob orientação de:

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2022

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DECLARE:

The PhD thesis entitled "Entomopathogenic fungi from Algarve region (Portugal): studies on their natural occurrence in the soil and multitrophic interactions that shape their biocontrol potential", presented by Francisco Ángel Bueno Pallero, MSc in Biology at the University of Sevilla (Spain), has been carried out at the University of Algarve (Portugal) under our direction. Francisco Ángel Bueno Pallero developed his research activities supported by the following assistantship grants: BI UAlg-2016/004 (associated to the grant number IF/00552/2014/CP1234/CT0007) and the BI UAlg-072/2016 (associated to the grant number UID/BIA/04325/2013 from MeditBio).

Francisco Ángel Bueno Pallero is the author of the thesis, and has developed the following functions, in each of the works:

- Development of an original idea, experimental design and performance of the studies included in the thesis. All these studies were financed by the grants “Mediterranean ecosystem as a model in the development of molecular and ecological approaches for the study of multitrophic interactions in the soil: ecosystem services and biocontrol enhancement” (ref. IF/00552/2014/CP1234/CT0007, FCT, Portugal) and “Soil Agroecology: characterizing multitrophic interactions to improve biocontrol of insect pests” (ref. RYC-2016-19939, Ministry of Science and Innovation, Government of Spain).

- Collection of samples and data processing

- Statistical analysis.

- Analysis of the results.

- Writing the first draft of the articles and producing the final version.

This thesis is organized as a compendium of publications.

Hence, we consider that this thesis meets the specific conditions to obtain PhD degree

Faro, May 1st 2022

Luís Neto Lídia Dionísio Raquel Campos Herrera

I DECLARE

That I am the author of the doctoral original thesis entitled "Entomopathogenic fungi from Algarve region (Portugal): studies on their natural occurrence in the soil and multitrophic interactions that shape their biocontrol potential".

Authors and papers consulted are duly mentioned in the text and are included in the list of references included.

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THESIS OVERVIEW

In accordance with the regulations in force at the University of Algarve (Regulations for the defense of doctoral thesis at the University of Algarve, approved by the Governing Council of Diário da República and, specifically with its Chapter nº 189/2020, 1º Suplemento, Série II de 2020-09-28), the thesis is presented as a compendium of scientific publications. Their full references are listed below in chronological order of publication. In addition, material suplementary is presented in the Annexes section.

This thesis has been configured from three scientific articles, all of them published in international journals included in the "Journal of Citation Reports-Science Edition" (JCR) lists and with Francisco Ángel Bueno Pallero as first (co)-author:

1. **Bueno-Pallero, F.A.,** Blanco-Pérez, R., Dionisio, L., Campos-Herrera, R. 2018. Simultaneous exposure of nematophagous fungi, entomopathogenic nematodes and entomopathogenic fungi can modulate belowground insect pest control. *Journal of Invertebrate Pathology*, 154, 85-94. doi: 10.1016/j.jip.2018.04.004.

   Research Area: Zoology. Impact Factor (2018): 2.101 (Q1, 18/166)

2. **Bueno-Pallero, F.A.,** Blanco-Pérez, R., Vicente-Díez, I., Rodríguez Martín, J.A., Dionísio, L., Campos-Herrera, R., 2020. Patterns of occurrence and activity of entomopathogenic fungi in the Algarve (Portugal) using different isolation methods. *Insects*, 11, 352; doi:10.3390/insects11060352.

   Research Area: Entomology. Impact Factor (2020): 2.769 (Q1, 18/102).

3. Castruita-Esparza, G., **Bueno-Pallero, F.A.,** Blanco-Pérez, R., Dionisio, L., Aquilino-Bolaños, T., Campos-Herrera, R., 2020. Activity of *Steinernema colombiense* in plant-based oils. *Journal of Nematology* 52, e2020-72. doi: 10.21307/jofnem-2020-072.

   Research Area: Zoology. Impact Factor (2020): 1.402 (Q3, 93/175).

ABSTRACT

The entomopathogenic fungi (EPF) are well-known beneficial soil organisms naturally occurring in the soil. Learning about their natural distribution in representative habitats and their interactions with other soil organisms can establish the best ecological scenario for the activity as biocontrol agents. Also, the co-formulation of two entomopathogens with plant-based products can be an affordable approach for farmers. With the Algarve region (Portugal) as a research frame, the objectives of this PhD thesis were: (1) to investigate the natural distribution of EPF in soils from the Algarve in four habitats (oak, pines, palmettoes, and citrus groves) and two soil eco-regions (calcareous and non-calcareous), (2) to evaluate whether the presence of entomopathogenic nematodes (EPN) or/and nematophagous fungi (NF) can affect the biocontrol potential of EPF, and (3) to investigate the impact of plant-based oil adjuvants on biocontrol activity of single or combined EPF and EPN, and the impact of time and temperature in the EPN survival and activity.

Firstly, we combined three isolation methods (fresh soil, dry soil, and selective media) to investigate the natural EPF distribution in the Algarve, using traditional and molecular tools for the identification. We established the assemblage of the EPF with the soil properties and EPN by using multivariate analysis. Second, we investigated the multitrophic interactions in single (EPF, EPN, NF), dual (EPN+EPF, EPF+NF, EPN+NF) and triple (EPN+EPF+NF) combinations of one EPF (*Beauveria bassiana*), one EPN (*Steinernema feltiae*), and two NF (*Arthrobotrys musiformis* and *Purpureocillium lilacinum*). Three different fungal applications (contact with mycelia, immersion, and injection) simulated different timing of fungal arrival. Also, we evaluated the interaction in the mycelia growth between EPF and NF species. We tested the impact on biocontrol of two plant-based oils (coconut and olive oils) when combined the EPF *B. bassiana* and the EPN *Steinernema colombiense*. Also, the viability and virulence of EPN incubated at five temperatures (4, 8, 14, 20, and 24°C) and five incubation times (1, 3, 7, 14, and 21 days) with both plant-based adjuvants was also evaluated.

The distribution of EPF in the Algarve region was not driven by botanical group or by ecoregion (calcareous *versus* non-calcareous). Five EPF species were identified, with *B. bassiana* as the dominant EPF (34% of the localities), followed by *Fusarium solani* (26%), and *F. oxysporum* (14%), both reported as EPF for the first time in Portugal. The species *P. lilacinus* (8%) and *Metarhizium anisopliae* (2%) were minority. Soil pH, organic matter, and Mg content explained most of the variability of the EPF community and EPN species-specific distribution. Second, we observed that EPF *B. bassiana* limited both NF species growth and *viceversa*. The EPF *B. bassiana* dominated triple-interaction when mycelia were exposed. The EPN *S. feltiae* dominated the triple-interaction in immersion exposure. The NF *A. musiformis* caused larval mortality if vectored inside the host. Finally, the plant-based oils tested were

compatible with the EPF and EPN. The combination of EPN+EPF produced an additive effect. EPN survival was higher in coconut than olive oil and water mixtures up to 7 days at 4ºC. Moreover, olive oil supported higher larval mortality caused by EPN than coconut oil at 4-20ºC and 14 days. Overall, this thesis provides new insights on the interactions occuring in the soil that can modulate their activity of EPF as biological control agents.

Keywords: entomopathogenic organisms, nematophagous fungi, natural distribution, biological control, soil ecology, plant-based oil adjuvants.

RESUMO

Os fungos entomopatogénicos (EPF) são organismos benéficos e bem conhecidos de ocorrência natural no solo. Estudar a sua distribuição natural em habitats representativos e as suas interações com outros organismos do solo pode estabelecer um melhor cenário ecológico para a atividade como agentes de biocontrolo. Além disso, a co-formulação de dois fungos entomopatogénicos com produtos à base de plantas pode ser uma abordagem acessível para os agricultores. Tendo a região do Algarve (Portugal) como quadro de investigação, os objetivos desta tese de doutoramento foram: (1) investigar a distribuição natural dos EPF em solos do Algarve em quatro habitats (carvalho, pinheiro, palmeira anã e citrinos) e duas ecorregiões de solo (calcário e não calcário), (2) avaliar se a presença de nemátodes entomopatogénicos (EPN) ou/e fungos nematófagos (NF) pode afetar o potencial biocontrolo dos EPF, e (3) investigar o impacto na atividade de biocontrole dos EPF de adjuvantes de óleo à base de plantas, EPF e EPN individual ou em combinação, e o impacto do tempo e da temperatura na sobrevivência e atividade da EPN.

Em primeiro lugar, combinámos três métodos de isolamento (solo fresco, solo seco e meios selectivos) para investigar a distribuição natural dos EPF no Algarve, utilizando ferramentas tradicionais e moleculares para a identificação. Estabelecemos a relação dos EPF com as propriedades do solo e dos EPN por meio de análise multivariada. Em segundo lugar, investigamos a interações multitróficas em combinações simples (EPF, EPN, NF), duplas (EPN + EPF, EPF + NF, EPN + NF) e triplas (EPN + EPF + NF) de um EPF (*Beauveria bassiana*), um EPN (*Steinernema feltiae*) e dois NF (*Arthrobotrys musiformis* e *Purpureocillium lilacinum*). Três aplicações diferentes de fungos (contacto com micélios, imersão e injeção) simularam tempos diferentes de chegada do fungo. Além disso, avaliamos a interação no crescimento de micélios entre espécies de EPF e NF. Testamos o impacto de dois óleos vegetais (óleo de coco e azeite) no biocontrolo, quando combinados o EPF *B. bassiana* e o EPN *Steinernema colombiense*. Além disso, foi também avaliada a viabilidade e virulência do EPN incubado a cinco temperaturas (4, 8, 14, 20 e 24 ° C) e cinco tempos de incubação (1, 3, 7, 14 e 21 dias) com ambos os adjuvantes à base de plantas.

A distribuição dos EPF na região do Algarve não foi determinada por grupo botânico ou ecorregião (calcário *versus* não calcário). Foram identificadas cinco espécies de EPF, sendo *B. bassiana* o EPF dominante (34% das localidades), seguida de *Fusarium solani* (26%) e *F. oxysporum* (14%), ambas reportadas como EPF pela primeira vez em Portugal. As espécies *P. lilacinus* (8%) e *Metarhizium anisopliae* (2%) foram as menos representadas. O pH do solo, a

matéria orgânica e o conteúdo de Mg explicaram a maior parte da variabilidade da comunidade dos EPF e da distribuição específica das espécies dos EPN. Em segundo lugar, observámos que o EPF *B. bassiana* limitou o crescimento das espécies de NF e *viceversa*. O EPF *B. bassiana* dominou a interação tripla aquando da exposição dos micélios. O EPN *S. feltiae* dominou a interação tripla na exposição por imersão. O NF *A. musiformis* causou mortalidade larval quando introduzido dentro do hospedeiro. Finalmente, os óleos vegetais testados foram compatíveis com os EPF e EPN. A combinação de EPN + EPF produziu um efeito aditivo. A sobrevivência de EPN foi maior em misturas de coco do que de azeite e água por até 7 dias a 4ºC. Além disso, o azeite suportou maior mortalidade larval causada por EPN do que o óleo de coco a 4-20ºC e 14 dias. No geral, esta tese fornece novas perspetivas sobre as interações que ocorrem no solo, as quais podem modular a atividade dos EPF como agentes de controlo biológico.

Palavras-chave: organismos entomopatogénicos, fungos nematófagos, distribuição natural, controlo biológico, ecologia do solo, adjuvantes de óleos vegetais.

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LIST OF ABBREVIATIONS

Am – *Arthrobotrys musiformis*

ANOVA – Analysis of Variance

Bb – *Beauveria bassiana*

Bbas – *Beauveria bassiana*

BCAs – Biological Control Agents

CCA – Canonical Correspondence Analisys

CFUs – Colonies Forming Units

CMA – Corn Meal Agar

EPF – Entomopathogenic Fungi

EPNs – Entomopathogenic Nematodes

GLM – Generalized Linear Models

HSD – Honestly Significant Difference in Tukey's Test

IJs – Infective Juvenils

IPM – Integrated Pest Management

ME – Expected Mortalities

MOTUs – Molecular Operational Taxonomic Units

MT – Mortalities

NF – Nemathopagous Fungi

NGS – Next Generation Sequencing

OM – Organic Matter

PDA – Potato Dextrose Agar

Pl – *Purpureocullium lilacinum*

Sf – *Steinernema feltiae*

Scol – *Steinernema colombiense*

SDF – Scavenger Deterrent Factor

SEM – Standard Error of the Mean

SOM – Soil Organic Matter

SPSS – Statistical Package for Social Sciences

UV - Ultraviolet

1

I
INTRODUCTION

CHAPTER I

1 INTRODUCTION

1.1 Sustainable agriculture and crop protection

Sustainable agriculture, by definition, seeks to guarantee quality agricultural production (food, textiles, biofuels, pharmaceuticals) for our generation and those to come. The challenge involves improving agricultural production not only in terms of yield but also environmental protection, efficiency in the use of natural resources (soil, water, air, and biota), and the resilience of ecosystems, guaranteeing their socio-economic viability.

In terms of crop protection and production, the farmers should ensure a balanced nutrition and protection against biotic threats that compromise the yield and the overall survival of the crop. In the European Union, thanks to the Directive 2009/128/CE, transposed in all States of the EU, the farmers are called to follow the guidelines of Integrated Pest Management (IPM), which entered strongly on January 1st, 2014. The main objective is to keep biotic threats (pests, diseases, weeds) below economic damage thresholds. Strategies are implemented to combine legal, biological, cultural, biotechnological (including plant breeding), and, ultimately, chemical measures. IPM is based on three fundamental pillars: 1) prevention, 2) monitoring/observation and 3) intervention. In any case, preventive or indirect control measures are a priority, such as favoring the biodiversity of agro-ecosystems or using biological control agents (augmentative or by conservation), and it uses direct control measures only if the economic threshold is exceeded, but in exceptional situations.

Despite the national and international regulations, in general, it is frequent that the management of pests and diseases is based on the use of short-lived agrochemicals, whose widespread use compromises their effectiveness (mainly due to the appearance of resistance) and poses serious environmental and human health problems (Pimentel, 1995; van der Werf, 1996; Nicholls et al., 2008). Hence, most of the agroecosystems are also governed by a IPM with inertia towards “Intelligent Pesticide Management” (Nicholls et al., 2008). In addition, it is expected that climate change may impact on the development of pests and diseases, increasing the number of cycles per year (multivoltine species) or the number of individuals generated. This phenomenon implies that it is plausible an increase on the number of doses/treatments with agrochemicals (Delcour et al., 2015). In

this line, a recent study calls for warning about the presence of pesticide residues in more than 80% of European agricultural soils, with mixtures of various products in more than 50%, with an unknown combined effect, and, therefore, difficult to assess in terms of risk to health and the environment (Silva et al., 2019). Altogether, these data highlight the urgent need to provide effective and non-polluting tools for the management of biotic threats, where the use of biological control agents arises as a promising tool.

1.2 Soil as a key resource in agriculture

Soil is a complex matrix, composed by unconsolidated mineral material derived from the bed-rock or transferred from adjacent areas, organic matter, air, and water, which sustain numerous organisms, claimed to gather a quarter of the planet known species (Decaëns, 2010; Thakur et al., 2020). The soil biodiversity comprises organisms from microscopic size, the microflora and microfauna (archaea, bacteria, oomycetes, fungi), to other of medium and large size, so called mesofauna and macrofauna (nematodes, mites, collembolan, earthworms, snails, moles, etc.) (Wall, 2012). In addition, plants are linked with the soil throughout the roots producing a special area of interaction with the soil ecosystem, denominated the rhizosphere, essential for their development and survival. The assemblage of soil organisms is dynamic, changing in time and space, and will depend on the forces that drive the limiting resources (water, nutrients, pollutans). These changes can be due to natural processes such as natural succession from a grassland to a forest, or due to anthropic actions such as agriculture that actively include inputs as fertilizers or pesticides as well as modify plant diversity and architecture, all of these affecting the soil and their inhabitants (Thakur et al., 2020). Numerous ecological theories on biodiversity (i.e. in land biogeography, niche delimitation, energy fluxes and species, species and functional food-webs) are still poorly implemented to understand soil biodiversity in a broad sense. Hence, expanding the knowledge on the natural occurrence of soil organisms and learning about the ecological scenarios that can modify their multitrophic interactions are critical to advance in the global soil ecology understanding.

It is increasingly recognized that soil biodiversity (organisms and their activities) is critical to human well-being, since they provide numerous key ecosystem services, such as aeration, regulation of hydrological processes, soil structure, the nature of organic matter, contribute to the regulation of various cycles of nutrient, and CO_2 fixation (Wall, 2012; Bardgett and van der Putten, 2014; Wall et al., 2015). Those processes are of extreme relevance in agricultural agroecosystems, as well as the function of other beneficial soil organisms associated with plant nutrition and protection. Unravelling about the possible drivers of interactions occurring in the soil among soil inhabitants will help to elucidate mechanisms that govern fundamental ecological processes. This knowledge can be implemented to develop rational, ecologically sound agricultural management tactics that enhance ecosystem services, such as biological control of soil dwelling insect pests (Bommarco et al., 2013).

1.3 Entomopathogenic fungi and other beneficial soil organisms

Entomopathogenic fungi are well-known beneficial soil organisms for multiples roles, from their traditional activity as biological control agents but also by their action as plant endophytes, rhizosphere colonizers, plant disease antagonists, and plant growth promoters (Lacey et al., 2015; Jaber and Ownley, 2018). More than 100 genera comprising about 1000 species are described as EPF (Barra-Bucarei et al., 2019). Mainly, the EPF species belong to the Order Hypocreales, in the Division Ascomycota, characterized to have a broad range of hosts. The Blastocladiomycota and Basidiomycota divisions have some EPF among their members, while the Division Entomophthoromycota only includes EPF (Figure 1.1). There are commercial species in most of the groups (Lacey et al., 2015), and the list is increasing year after year.

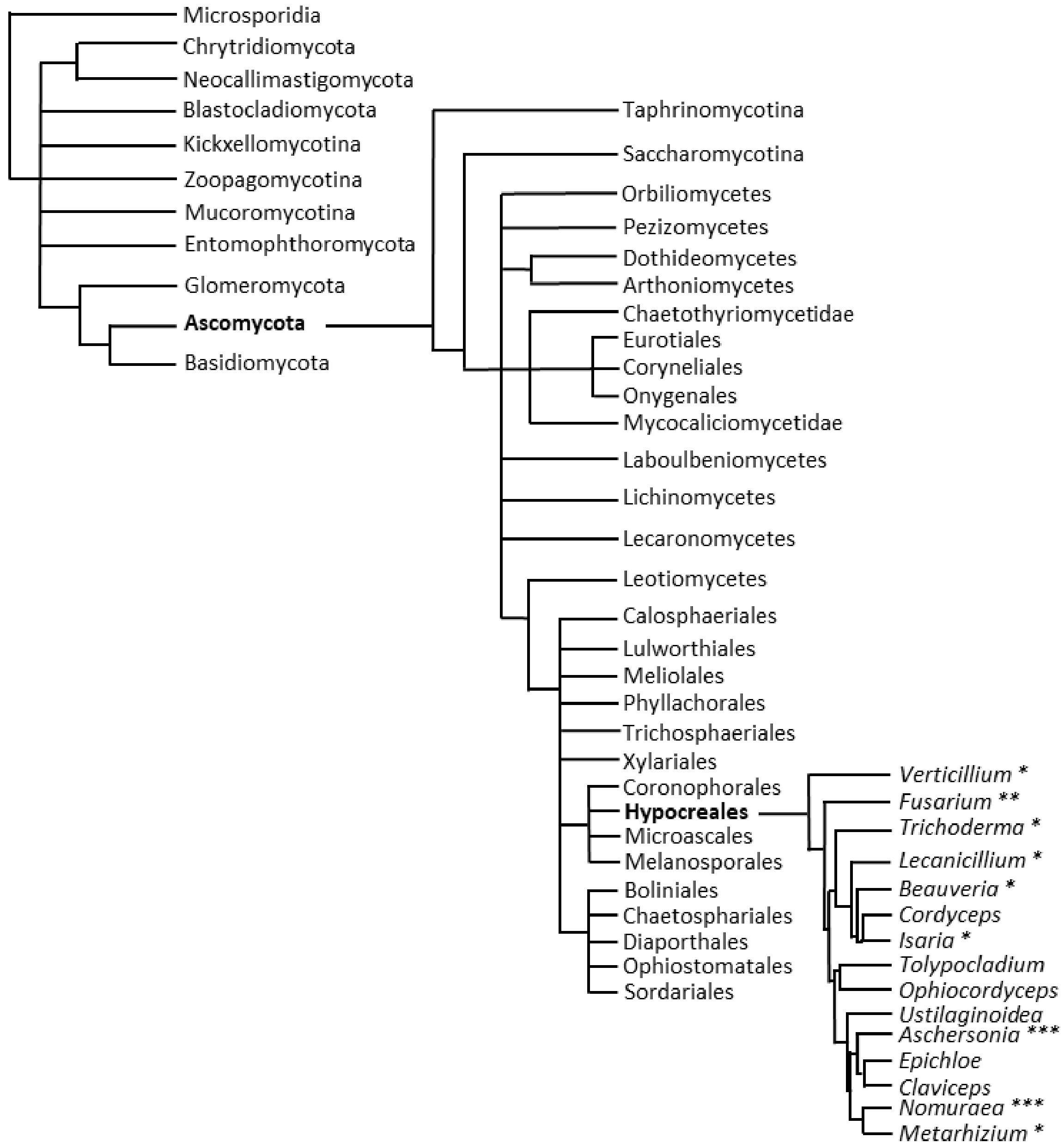

Figure 1.1. **Taxonomy of the entomopathogenic fungi**, following Hibbet et al., (2007), Humber (2012), Gryganskyi et al., (2012; 2013), Wang et al., (2016), and addapted from Fernández-Bravo (2017). From each group, a representative species with commercial use is presented *Commercialized in conidial formula; **Micotoxins formula commercialized; ***No longer commercially available, no data (Lacey et al., 2015; Hernández-Rosas et al., 2020; Faria and Wraight, 2020; Domingues et al., 2020).

Overall, one important aspect in the biology of the EPF is that they can act as obligate or facultative arthropod pathogens (Goettel et al., 2006), linked to two modes of action, respectively, acting as parasites or as saprophytes of arthropods hosts (Charnley and Collins, 2007).

The parasitic phase starts when the EPF spores establish contact with the host, penetrating the cuticle and reaching the body cavity (Oreste et al., 2012). In this phase, the EPF produces certain metabolites and antibiotics that contribute to the dead of the arthropod at the same time that limit the proliferation of other undesired organisms, respectively (Strasser et al., 2000; Charnley and Collins, 2007; Donatti et al, 2008; Parine et al., 2010). In particular, the EPF can produce secondary metabolites with insecticidal activity, such as destruxins, oosporein, beauvericin, bassianolide, beauveriolides, etc. (Hamil et al., 1969; Quesada-Moraga et al., 2006; Wang et al., 2017), which are responsible of changes in the host, comprising behavioral fever, reduced or enhanced activity, modification in the reproduction and death (Roy et al., 2006). When the arthropod is killed, the EPF display the saprohytic phase, when the fungus grows the hyphae, generate the reproductive structures, and finally, produces the aerial mycelia to allow the dispersion of spore (Figure 1.2).

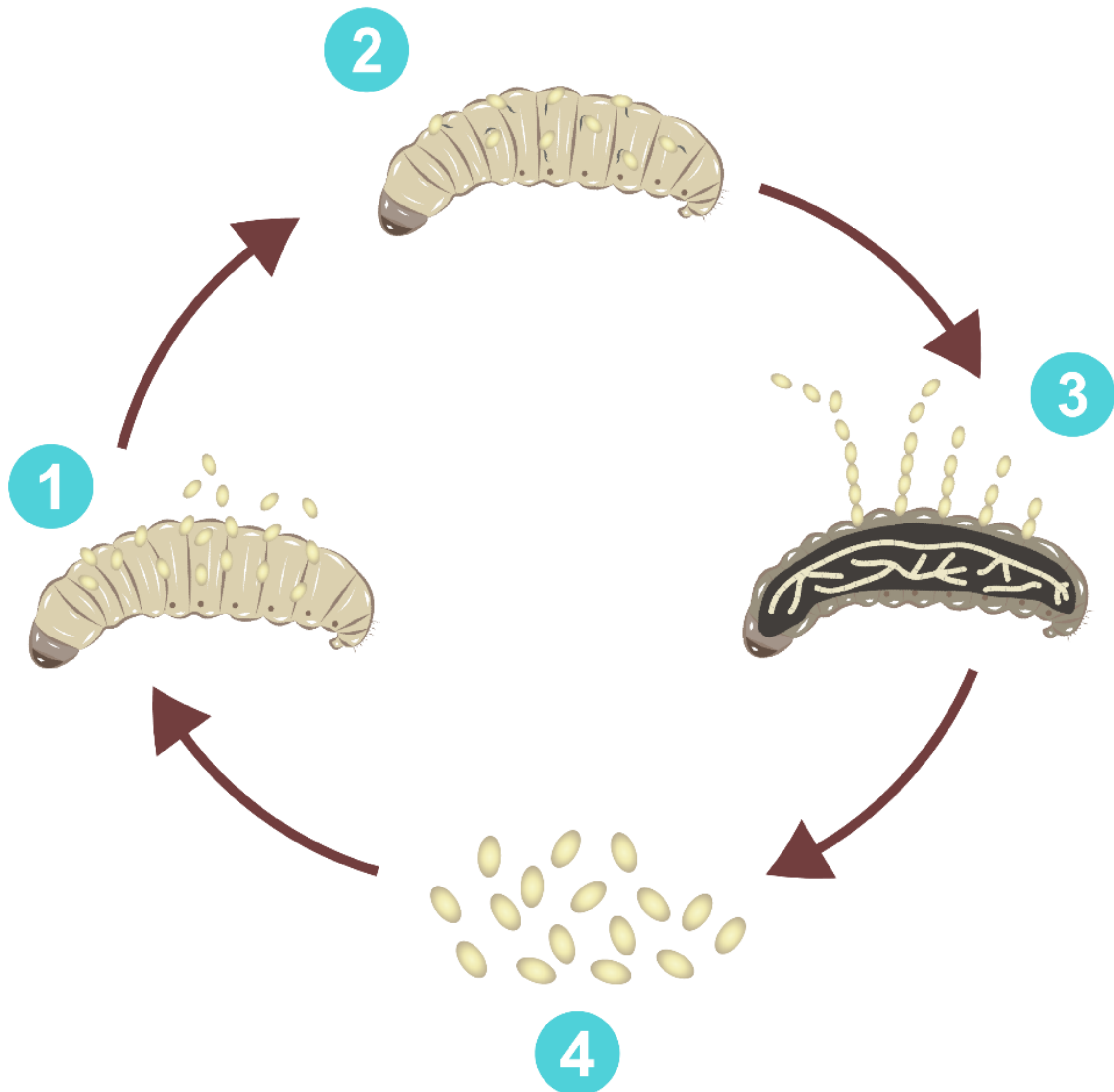

Figure 1.2. **Schematic life cycle of an entomopathogenic fungi**. Generally, the cycle starts when conidia contact with insect cuticle (1), moment in which germination begins. The germination tube penetrates the arthropod hemocoel (2) leading to cell proliferation inside of it until the insect dies (3). Hyphae go through corpse insect cuticle and produce conidiophores in the cadaver exterior, to help conidia dispersion (4).

Besides the EPF, many members of the soil biota are considered as beneficial soil organisms. In the context of the present study, we would like to highlight two: entomopathogenic nematodes (EPN) and nematophagous fungi (NF). The nematodes in the genera Steinernema and Heterorhabditis (Nematoda: Rhabditida) are the traditional EPN members (Dillman et al., 2012; Lacey et al., 2015). They are considered excellent biological control agents because they selectively search their insect hosts and kill them within 2-3 days with the aid of mutualistic bacteria of the genera *Xenorhabdus* and Photorhabdus, respectively (Boemare, 2002; Stock, 2015). The stress-resistant stage denominated "infective juvenile" (IJ) is in charge of the displacement and location of the host (mainly insects, but also certain arthropods) (Campos-Herrera, 2015). Once they penetrate in the body cavity, the IJs release the bacteria, and together, they kill the host in 24-48 h while producing certain compounds to limit the growth of other competitors of the resource (Boemare, 2002; Bode, 2009; Lu et al., 2017). Inside the host, the EPNs complete 1 to various life cycles and emerge from the cadaver carcase as IJ once the conditions are limiting (Stock, 2015; Griffin, 2015). Because they are naturally distributed in soils throughout the world, both in natural and agricultural areas (Adams et al., 2006) and the availability of commercial products (Lacey et al., 2015), they are also considered excellent products in IPM programs and even in organic production (Campos-Herrera, 2015).

The NF are present in natural and agricultural soil worldwide and can be considered beneficial soil organisms for their potential to control harmful nematodes of crops and livestock (Nordbring-Hertz et al., 2011; Braga and Araújo, 2014; Li et al., 2015). Briefly, the NF are classified in three main groups: egg- and cyst-parasitic, nematode-trapping, and endoparasitic (López-Llorca et al., 2008). Overall, the NF acting as eggs-cyst-parasitic or trapping NF have the dual lifestyle, saprophytic and parasitic, while the endoparasitic are obligated to use nematodes as food resource for their development. Each NF species displays a specific strategy to capture the nematodes, such as adhesive nets, branches, knobs or hyphae, constricting rings, zoospores or appressoria (Nordbring-Hertz et al., 2011). Whether the NF activate these structures or not can be a spontaneous action or promoted by the presence of certain stimuli, including the recognition of nematodes (Nordbring-Hertz et al., 2011). The fact that the NF can attack a broad variety of

nematodes, including other beneficial soil organisms such as EPF and EPNs (Jaffee and Strong, 2005; El-Borai et al., 2007, 2009; Nordbring-Hertz et al., 2011; Pathak et al., 2012, 2017), highlight the necessity of expanding the knowledge on the multitrophic interactions occurring in the soil.

1.4 Natural occurrence of entomopathogenic fungi in the soil

Overall, soil is considered as the main reservoir for EPF (Keller and Zimmerman, 1989; Hajek, 1997), where most of the species are represented with varying proportions and active stages (Keller et al., 2003; Meyling and Eilenberg, 2006; Quesada-Moraga et al., 2007). EPF are closely associated with soil arthropods, being considered as natural regulators of their populations in the ecosystem (Inglis et al., 2001). For decades, many efforts have been invested in learning about the natural occurrence of EPF in soils all around the world. The prevalence of the EPF, meaning the percentage of positive samples/sites over the total, is quite variable. There are studies that detected EPF in more than 90% of the sites, such as in Ontario (Canada) or Switzerland (Bidochka et al., 1998; Keller et al., 2003). On the contrary, there are studies that report only 20-30% of detections reported in the UK, Mexico, Turkey, and Tasmania (Sánchez-Peña et al., 2011; Rath et al., 1992; Chandler et al., 1998; Sevim et al., 2009). Similarly, the number of species detected also varied depending on the surveys. For example, some surveys have detected 8 or more species in agricultural areas in Portugal and Spain (Oliveira et al., 2013; Garrido-Jurado et al., 2015; Sharma et al., 2018), while other studies only detected two species in Mexico and Spain (Quesada-Moraga et al., 2007; Sánchez-Peña et al., 2011).

One of the most extended approaches to unravel the natural distribution of EPF has been the evaluation of their presence in various habitats, such as oak forest, olive groves or vineyards. The term habitat comprises the mixture of various biotic and abiotic conditions that overall translate in a similar landscape and functions. Overall, the EPF natural occurrence and activity is strongly affected by the degree of habitat management and disturbance (Quesada-Moraga et al., 2007). In this regard, traditionally, some species have been linked to certain habitats. For example, *Beauveria bassiana* was considered prevalent in shaded semi-natural areas (oaks or pines habitats), while *Metarhizium*

anisopliae resulted more abundant in altered soils such as agricultural areas (Meyling and Eilenberg, 2006; Quesada-Moraga et al., 2007; Steinwender et al., 2014). Further studies have expanded this traditional view, with *B. bassiana* being present as the dominant EPF species in many Mediterranean agricultural areas, including olive groves and vineyards (Oliveira et al., 2013; Garrido-Jurado et al., 2015; Sharma et al., 2018). In the Iberian Peninsula, the presence of EPF has been explored in natural and agricultural areas, providing a solid background on the presence and diversity of these fungi in various soils (Asensio et al., 2003; Quesada-Moraga et al., 2007; Oliveira et al., 2013; Garrido-Jurado et al., 2015; Sharma et al., 2018). Despite the differences in the methods of isolation and identification, overall, all these studies were in agreement that the most prevalent EPF species in the Iberian Peninsula was *B. bassiana*, followed in many of the species *M. anisopliae*. However, the biodiversity of EPF species strongly varied among these studies, from 2 (Quesada-Moraga et al., 2007) to 12 species (Sharma et al., 2018). Differences in the habitat surveyed could explain these low or high biodiversity, but also, the effort of sampling, the method of isolation and the tools for specific identification (molecular *versus* traditional) might contribute to these contrasting observations (Meyling et al., 2006).

1.5 Entomopathogenic fungi isolation and identification

To investigate the natural occurrence of EPF in the soil, the first steps are the isolation and the identification. Because the soil is a complex matrix from which organism should be separate to be identified, various methods have been proposed to isolate the EPF. The most extended method, denominated "insect-bait", applies the ability to kill the arthropods displayed by the EPF species for their isolation (Zimmermann, 1986; Meyling 2007; Uzman et al., 2018) (Figure 1.3A and 1.3B). Briefly, an aliquot of soil under study is exposed to certain number of arthropods, allowing the EPF to act during a certain timeframe, often 6-14 days. After this period of incubation at dark, the mortality is assessed and the arthropods with symptom of EPF infection are incubated for further analysis to confirm the Koch's postulates (Campos-Herrera and Lacey, 2018). The most extended arthropod used as insect bait is *Galleria melonella* (Lepidoptera: Pyralidae). However, in light of searching for the discovery of new or rare species (Goble et al., 2010), other arthropod hosts have been also employed, such as *Tenebrio mollitor* (Coleoptera, Tenebrionidae), in some cases even combining both hosts (Sánchez-Peña et

al., 2011; Meyling et al., 2012; Steinwender et al., 2014; Sharma et al., 2018). Although extended, this methodology has some limitations, such as detecting only those EPF that are actives at the moment of the experiment, or those that are compatible with the selected host. Hence, is possible that EPF species specific for certain hosts are not detected with the insect models commonly used in the bait. Similarly, the co-occurrence of various EPF species in the same sample can be masked by simple competition for the hosts, detecting only the dominant species.

The use of soil dilution and culture in selective media is another method of EPF isolation that might contribute to overcome some of these limitations (Meyling and Eilenberg, 2007; Campos-Herrera and Lacey, 2018) (Figure 1.3C). In this approach, the selection of the selective media is critical. In this regard, the selection of specific antibiotic or toxic and their proportion in the base media will imply differences in fungal growth, and hence, the detection of EPF. Many EPF studies using soil dilution and selective media have used the combination of the fungicide dodine (N-dodecylguanidinemonoacetate) with various antibiotics (ampicillin, chloramphenicol) in potato dextrose agar (PDA) (Shin et al., 2010; Inglis et al., 2012; Shapiro-Ilan et al., 2012; Campos-Herrera and Lacey 2018). In addition, other dodine-free media have been proposed as alternative, such as supplementing of chloramphenicol, thiabendazole and cycloheximide (CTC medium) (Fernandes et al., 2010). In rare occasion, EPF species-specific media has been employed in search of *B. bassiana* isolates (Doberski and Tribe, 1980; Shapiro-Ilan and Brown, 2013). However, the selective media also have some limitations, such as the fine tuning of the soil dilution selection to allow recovering colonies forming units (CFUs) but at the same time detecting rare species, the competition in the plate and the difficulties of accomplishing the Koch’s postulates. The combinations of both basic techniques, insect-baits and selective media arises as the optimal procedure to ensure maximizing the EPF diversity recovery (Korosi et al., 2019).

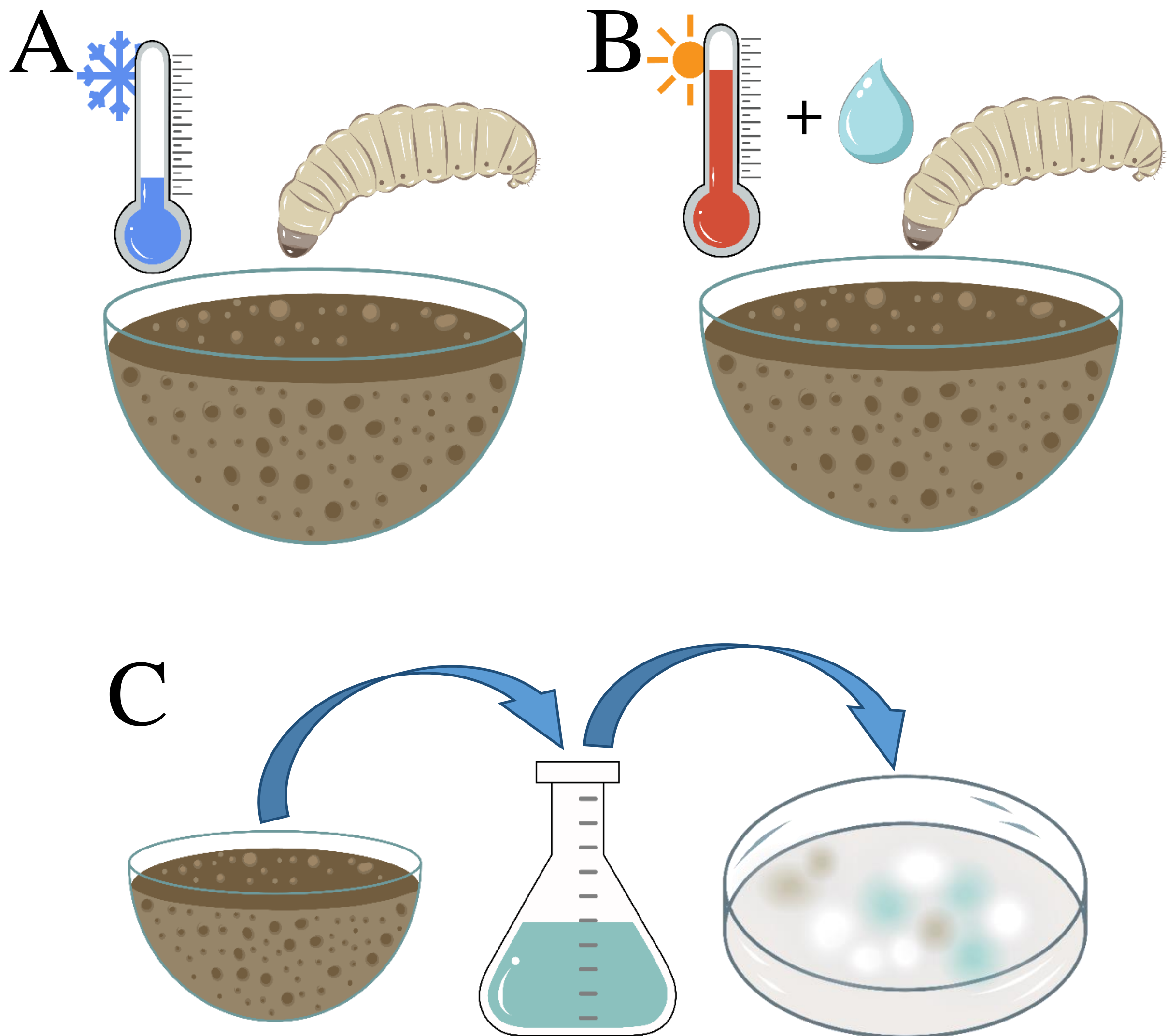

Figure 1.3. **Isolation methods of EPF**. A. Traditional insect bait, aliquot of soil is exposed to a certain number of alive insect larvae during 6-14 days in dark conditions, controlled room temperature and turning upside-down every day. B. Traditional insect bait, with heat-pre-dried and remoistened soil aliquot exposed to a certain number of alive insect larvae during 6-14 days in dark conditions, controlled room temperature and turning upside-down every day. C. Soil dilution and culture in selective media

Once the EPF has been isolated and verified the Koch's postulates, the next step is the accurate identification and classification. The morphological/morphometrical characterization has been the traditional methods for the EPF identification (Zimmermann, 1986). However, during the last decades, various molecular tools have been applied that solve some of the inaccuracy detected in the traditional method. Although there are many molecular tools that can be used for the study of biology and ecology of the EPF, such as transcriptomic, metabolomics, proteomic, etc., the most extended in the identification of EPF and studies related to their biodiversity use molecular tools. These tools are based on the detection of the variability of certain areas of the genome by using general primers to produce an amplicon that is then sequenced by traditional Sanger reaction and submitted to GenBank or other open resources dataset available. Some of the most frequently used regions are the Internal Transcribed Spacers (ITS) of ribosomal DNA, the Elongation Factor 1-alpha region (EF-1α), and the region intergenic nuclear Bloc, which have been also combined in both phylogenetic and biogeographical approaches (White et al., 1990; Bischoff et al., 2009; Rehner et al., 2011; Garrido-Jurado et al., 2015). In some cases, these sequences are further processed, for example, with the digestion of specific restriction enzymes (PCR-RFLP) to establish the variability of the EPF (Wyrebek et al., 2011; Nishi and Sato, 2017). In addition, species-specific primers have been designed for certain complex group of species, such as *Metarhizium* spp. (Schneider et al., 2011; Kabaluk et al., 2017; Mayerhofer et al., 2019). Finally, new approaches using Next Generation Sequencing (NGS), also called high-throughput sequencing, are expanding the knowledge on the presence of EPF at the same time that place into context of the presence of other soil microorganisms (Deaver et al., 2019; Mayerhofer et al., 2017). Briefly, NGS approach generates a full sequence derived from a target area of the genome. The size of the sequences or "amplicons" is now over 400 bp, and hence, allowing to retrieve enough information to discriminate to species level. This approach requires good bioinformatic skills to manage thousands of sequences, clean and detect chimeras, and standardize the units to the Molecular Operational Taxonomic Units (MOTUs). However, despite their versatility and huge potential, one of the limitations in ecological studies is the necessity of link those MOTUs to real organisms. Moreover, the detection of these EPF by NGS do not implies that they are active and also cultures are not available for further characterizations. Overall, the use of specific molecular markers associated to known regions can help the identification of complex systems, detecting close-related and cryptic species that before we considered

the just same (Glare, 2004; Schneider et al., 2011; Campos-Herrera and Lacey, 2018). However, only when traditional and molecular methods are combined is possible to achieve a more comprehensive understanding of target systems (Campos-Herrera and Lacey, 2018).

1.6 Entomopathogenic fungi and their interactions with soil abiotic and biotic components

The EPF diversity, prevalence and activity can be modulated by both abiotic and biotic factors (Asensio et al., 2003; Jaronski, 2007, 2010; Lacey et al., 2015). From one side, abiotic factors such as soil texture (clay, silt and sand content), can lead to maintain differential EPF community. For example, sandy soils characterized by higher water content and air mobility generally enhance the movement of fungal propagules. On the contrary, soils with high clay content, can support higher adsorption of conidia (Eilenberg and biogeographical approaches (White et al., 1990; Bischoff et al., 2009; Rehner et al., 2011; Eilenberg and Hokkanen, 2006; Jaronski, 2007). Other important abiotic factor is the soil organic matter (SOM). It was proposed that the EPF diversity can be modulated by the SOM by promoting the presence of certain arthropod as host, but on the contrary, it can also increase the presence of EPF competitors and natural enemies (Thiele-Bruhn et al., 2012). Moreover, of critical relevance, the abiotic factors such as temperature and moisture are also key modulators of EPF presence and activity in soils, since these will establish the rank of viability and life cycle completion (Jaronski, 2007).

In regard to the biotic factors, it is well-known that many soil organisms interact with EPF in different ways (Jaronski, 2007). For example, EPF growth and germination can be altered by the presence of certain microorganisms that secretes toxic secondary metabolites (Jaronski, 2007). Also, other soil organisms from the mesofauna (collembolan, mites) and macrofauna (earthworms) can predate on them (Broza et al., 2001; Dromph, 2003; Brownbridge and Glare, 2007; Jaronski, 2007; Shapiro-Ilan and Brown, 2013; Chelkha et al., 2021). In addition, the EPF can compete for the arthropod host as a food resource with other entomopathogens, such as EPN. Augmentation experiments where both EPF and EPN were combined, showed that the final output depend on the EPN and EPF species, the concentrations and the timing, resulting in varying results from synergistic to antagonist effects (Shapiro-Ilan et al., 2004; Jabbour et al., 2011; Hajek and Meyling, 2018). Finally, some of these relationships can be also

positive, such as the phoretic dissemination of the EPF by Collembolla or earthworms that can enhance they distribution in the soil and hence, the ability to be in contact with arthropod host (Dromph, 2003; Shapiro-Ilan and Brown, 2013).

1.7 Entomopathogenic fungi in sustainable agriculture

The EPF display several attributes that make their use ecologically compatible and environmentally friendly strategy, hence, are of choice in IPM and organic production systems. Among those traits we can highlight: (i) they attack a wide number of arthropods, some being specialist and other more generalist (Lacey et al., 2015; Barra-Bucarei, et al., 2019); (ii) they persist in the soil for long periods, even years, and can produce natural hot spots of infection (Lacey et al., 2015); (iii) they can be mass-produced in various systems, such as solid, semi-solid and liquid (Lacey et al., 2015; Jaronski and Mascarin, 2017); (iv) they can be easily applied to the agroecosystem with regular equipments and under the safe conditions as for agrochemical use, and the residues are minimal (Lacey et al., 2015); (v) they are compatible with other chemicals, such as insecticides, herbicides and certain fungicides (Yáñez and France 2010; Schumacher and Poehling 2012), (vi) to date, there is not report on resistance development to EPF, although some insects have shown the ability to produce detoxification enzymes, antibiotic secretions, and immune responses (Serebrov et al., 2006; Vega and Kaya, 2012), and (vii) they are safe for human, although there are some reports of issues produced by certain species of *Metarhizium* in immunodepressed individuals (Nourrisson et al., 2017), and harmless to beneficial non-target organisms such as bees, earthworms and Collembola (Goettel et al., 2001; Traugott et al., 2005; Brownbridge and Glare, 2007; O'Callaghan and Brownbridge, 2009). Altogether, EPF are successful biological control agents for which several products and companies have developed commercial products (Lacey et al., 2015). In addition, the extra-role described recently, such as plant disease antagonists, plant growth promoters and even biofertilizers (Goettel et al., 2008; Kim et al., 2009; Koike et al., 2011; Kabaluk and Ericsson, 2007; Behie et al., 2012), enhance their beneficial action in the agroecosystems.

One of the critical points in the release of the EPF in the environment is the formulation in a suitable medium that maintain or enhance the fungal viability and efficacy while allow easy and optimal application (Wraight et al., 2001; Brownbridge and Glare, 2007; Jackson et al., 2006; Thompson et al., 2006; Charnley and Collins, 2007;

Jaronski, 2007, 2010). The selection of the formulation should take into account the target pest and the most suitable developmental stage, the area where will be applied (aerial/foliar or soil/roots), and the environmental conditions (temperature, relative humidity, wild, UV exposure). In this regard, one of the current challenges is the enhancement of aerial/foliar applications, in which the combination with certain adjuvants and surfactants can enhance the persistence of the EPF in the desired region. (Lacey et al., 2015). Oil formulations have shown good results when formulating with certain species. For example, the use of various vegetable oils to formulate *M. anisopliae* var. *acridum* to fight against locusts and grasshoppers, allowed overcoming the environmental limitation of the dry habitats in which it should be applied (Lomer et al., 2001; Bateman, 2004; Moore, 2008). More recently, the formulation of *Zoophthora radicans* with low-cost oil based products to fight against *Plutella xylostella*, significantly increased the efficacy and provide a novel alternative tool to insecticides in this pest management (Batta et al., 2010). Exploring other oil formulations can expand the use and versatility of the EPF at the same time that provide suitable media for local shipment. In this regard, certain plant-based products such as coconut oil and olive oil can be an excellent alternative. They are easily accessible in local markets, relatively low price and also can provide certain additional characteristics to the product. For example, the coconut oil remains solid until 24ºC, and hence, can be an excellent complement to EPF formulation and shipment in local regions. Similarly, the olive oil is well-known for their conservation properties due the high presence of antioxidant compounds, often used in food-industry. This feature might also apply to the conservation of EPF viability, although there is not evidence relating olive oil combination with EPF efficiency.

Besides single application of EPF, the co-formulation of EPF with other beneficial organisms has been explored, in search for synergistic effects in the control of the target pest. For example, it is possible to combine different species or strains of EPF, such as *B. bassiana* and *M. acridum* (identified as *M. flavoviride*), with the aim to overcome the fluctuating or extreme temperatures over a period of time (Inglis et al., 1997). Similarly, various studies have tested the combination of EPF with other entomopathogens, such as EPN and entomopathogenic bacteria, *Bacillus thuringiensis* (Ansari et al., 2008, 2010; Wraight et al., 2009). However, the nature of their interaction (additive, synergic, or antagonistic) has varied depending on the species combined, the concentration and the timing, highlighting the necessity of the fine tuning of these combined formulations. For

example, Ansari et al. (2008) showed that when *M. anisopliae* was combined with different EPN species, the effect was synergic but depended on the time of application. In specific, the mix of *M. anisopliae - H. bacteriophora* resulted in a synergic effect, independently of the timing of application. However, the combination with *S. kraussei* and *S. feltiae* resulted synergistic only under certain timings. Similarly, Wu et al. (2014) observed an additive interaction with *H. bacteriophora* and *H. megidis* co-applied with *B. bassiana* and *M. anisopliae*, respectively. Finally, there are also studies that observe contrary results, observing that, with few exceptions, several EPN-EPF combinations produced antagonist interactions (Shapiro-Ilan et al., 2004; Acevedo et al., 2007). Hence, the combination of EPN and EPF is still poorly understood and requires further research to unravel the best conditions for their co-application. In addition to the compatibility of EPF with other organisms for soil applications, other studies have focused the combined use of EPF with predators or parasitoids (Roy and Pell, 2000; Wraight, 2003). Also, *B. bassiana* resulted compatible with the co-application of the parasitoid *Encarsia formosa*, as well as with the generalist predator, *Dicyphus hesperus* for the fight against greenhouse whiteflies (*Trialeurodes vaporariorum*) (Labbé et al., 2009). More recent studies have shown that the combination of EPF with certain chemical such as specific pheromones can be a natural enhancer of the activity (Abdellaoui et al., 2020). Therefore, exploring new formulations and combinations of beneficial organisms are a promising strategy to enhance the activity of EPF to be implemented in IPM and organic production systems.

1.8 Thesis outline, hypothesis and objectives

Learning about the natural presence and distribution of EPF in natural areas in relationship with other beneficial soil organisms is critical in the context of sustainable agriculture, to allow the establishment of efficient strategies of pest and pathogen control based on biocontrol agents (Bommarco et al., 2013; Ciancio et al., 2016). However, this information is still scarce. Similarly, there is little information of the interaction of these EPF with other organisms and their impact in the biocontrol, in conservation or augmentative (co-formulation) approaches (Shapiro-Ilan et al., 2004; Navarro et al., 2014; Shaurub et al., 2016). In this thesis, the natural occurrence of EPF in soils from Algarve (Southern Portugal) and how the EPF activity as biocontrol can be modulated when combined with other soil organisms was investigated. The hypothesis were: (i) the presence of EPF will be modulated by vegetation type, expecting that forest habitat (oak

and pine semi-natural areas) and acidic-ecoregions in the Algarve will favor their presence, (ii) the simultaneous application of various biological controls (EPF, EPN and NF) will result in synergic, additive or antagonist interaction, modulated by the time of arrival, and (iii) the combination of EPF and EPN with plant-based oils generates a suitable product that maintain viability of both biocontrol agents. In detail, the findings were organized in three chapters:

In the Chapter II, it was investigated the natural distribution of EPF in soils from the Algarve (Portugal) in four of the most characteristics habitats: oak, pines, palmettoes and citrus groves, also distributed in two soil eco-regions, calcareous and no calcareous. Furthermore, three methods of isolation (fresh soil, dry soil and selective media) were combined, and the isolates were identified by traditional (morphology and morphometric studies) and molecular tools. Finally, the assemblage of the EPF with the soil properties was discussed in terms of their occurrence and relationship with the presence of other entomopathogen, the EPN.

In the Chapter III, it was investigated whether the presence of EPN or/and NF can affect the biocontrol potential of the EPF. Under laboratory conditions, it was evaluated single (EPF, EPN, NF), dual (EPN+EPF, EPF+NF, EPN+NF) and triple (EPN+EPF+NF) combinations of one EPF, *Beauveria bassiana* (Hypocreales: Clavicipitaceae), one EPN species, *Steinernema feltiae* (Rhabditida: Steinernematidae), and two NF, *Arthrobotrys musiformis* (Orbiliales: Orbiliaceae) and *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae). To establish different timing of arrival, three different fungal applications (contact with mycelia-conidia, immersion in conidial suspension, and injection of conidial suspension) were tested, using *Galleria melonella* (Lepidoptera: Pyralidae) as model insect to estimate larval mortality and time to kill by each of the agents.

In the Chapter IV, it was investigated the possible combination of EPF and EPN with plant-based oils as natural adjuvants and their impact in the biocontrol potential. Specifically, it was combined the EPF *B. bassiana* and the EPN *S. colombiense,* a nematode from tropical and subtropical areas (San-Blas et al., 2019). In addition, it was tested whether the incubation of this EPN with plant-based oils at five different temperatures (4, 8, 14, 20, and 24°C) and five incubation times (1, 3, 7, 14, and 21 days) can serve as storage-shipping system in countries of limited resources.

II
PATTERNS OF OCCURRENCE AND ACTIVITY OF ENTOMOPATHOGENIC FUNGI IN THE ALGARVE (PORTUGAL) USING DIFFERENT ISOLATION METHODS

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CHAPTER II

2 PATTERNS OF OCCURRENCE AND ACTIVITY OF ENTOMOPATHOGENIC FUNGI IN THE ALGARVE (PORTUGAL) USING DIFFERENT ISOLATION METHODS

2.1 Abstract

Entomopathogenic fungi (EPF) are distributed in natural and agricultural soils worldwide. To investigate EPF occurrence in different botanical habitats and soil-ecoregions, we surveyed 50 georeferenced localities in the spring of 2016 across the Algarve region (South Portugal). Additionally, we compared three EPF isolation methods: insect baiting in untreated or pre-dried-soil and soil dilution plating on a selective medium. We hypothesized that forest habitats (oak and pine semi-natural areas) and the acidic soil ecoregion may favor EPF occurrence. Overall, EPF species were present in 68% of sites, widely distributed throughout the Algarve. The use of selective media resulted in higher recovery of EPF than did either soil-baiting method. Contrary to our hypothesis, neither vegetation type nor ecoregion appeared to influence EPF occurrence. Traditional and molecular methods confirmed the presence of five EPF species. *Beauveria bassiana* (34% of sites), was the most frequently detected EPF, using pre-dried soil baiting and soil dilution methods. However, baiting untreated soil recovered *Fusarium solani* more frequently (26% of sites), demonstrating the utility of using multiple isolation methods. We also found *Fusarium oxysporum*, *Purpureocillium lilacinum* and *Metarhizium anisopliae* in 14%, 8% and 2% of the sites, respectively. Three abiotic variables (pH, soil organic matter and Mg) explained 96% of the variability of the entomopathogen community (EPF and entomopathogenic nematodes) in a canonical correspondence analysis, confirming the congruence of the soil properties that drive the assemblage of both entomopathogens. This study expands the knowledge of EPF distribution in natural and cultivated Mediterranean habitats.

2.2 Introduction

Fungi are the predominant natural pathogens of arthropods (Lacey et al., 2015). Hypocrealean entomopathogenic fungi (EPF), such as those in the genera *Beauveria* and *Metarhizium*, are natural inhabitants of most terrestrial ecosystems, including natural and agricultural areas (Bidochka et al., 1998; Meyling and Eilenberg, 2006; McGuire and

Northfield, 2020). EPF can interact with arthropod hosts as parasites or saprophytes (Charnley and Collins, 2007). During the parasitic phase, after the conidia infect the host, the fungus produces various compounds responsible for host death (Oreste et al., 2012; Altinok et al., 2019) and other secondary metabolites with an antibiotic or antagonistic response to defend the cadaver from opportunistic organisms (Strasser et al., 2000; Charnley and Collins, 2007; Donatti et al., 2008). During the saprophytic phase, mycelia invade the entire body cavity to finally generate emergent mycelia and conidiophores for passive dispersion of the spores (Vega et al., 2007; Bueno-Pallero et al., 2018).

Among different methods to isolate EPF from the soil is the traditional insect-bait using *Galleria melonella* (Lepidoptera: Pyralidae) as a host (Zimmerman, 1986; Meyling 2007; Uzman et al., 2018). With the expectation of recovering additional EPF species (Goble, 2010), some studies have employed other insects such as *Tenebrio mollitor* (Coleoptera, Tenebrionidae) or combination of different hosts (Sanchez-Peña et al., 2011; Meyling et al., 2012; Steinwender et al., 2014; Sharma et al., 2018). Other studies have used soil dilution and selective media to isolate EPF from soil (Meyling and Eilenberg, 2007; Campos-Herrera and Lacey, 2018). Although alternative selective media recipes that lack dodine (N-dodecylguanidinemonoacetate) have been suggested (Fernandes et al., 2010), this fungicide, in combination with other antibiotics, is commonly used to isolate EPF and minimize contamination (Campos-Herrera and Lacey, 2018; Shin et al., 2010; Inglis et al., 2012; Shapiro-Ilan et al., 2012).

Combinations of insect-baits and selective media procedures are recommended to capture a wider range of EPF species (Korosi et al., 2019). Once isolated, fungal classification is often based on morphologic/morphometrical examinations that can be inaccurate when resolving closely related species. However, molecular tools are increasingly available for identification (Campos-Herrera and Lacey, 2018; Hibbet et al., 2007; Schneider et al., 2011).

As natural regulators of arthropod populations in the ecosystem (Inglis et al., 2001), EPF are well-known biologic control agents against a broad variety of arthropod pests (Lacey et al., 2015; Hajek and Leger, 1994; McLaughlin et al., 2009; Wall, 2012). Active research has developed commercial products based on EPF for decades including mycoinsecticides derived from their active metabolites (Donatti et al., 2008; Inglis et al.,

2001; Jeffs and Khachatourians, 1997; Jaronski, 2010). However, abiotic factors and biotic interactions that occur in the soil can affect their performance as biocontrol agents (Asensio et al., 2003; Jaronski, 2007; Jaronski, 2010; Lacey et al., 2015). For example, soil granulometry can affect EPF communities: the greater porosity of sandy soils enhances the movement of fungal propagules, while clay substrates cause adsorption of conidia (Jaronski, 2007; Eilenberg and Hokkanen, 2006). Similarly, the soil organic matter (SOM) content modulates EPF diversity by promoting the presence of insect hosts, while favoring some EPF antagonists (Thiele-Bruhn et al., 2012). Temperature and moisture are also key drivers of EPF presence and activity in soils (Jaronski, 2007).

Many soil organisms interact with EPF and *viceversa* (Jaronski, 2007). Some microorganisms produce secondary metabolites that are toxic to EPF or inhibit their germination and growth (Bueno-Pallero et al., 2018; Jaronski, 2007). EPF are weak saprophytes in natural conditions (Lacey et al., 2015) and resource competition from other entomopathogens, such as the entomopathogenic nematodes (EPNs), may also restrict their occurrence. Several combined application studies using EPN and EPF reported that their interactions are not only species dependent (including the target host), but different concentrations and timing of application (simultaneous or sequential) are factors that can alter the outcomes (Shapiro-Ilan et al., 2004; Jabbour et al., 2011; Hajek and Meyling, 2018). Interactions of a similar kind occur among other microorganisms in the soil, such as nematophagous fungi (Bueno-Pallero et al., 2018). EPF also establish complex and highly differentiated interactions with soil macroinvertebrates that can enhance or reduce EPF occurrence, such as earthworms which favor EPF dissemination and activity or collembolans that feed on them (Jaronski, 2007; Shapiro-Ilan and Brown, 2013).

Habitats synthesize a combination of diverse biotic and abiotic properties and have been proposed as one of the main drivers for the EPF natural occurrence. *Beauveria bassiana* was reported linked with shaded seminatural areas (oaks or pines) and *Metarhizium anisopliae* with crops soils (Meyling and Eilenberg, 2006; Steinwender et al., 2014; Quesada-Moraga et al., 2007). However, *B. bassiana* is also a dominant EPF species in many Mediterranean agricultural areas (Sharma et al., 2018; Oliveira et al., 2013; Garrido-Jurado et al., 2015).

Both natural and agricultural areas have been explored for the presence of EPF species in Spain (Asensio et al., 2003; Quesada-Moraga et al., 2007; Garrido-Jurado et al., 2015). Conversely, in Portugal, little is known in this regard except for a few surveys mainly focused on perennial crops such as olives and grapes (Sharma et al., 2018; Oliveira et al., 2013). Four main botanical groups characterize the Algarve region (South Portugal): oak (some still under cork production), pine (native populations or replanted areas), palmetto (wild areas) and citrus (the main agricultural sector in the region). This region also comprises two main soil-ecoregions: "calcareous" or "Barrocal" (predominantly basic soil with a high percentage of carbonates, poor in Fe and mainly located in the low-inland areas) and "non-calcareous" or "Serra and Littoral" (more acidic soils, not limited in Fe availability and mainly surrounding the Barrocal). Campos-Herrera et al., 2019, investigated the natural occurrence of EPNs in the Algarve region and concluded that botanical group and some abiotic factors (particularly soil pH and clay content), appeared to modulate communities of EPNs and associated microorganisms.

Herein, we extend that study to include the natural occurrence of EPF species. We speculated that vegetation type will drive EPF occurrence and activity, being especially favored in oak and pine semi-natural habitats (Meyling and Eilenberg, 2006; Quesada-Moraga et al., 2007) and that the dominant species will be *B. bassiana* as shown in previous studies in Mediterranean areas (Sharma et al., 2018; Garrido-Jurado et al., 2015). Based on the general premise that fungi tolerate acidic soils better than basic soils (Foth, 1984), we also hypothesized that non-calcareous soils would favor the presence of EPF.

Finally, we predicted that those soil properties associated with the EPN soil food web assemblage (Campos-Herrera et al., 2019) would also define the EPF community. We also expect different methods of EPF isolation to detect different patterns of recovery occurrence, activity and biodiversity (Korosi et al., 2019). Hence, this study aimed to explore ecological drivers of EPF species in the Algarve and to identify abiotic factors associated with their natural occurrence, based on the results provided by three isolation methods: untreated soil bait, pre-dried soil bait and soil dilution and culture in selective media. Thus, the specific objectives of this study were: (1) compare the natural EPF distribution and species composition derived from the three methods of isolation, (2) investigate the effect habitat type and soil-ecoregion on EPF distribution and species composition and (3) discriminate the abiotic factors that drive EPF and EPN assemblages.

2.3 Materials and methods

2.3.1 Study area, sampling method and soil parameters.

A total of 50 geographical localities distributed throughout the south coast and the interior of Portugal in the Algarve region (Southwest of continental Europe) were surveyed during spring 2016 (Figure 2.1). These localities represented four of the most widespread habitats in the region driven by the following botanical groups: oak (*Quercus* spp., Fagales, Fagaceae, n = 14), pine (*Pinus* spp. Pinales, Pinaceae, n = 14), palmetto (Arecales, Arecaceae, n = 7) and citrus orchard (*Citrus* spp., Sapindales, Rutaceae, n = 15). These localities were also assigned to one of the two typical soil-driven ecoregions knowns as “Barrocal” (n = 20) and “Serra and Littoral (n = 30), characterized as “calcareous” (alkaline soils with low Fe content) and “non-calcareous” (lower pH and higher Fe content). Campos-Herrera et al., 2019 reported the details for geographical coordinates, localities and sampling schemes. Briefly, we collected two composite samples per locality in an area ~ 0.5 ha, each comprised of 20 single soil cores (2.5 cm ø × 20 cm depth) and store them at 4 ºC in dark conditions until further processing (<5 days). Hereafter, we well mixed all the cores of each sample in the laboratory and divided them into subsamples of 200 g of fresh soil employed for subsequent analyses. Campos-Herrera et al. (2019) reported the soil parameters (pH, electric conductivity, organic matter, macro- and micronutrient elements and granulometry) analyzed by the Laboratório de Análises Químicas, LAQ (Universidade do Algarve, Faro, Portugal).

2.3.2 Entomopathogenic fungi isolation

We examined the EPF occurrence in all the samples (n = 100) by using three types of soil processing in each one to ensure a balanced analysis: (i) untreated soil, (ii) pre-dried soil and (iii) soil dilution and culture in selective medium. Hence, from each sample, we had three types of measurement of EPF natural occurrence. Overall, we baited the untreated and pre-dried soil samples following Zimmerman, 1986 and Meyling, 2007 procedures. First, the untreated soil samples employed in this study were the same as described by Campos-Herrera et al. (2019) but separating the dead insect larvae which confirmed nematode emergences from those with signs of fungal death (Shapiro-Ilan et al., 2003; Campos-Herrera et al., 2018). Second, the pre-dried soil samples were first lightly dried at 35 ºC over a week to avoid any EPN infestation (Quesada-Moraga et al., 2007), then remoistened to half field capacity with distilled water (Zimmermann, 1986; Meyling, 2007). For both bait tests, we used the final instar larva of *G. mellonella* as host, reared at Universidade do Algarve (Faro, Portugal), by performing two independent rounds of 10 larvae each per sample. Then, we incubated them for four days at 22-24 ºC in dark conditions, inverting the containers daily to ensure the movement of the larvae through the soil regularly. Following Quesada-Moraga et al., 2007, after assessing larval mortality, we disinfected each dead larva with 1-2% sodium hypochlorite solution for 3 minutes (rinsing with sterile distilled water between washes) and subsequently placed them into independent moist chambers with sterilized filter papers (RH > 90%, 27 +/- 1ºC in dark conditions). Live larvae were incubated in the same experimental conditions for an additional 72 h to record possible late mortality.

We revised the insect cadavers every 2-3 days during 16-20 days for the detection of fungal mycelium growth. Cadavers that showed abundant emerging mycelium (i.e., from intersegmental regions and natural holes) were considered as likely infected by EPF, while the presence of little to no mycelium on the surfaces of cadavers was associated with saprophytes and these were discarded (Asensio et al., 2003).

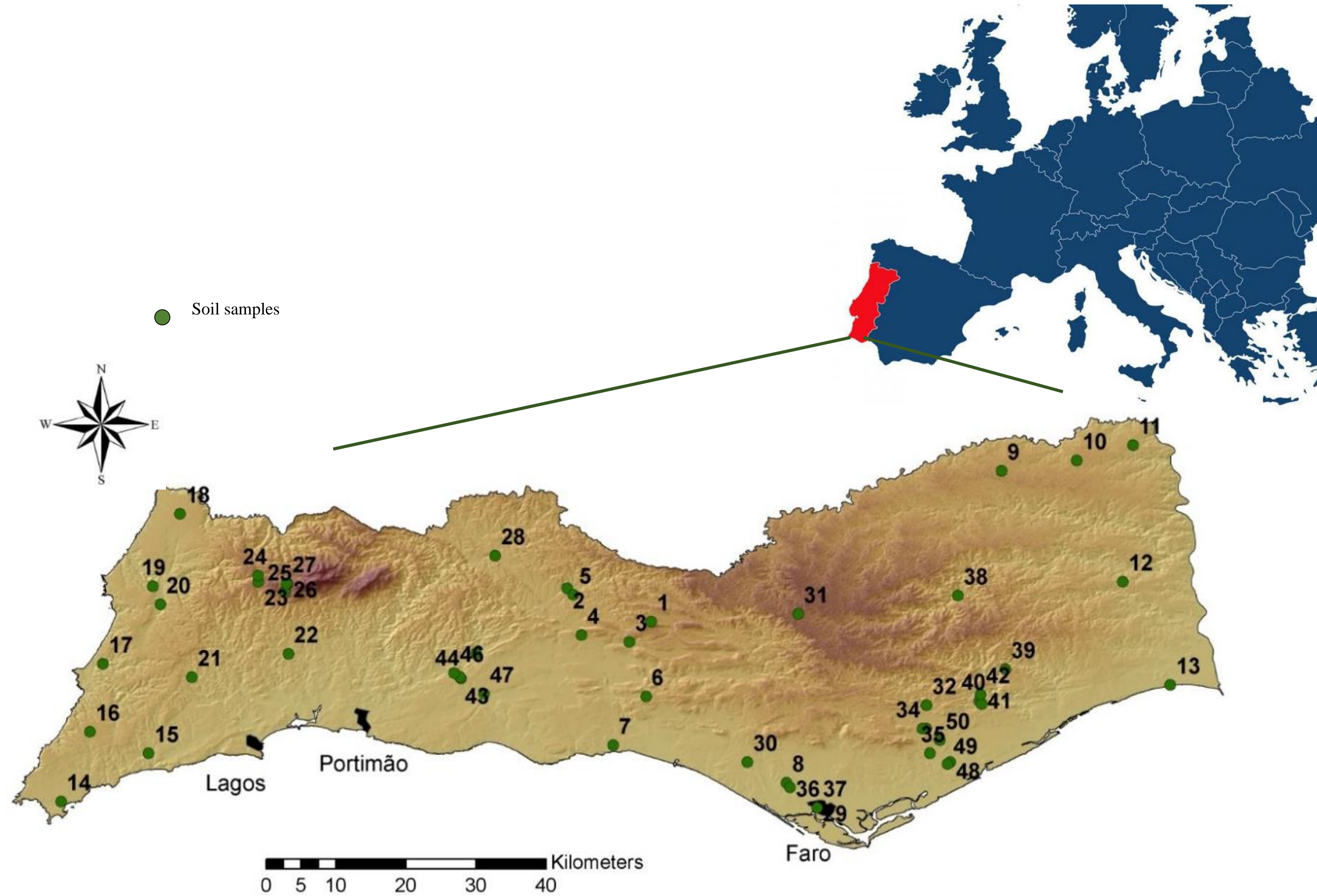


Figure 2.1. **Distribution of the sampling sites** along the Algarve region, Southern Portugal (see Campos-Herrera et al. 2019, for additional details).

For the third method of EPF isolation, we followed the procedure adapted by Shin et al., 2010 and Korosi et al., 2019. The selective media was prepared with potato dextrose agar (PDA, Biokar, Lardero, La Rioja, Spain) supplemented with 0.1 g/L dodine (Sigma Aldrich, San Luis, MO, USA) and 0.1 g/L chloramphenicol (Sigma Aldrich) (Shin et al., 2010; Korosi et al., 2019). Briefly, five grams of dried soil from each subsample was suspended in Falcon® tube with 45 mL sterile half-strength Ringer solution and 0.05% Tween 80 (Klingen et al., 2002). The soil suspension was homogenized (Vortex®, Darmstadt, Germany) for 3 min. Preliminary tests on serial dilutions confirmed satisfactory results in terms of the facility to identify and remove individual fungal colonies from the plates when plating 100 mL of 10^{0} suspension. We prepared 3 plates with selective media per sample. Thereafter, the plates were sealed with Parafilm® and maintained at 27 ºC in the dark until fungal growth was observed (7-10 days). Finally, we estimated the number of putative EPF colonies forming units (CFUs) and richness per plate.

A pure fungal culture was established in PDA from each cadaver with evidence of EPF growth derived from the untreated or pre-dried soil test as well as from the CFU retrieved from the selective media. To identify the causal agent of the death, we evaluated the pathogenicity of those pure cultures by confirming the Koch's postulates. We placed three last instar *G. mellonella* into Petri dishes with PDA and actively growing mycelia (2 h, 27 +/-1 ºC in dark conditions). We prepared two plates per isolate and another two control PDA plates for the control. Then, we placed the larvae independently in new Petri dishes with moistened study filters to incubate them in a humid chamber (RH > 90%, 27 +/-1 ºC in dark conditions). After three days of larvae-fungus contact, we evaluated the larval mortality daily for a total of 8 days, processing the insect cadavers following the disinfecting and cleaning protocol previously described for the baiting methods (adapted protocol from Cabrera-Mora et al., 2019). During this period, larvae did not receive any supplemented food. To ensure that the larvae were healthy, not dying because of starvation and the experimental conditions were appropriate, we verified that the surviving larvae pupated and emerged as adults.

2.3.3 Fungal identification

All fungi that confirmed Koch's postulates were considered EPF. For their identification, we first performed a preliminary macro-/microscopical description using taxonomic keys based on morphologic characteristics (Barnett and Hunter, 1987; Humber 1997; Humber, 2005). Semi-permanent slide mounts of lactophenol cotton blue were prepared and observed in the microscope. Pictures of representative species were also recorded. We recovered the mycelium of the selected fungi for the establishment of an EPF collection and to store at -20 ºC for further molecular identification. For DNA extraction, we first mechanically disaggregated the mycelia for 15 seconds by using a sterile blue pestle assembled to a pellet mixer (VWR International, Lutterworth, UK). Then, we followed the maximum yield protocol of the Speedtools tissue DN extraction kit (Biotools, Madrid, Spain). DNA of each sample was eluted in 50 mL of Milli-Q water (Milli-QWater Systems, Millipore S.A., Molsheim, France), analyzed for quality and quantity (nanodrop system, Thermo Scientific 200 ºC spectrophotometer) and stored at -20 ºC until subsequent analysis.

By using the universal primers ITS1 and ITS4 (White, 1990), we evaluated the ITS region (ITS1, 5.8S and ITS2) as barcoding for the molecular identification of the selected fungi (Sharma et al., 2018). For ensuring optimal amplification in conventional PCR, all the DNA samples were previously diluted to a range between 0.5-1 ng/µL. Each reaction was performed in a final volume of 20 µL and included 1 µL DNA template, 200 nM dNTP mix (Promega, provided by Promega Biotech Iberica SL, Alcobendas, Madrid, Spain), 1× PCR buffer (5×ColorlessGoTaq®Reaction buffer, Promega), 400 nM of each primer (Biosearch Technologies, supplied by Biotools, Spain) with 0.68 U GoTaq® G2 DNA Polymerase (Promega). Amplification's procedures were performed as described by Luo and Mitchell (2002), employing the thermocycler Biometra T gradient (Biolabs, France). Each PCR product was verified for expected size by visualization after electrophoresis in 2% agarose gel in TAE (pH 8.3 +/- 0.1) (Fisher Bioreagents, Ltd., Madrid, Spain) run along the reference BenchTop 100 bp DNA ladder (Promega). Individual bands were purified by QIAquick Gel Extraction (Qiagen®, Hilden, Germany) and the occurrence of the expected band verified in a TAE 0.8% agarose gel. All the

samples were sequenced at Macrogen (Macrogen Europe Laboratory, Inc., Madrid, Spain). For each sample, the sequences (forward and reverse) were assembled (Geneious, R.5.6.5., Biomatters, Inc., Auckland, New Zealand), compared to reported sequences using Blast (http://blast.ncbi.nlm.nih.gov) and submitted to Genbank.

2.3.4 Comparison of the detection methods and identification of ecological drivers for the entomopathogenic fungi natural occurrence

For each EPF isolation method, we included all fungal isolates that confirmed Koch's postulates for the estimation of the frequency of occurrence (positive sites per total sites, expressed as percentage). For the insect baits methods, we also analyzed the larval mortality percentage. After examining for possible correlations (Pearson test) among the results obtained by different methods, we analyzed whether these variables were statistically significant among those methodologies by using one-way ANOVA and Tukey's HSD test ($p < 0.05$) performed with SPSS 25.0 (SPSS Statistics, SPSS, Inc., Chicago, IL, USA). We also estimate the percentage of total EPF occurrence by combining the data obtained for all three EPF isolation methods (Sharma et al., 2018; Korosi et al., 2019; Klingen et al., 2002). For this combined data, we counted a site as positive for the EPF detection independently that resulted positive in one, two or the three methods and if one or more species were detected. Moreover, to provide additional data on the virulence of the isolates, we calculated the average time the isolates derived from each detection method took to kill the larvae in Koch's postulate tests for all isolation methods. We visualized in maps the regional data derived from (i) the EPF occurrence values depending on the isolation method, (ii) the total EPF presence/absence and (iii) species isolated per site by using the SPSS statistical package for Windows V19 and ArcGis 10.

Additionally, we analyzed the impact of the botanical habitats (oak, pine, palmetto and citrus grove) and soil-ecoregions (calcareous and non-calcareous) on the percentages of EPF occurrence and compared the results obtained among the three isolation methods. We assessed significant differences among habitat types or ecoregions, as well as among the isolation method by using one-way ANOVA and Tukey's HSD test and t-test ($p < 0.05$) performed with SPSS 25.0. Before statistical analysis, all the variables were arcsine transformed. We used least-square means +/- S.E. as descriptive statistics.

2.3.5 Relationships of entomopathogenic fungi within abiotic factors and multivariate analysis of the entomopathogen community assemblage

First, we established the range of the abiotic variables for the natural occurrence of each EPF species identified in this survey. Then, multivariate analyses of the selected entomopathogen organisms and environmental factors were performed using CANOCO 5 (Ter Braak et al., 2009; Šmilauer and Lepš, 2014). We employed the eight abiotic variables selected by Campos-Herrera et al., 2019, to avoid strong co-linearity: elevation and the soil properties of pH and clay, OM, P, Mg, Zn and Fe content, to be included as explanatory (predictors) variables in CANOCO. For the dependent (response) variables, we selected target species that were present in at least 10% of the field sites (Lepš and Hadincová, 1992). We explored the assemblage of two kinds of soil entomopathogen organisms: fungi and nematodes. We retrieved the EPN inputs from the dataset presented by Campos-Herrera et al., 2019, while for EPF we used the total occurrence (the combination of the numbers obtained for the three methods) of each fungal species. Prior to analysis, both biotic and abiotic variables were standardized by dividing by the highest values, ranking all values 0-1 (Lepš and Šmilauer, 2003). As described by Campos-Herrera et al., 2019, Detrended Canonical Correspondence Analysis (DCCA) was used to estimate the length of the system, selecting a Canonical Correspondence Analysis (CCA, constrained axes) when heterogeneous communities were detected (Ter Braak et al., 2009). We used the CCA (interspecies correlations) with Monte Carlo permutation (n = 499) and automatic forward selection for the assignment of significant environmental variables, using the p-values derived from the forward selection. The graphical results of the CCA were presented with bi-plot scaling (CANOCO 5).

2.4 Results

2.4.1 Distribution of entomopathogenic fungi across the Algarve region: comparison of three methods of isolation

The EPF occurrence detected by the selective medium method were significantly higher than those reported through bait methods ($F_{2,147} = 4.70$, $p = 0.011$; Figure 2.2A). The average larval mortality percentage detected by the untreated soil bait was 0.9% ± 0.26 and by the pre-dried 1.6% ± 0.70 and differences between methods were not

significant. The isolates cultured in PDA and derived from the untreated soil method resulted in a significantly longer time to kill *G. mellonella* larvae than those from the pre-dried soil bait and selective medium ($F_{2,74} = 6.44$, $p = 0.003$; Figure 2.2B). The results for the isolation methods were not significantly correlated, neither the EPF occurrence (the three methods) nor the larval mortality (only the two bait methods) (in annexes 1 as Supplementary Materials, Annex 1.A). In addition, we found certain geographical differences for the EPF detection efficiency among isolation methods, as shown by the almost exclusive EPF detection in the eastern areas of the Algarve region by the selective medium method (Figure 2.3A).

Overall, 68% of the sites, widespread all along the sampling area, were positive for the occurrence of any EPF species (Figure 2.2A, Figure 2.3B). Five fungal species that confirmed Koch's postulates were identified by morphologic and molecular identifications (Table 2.1). The species *Beauveria bassiana* and *Fusarium solani* were the most prevalent (Table 2.1) and widely distributed EPF (Figure 2.3C), detected in 34% and 26% of the sites, respectively. The distribution of *F. oxysporum*, present in 14% of the sites (Table 2.1), was mainly restricted to the central area of the Algarve region (Figure 2.3C). All the isolation methods identified those three abundant fungal species (Table 2.1; Figure 2.2A) with similar efficiency of recovery (Supplementary Materials, Annex 1.B). Conversely, *Purpureocillium lilacinum* was reported only in four sites (none in untreated soil baits), mainly located in the northwest of the Algarve (Figure 2.3C) and its detection slightly differed among methods (Annex 1.B). Finally, we detected the species *Metarhizium anisopliae* in just one site using the pre-dried soil (Table 2.1, Figure 2.3C). In summary, the pattern of EPF occurrence by species was different among methods (Figure 2.2A). We detected all five EPF species only through pre-dried soil baits, but only three species in untreated soil baits. Accordingly, the method of isolation, *F. oxysporum* was the dominant species in untreated soil baits, while *B. bassiana* resulted the dominant in pre-dried soil baits and selective media method (Figure 2.2B).

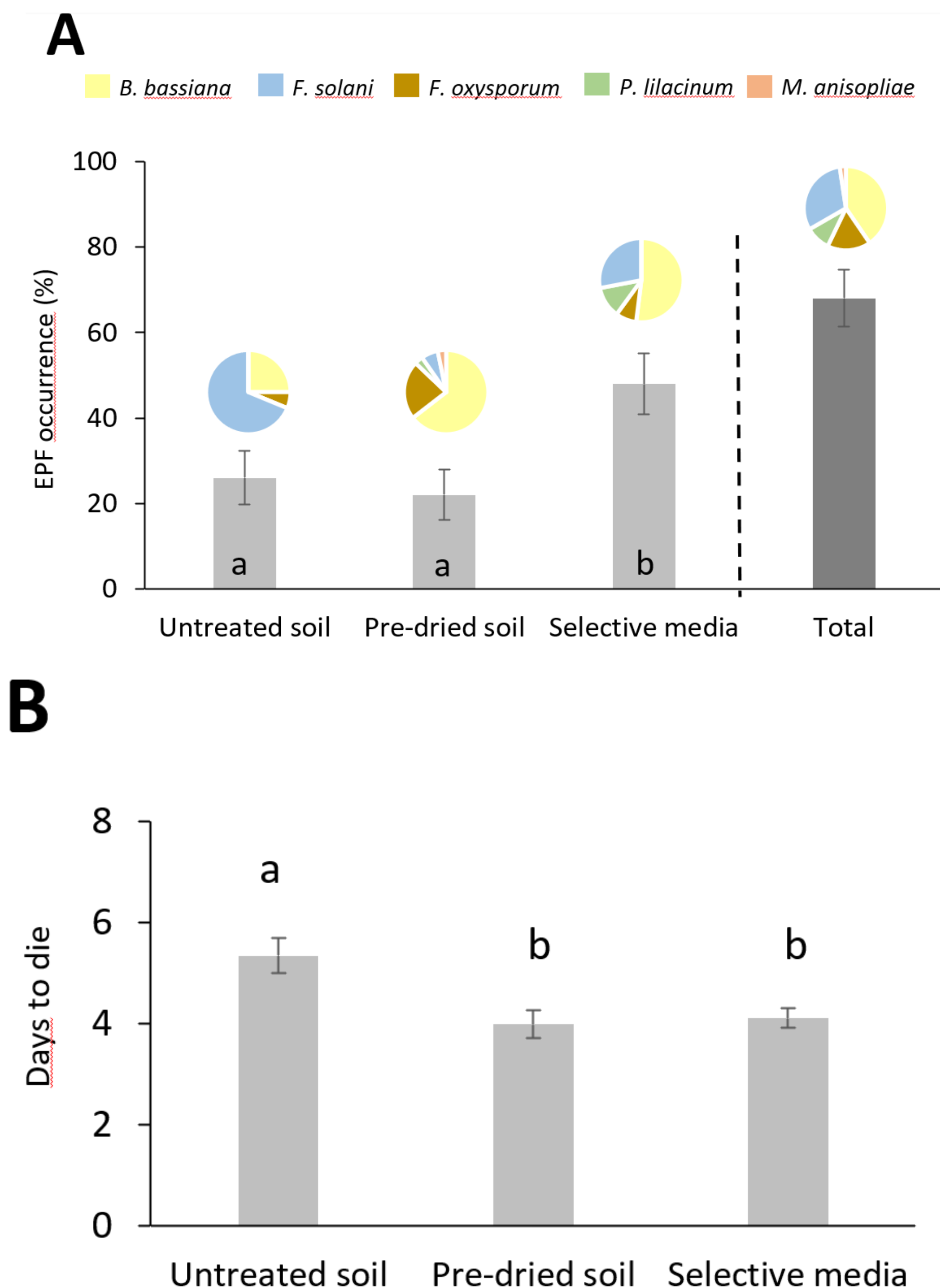


Figure 2.2. **Entomopathogenic fungi (EPF) recorded by using three isolation methods and total EPF recovery** (data from all methods combined) (A) Recovery frequency in the overall survey. EPF species averages are proportionally represented in pies; (B) number of days to kill *Galleria melonella* larvae by the isolates derived from each of the isolation methods. Data derived from pure cultures in PDA and evaluated by Koch's postulates test. Different letters indicate significant differences ($p < 0.05$) in Tukey's test (HSD). Values are least-square means ± SE.

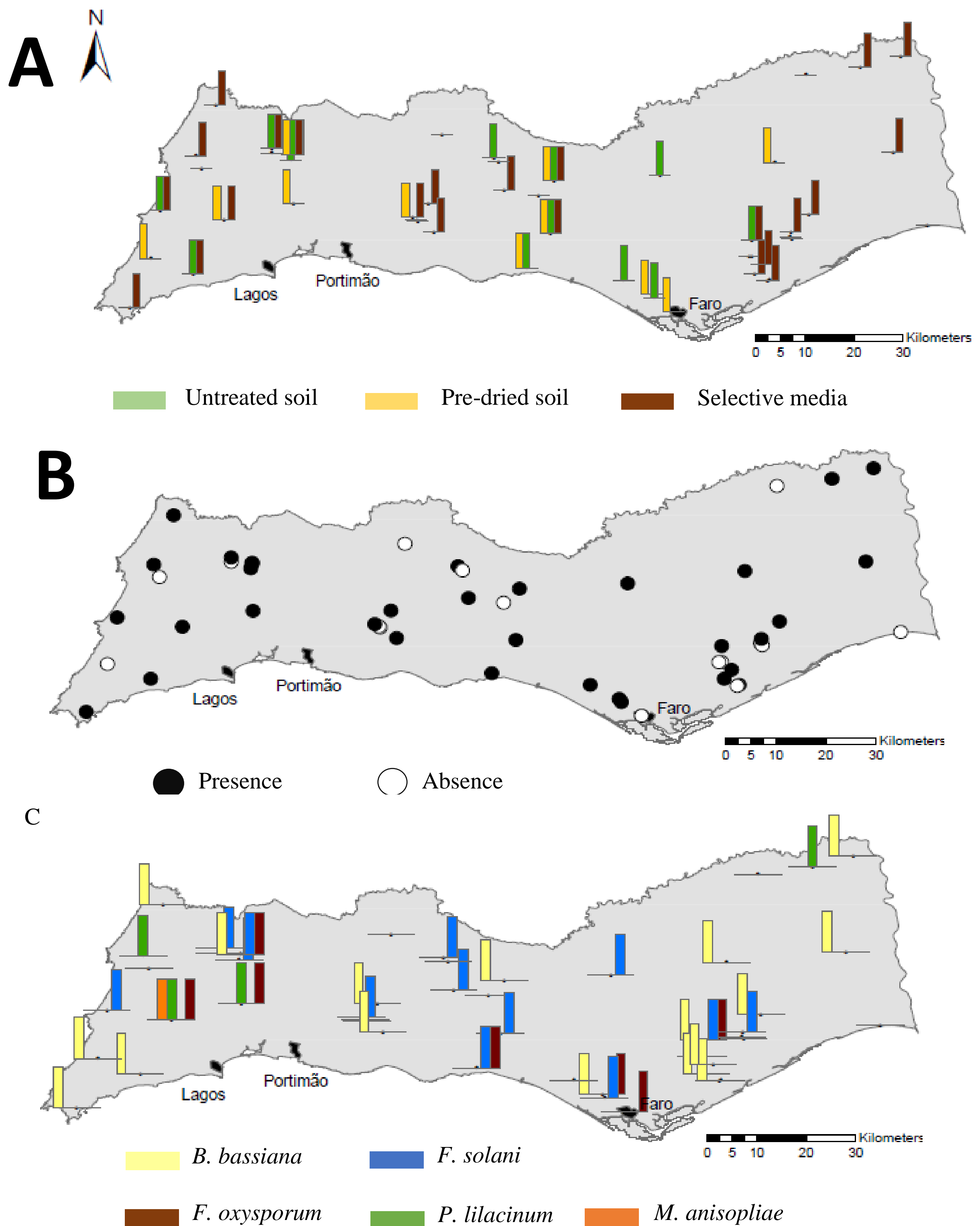


Figure 2.3. **Geographical distribution for entomopathogenic fungi (EPF) occurrence in the region of Algarve** (Southern Portugal) (A) Positive sites for each isolation method; (B) positive sites for any isolation method; (C) distribution of EPF species.

Table 2.1. **Summary of soil properties** and general characteristics associated with entomopathogenic fungi (that confirmed Koch's postulates) isolated in the Algarve (Portugal).

Entomopathogenic fungi species					
Variables for Characterization	*B. bassiana*	*F. solani*	*F. oxysporum*	*P. lilacinum*	*M. anisopliae*
General characteristics					
Isolation method[a]	U, D, S	U, D, S	U, D, S	D, S	D
No. positive sites	17 (34%)	13 (26%)	7(14%)	4 (8%)	1 (2%)
GenBank accession number[a]	MN808334	MN808329, MN808331	MN808330, MN808332	MN808335	MN808333
General properties					
Habitat type	Oak, Pinus, Palmitto, Citrus	Oak, Pinus, Palmitto, Citrus	Oak, Pinus, Citrus	Oak, Pinus	Oak
Ecoregion	Calcareous, No-calcareous	Calcareous, No-calcareous	Calcareous, No-calcareous	No-calcareous	No-calcareous
Altitude (m.a.s.l.)	23-527	9-500	4-527	99-215	99
pH	5.0-8.1	4.9-8.1	5.1-8.0	5.1-6.1	6.1
CE (µS/cm)	65-523	82-430	65-402	66-374	374
SOM (%)	1.6-11.1	2.3-17.6	1.8-10.8	3.8-10.8	10.8
Sand (%)	25-87	25-80	25-80	26-47	33
Silt (%)	10-44	7-49	15-39	31-43	39
Clay (%)	3-48	3-37	5-39	18-35	28
Soil fertility					
P ($mg \cdot kg^{-1}$)	0.02-47.5	0.17-49.1	0.02-25.2	0.02-3.9	0.01
K ($mg \cdot kg^{-1}$)	23-110	25-165	25-141	25-127	127
Mg ($mg \cdot kg^{-1}$)	49-1160	4-1068	49-746	220-646	646
Ca ($mg \cdot kg^{-1}$)	529-3867	545-4851	542-2589	584-1510	1510
Zn ($mg \cdot kg^{-1}$)	0.04-7.22	0.03-9.60	0.06-4.73	0.90-3.10	3.11
Mn ($mg \cdot kg^{-1}$)	7.7-34.6	2.7-35.0	11.5-34.6	33.5-34.6	34.6
Fe ($mg \cdot kg^{-1}$)	3.8-76.7	0.9-67.9	3.3-62.1	40.3-73.7	40.3
Cu ($mg \cdot kg^{-1}$)	0.01-4.97	0.02-8.13	0.01-2.75	0.5-1.6	1.40

[a] Method of isolation code: U - untreated soil; D - pre-dried soil; S - soil dilution and plating in selective medium. [b] Representative isolates deposited at UAlg – Universidade do Algarve and ICVV - Instituto de Ciencias de la Vid y del Vino -collections.

2.4.2 Impact of ecological drivers on entomopathogenic fungi natural occurrence.

We obtained similar ecological trends independently of the EPF isolation method employed, except for higher EPF occurrence using untreated soil baits in non-calcareous soils (Figure 2.4B, Annex 1.C). EPF occurrence was also similar for all isolation methodologies except in calcareous soils, where the EPF recovery was significantly higher using the selective medium method (Figure 2.4B, Annex 1.D). Larval mortality percentage was not significantly different between soil bait methods regardless of botanical group or ecoregion. The number of colonies and richness per sample observed by the selective medium method did not differ among habitat types or ecoregions.

We found the species *B. bassiana* and *F. oxysporum* in similar percentages (Annex 1.B) in all botanical groups and both soil ecoregions (Annex 1.E). Only *P. lilacinus* showed higher recoveries in non-calcareous soils than in calcareous (Annex 1.E; Annex 1.B), while *M. anisopliae* was only detected once, in the semi-natural oak habitat of non-calcareous soils.

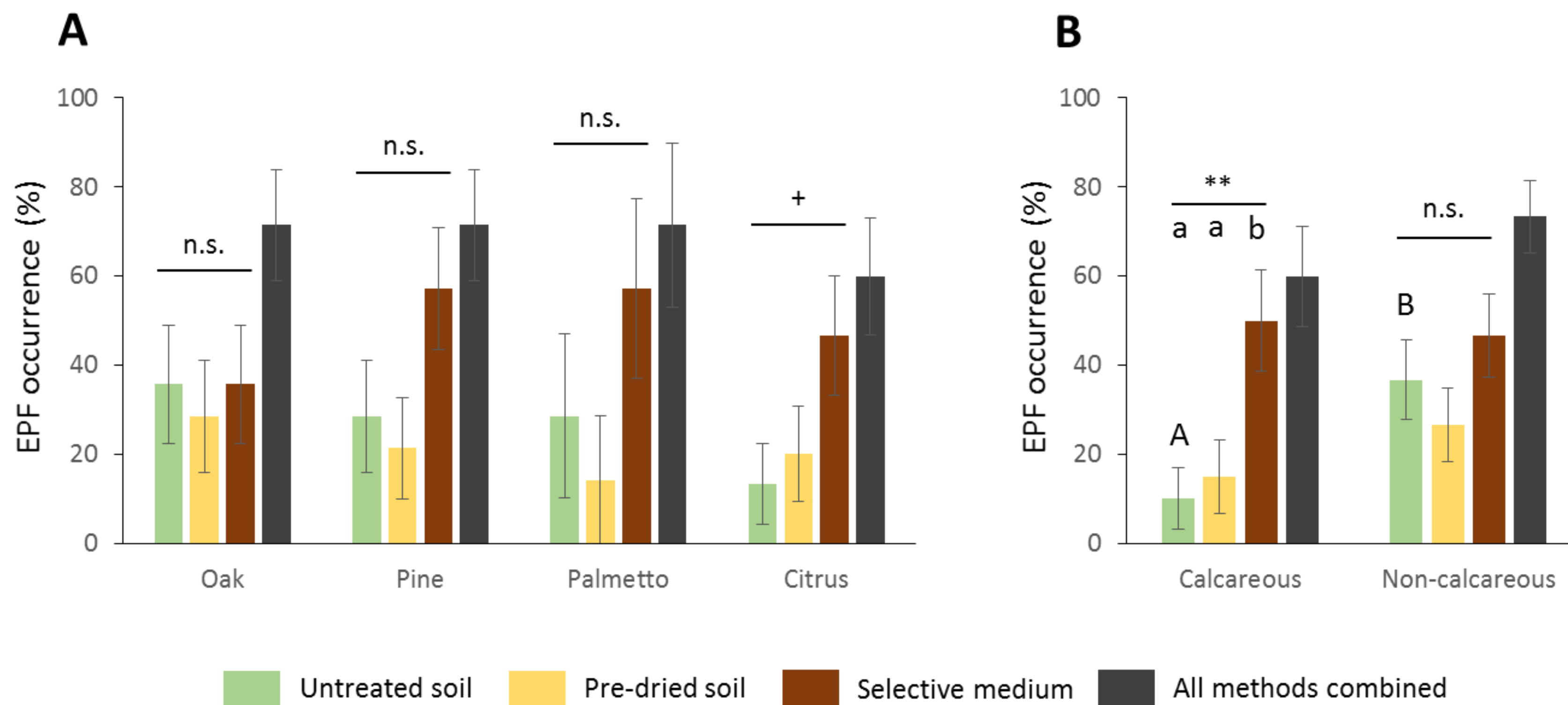


Figure 2.4. **Comparison of entomopathogenic fungi (EPF)** recovery frequency using different isolation methods depending on two ecological drivers (A) botanical habitats; (B) soil ecoregion. Asterisks indicate significant differences within treatment comparisons at ** $p < 0.01$, + $p < 0.1$ and n.s., not significant. Different letters indicate significant differences in Tukey's test (HSD) ($p < 0.05$). Values are least-square means ±SE.

2.4.3 Abiotic ranges for the occurrence of entomopathogenic fungi and patterns of assemblage into selected members of the entomopathogenic community

Overall, EPF occurred broadly among all the soil fertility parameters (Table 2.1). The three most abundant fungal species, *B. bassiana*, *F. solani* and *F. oxysporum* were detected in all botanical groups (except *F. oxysporium* not encountered in palmetto habitats) and both soil ecoregions, from sea level up to 500 m ASL and for wide ranges of soil pH and granulometry. The species *P. lilacinus*, only reported in 8% of the sites, was present in oak and pine habitats and non-calcareous soils, with loam/clay loam texture, lightly acidic soils and high SOM and Fe content. The only positive site for *M. anisopliae* was located in the west of the Algarve region at high altitude (around 100 m ASL), in oak habitat and non-calcareous soils with clay loam texture, lightly acidic soils and high SOM and Fe content.

We selected eight abiotic factors (elevation, soil pH and clay, SOM, P, Mg, Zn and Fe content) as explanatory variables (see Campos-Herrera et al., 2019, for more details) in the multivariate analysis of the soil entomopathogen species present in at least 10% of the sites (three EPF and two EPNs). CCA was conducted since DCCA maximum length was 4.1. The first two axes accounted for 95.6% of the explained fitted variation in species-environment relationships (Figure 2.5). The explanatory variables SOM and soil pH significantly contributed to explaining the biotic assemblage ($p < 0.05$), defining mainly the axis 2, while Mg content marginally contributed ($p = 0.07$) and slightly defined axis 1. Overall, the EPF species were more associated with the axis 1, with *B. bassiana* and *F. oxysporum* defined by high Mg concentrations and hence, associated with calcareous soils, but *F. solani* showing the opposite association. By contrast, the contrary, the EPN species were more associated with axis 2: *H. bacteriophora* with high soil pH and *S. feltiae* with high SOM.

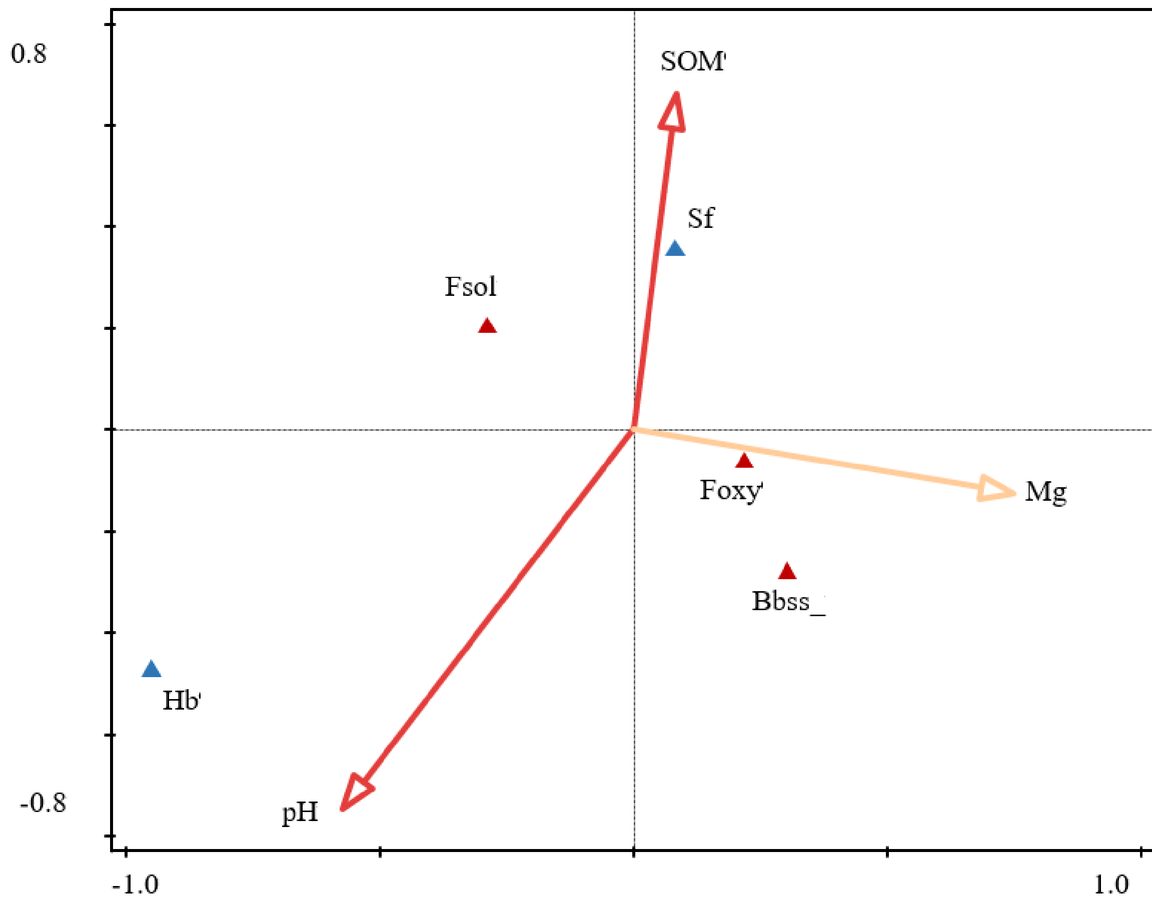


Figure 2.5. **Canonical correspondence analysis depicting the regional distribution and relationships between significant abiotic factors and soil organisms** ($p < 0.05$ in red and $p < 0.1$ in orange) in the Algarve region. Codes for abiotic factors (arrows): soil organic matter (SOM), soil pH (pH) and Mg content (Mg). Codes for biotic factors (triangles): the entomopathogenic fungi species (brown triangles) *Beauveria bassiana* (Bbss), *Fusarium solani* (Fsol) and *F. oxysporum* (Foxy); and the entomopathogenic nematodes (blue triangles) *Heterorhabditis bacteriophora* (Hb) and *Steinernema feltiae* (Sf).

2.5 Discussion

2.5.1 Distribution of entomopathogenic fungi across the Algarve region and comparison of three isolation methods

Overall, 68% of the sites were positive for the presence of EPF species in the Algarve region. Previous surveys found similar percentages, for example, 52% in the Pacific Northwest (Bruck, 2004) and 72% in Spain (Quesada-Moraga et al., 2007). However, EPF occurrence was quite variable in other studies, with only 20-30% of detections reported in the UK, Mexico, Turkey and Tasmania (Chandler et al., 1998; Sánchez-Peña et al., 2011; Rath et al., 2012; Sevim et al., 2009) or over 90% in Ontario (Canada) and Switzerland (Bidochka et al., 1998; Keller et al., 2003). Those frequencies are difficult to compare due to the lack of methodological uniformity among surveys. Not all the studies made the same effort in recovering the fungi. While Quesada-Moraga et al., 2007, baited 50 *G. mellonella* larvae *per site* in five independent soil subsamples, Chandler et al., 1998, employed just one larva per sample. In our study, by using three different EPF isolation methods and employing 20 *G. mellonella* larvae per sample in two independent baiting rounds, we increased the likelihood of recovering different entomopathogens (Hominick, 2002), including those of distinct guilds (EPF and EPNs) in the same sample (Campos-Herrera et al., 2019). Similarly, it is possible that increasing the size of the samples and the number of samples per site and repeating the study at various times (Meyling and Eilenberg, 2006), we could have detected a higher incidence of EPF in the Algarve region. However, we collected all samples in springtime, which may be the most favorable season for surveying EPF in Mediterranean habitats (Oliveira et al., 2013; Garrido-Jurado et al., 2015) and hence, the overall picture of their natural occurrence can be detected. In any case, further studies including other habitat types and seasonal pattern can provide new insights on the EPF natural distribution in Algarve and other Mediterranean areas.

As we hypothesized, we observed different patterns of EPF occurrence by using different isolation methods, particularly between insect baits and the selective medium methodology. Although the serial soil dilutions plating on selective media is considered a quantitative method and insect baits provide semi-quantitative data (Jaronski, 2007), we analyzed variables that allowed comparisons among the three methodologies. Although selective media recover pathogenic and saprophytic phases of EPF (Meyling, 2007), we resolved this methodological restriction by confirming the pathogenic capability of the

isolates via Koch´s postulates. Two independent rounds in soil baits allowed the isolation of EPF that remained inactive in the first round, as recommended for retrieving inactive EPNs (Hominick, 2002). Despite these protocols, the EPF occurrence and larval mortality detected by both soil baits were significantly lower than found using selective media. Especially low EPF occurrence and pathogenicity rates occurred using untreated soil baits, suggesting that antagonism or competition with other entomopathogens such as EPNs could interfere with efficient EPF isolation compared with pre-dried baits (Campos-Herrera et al., 2019). Additionally, the incubation of the soil bait samples at different temperatures could increase the recovery of different EPF species and provide differential patterns of distribution (Mietkiewski and Tkaczuk, 1998). In any case, no correlations among methodologies were established for any of the variables studied (as observed by Kessler et al., 2003), suggesting that each method may be detecting EPF in different active stages (Jaronski, 2007). Consequently, the combined results of all methodologies likely provided a more realistic distribution of EPF in the Algarve region (Korosi et al., 2019).

2.5.2 Entomopathogenic fungi species composition and the ecological drivers of their natural occurrence

Our detection of five EPF species is similar to previous studies: four in Spain (Asensio et al., 2003), six in Turkey (Sevim et al., 2009) and in Egypt (El-Ghany et al., 2012) and seven in Denmark (Meyling and Eilenberg, 2006). However, some studies also reported a higher or lower number of isolates, such as 8 species in olive groves in Portugal (Oliveira et al., 2013), 9 in agricultural areas in Spain (Garrido-Jurado et al., 2015), 12 in Portuguese vineyard soils (Sharma et al., 2018) or the only two species (*B. bassiana* and *M. anisopliae*) in Spain and Mexico (Quesada-Moraga et al., 2007; Sánchez-Peña et al., 2011), respectively. It is noteworthy that Campos-Herrera et al., 2019, employed the same untreated soil samples for evaluating the presence of EPNs, so the higher abundance of *F. solani* found through this particular methodology may be related to its interaction with EPNs. For example, Wu et al., 2018, observed that *F. solani* increased the efficacy of the EPN species *Steinernema diaprepesi*.

Our observations could support the hypothesis that both entomopathogenic organisms cooperate in a mutual strategy for the control of insect pests, but additional studies are needed for confirmation. Finally, *P. lilacinum* was the only EPF species that

showed significant differences among isolation methodologies, being favored by the selective medium method. These results highlight the necessity of using various isolation methods to unravel as much as possible the presence of different EPF species in soil communities.

In agreement with previous surveys conducted in the Iberian Peninsula (Asensio et al., 2003; Quesada-Moraga et al., 2009; Oliveira et al., 2013; Garrido-Jurado et al., 2015; Sharma et al., 2018), *B. bassiana*, identified in 34% of the sites, was the prevalent EPF species in Algarve's soils. The following two most common fungal species were *F. solani* and *F. oxysporum*, present in 26% and 14% of the sites, respectively.

In addition, the species *P. lilacinum* and *M. anisopliae*, isolated just in a few sites, were previously detected in low numbers in the Iberian Peninsula (Oliveira et al., 2013; Garrido-Jurado et al., 2015). The EPF species *B. bassiana*, widespread in soils, is commonly used in commercial products (Lacey et al., 2015). Sharma et al., 2018, also describe a *P. lilacinum* strain isolated from soils of Northern Portugal as an entomopathogen. Although *F. solani* and *F. oxysporium* are not frequently reported as entomopathogen organisms, there are some exceptions, such as the isolates from Egyptian soils of both fungal species described by El-Ghany et al., 2012, using *G. mellonella* as bait. In addition, Wu et al., 2018, isolated *F. solani* in *Diaprepes abbreviatus* (Coleoptera: Curculionidae) soil bait. Moreover, a recent review by Sharma and Marques, 2018, disentangled the complex nature of various *Fusarium* spp., observing opportunistic, saprobic and entomopathogenic behaviors. Indeed, *F. oxysporum* and *F. solani* are suggested to have insecticidal properties and the ability to develop inside hosts (Sharma and Marques, 2018).

Concerning the ecological drivers and contrary to our hypothesis, botanical habitats did not affect EPF occurrences nor larval mortalities in soils of the Algarve region. Previous studies obtained contrasting results. Quesada-Moraga et al., 2007, in agreement with our observations, did not find significant differences among habitats (natural *versus* agricultural) for the overall EPF presence, but observed species-specific habitat preferences. On the contrary, Sánchez-Peña et al., 2011, observed overall higher EPF occurrence in oak areas (51% of the sites) than other botanical habitats (less than 18% of the sites in pine, chaparral and agricultural areas). The high number of soil samples (280)

analyzed by Sánchez-Peña et al., 2011, may have favored the observation of statistical significance.

Since fungi generally tolerate acidic soils better than basic soils (Foth, 1984), we expected higher EPF detections in the non-calcareous region. Indeed, the three predominant EPF species in the Algarve (*B. bassiana*, *F. solani* and *F. oxysporum*) were retrieved in soils ranging from pH 5 to 8, but for the total fungal species isolated through all methodologies, this general trend was not supported. Optimal growth ranges may be relevant in determining the predominance of some species over others (Quesada-Moraga et al., 2007). Providing information on optimal ranges for EPF occurrences could improve predictive models useful in biologic control strategies.

2.5.3 Entomopathogenic fungi assemblage patterns

Many environmental factors can affect the EPF natural occurrence. Besides temperature and humidity, key factors for the EPF reproduction and activity (Jaronski, 2007), soil properties that provide the microhabitat conditions associated with the EPF are very important. Several authors have discussed how soil properties can affect the EPF natural occurrence (Jaronski, 2007; Quesada-Moraga et al., 2007; Jabbour and Barbercheck, 2009; Campos-Herrera et al., 2016; Campos-Herrera and Lacey, 2018). The soil pH and soil organic matter content were the two soil properties that mainly explained the variability of the EPF and EPN species included in our multivariate linkages. These two variables were also reported as relevant factors in previous studies of similar nature (Quesada-Moraga et al., 2007; Jabbour and Barbercheck, 2009; Campos-Herrera et al., 2016; Campos-Herrera et al., 2019). The typical consideration that fungus tolerate acidic soils better than basic soils (Foth, 1984), was also shown to some extend with the EPF detected in Algarve.

Despite some exceptions to the most detected species: *B. bassiana*, *F. solani* and *F. oxysporum*, results in line with the ecoregion pattern commented before, the multivariate analysis showed that the EPF species were mainly distributed in areas with acidic pH. However, previous studies in the Iberian Peninsula showed a higher detection in basic soils (Quesada-Moraga et al., 2007). However, the fact that in this study the most

prevalent species were *B. bassiana*, *F. solani* and *F. oxysporum* can explain the differences observed in the preferred pH recorded by Quesada-Moraga et al. (2007), where only *B. bassiana* and *M. anisopliae* were recorded. In addition, low soil organic matter content is often associated with higher EPF occurrence (Quesada-Moraga et al., 2007; Jabbour and Barbercheck, 2009). It is noteworthy that different EPF and EPN assembled differentially, occupying different quadrants in the CCA analysis. Thus, while the EPF species *B. bassiana* and *F. oxysporum* were slightly linked to calcareous soils, *F. solani* was more associated with non-calcareous soils. Finally, EPNs followed a similar pattern that was observed by Campos-Herrera et al (2019) when other soil organisms, excluding EPF, were included in their CCA analyses.

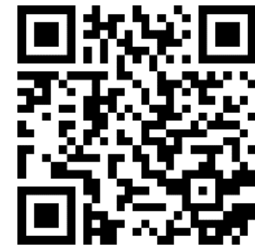
2.6 Conclusions

This study expands the understanding of EPF natural distribution and increases the number of EPF species previously described in the Iberian Peninsula (Sharma et al., 2018; Asensio et al., 2003; Quesada-Moraga et al., 2007; Oliveira et al., 2013; Garrido-Jurado et al., 2015). It also explores the ecological drivers that could modulate their occurrence in the Algarve region. The study provides a more comprehensive characterization of the EPF occurrence by using three different isolation methodologies, combining traditional and molecular tools for species identification. Moreover, the description of the EPF/EPN community assemblage by the soil properties provides new insights on shared niches among different guilds. Under a restrictive and prohibitive context of many agrochemical products, identifying the best ecological scenarios for the mutual use of different beneficial soil organisms (Imperiali et al., 2017; Jaffuel et al., 2019), promoting cooperation and avoiding competition for hosts (Bueno-Pallero et al., 2018), will provide more effective bio-tools that can contribute to a more sustainable agriculture.

2.7 Supplementary materials

The following are available in annexes 2 and online at http://www.mdpi.com/2075-4450/11/6/352/s1, Annex 1.E. Comparison of entomopathogenic fungi (EPF) recovery frequency by species depending on two ecological drivers. A. Botanical habitats. B. Soil ecoregion. Different letters indicate significant differences in T-test ($p < 0.05$). Values are least-square means ± SE, Annex 1.A. Pearson correlations ($p < 0.05$) to compare the recovery occurrence and larval mortality percentages among different isolation methods of entomopathogenic fungi (EPF); n.s., no significant, Annex 1.B. Statistical analysis (One-way ANOVA and T-test) for the occurrence of fungal that confirmed Koch´s postulates accordingly the variables isolation method, vegetation type, and soil eco-region, Annex 1.C. Statistical analysis (One-way ANOVA and T-test) of the impact of the variables vegetation type or soil ecoregion on the occurrence of entomopathogenic fungi (EPF) and larval mortality recorded for each of EPF isolation method, Annex 1.D. Statistical analysis (One-way ANOVA) of the efficiency among isolation methods for the occurrence of entomopathogenic fungi (EPF) and larval mortality recoveries efficacy depending on the factor's vegetation type or ecoregion.

III
SIMULTANEOUS EXPOSURE OF NEMATOPHAGOUS FUNGI, ENTOMOPATHOGENIC NEMATODES AND ENTOMOPATHOGENIC FUNGI CAN MODULATE BELOWGROUND INSECT PEST CONTROL



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CHAPTER III

3 SIMULTANEOUS EXPOSURE OF NEMATOPHAGOUS FUNGI, ENTOMOPATHOGENIC NEMATODES AND ENTOMOPATHOGENIC FUNGI CAN MODULATE BELOWGROUND INSECT PEST CONTROL

3.1 Abstract

Entomopathogenic nematodes (EPNs) and fungi (EPF) are well known biological control agents (BCAs) against insect pests. Similarly, the nematophagous fungi (NF) are considered good BCA candidates for controlling plant parasitic nematodes. Because NF can employ EPNs as food and interact with EPF, we speculate that the simultaneous application of EPNs and EPF might result in higher insect mortality, whereas the triple species combination with NF will reduce the EPN and EPF activity by predation or inhibition. Here we evaluated single, dual (EPN + EPF, EPF + NF, EPN + NF) and triple (EPN + EPF + NF) combinations of one EPN, *Steinernema feltiae* (Rhabditida: Steinernematidae), one EPF, *Beauveria bassiana* (Hypocreales: Clavicipitaceae), and two NF, *Arthrobotrys musiformis* (Orbiliales: Orbiliaceae) and *Purpureocillium lilacinum* (Hypocreales: Ophiocordycipitaceae) under laboratory conditions. First, we showed that EPF reduced the growth rate of NF and *viceversa* when combined in both rich and limiting media, suggesting a negative interaction when combining both fungi. Three different fungal applications (contact with mycelia-conidia, immersion in conidial suspension, and injection of conidial suspension) were tested in single, dual and triple species combinations, evaluating *Galleria melonella* (Lepidoptera: Pyralidae) larval mortality and time to kill. When mycelia were presented, the EPF appeared to be the dominant in combined treatments, whereas in immersion exposure was the EPN. In both types of exposure, NF alone did not produce any effect on larvae. However, when *A. musiformis* was injected, it produced larval mortalities > 70% in the same time span as EPN. Overall, additive effects dominated the dual and triple combinations, with the exception of injection method, where synergisms occurred for both NF species combined with EPN + EPF. This study illustrates how differences in species combination and timing of fungal arrival can modulate the action of BCAs when augmented in the soil. Further studies are required to fine-tune these multitrophic interactions to provide successful, sustainable and resilient pest management in agroecosystems.

3.2 Introduction

Soil organisms are responsible for numerous beneficial ecological goods and services (Cavigelli et al., 2012). The soil supports a great diversity of organisms, connected by complex food webs, and subjected to environmental fluctuations that drive changes in the soil communities in time and space (Ritz and van der Putten, 2012). Understanding the interactions occurring among soil inhabitants will help to elucidate mechanisms that govern fundamental ecological processes. This knowledge can be implemented to develop rational, ecologically sound agricultural management tactics that enhance ecosystem services, such as biological control of soil dwelling insect pests (Bommarco et al., 2013).

Entomopathogenic nematodes (EPNs) and fungi (EPF) are well known biological control agents (BCAs) (Lacey et al., 2015), widely distributed in soils ranging from natural areas to agroecosystem (Charnley and Collins, 2007; Campos-Herrera, 2015; Lacey et al., 2015). The traditional EPNs in the families Steinermatidae and Heterorhabditidae are well-studied insect entomopathogens, cosmopolitan, and already commercially used against soil insect pests (Hominick, 2002; Lacey et al., 2015; Stock, 2015). In the soil, individuals in the stage called “infective juvenile” (IJ) locate soil dwelling insect hosts, penetrating through natural openings (spiracles, mouth and anus) or sometimes through the cuticle. The IJs host an enteric γ- Proteobacteria in mutualistic association (Boemare, 2002); hence, the nematode is a vector transporting the bacteria to the insect hemocoel. Once inside the host, IJs release the bacteria and both organisms con- tribute to insect death (Dillman et al., 2012; Sugar et al., 2012). The production of Scavenger Deterrent Factor (SDF) by the bacteria protects the insect cadaver from other opportunistic microorganisms and in- vertebrate scavengers (Gulcu et al., 2012; Lewis et al., 2015). Both nematode and bacteria complete several generations inside the insect cadaver until food and waste products became limiting (Boemare, 2002; San-Blas et al., 2008). At this point, EPN development arrest at the IJ stage, which incorporate bacteria and leave the cadaver in search of new hosts (Boemare, 2002). Development of IJs in the cadaver occurs during 1-3 weeks, depending on the EPN species, the host, and variables such as temperature or moisture (Kaya and Gaugler, 1993; Griffin, 2015).

The EPF are also widespread in the soil and infect a wide range of arthropods (Hajek and St. Leger, 1994; Inglis et al., 2001). Commercial EPF are also widely produced as BCAs (Jeffs and Khachatourians, 1997; Donatti et al., 2008; Lacey et al., 2015). Most of the EPF display distinct parasitic and saprophytic phases of interaction with arthropod hosts (Charnley and Collins, 2007). The parasitic phase starts with fungal infection, which occurs by contact of the EPF spore with the host (Oreste et al., 2012). The fungus penetrates the cuticle and inside the insect body cavity, secretes toxic secondary metabolites to kill the in- sect and antibiotics to prevent bacteria proliferation in the cadaver (Strasser et al., 2000; Charnley and Collins, 2007; Donatti et al, 2008). After insect death, EPF switch to a saprophytic phase, with active hyphal growth and production of reproductive structures in the emergent aerial mycelia for dispersion to complete their life cycle in new hosts.

Like EPNs and EPF, nematophagous fungi (NF) are ubiquitous in natural and agricultural soils (Nordbring-Hertz et al., 2011). The NF are classified in three main groups: nematode-trapping, endoparasitic and egg- and cyst-parasitic (López-Llorca et al., 2008). Generally, trapping and eggs-cyst-parasitic NF can also survive saprophytically, while endoparasitic NF are obliged to use nematodes to complete development. Depending on the NF species, there are a great variety of structures that can be developed to capture the nematode, such as adhesive nets, branches, knobs or hyphae, constricting rings, zoospores or appressoria (Nordbring-Hertz et al., 2011). The generation of these structures can be spontaneous or the result of environmental stimuli, including nematode presence (Nordbring-Hertz et al., 2011). Various NF members are considered good candidates for controlling harmful nematodes of crops and livestock (Nordbring-Hertz et al., 2011; Braga and Araújo, 2014; Li et al., 2015). However, NF are natural enemies of all types of nematodes, including those that provide human bene fit such as EPNs (Jaffee and Strong, 2005; El-Borai et al., 2007, 2009; Nordbring-Hertz et al., 2011).

Both EPNs and EPF exploit the same resource, the insect. The final host mortality and successful entomopathogenic reproduction will de- pend on the target insect (species/developmental stage), exposure methods, the concentration, the timing, the sequence of arrival, and the EPN/EPF species combined (Shapiro-Ilan et al., 2004; Acevedo et al., 2007; Jabbour et al., 2011; Tarasco et al., 2011; Hajek and Meyling, 2018).

The complete exclusion of one of the entomopathogens is possible (Barbercheck and Kaya, 1990; Kaya, 2002), since both EPF and EPNs produce specific metabolites and bio-products to prevent the attack of the cadaver by other opportunistic microorganisms and scavengers (Strasser et al., 2000; Lewis et al., 2015). However, under different timing/concentration/species combination scenario, additive and synergistic effects can be also displayed (Shapiro-Ilan et al., 2004; Jabbour et al., 2011; Tarasco et al., 2011; Navarro et al., 2014; Shaurub et al., 2016). Similarly, the co-occurrence of EPF and NF can produce an antagonist effect by inhibiting the normal growth of other fungi (Asensio et al., 2007; Andaló et al., 2008; Sahab, 2012). However, to our knowledge, there are no reports testing whether NF presence inhibits EPF ability to kill the insect. In addition, NF are natural enemies of EPNs (Jaffee and Strong, 2005; El-Borai et al.,2007; Andaló et al., 2008), and hence, the NF-EPN interaction can regulate the ability to control insect pests (Ram et al., 2008; Stuart et al., 2008). Although early studies reported heterorhabditids more resistant to NF attack (Timper and Kaya, 1989), further investigations revealed EPN susceptibility to NF to be species and environmentally dependent (El-Borai et al., 2009; El-Borai et al., 2011).

To our knowledge, the extent to which the simultaneous presence of EPN, EPF and NF modulates insect control potential is completely unexplored. We hypothesized that a simultaneous application of EPN and EPF at levels balanced to produce moderate larval mortality by either guild will display a positive effect (ranging from additive to synergistic). We also expected that NF growth would be strongly inhibited by the presence of EPF and *viceversa*. Hence, when exposed to an insect, the presence of NF would decrease the ability to kill the insect by either entomopathogen (Asensio et al., 2007; Andaló et al., 2008; El-Borai et al., 2009, 2011; Sahab, 2012; Navarro et al., 2014). Here we assessed the extent to which each organism affects the others in the control of an insect. The aims of this study were: (1) to assess the growth rates of EPF and NF when cultured alone or combined in either nutrient limiting or rich media; and (2) to evaluate the insecticidal ability of EPNs, EPF and NF when exposed alone or combined.

3.3 Material and methods

3.3.1 Insects, nematodes and fungi

Last instar larvae of *Galleria melonella* L. (Lepidoptera: Pyralidae) reared at the University of Algarve (Portugal) were employed to maintain the EPN species *Steinernema feltiae* (Filipjev) (Rhabditida: Steinernematidae; AM-25 population, native from Algarve; GenBank accession number MG551674). The culture was regularly refreshed (Woodring and Kaya, 1988) and upon IJ emergence, nematodes were recovered in mineral water, stored at 14 °C, and used within 2 weeks of harvest. The EPF species *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae; native from Algarve; GenBank access number MG515530) was isolated from a naturally infected adult of *Rhynchophorus ferrugineous* Olivier (Coleoptera: Curculionidae) (University of Algarve, Campus of Gambelas). Two NF species from Dr. L.W. Duncan's collection (University of Florida, USA) were tested: one trapping NF, *Arthrobotrys musiformis* Drechsler (Orbiliales: Orbiliaceae; strain 11, GenBank accession number KJ938572) and one egg/cyst parasite, *Purpureocilium lilacinum* (Thom) (Hypocreales: Ophiocordycipitaceae; strain 9357, GenBank accession number KJ938575). EPF and NF were cultured on 90 mm diam. Petri dishes with Potato Dextrose Agar (PDA, Biokar) at 25 ± 1 °C and were not sub-cultured more than once during the study (Shapiro-Ilan et al., 2004). Fungal material was stored at 4 °C until use (Goettel and Inglis, 1997).

3.3.2 Mycelia growth of concomitant entomopathogenic fungi and nematophagous fungi.

The direct opposition method (adapted from Dennis and Webster, 1971; Chen et al., 2016) evaluated the interaction between EPF and each of the two NF. The differential growths were tested in two media, PDA as rich medium and Corn Meal Agar (CMA) ¼ (Fluka analytical, Sigma-Aldrich, CO, USA) as limiting medium. Discs (6.5 mm of diam.) of actively growing mycelia in PDA and CMA, respectively, of each of the fungi were taken with a sterilized metallic cylinder and placed on 90-mm Petri dishes containing 13 mL of PDA or CMA. In the plates with combined exposure of the EPF and NF, discs were confronted at 30 mm distance, whereas control treatments only contained the disc of one of the fungi placed in the same position ($n = 5$; 2

independent trials). The fungal growth was measured daily until any fungus (control or in pair) reached the maximum possible growth (i.e., farther border of the plate).

3.3.3 Larval mortality from individual or concomitant nematophagous fungi, entomopathogenic nematodes and entomopathogenic fungi

Three experiments investigated different strategies of EPF/NF exposure to the host: (i) larval direct contact with mycelia (Leemon and Jonsson, 2008), (ii) larval immersion in conidial suspension (Bacca and Lagos, 2014), and (iii) larval injection of conidial suspension (Tarasco et al., 2011). On the contrary, the EPN was directly released in arena in all the cases, independently of the fungal method of exposure. All the experiments were conducted in 24-wells plates (Falcon Multiwell, 24 well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA) filled with 1.0 g of sterile oven-dried sand (pure sand, Vale do Lobo, Loulé, Portugal). Distilled water (200 µL) and one last instar *G. mellonella* were added to each well (n = 20 per treatment).

To allow detecting interactions in the combined treatments (Koppenhöfer and Grewal, 2005), preliminary immersion/injection experiments evaluated single EPF or NF application at various concentrations (0, 100-108 conidia) (n = 15 per treatment, 2 independent trials) (Wang et al., 2002; Shapiro-Ilan et al., 2004; Leemon and Jonsson, 2008; Oreste et al. 2012; Akmal et al., 2013; Akbari et al., 2014; Castillo-López et al., 2013; Navarro et al., 2014). Similarly, for the EPN we evaluated the larval mortality in the range of 0, 1, 5, 10, 20, 50 and 100 IJs/well (n = 20 per treatment, 2 independent trials). For all the organisms, final experimental concentrations were adjusted to avoid single species treatments with over 75% larval mortality and readjusted, if needed (Table 3.1). In the case of the direct-contact with mycelia-conidia, the dosage was equivalent to the disc applied per treatment, and hence, there were not preliminary concentration experiments. Plates were randomly distributed in humidity chambers (> 90% relative humidity) and maintained at 24 ± 1 °C (Acevedo et al., 2007). Larval mortality was determined daily for up to five days (Dillman et al., 2012). In all the experiments, the dead larvae were rinsed in tap water, placed in White traps (White, 1927) and inspected to evaluate the progress of the most probable causing agent, comparing cadaver's morphology and consistence that resulted from treatments with single species application

and in combinations. Because dual and triple species combinations might produce "hidden" actions, we only registered the most apparent causing agent of the larval dead, but without consideration for the statistical analysis, only for the general observation. All experiments were repeated twice, with newly produced/prepared material.

In all experiments EPNs were hand-picked into the final volume per well prior to addition of the sand, the fungi and the larva. For the experiments involving larval direct-contact with mycelia-conidia (Leemon and Jonsson, 2008), discs of each fungus were retrieved from several plates of the same age. For both larval immersion and larval injection experiments (Tarasco et al., 2011; Bacca and Lagos, 2014), conidial suspensions were prepared by using a sterile swab to transfer the conidia from PDA plates to a Falcon® tube with sterile half-strength Ringer solution and 0.05% Tween 80 (Klingen et al., 2002). The conidial suspension was homogenized (Vortex®) for 2-3 min and concentrations estimated by haemocytometer (Neubauer improved). Each suspension was adjusted to the final concentration depending on the type of fungal exposure test (Table 3.1). As additional control for the preparation of conidial concentrations, PDA plates were inoculated with serial dilution of each of the fungal suspensions employed in each of the experiments (data not shown). For the immersion method, larvae were immersed for 5 s in fungal suspensions with continuous movement to avoid sedi- mentation (Acevedo et al., 2007; Bacca and Lagos, 2014; Wakil et al., 2017). For the injection method, we applied either 10 µL of fungal suspensions or sterile Ringer solution as controls in the first pseudolegs pair of the larva by using a disinfected microsyringe (Griesch and Vilcinskas, 1998; Tarasco et al., 2011).

3.3.4 Statistical analysis

In the study about the mycelia growth interactions between EPF and EF, fungal growth was expressed as mm × day^{-1}. Student-T test ($p < 0.05$) assessed the differences in the growth rate of each of the fungus exposed alone (control) or combined (EPF + NF) for each of the media as well as changes in the growth rate between the two media (PDA and CMA) for the same fungal exposure (Miyashira et al., 2010). The appropriate concentration for the EPN, EPF and NF for all the exposure tests (Table 3.1) was estimated by Probit analysis, pooling the data of two independent trials per species and adjusting as required (data not shown). In the study about the interaction among EPN,

EPF and NF, we considered two variables: larval mortality percentage and days after exposure to kill the insect. Prior to statistical analysis, the percentages were arcsine transformed. We checked that the data of the independent trials could be pooled by two-ways ANOVA, and when confirmed, we employed one-way ANOVA followed by Tukey's test (HSD) ($p < 0.05$) for mean comparisons. In the analysis of time to kill the insect, treatments with only one value were discarded to avoid considering not representative data. All the statistical analyses were performed with the software SPSS (version 21.0, SPSS Statistics, SPSS Inc., Chicago, IL, USA). Data presented as mean ± SEM of untransformed values.

To evaluate the nature of EPN, EPF and NF interactions (antagonistic, no-interaction/additive, or synergistic), we followed and adapted (for the triple interaction) the formula proposed by Shapiro-Ilan et al. (2004) and Ansari et al. (2010). We compared the expected and observed mortalities for each of the fungal exposure experiments. The expected mortalities (ME) were calculated as: ME = MT1 + [MT2 × (1 − MT1)] for combination of two organisms and as ME = MT1 + [MT2 × (1 − MT1 − MT3)] + [MT3 × (1 − MT1 − MT2)] for combinations of three organisms, where MT1, MT2 and MT3 are the mortalities observed from each organism alone. Results from a X2 test applied for the expected and observed mortalities [i.e., X2 = (MT1T2 − ME)2/ME, where MT1T2 is the observed mortality for two organisms alone] were compared to the χ^2 table value for 1 degree of freedom ($p = 0.05$). Values of $\chi^2 < 3.8415$ indicated additive interactions and non-additive if $\chi^2 > 3.8415$ (antagonist or synergist). If the differences MT1T2 − ME > 0 the interaction was considered synergistic, while antagonistic if MT1T2 − ME < 0 (Shapiro-Ilan et al., 2004; Ansari et al., 2010).

Table 3.1. **Experimental design to evaluate the interaction** among nematophagous fungi (NF) and entomopathogens (nematode -EPN- and fungus -EPF-) in the larval mortality rate.

Treatment code	Type of organism	Species	Mycelia exposure Application[a]	Immersion Concentration[b]	Injection Concentration[b]
Blank[c]	–	–	–	–	–
Control[c]	–	–	–	–	–
Sf	EPN	*S. feltiae*	1–2	1-2	1-2
Sf+Disc[d]	EPN	*S. feltiae*	1–2	Not included	Not included
Bb	EPF	*B. bassiana*	6.5	$1\times10^{6-7}$	$6\times10^{3-4}$
Am	NF	*A. musiformis*	6.5	1×10^{4}	1×10^{1}
Pl	NF	*P. lilacinum*	6.5	1×10^{8}	$2\text{-}3\times10^{6}$
Sf+Bb	EPN+EPF	*S. feltiae/B. bassiana*	1–2/6.5	$1\text{-}2/1\times10^{6-7}$	$1\text{-}2/6\times10^{3\text{-}4}$
Sf+Am	EPN+NF	*S. feltiae /A. musiformis*	1–2/6.5	$1\text{-}2/1\times10^{4}$	$1\text{-}2/1\times10^{1}$
Sf+Pl	EPN+NF	*S. feltiae/P. lilacinum*	1–2/6.5	$1\text{-}2/1\times10^{8}$	$1\text{-}2/2–3\times10^{6}$
Bb+Am	EPF-NF	*B. bassiana /A. musiformis*	6.5/6.5	$1\times10^{6}/1\times10^{4}$	$6\times 10^{3}/ 1\times10^{1}$
Bb+Pl	EPF-NF	*B. bassiana/P. lilacinum*	6.5/6.5	$1\times10^{7}/1\times10^{8}$	$6\times 10^{4}/2–3\times10^{6}$
Sf+Bb+Am	EPN+EPF+NF	*S. feltiae / B. bassiana /A. musiformis*	1–2/6.5/6.5	$1\text{-}2/ 1\times10^{6}/1\times10^{4}$	$1\text{-}2/6\times10^{3}/1\times10^{1}$
Sf+Bb+Pl	EPN+EPF+NE	*S. feltiae/B. bassiana/P. lilacinum*	1–2/6.5/6.5	$1\text{-}2/1\times10^{7}/1\times10^{8}$	$1\text{-}2/6\times10^{4}/2\text{-}3\times10^{6}$

a EPN concentrations in number of infective juveniles (IJs) per well; for EPF and NF, application was performed with discs (mm diameter) of mycelia and conidia. In single fungal application, one disc of sterile medium was applied to maintain the proportion of two discs per well.
b EPN concentrations in number of infective juveniles (IJs) per well (half of the wells received one IJ and the other half 2 IJs to achieve the general concentration of 1.5 IJs); EPF and NF concentrations expressed as number of conidia/ml per larva.
c Blank treatment considers only the presence of insect larvae; control treatment implies that the larva was handled as the larva in each of the treatments, but with sterile solution or discs of sterile medium.
d Sf+Disc includes the assigned number of nematodes per well with two discs of sterile medium to ensure same conditions than in treatments with fungi.

3.4 Results

3.4.1 Mycelia growth of concomitant entomopathogenic fungi and nematophagous fungi

The growth rates of all fungi were reduced when exposed to direct opposition compared to their corresponding controls (Figure 3.1) ($p < 0.01$), with the exception of *B. bassiana* combined with *A. musiformis* in rich medium (Figure 3.1C). Overall, growth of either NF was more inhibited (Figure 3.1A and 3.1B) than that of *B. bassiana (*Figure 3.1C and D). These differences were greater when the fungi were grown in rich medium (PDA) than in limiting medium (CMA ¼). All the interactions showed inhibition by contact (distance between mycelia < 2 mm) (Wheeler and Hocking, 1993) (data not shown).

3.4.2 Larval mortality from individual or concomitant nematophagous fungi, entomopathogenic nematodes and entomopathogenic fungi

The presence of discs of media and NF did not significantly reduce the larval mortality or time to kill by *S. feltiae* (Figure 3.2). The application of *B. bassiana* alone caused $> 90\%$ larval mortality in 4 days, whereas neither NF applied alone caused any larval mortality (Figure 3.2). When the NF were combined with any of the entomopathogens in dual applications, neither the larval mortality nor the time to kill the insect differed from the mortality caused by single applications of the EPN or EPF (Figure 3.2). The larval mortality reached 100% in all the treatments where *B. bassiana* was combined with *S. feltiae,* or in the triple species addition with the any of the NF, producing the insect mortality in intermediate time between the single application of EPF and EPN ($\sim$3.5 days; Figure 3.2). Similar trends were observed in trial 2 (Annex 2). All interactions when larvae were in direct contact with mycelia-conidia were additive, with the exception of an antagonistic effect for the combination of *S. feltiae* and *P. lilacinus* (Table 3.2).

When larvae were immersed in conidial suspension, the EPN single application produced the same trend as in the mycelial-conidial exposure, reaching ~60% mortality in 2.5–3.5 days (Figure 3.3). The larval mortality caused by *B. bassiana* alone ranged from 15 to 60%, employing at least 4 days to kill the insect.

Both NF species single suspension produced < 10% mortality (Figure 3.3). As in the mycelial-conidial exposure, in dual exposition of EPN and any of the NF, neither the larval mortality nor the time to kill the insect were significantly different from the mortality observed in the single application of the EPN (Figure 3.3). The combination of both entomopathogens caused larval mortality and time to kill the insect apparently driven by the EPN (Figure 3.3). In the experiments with *A. musiformis*, an antagonist effect was observed (Table 3.2) probably due to the use of a slightly lower *B. bassiana* final concentration (Table 3.1). The dual exposure of *B. bassiana* with either NF caused opposite effects: synergism when combined with *A. musiformis* and antagonism with *P. lilacinum* (Table 3.2, Figure 3.3A and B). The larval mortality and time to kill the insect in triple species combination with *P. lilacinum* were apparently driven by the EPN (Figure 3.3A and C), producing an additive effect (Table 3.2). In the case of *A. musiformis*, the larval mortality in the triple species treatment was significantly higher than any of the three individual applications (Figure 3.3B), overall producing an additive effect (Table 3.2), but in a time as short as for the EPN (~2.8 days; Figure 3.3D).

In the experiment involving larval injection of conidial suspensions, the EPN single species application also produced 50-60% mortality in ~2.5 - 3.5 days (Figure 3.4). The larval mortality caused *B. bassiana* alone ranged from ~20-40%, employing > 4 days to kill the insect (Figure 3.4). Similarly, *P. lilacinum* injected alone was adjusted to produce larval mortality 30% in > 4 days (Figure 3.4B and D). However, the injection of 10^1 conidia/mL of *A. musiformis,* the lowest concentration we could prepare, produced larval mortalities > 70% in just 3 days (Figure 3.4A and C). The combination of both entomopathogens increased the larval mortality (Figure 3.4), producing a synergist effect in the *A. musiformis* trials as result of using a slightly lower *B. bassiana* final concentration (Tables 3.1 and 3.2). The dual infection by EPN and *A. musiformis* was apparently driven by the NF (Figure 3.4A and C), without significant differences with the single *A. musiformis*

application, overall producing an additive effect (Table 3.2). *P. lilacinum* combined with *S. feltiae* did not increase the larval mortality or the time to kill above that for the EPN alone (Figure 3.4B and D). When *B. bassiana* was combined with *A. musiformis,* the larval mortality increased synergistically (Figure 3.4A and C, Table 3.2). When *B. bassiana* was combined with *P. lilacinum*, larval mortality was additive (Table 3.2) and not significantly higher than that for either fungus (Figure 3.4B) nor was the time to kill affected (Figure 3.4D). Overall, when the fungi were injected, the triple species combination produced a synergistic effect (Table 3.2).

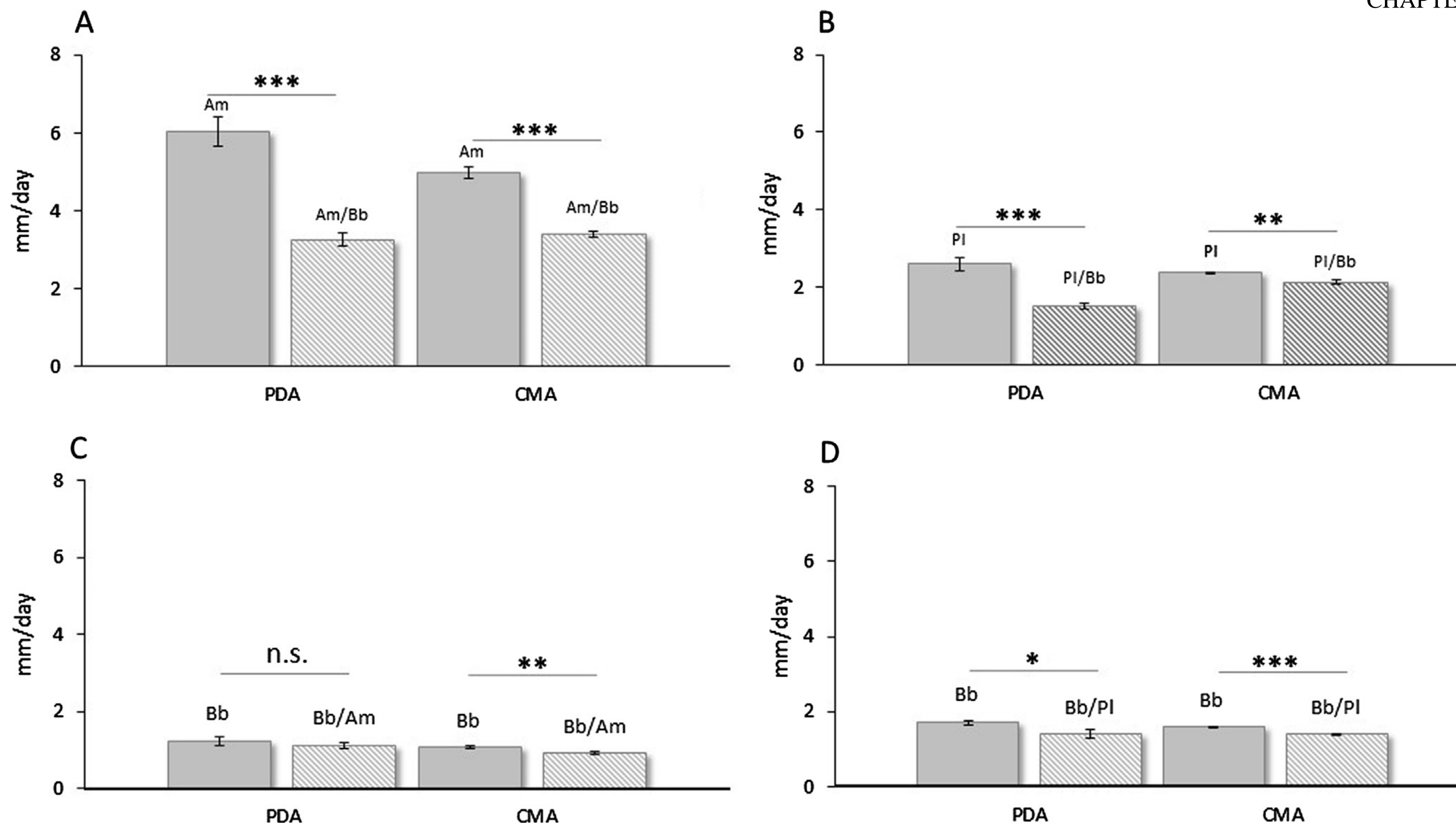

Figure. 3.1. ***In vitro* growth rates of entomopathogenic fungi** (EPF) *Beauveria bassiana* (Bb) and nematophagous fungi (NF) *Arthrobotrys musiformis* (Am) and *Purpureocillium lilacinum* (Pl) in rich medium (PDA) and limiting medium (CMA ¼). A. Growth rates of Am, alone or combined with Bb. B. Growth rates of Pl, alone or combined with Bb. C. Growth rates of Bb, alone or combined with Am. D. Growth rates of Bb, alone or combined with Pl. Differences between pair treatments (alone or combined) for each media assessed by Student-T-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, n.s. no significant. Data are expressed as average ± SEM.

Table 3.2. **Interactions observed when combining entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPF) and/or nematophagous fungi (NF)** for supression of *Galleria mellonella* larvae in 5 days

	Mycelia-conidia exposure				Immersion				Injection			
Combination[a]	Observed mortality[b]	Expected mortality[c]	χ^2	Interaction[d]	Observed mortality[b]	Expected mortality[c]	χ^2	Interaction[d]	Observed mortality[b]	Expected mortality[c]	χ^2	Interaction[d]
Sf + Bb	100/100	94/94	0.38/0.38	Additive/Additive	80/45	85/70.3	0.29/9.08	Additive/Antagonism	80/77.5	77.5/54.6	0.08/9.58	Additive/Synergistic
Sf + Pl	25	40	5.63	Antagonism	52.5	61	1.18	Additive	75	73.8	0.02	Additive
Sf + Am	35	40	0.63	Additive	72.5	67.6	0.35	Additive	95	84.9	1.21	Additive
Bb + Pl	90	90	0.00	Additive	87.5	63.4	9.13	Synergistic	52.5	58	0.52	Additive
Bb + Am	100	90	1.11	Additive	10	21.4	6.05	Antagonism	100	77.3	6.66	Synergistic
Sf + Bb + Pl	100	94	0.38	Additive	92.5	82.9	1.12	Additive	82.5	64.8	4.87	Synergistic
Sf + Bb + Am	100	94	0.38	Additive	65	67.6	0.10	Additive	100	69.1	13.79	Synergistic

[a] The EPN *Steinernema feltiae*, Sf; the EPF *Beauveria bassiana*, Bb; the NF *Arthrobotrys musiformis*, Am and *Purpureocillium lilacinus*, Pl.

[b] Observed mortality (%), average of 20 replicates of *G.mellonella* in two trials (40 total).

[c] Expected mortality in %, calculated $M_E=M_{T1}+M_{T2}*(1-M_{T1})$ for combination of two organisms applied on the larvae; and $M_E=M_{T1}+M_{T2}\times(1- M_{T1}+M_{T3}) + M_{T3}\times(1- M_{T1}+M_{T2})$ for combination of three organisms applied on the larvae simultaneously; where M_{T1}, M_{T2} and M_{T3} are the mortalities for each organism alone, respectively.

[d] Interaction was based on χ^2 ratio between expected and observed mortalities.

[e] Data from the experiment with Pl in the left and from the Am in the right.

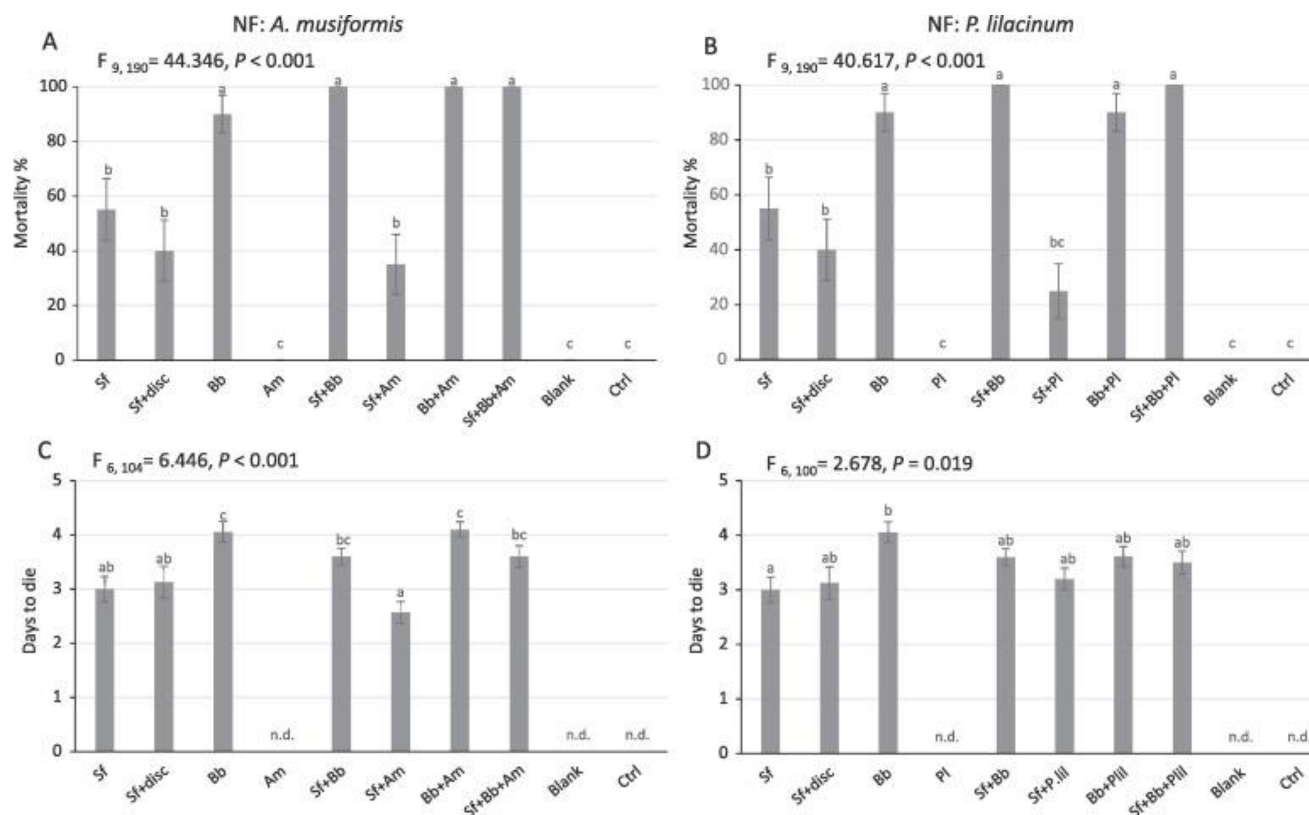

Figure 3.2. **Evaluation of the interaction among nematophagous fungi (NF) and entomopathogens (nematode and fungi) in the direct-contact with mycelia-conidia** experiment (trial 1). A and B. Larval mortality percentage. C and D. Number of days until larval death. Different letters above bars indicate statistical differences (one-way ANOVA and Tukey's test (HSD), $p < 0.05$). Treatments code: control (Ctrl, larvae exposed to two sterile discs), Blank (untreated larvae), *Steinernema feltiae* (Sf), *Beauveria bassiana*, (Bb), *Arthrobotrys musiformis* (Am), *Purpureocillium lilacinum* (Pl), and the double or triple combinations of these organisms. Data are expressed as average ± SEM.

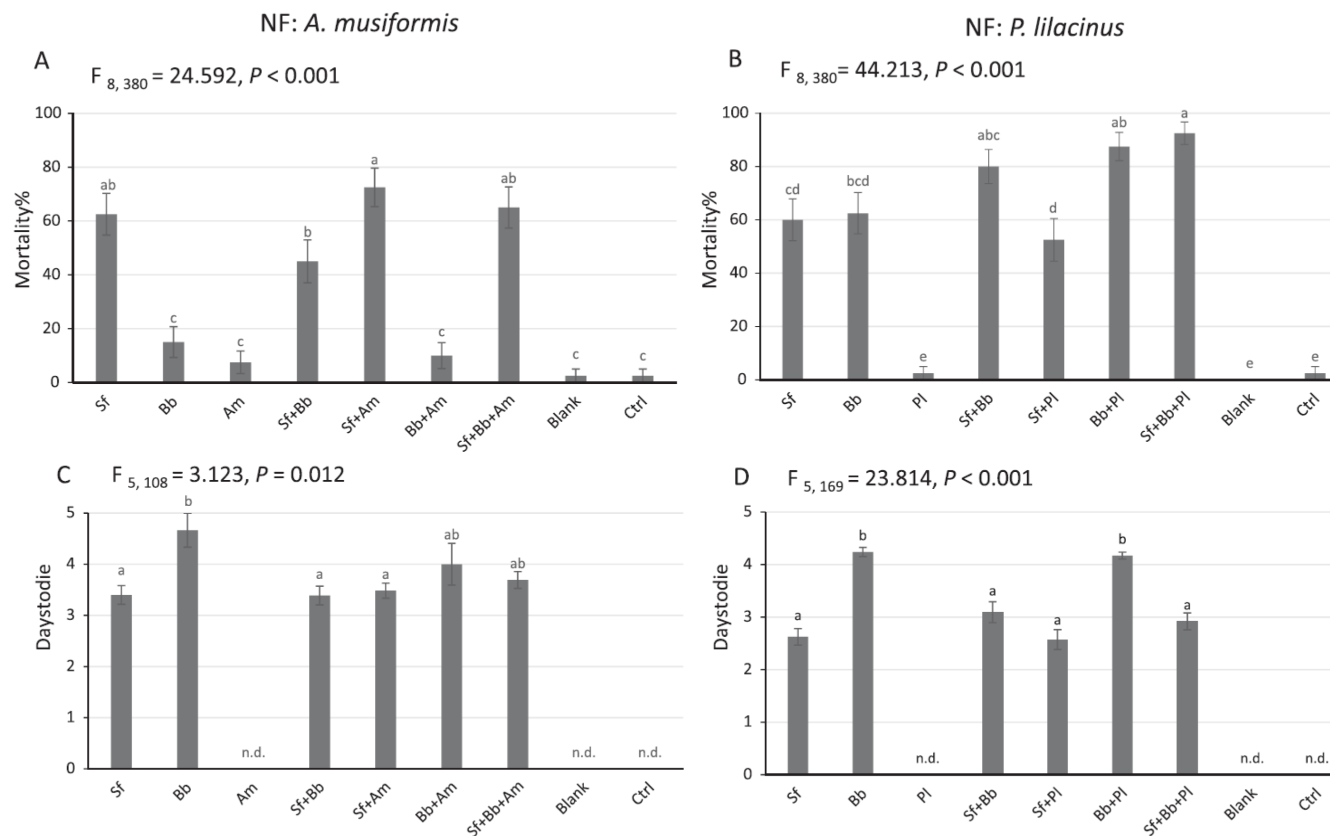

Figure 3.3. **Evaluation of the interaction among nematophagous fungi (NF) and entomopathogens (nematode and fungi) in the immersion fungi exposure** experiment (data from trial 1 and 2 combined). A and B. Larval mortality percentage. C and D. Number of days until larval death. Different letters above bars indicate statistical differences (one-way ANOVA and Tukey's test (HSD), $p < 0.05$). Treatments code: control (Ctrl, larvae immersed in sterile solution), Blank (untreated larvae), *Steinernema feltiae* (Sf), *Beauveria bassiana*, (Bb), *Arthrobotrys musiformis* (Am), *Purpureocillium lilacinum* (Pl), and the double or triple combinations of these organisms. Data are expressed as average ± SEM.

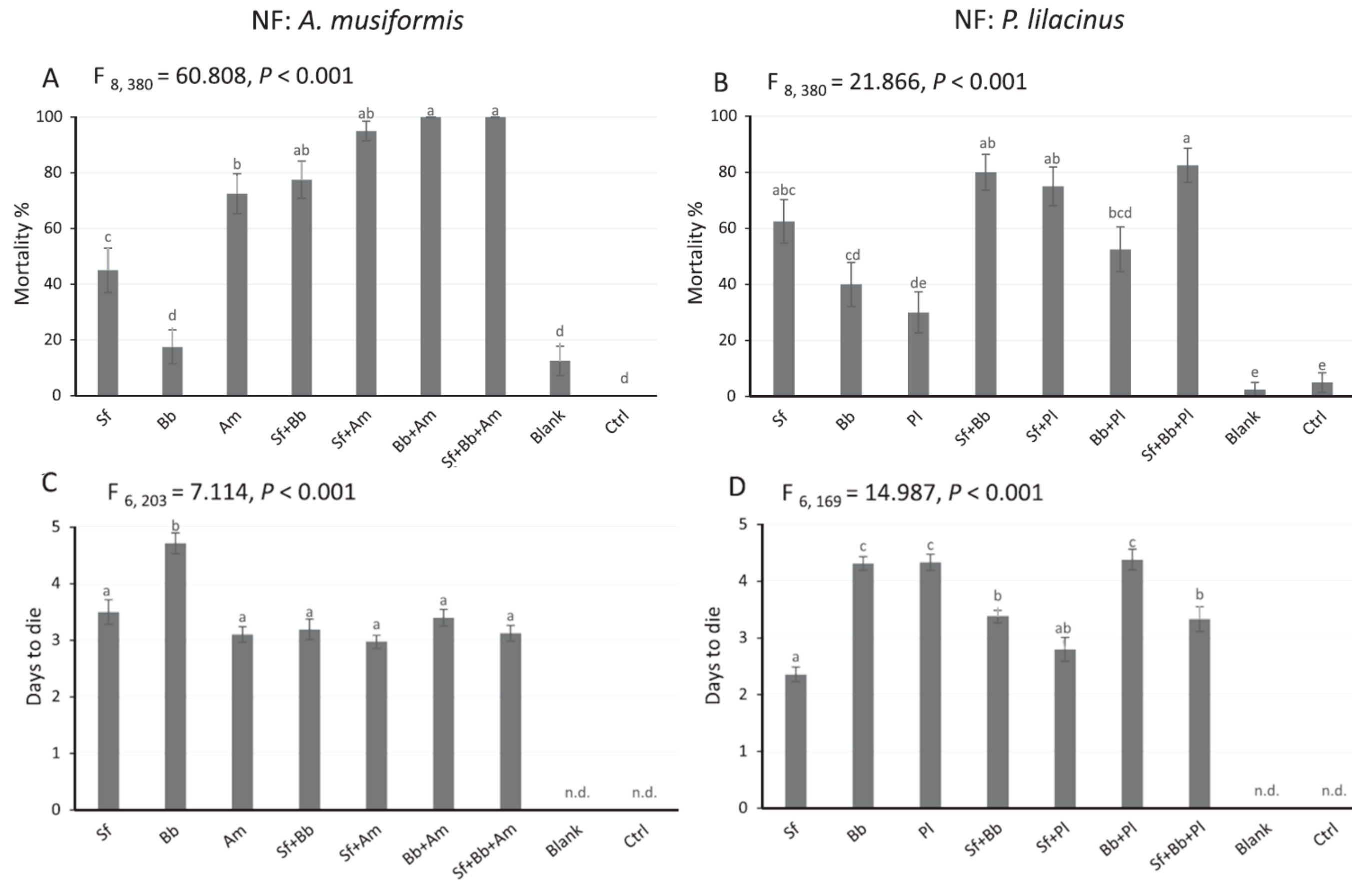

Figure 3.4. **Evaluation of the interaction among nematophagous fungi (NF) and entomopathogens (nematode and fungi)** in the injection fungi exposure experiment (data from trial 1 and 2 combined). A and B. Larval mortality percentage. C and D. Number of days until larval death. Different letters above bars indicate statistical differences (one-way ANOVA and Tukey's test (HSD), $p < 0.05$). Treatments code: control (Ctrl, larvae injected with sterile solution), Blank (untreated larvae), *Steinernema feltiae* (Sf), *Beauveria bassiana*, (Bb), *Arthrobotrys musiformis* (Am), *Purpureocillium lilacinum* (Pl), and the double or triple combinations of these organisms. Data are expressed as average ± SEM.

3.5 Discussion

This study illustrates, for the first time, how the presence and timing of natural enemies/competitors can modulate the action of EPNs and EPF as BCAs. First, we observed that both types of fungi (EPF and NF) produced antagonistic effect in mycelia growth when both shared the growth media, producing inhibition by contact (Wheeler and Hocking, 1993). Overall, the impact in growth rate differed on type of media, with slower effect in the limiting medium (CMA ¼) than in the rich one (PDA), suggesting as first modulator factor in the dual EPN-NF interaction the availability of resources in the soil. Moreover, in agreement with Asensio et al. (2007), the growth inhibition induced by *B. bassiana* towards the NF was stronger than the impact of either NF against the EPF. The species *B. bassiana* is a well-known antagonist of other fungi (Ownley et al., 2008; Sahab, 2012). In our *in vitro* assays, *B. bassiana* effectively inhibited both NF growth, probably due to its ability to produce secondary metabolites, including antifungal compounds (Strasser et al., 2000; Ownley et al., 2008; Sahab, 2012). Similarly, the NF exhibit the capability to interact with other fungi using same structures used to prey on nematodes (Nordbring-Hertz et al., 2011). Here we tested two type of NF, *A. musiformis* as trapping NF and *P. lilacinum* as egg-cyst-parasite NF, both with capability to survive saprophytically. We observed that *A. musiformis* growth rate was higher than *P. lilacinum*, which could have influenced in a higher growth inhibition when exposed against the EPF *B. bassiana.* Hence, as we expected, dual combination of EPF and NF produced inhibition, which strength will be mediated by the species and the media, leading to vary in the capability of the EPF to kill the insect.

Type of fungal exposure, concentration, and species (Shapiro-Ilan et al., 2004) affected the insect mortality rate and time to kill when dual and triple species combinations occurred. Although all the organisms were simultaneously applied, the use of three methods of exposure were intended to simulate different timing of fungal arrival. The mycelial- conidial exposure recreated the local active mycelia growth (NF) and/ or presence of an infested host (EPF) (Meyling and Eilenberg, 2007); the immersion treatment was a proxy of an inundation treatment (Oreste et al., 2012; Lacey et al., 2015); and the injection experiments provided a priority of penetration in the insect, which is

often encountered for the EPF and considered naturally unexpected to NF, unless is acting as scavenger. However, the application of the injection to both fungi al- lowed us to provide the same experimental conditions against the EPN as probe of concept. This fungal exposure determined A priori the main possible actor driving larval mortality in dual and triple species combinations. Overall, the EPF appeared to dominate the interactions in mycelial-conidial exposure, the EPN in immersion exposure, and the NF *A. musiformis* or the EPN (in *P. lilacinum* experiments) in the injection trials. However, the degree of final interactions (antagonism, additive or synergism) varied among fungal exposure, treatments, and species.

We hypothesized that the presence of NF would decrease the ability to kill the insect by both entomopathogens, irrespective of the application methods (Asensio et al., 2007; Andaló et al., 2008; Ownley et al., 2008; El-Borai et al., 2009, 2011; Sahab, 2012; Navarro et al., 2014). Contrary to our expectations, NF inhibited entomopathogens only when mycelia of *P. lilacinum* were combined with EPN and when *B. bassiana* was combined with *A. musiformis* . Moreover, synergistic effect was re- corded when EPF- *P. lilacinum* were exposed by immersion or injection, and in all the triple species combinations in injection method. In agreement with Andaló et al. (2008), when the NF were applied alone against *G. mellonella* in mycelial-conidial and immersion exposure, both NF were innocuous. However, when NF were injected in the single treatment, both were able to produce larval mortality, ranging from ~30% in *P. lilacinum* up to > 70% in *A. musiformis* . This pathogenic behavior, reported herein for the first time in the trapping NF *A. musiformis*, required a minimal concentration, equivalent to just one conidia, to kill the insect. Hence, it is plausible that the presence/action of an entomopathogen might support the NF entrance in the host, producing additive or even synergistic effect in mortality.

The EPF appeared to dominate their dual and triple species interactions in mycelial-conidial exposure. Despite the presence of mycelia on larvae or in soils surface is not a frequent and durable form of the EPF (Meyling and Eilenberg, 2007), some successful biocontrol attempts incorporated mycelial pellets and propagules with conidia (Charnley and Collins, 2007; Jaronski, 2008; Lacey et al., 2015). When the mycelial-conidial

exposure method was employed, the EPF *B. bassiana* produced ~90% larval mortality in just 4 days, the shorter time recorded in any exposure type for this fungus. During the assay, the insects were able to move for more than three days, so the disper- sion of the fungus would be plausible under natural conditions (Hajek, 1997; Meyling and Eilenberg, 2007). In the treatments involving EPN alone or combined with NF, both larval mortality and time to kill apparently resulted from the activity of the EPN. Overall, most of the effect in dual and triple species combined treatments resulted additive, with the exception commented above between EPN - *P. lilacinum*.

The highest variability of interactions in the dual and triple species combinations occurred in the immersion experiments. Minimal changes in the initial concentration were critical to modulate the interaction (antagonism, additive or synergism), even producing opposite trends (Shapiro-Ilan et al., 2004). Overall, previous studies involving larval immersion showed that the simultaneous combination of EPF and EPN produced an additive effect. For example, Bacca and Lagos (2014) observed that the simultaneous application of the EPF *B. bassiana* and the EPN *Steinernema sp.* resulted in additive interaction against *G. mellonella* larvae, using considerably higher concentrations that our conditions (10^9 spores/mL and > 100 IJs of EPN per larvae). However, the development of both entomopathogens was negatively affected. In their study, Bacca and Lagos (2014) observed that the EPN penetration rate and the IJs production decreased in combined treatments as well as the EPF sporulation, which was significantly reduced. Similarly, during the first week post-exposure, Wakil et al. (2017) observed an additive effect in the simultaneous application of the EPF *B. bassiana* or *M. anisopliae* (1 × 106/mL, each larva immersed in 100 mL) and the EPN *Heterorhabditis bacteriophora* (10 IJs per larvae) independently of the developmental stage of the *R. ferrugineus*. In our study, the additive effect was observed when the larvae were immersed in 1 × 107 conidia/mL, while when this concentration was reduced to 1 × 106 conidia/mL, the resulting effect was antagonist. This observation is in agreement with Shapiro-Ilan et al. (2004), where the reduction in half of the concentration of EPF *B. bassiana* and the EPN *S. carpocapsae* modulated from additive to antagonism. However, when Shapiro-Ilan et al. (2004) employed a heterorhabditids, *H. indica*, the final result was antagonistic, independently of the initial concentration. Despite the methodological differences in our conditions in immersion experiments and Shapiro-Ilan et al. (2004)

application as suspension, both studies highlight the relevance of the fine tuning between species and concentration combination to achieve the desired effect, additive or synergistic, rather than antagonistic.

Once inside the insect, both EPF and EPN (mainly the symbiotic bacteria) have the ability to release specific metabolites to prevent the attack of the cadaver by other opportunistic microorganisms and scavengers (Strasser et al., 2000; Lewis et al., 2015). This activity can produce the exclusion of one of the organisms, although little is known about the speci fic mechanisms mediating this interaction. For example, Barbercheck and Kaya (1990) showed that the presence of *Xenorhabdus* inhibited the growth of *B. bassiana*, when the EPF had advanced timing in application. Similarly, Tarasco et al. (2011) observed a strong competition in the host hemocoel between the EPN and EPF, suggesting compounds produced by the EPF and the EPN bacterial partner as the main possible cause of the antagonist effect. In the injection experiments, we gave priority to the presence of the EPF inside the hemocoel, expecting to favour the battle inside the insect to the fungal action. However, contrary to the possible exclusion observed by Barbercheck and Kaya (1990) and Tarasco et al. (2011), the combination of EPN and EPF in the injection exposure resulted either additive or synergistic effect (in the triple species combination). In our study, it is plausible that the insect already developing fungal infection were stressed en- ough to result potentially more susceptible to EPN infection (Barbercheck and Kaya, 1991; Ansari et al., 2006). Hence, more studies involving other species or/and concentration could unravel under which scenario the interaction is balanced toward antagonistic or additive/synergistic effect.

The simultaneous application of EPN and EPF have produced very contrasting effect depending of the concentration and species involved, ranging from majority of antagonisms (Shapiro-Ilan et al., 2004), mainly additive effects (Jabbour et al., 2011; Bacca and Lagos, 2014; Wakil et al., 2017) or even synergisms (Ansari et al., 2010; Shaurub et al., 2016). In our study, most of the interactions resulted additive, even when the presence of A priori natural enemy/competitor, such as NF. Contrary to the expected reduction of the biocontrol activity produced by entomopathogens in presence of the NF, triple species combinations EPN + EPN+NF resulted additive or even synergistic if the NF could be inside the insect. In a massive presence of EPNs in the soil, such as augmentation biological control approach, the presence of NF can also increase (El-Borai et al., 2007). In this scenario, it is possible that EPNs could penetrate the insects with

some propagules of NF, and hence, triggering an additive or synergistic effect against the insect pest if EPN is combined with EPF release. In addition to the biotic factors modulating these interactions (i.e. species, concentration, timing), the abiotic factors such as temperature, humidity and texture can frame the final output of biological control potential (Barbercheck and Kaya, 1990; 1991; Jabbour et al., 2011). This is particularly relevant in the context of climate change, which largely reshape species distribution (Schröter et al., 2005). Further research involving other EPN, EPF and NF species, applied at different concentrations, timing, and testing various hosts under different abiotic conditions can settle the ecological conditions that drive towards the best combination of BCAs.

3.6 Acknowledgements

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IV
ACTIVITY OF *STEINERNEMA COLOMBIENSE* IN PLANT-BASED OILS

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CHAPTER IV

4 ACTIVITY OF *STEINERNEMA COLOMBIENSE* IN PLANT-BASED OILS

4.1 Abstract

Entomopathogenic nematodes (EPNs) are excellent biological control agents. Although traditionally EPN application targeted belowground insects, their aboveground use can be supported if combined with adjuvants. We hypothesized that EPN infective juveniles (IJs) could be combined with plant-based oils as adjuvants, without decreasing their efficacy against insect larvae under various scenarios. Specifically, our objectives were to evaluate the activity of *Steinernema colombiense* (Nematoda: Steinernematidae) when mixed with two plant-based oils (coconut and olive oils) and maintained at different temperatures and times, or combined with entomopathogenic fungi. First, we evaluated how these oils affected IJ survival and virulence against last instar *Galleria melonella* (Lepidoptera: Pyralidae) larvae when maintained at five different temperatures (4, 8, 14, 20, and 24°C) and five incubation times (1, 3, 7, 14, and 21 days), using water as control treatment. Second, we evaluated virulence when combined with these two oils as well as with water (control) and combined with the entomopathogenic fungi (EPF), *Beauveria bassiana* (Hypocreales: Clavicipitaceae). Infective juvenile survival was higher in coconut than olive oil and water mixtures up to 7 days at 4°C. Conversely, olive oil supported higher larval mortality than coconut oil at 4 to 20°C and 14 days. Similarly, the number of days needed to kill insect larvae increased at extreme temperatures (4 and 24°C) after 14 days. Finally, the EPN + EPF combination showed an additive effect compared to EPN and EPF single treatments. Our findings indicate that our plant-based oil mixtures maintain viable IJs at moderate temperatures and up to 7 to 14 days and can be used in single EPN mixtures or combined with EPF.

Keywords: *Beauveria bassiana*, biological control, coconut oil, olive oil, *Steinernema*, temperature.

4.2 Introduction

Entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are well-known biological control agents used against many arthropod species (Campos-Herrera, 2015; Lacey et al., 2015). They selectively search for insect hosts and kill them within 2 to 3 days with the aid of mutualistic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Adams et al., 2006; Dillman et al., 2012). Their worldwide distribution in soils (Kaya et al., 2006) and the availability of commercial products (Lacey et al., 2015) make the EPNs excellent agents to employ them in integrated pest management (IPM) programs and under organic production (Campos-Herrera, 2015).

Formulation and application technique are key aspects of the use of EPNs as biological control agents. The selection of the best formulation and application technique may differ depending on the target pest and its location, above or belowground (Hiltpold, 2015). The most widespread practice for EPNs use in biocontrol agent is the massive application of infective juveniles (IJs) stages in belowground agroecosystems, where they are naturally adapted (Lacey et al., 2015). Numerous substrates such as vermiculite, clay, activated charcoal, polyacrylamide, alginate capsules, or simple water-dispersible granules have been tested as formulation agents with variable shelf life and storage limitations (Hiltpold, 2015; Ramakuwela et al., 2015; Kary et al., 2018; Leite et al., 2018; Touray et al., 2020). Then, EPN is applied in the soil by creating a simple suspension in water, which is easy to manage and cheap for growers to use. Survival and activity of EPNs on soils will differ depending on the substrate selected for their formulation and the environmental conditions of storage (temperature, humidity, and time) (Grewal, 2000; Kim et al., 2015; Ramakuwela et al., 2015; Leite et al., 2018; Touray et al., 2020).

EPN application for targeting aerial pests requires extra adjustments to keep them located on the target plant part (Brusselman et al., 2012; Shapiro-Ilan and Dolinski, 2015). Often aerial application approaches include adjuvants or surfactants to enhance IJ survival under stressing factors, such as desiccation and UV exposure (Schroer and Ehlers, 2005; Beck et al., 2013; De Waal et al., 2013; Hiltpold, 2015; Dito et al., 2016; Noosidum et al., 2016), but also to maintain virulence and reduce drippage from leafs. Polymers as Zeba and sprayable fire-gels are among the synthetic compounds tested for

this purpose (Lacey et al., 2010; Shapiro-Ilan et al., 2010, 2015; De Waal et al., 2013; Portman et al., 2016; Noosidum et al., 2016). Also, mixtures based on plant-based oils reported promising results (Krishnayya and Grewal, 2002; Qiu et al., 2008; Moreira et al., 2013; Monteiro et al., 2014; Alves et al., 2017; Aquino-Bolaños, Morales-García and Martínez-Gutiérrez, 2019; Aquino-Bolaños, et al., 2019), expanding the range of specific physical-chemical properties to consider. For example, since certain plant-based oils such as coconut oil remain solid at relative high temperatures (~24°C), this could be of interest for producing stable shipments. Hence, exploring the potential of using oil-based adjuvants such as olive and coconut oils, both often accessible in regular stores, would provide an alternative for local EPN producers.

There are several options to enhance the efficiency of EPN formulations and application approaches. An example could be the additions of chemical compounds such as pheromones (Shapiro-Ilan et al., 2019). Also, the simultaneous application of EPNs with other beneficial soil organisms has shown enhanced biocontrol and plant protection activity. Previous studies have reported successful co-applications with entomopathogenic fungi (EPF), arbuscular mycorrhizal soil fungi, and bacteria in the genus *Pseudomonas* (Shapiro-Ilan et al., 2004; Molina-Acevedo et al., 2007; Ansari et al., 2008; Imperiali et al., 2017; Jaffuel et al., 2019). This co-application approach is a promising tool for sustainable agriculture that, once optimized, could save costs and time to growers. The EPF *Beauveria bassiana* (Hypocreales: Clavicipitaceae), which occurs in natural and agricultural soils worldwide, is one of the most prominent biological control agents used among commercial products (Lacey et al., 2015), and thus, can be an excellent candidate for combinations of this kind.

Regional programs IPM often promote local EPN productions (San-Blas et al., 2019). In this context, new systems that guarantee high IJ survival and virulence after medium-term storage must be developed without significantly increasing costs and with relative accessible products. Herein, we explored two plant-based oils used as model of possible adjuvant: coconut (*Cocos nucifera*) and olive (*Olea europaea*). We selected the EPN species *Steinernema colombiense*, described by López-Núñez et al. (2008). This nematode is naturally occurring in several countries of Latin America and has shown promising results against several below and aboveground insect pests (Delgado-Ochica and Sáenz-Aponte, 2012; Rosero-Guerrero et al., 2012; Aristizábal et al., 2015). We hypothesized that coconut and olive oils, as models plant oil-based adjuvants, can be

combined with EPN to be used as mixing media, single applied or combined with the EPF species *B. bassiana*, without any deleterious effect or negative impact on virulence. Therefore, the objectives of this study were: (i) to evaluate the survival and virulence of *S. colombiense* against last instar larvae of *Galleria melonella* (Lepidoptera: Pyralidae) when combined with coconut and olive oils as model adjuvants at different temperatures and times, and (ii) to evaluate the efficacy of using these plant-based oil combined with *S. colombiense* and *B. bassiana*.

4.3 Material and methods

4.3.1 Organisms, oils, and substrates

We conducted the experiments by employing *S. colombiense* isolated from Mexico and donated by Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional, Unidad Sinaloa - CIIDIR Sinaloa (México) (ITS region, GenBank accession number MG551678). The EPF *B. bassiana* was isolated in Algarve (Portugal) (GenBank access number MG515530, Bueno-Pallero et al., 2018). The insect *Galleria melonella* (Lepidoptera: Pyralidae) was used as insect hosts, reared at the University of Algarve (Portugal). The nematodes were regularly refreshed in vivo using last instar *G. mellonella* as the host (Woodring and Kaya, 1988). The IJs were recovered upon emergence and stored in mineral water at 14°C. These were used within 2 weeks of harvest (Bueno-Pallero et al., 2018). *Beauveria bassiana* was isolated from a naturally infected adult of *Rhynchophorus ferrugineous* (Coleoptera: Curculionidae), cultured on 90-mm diam. petri dishes with potato dextrose agar (PDA, Biokar) at 25 ± 1°C, avoiding sub-culture more than once during the study (Shapiro-Ilan et al., 2004), and stored at 4°C until use (Goettel and Inglis, 1997). The coconut and olive oils tested were obtained from commercial products (San Lucas® and Carbonell®, respectively), and maintained in cold at dark conditions until use. Finally, we used pure mineral sand (Vale do Lobo, Loulé, Portugal) as a substrate in the infectivity test. To prepare the sand for each experiment, it was washed several times with running water, autoclaved for 1 h (two times in two consecutive days as suggested by Elhady et al., 2018), oven-dried at 40°C with ventilation, and stored in laboratory conditions at least for a week before being used (Chiriboga et al., 2017).

4.3.2 Combination of entomopathogenic nematodes with plant-based oils at different temperatures and times

We assessed for the survival and virulence of *S. colombiense* combined with coconut oil, olive oil, or water (control) at five temperatures (4, 8, 14, 20, and 24°C) and five incubation times (1, 3, 7, 14, and 21 days). We used 24-wells plates (Falcon Multiwell, 24- well Polystyrene, Corning Incorporated-Life Sciences, Duham, USA), designating eight wells per treatment (coconut, olive, or water) per plate. The treatments consisted of 20 IJs released in 200 µL final volume per well of coconut/olive oil mix (60 µL of oil and 140 µL of water) or distilled water as a negative control (Glazer and Lewis, 2000). Because the oils do not mix with water, we prepared the corresponding mixtures by stirring the proportional quantities of water/ nematodes and oils in a big beaker with a stirring bar. The oil mixtures were applied when the combination appeared well mixed and the size of the oil particles were minimal. In addition, in the case of the coconut oil, we warmed this suspension to 25 to 28°C to ensure that the oil was liquid since the coconut is solidat temperatures below 24°C (https://en.wikipedia. org/wiki/Coconut_oil). The plates were closed with parafilm and stored in the corresponding temperature in a plastic container with moistened towels to prevent evaporation. We estimated nematode survival rates by counting the number of live IJs per well at the inoculation time and after incubation times, touching straight nematodes to verify their capacity to move. In the case of the coconut treatments, we warmed for few minutes in a metal plate at 25°C to merge the oil. After the nematode survival estimation, we added 2 g of sterilized sand and one last instar *G. mellonella* larva per well to evaluate the EPN infectivity, incorporating additional plates with adjuvant treatments but no nematodes as controls. We revised daily larval mortality for an incubation period of five days at 24°C in dark conditions (Dillman et al., 2012). To verify that mortality was due to EPN activity, we transferred all insect cadavers individually to White traps (White, 1927) and incubated them in the same conditions, until confirming nematode emergences. The whole experiment was performed twice using new nematodes, insects, and adjuvants.

4.3.3 Combination of entomopathogenic nematodes and fungi with plant-based oils

To determinate the nature of the interaction between *S. colombiense* and *B. bassiana*, we first evaluated single applications at different concentrations (Koppenhöfer and Grewal, 2005). We employed 24-wells plates, inoculating 100 µL of each suspension per well (n = 20 per treatment, two independent trials). The EPN concentrations tested were 0, 1, 5, 10, 20, 50, and 100 IJs/well, all applied from volumetric suspension accordingly adjusted, except 1 and 5 IJs/well, handpicked (Blanco-Pérez, et al., 2019). For *B. bassiana*, we prepared conidia suspensions using a sterile swab to transfer the conidia from PDA massive production plates to a Falcon® tube with sterile half-strength Ringer solution and 0.05% Tween 80 (Klingen et al., 2002). The concentrations used (0, 10^0-10^8 conidia) were estimated by counting in a hemocytometer (Neubauer improved) after the conidia suspension was homogenized (Vortex®) for 2 to 3 min (Bueno-Pallero et al., 2018). After EPN or EPF application of the corresponding concentration, we placed 2 g sterilized soil and one last instar *G. mellonella* larvae per well. All plates were closed with parafilm, incubated at 24°C in dark conditions in a plastic container with moistened towels to prevent evaporation. Larval mortality was checked daily fora week. In addition, as technical control for EPF, we inoculated PDA plates with all the prepared conidia concentrations for ensuring viability.

We estimated that the inoculation of 2 IJs of *S. colombiense* and 1×10^7 spores of *B. bassiana* per well was required to kill ~50% of the *G. mellonella* larvae. We added those quantities in a final volume of 200 µL per well of the coconut/ olive oil mixture (60 µL of oil and 140 µL of water) or distilled water (control). As described before, the oil mixtures were prepared in a big beaker with a stirring bar and applied when the combination appeared well mixed. In addition, in the case of the coconut oil, we warmed this suspension to 25 to 28°C. The corresponding suspensions were inoculated in 24-wells plates as previously described (eight wells per adjuvant, two 24-plates per organism: EPN, EPF, EPN + EPF, and none organism as control). Then, we added 2 g of sterilized sand and one last instar *G. mellonella* per well. Plates were closed with parafilm, incubated at 24°C in the dark, and introduced in a plastic container with moistened towels to prevent evaporation. Larval mortality was monitored daily for 5 days. As before, we transferred all insect cadavers individually to modified White traps and incubated them in the same conditions, until confirming nematode/fungi emergences. The whole

experiment was performed twice using new nematodes, fungi, insects, and adjuvants.

4.3.4 Statistical analyses

First, the appropriate concentration for the EPN and EPF co-application test was estimated by Probit analysis (SPSS 25.0), pooling the data of two independent trials (data not shown). We calculated the percentage of live (mobile) IJs after the incubation times for each treatment, considering the total mobile IJs before and after application. Similarly, we estimated the virulence for each treatment by calculating the frequency of larval mortality and the number of days needed to kill. Before statistical analysis, percentage values were arcsine transformed, and data from different trials were combined after checking the statistical similarity of the results (data not shown). We ran generalized linear models (GLM, $P < 0.05$) for the analysis of the variables percentage of alive IJs, larval mortality, and days needed to die. For the first experiment, the factors tested were the adjuvant (three levels: control/water, coconut oil, and olive oil), the temperature (five levels: 4, 8, 14, 20, and 24°C), the time (five levels: 1, 3, 7, 14, and 21 days), and their interactions. In addition, we performed an one-way ANOVA and Tukey test ($P < 0.05$) for each temperature and time of incubation to disentangle the specific impact of the adjuvants. For the second experiment, EPN-EPF combination, the factors tested were the adjuvant (three levels: control/ water, coconut oil, and olive oil), organisms applied (four levels: EPNs, EPF and EPNs + EPF, and control) and their interactions.

We followed the formulae proposed by Shapiro- Ilan et al. (2004) and Ansari et al. (2010) to evaluate the nature of the EPN-EPF interaction (antagonistic, no-interaction/additive, or synergistic), comparing the expected and observed mortalities for each adjuvant (coconut/olive oil or distilled water). The expected mortalities (ME) were calculated as $ME = MT1 + [MT2 \times (1 - MT1)]$ when EPNs and EPF were combined. We run a $\chi2$ test for the expected and observed mortalities [i.e. $\chi2 = (MT1T2 - ME)2/ME$, where MT1T2 is the observed mortality for each organism single applied]. Those values were matched with the $\chi2$ table for 1 degree of freedom ($p = 0.05$) so that $\chi2 < 3.8415$ indicated additive interaction and $\chi2 > 3.8415$ non-additive (antagonist or synergist) interaction. Thus, the interaction was considered synergistic if $MT1T2 - ME > 0$, and antagonistic if $MT1T2 - ME < 0$ (Shapiro-Ilan et al., 2004; Ansari et al., 2010).

We performed all the analyses with SPSS 25.0 (SPSS Statistics, SPSS Inc., Chicago, IL, USA). We used least-square means ± S.E. as descriptive statistics.

4.4 Results

4.4.1 Impact of temperature and time on the survival and virulence of the entomopathogenic nematode combined with plant-based oils

EPN survival measured as percentages of live IJs resulted in statistically significant for all factors (adjuvant and temperature and time of incubation) and their interactions except for adjuvants (Table 4.1, Figure 4.1.1). We analyzed all the three factors individually to disentangle for specific effects (Figure 4.1.1). Overall, we observed most of the statistical differences among adjuvants for short periods (1-7 days). Particularly, we found more live IJs in coconut mix than in water after 1 day and at extreme temperatures (4 and 25°C) (Figure 4.1.1A), a trend also observed at 4°C after 3 and 7 days (Figure 4.1.1B, C) and at 14°C for day 7 (Figure 4.1.1C). Conversely, for moderate temperatures (8, 14, and 20°C), higher percentages of live IJs were more often reported for water than for plant-based oils (Figure 4.1.1A-C). The temperature of 25°C drastically reduced IJ survival after 7 days, independently of the adjuvant used (Figure 4.1.1C-E). In any case, long periods considerably decreased the percentage of live IJs (below 20 and 2% for 14 and 21 days, respectively: Figure 4.1.1D, E), only significantly higher for olive oil than for coconut oil for 14 days at 20°C (Figure 4.1.1D).

Larval mortality was statistically significant for all factors and their interactions except for the adjuvant × time × temperature triple interaction (Table 4.1, Figure 4.1.2). We observed in the individualized analysis that larval mortality was over 85% for 1 to 7 days (except for 7 days at 25°C) without differences among adjuvants (Figure 4.1.2A-C). For 14 days, we recorded significantly lower larval mortalities for coconut oil than for olive oil (at 4-20°C) and for water (at 4 and 8°C) (Figure 4.1.2D). At 24°C, 7 or more days of incubation resulted in a considerable reduction of the larval mortality (~50% or less; Figure 4.1.2C-E). After 21 days, larval mortality was significantly higher for water than for plant-based oils at 4°C (Figure 4.1.2E).

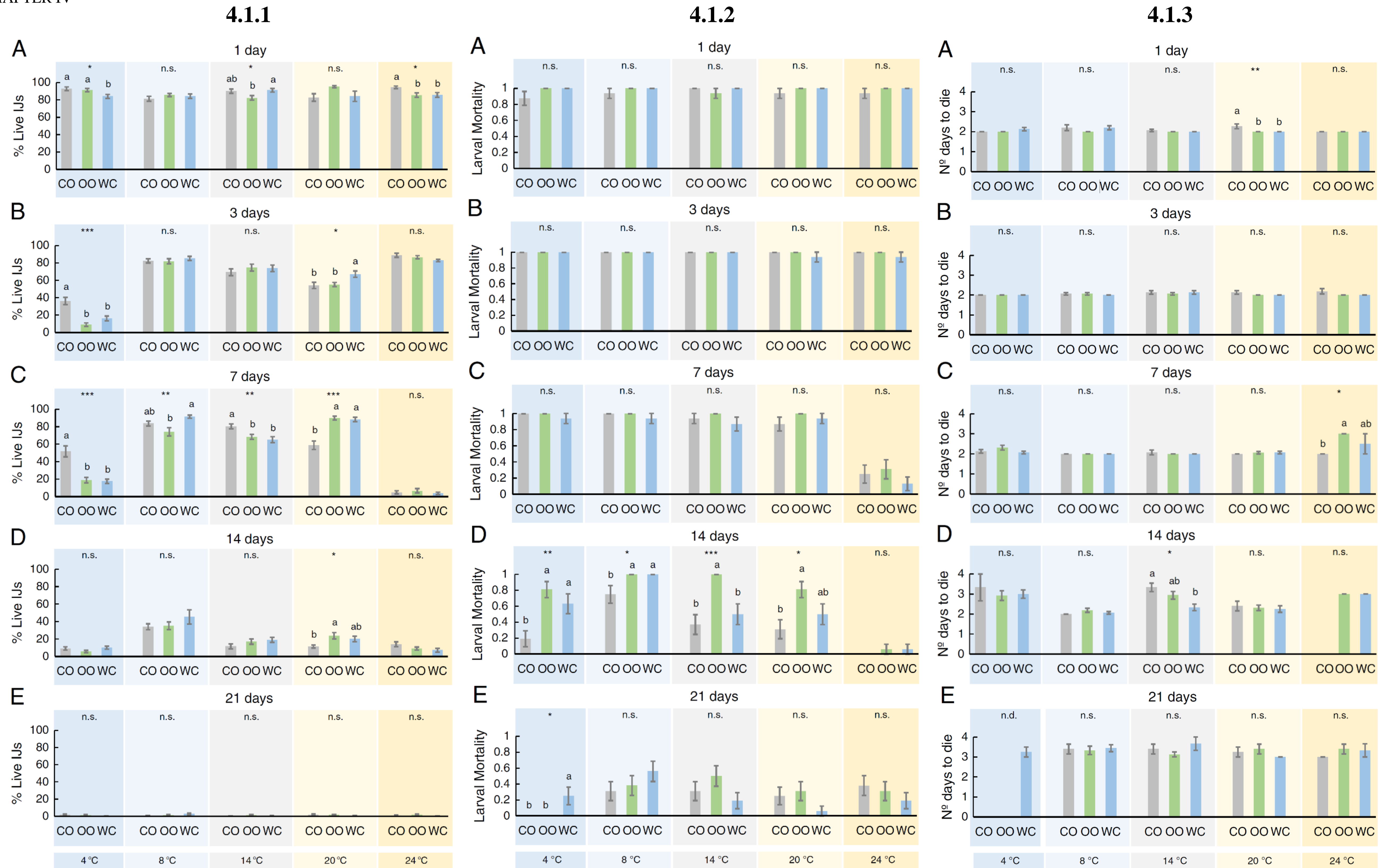

Figure 4.1. **Effect of adjuvants employed in *Steinernema colombiense* combination**, coconut oil (CO), olive oil (OO), and distilled water (WC), previously incubated at five different temperatures (4-24°C) and for five different times (1-21 days); **Figure 4.1.1**- percentage of live (mobile) infective juveniles (IJs); **Figure 4.1.2.-** frequency of larval mortality of last instar *Galleria melonella;* **Figure 4.1.3.** number of days needed to kill last instar *Galleria melonella* larvae. Asterisks indicate significant differences within treatment comparisons at *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n.s., not significant and n.d. no data. Different letters indicate significant differences in Tukey's test (HSD). Values are least-square means ± SE.

The number of days needed to kill the *G. mellonella* larvae was statistically significant for all factors and their interactions except for the adjuvant employed and its interaction with temperature (Table 4.1). We found few differences among adjuvants in the individualized analyses. Essentially, IJs killed slower in the coconut mix for 1 and 14 days at 20 and 14°C, respectively (Figure 4.1.3A, D), but faster for 7 days at 25°C (Figure 4.1.3C). Overall, IJs stored up to 14 days needed 2 days on average to kill (Figure 4.1.3A-D), while ~3 days were needed for IJs stored for 21 days (Figure 4.1.3E) or for 14 days at extreme temperatures (4 and 24°C; Figure 4.1.3D)

4.4.2 Combination of entomopathogenic nematodes and fungi with plant-based oils.

The entomopathogenic activity was not affected by the adjuvant employed, but for the organisms (EPN, EPF, or EPN + EPF) applied (Figure 4.2). The combined application of both entomopathogenic organisms, *S. colombiense* and *B. bassiana*, resulted in significantly higher larval mortalities than single application (Figure 4.2A). The evaluation of the EPN-EPF interaction confirmed an additive effect for their co-application independently of the adjuvant employed (Table 4.1.2). Additionally, the combined application of both entomopathogens significantly reduced the number of days needed to kill insect larvae than *B. bassiana* single applied (Figure 4.2B). However, those numbers remained significantly higher than recorded for EPNs single applied except if formulated with olive oil (Figure 4.2B).

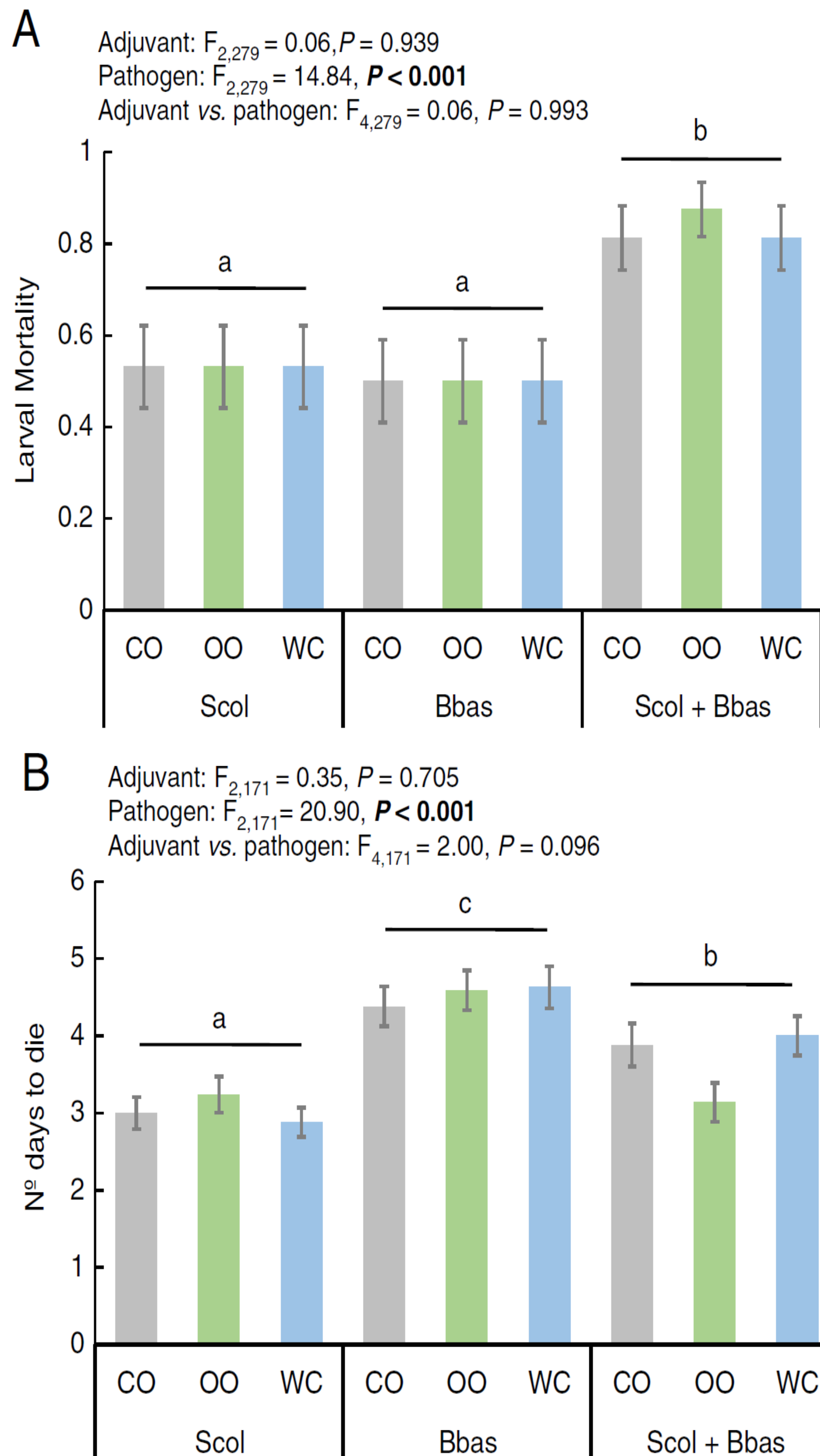

Figure 4.2 **Virulence of *Steinernema colombiense* (Scol) and *Beauveria bassiana* (Bbas),** single applied or combined (Scol+Bbas), combined with coconut oil (CO), olive oil (OO), and distilled water (WC). A: Frequency of last instar *Galleria melonella* larval mortality. B: Number of days needed to kill the insect larva. Different letters indicate significant differences in Tukey's test (HSD). Values are least-square means ± SE. Significant differences for GLM analysis ($p < 0.05$) are highlighted in bold.

Table 4.1. **Statistical analysis of the effect of three factors (adjuvants, temperature and time of storage) and their interactions** (GLM, $p < 0.05$) in the infective juvenile (IJ) survival measured as percentage of live IJ, and its virulence measured as larval mortality and number of days to kill the insects.

Fixed factors	% Live IJs	% Dead larvae	No. days to die
Adjuvant (Adj)	$F_{2, 1,125} = 1.53$; $p = 0.217$	$F_{2, 1,125} = 15.31$; $p <$ **0.001**	$F_{2,786} = 0.69$; $p = 0.513$
Incubation temperature (Temp)	$F_{4, 1,125} = 228.95$; $p <$ **0.001**	$F_{4, 1,125} = 49.31$; $p <$ **0.001**	$F_{4,786} = 9.24$; $p <$ **0.001**
Incubation time (Time)	$F_{4, 1,125} = 2,309.61$; $p <$ **0.001**	$F_{4, 1,125} = 274.73$; $p <$ **0.001**	$F_{4,786} = 185.90$; $p <$ **0.001**
Adj × Temp	$F_{8, 1,125} = 17.40$; $p <$ **0.001**	$F_{8, 1,125} = 2.67$; $p =$ **0.007**	$F_{8, 786} = 1.86$; $p = 0.063$
Adj × Time	$F_{8, 1,125} = 2.31$; $p =$ **0.018**	$F_{8, 1,125} = 7.39$; $p <$ **0.001**	$F_{8, 786} = 3.26$; $p =$ **0.001**
Tem × Time	$F_{16, 1,125} = 136.59$; $p <$ **0.001**	$F_{16, 1,125} = 19.43$; $p <$ **0.001**	$F_{16, 786} = 10.89$; $p <$ **0.001**
Adj × Temp × Time	$F_{32, 1,125} = 5.52$; $p <$ **0.001**	$F_{32, 1,125} = 1.42$; $p = 0.061$	$F_{32, 786} = 2.11$; $p =$ **0.001**

Note: The p values below 0.05 are highlighted in bold.

Table 4.2. **Interactions of the co-application of entomopathogenic nematodes and entomopathogenic fungi** formulated in different adjuvants for the suppression of *Galleria melonella* larvae after.

Adjuvant	Observed mortality$_a$	Expected mortality$_b$	$\chi 2$	Interaction
Distilled water	81.3	76.6	0.28	Additive
Cocoa oil	81.5	76.6	0.31	Additive
Olive oil	87.8	76.6	1.58	Additive

Notes: a Observed mortality (%), average of 16 replicates in two trials (32 total); b expected mortality (%), calculated ME = MT1 + MT2 × (1 − MT1) for combination of two organisms applied on the larvae, where MT1 and MT2 are the mortalities from *Steinernema colombiense* and *Beauveria bassiana* single applied, respectively.

4.5 Discussion

As we hypothesized, overall, *S. colombiense* did not suffer significant deleterious effects in survival nor in infectivity when combined with the two plant-based oils than in treatment with water. Specifically, we recorded similar survival *S. colombiense* of IJs formulated with coconut and olive oils and water (control treatment) for 3 to 7 days at 8 to 20°C. In agreement with Kary et al. (2018), the adjuvantand 20°C), but only up to 7 days. IJ survival reduced significantly after 14 days, even below 2% at 21 days. EPN species-specific differences could explain the disparities observed between these studies (Shapiro- Ilan and Dolinski, 2015). Also, studies that investigate plant-based oil mixtures registered contrasting results. For example, over 95% of *S. feltiae* IJs survived if mixed with plant-based oil (Oxiquímica Agrociência, Ltda) and stored at 24°C for 5 days (Moreira et al., 2013). Under similar conditions (7 days at 24°C), *S. colombiense* IJs survived no more 5% independently of the adjuvant tested. This difference can be due to the fact that Moreira et al. (2013) employed tanks with specific pressure and a mix of 1% of the adjuvants, while our proportion was significantly higher, and no pressure was applied. In addition, we did not employ any emulsifier, which could contribute to the formation of two layers, one below with water and the nematodes and one above with the oils. These two layers could limit the air exchange in the well, and hence, could have promoted the reduction of the IJs survival after 7 days. On the other hand, the survival of *S. colombiense* after 7 days at 20°C were higher than recorded for *S. websteri* mixed with citronella (*Cymbopogon citratus*) and red cedar (*Juniperus virginiana*) at similar exposure conditions (6 days at 22°C), but lower if compared with the results obtained for *S., carpocapsae* in the same study (Aquino-Bolaños, Morales-García and Martínez-Gutiérrez, 2019). Finally, Alves et al. (2017) showed that certain plant-based oils could be incompatible with EPN formulations. In their study, the combination of *Heterorhabditis* sp. CB40 with TEK-F® resulted in 16.4% IJ survival, while the results with Aureo® were similar that water controls (97.4 and 93.2%, respectively). Consequently, different plant-based oils can have positive, negative, or neutral effects (as for the coconut and olive oil mix showed in our study). Hence, testing the efficacy of the combination of EPN species with a new plant-based oil adjuvant is highly recommended before application.

Similarly, nematode virulence (larval mortality and time to kill insect larvae) considerably decreased from 14 days. From 1 to 7 days, independently of the adjuvant

employed, we recorded over 80% of larval mortalities at most of the temperatures. This high larval mortality can be explained by the fact that only one nematode is required to survive to kill an insect (Stock, 2015). Aquino-Bolaños, Morales-García and Martínez-Gutiérrez (2019) reported similar values for the EPN species *S. wesbteri* and *H. bacteriophora* formulated with *C. citratus* and *J. virginiana*, while *S. carpocapsae* formulated with *C. citratus* only provided 60% of larval mortality. Conversely, we obtained less than 50% of larval mortality for IJs stored for 7 days at 24°C, which is a result that contrasts with the 100% mortality reported by Moreira et al. (2013) for plant-based oil adjuvants under similar conditions (5 days at 24°C). The larval mortalities of *Hedypathes betulinus* (Coleoptera: Cerambycidae) registered by Alves et al. (2017) were 80% and below 20% for *Heterorhabditis* sp. formulated with Aureo® and TEK-F®, respectively. Finally, IJs mixed with coconut oil improved their virulence (survival and time to kill rates) from 1 to 7 days at low and high temperatures (4 and 24°C), although without implying higher larval mortality rates. In the case of olive oil mixtures, we recorded higher mortality rates at 14 days at 14°C. We demonstrate the compatibility of both plant-based oils for the combination with the EPN species *S. colombiense* and the EPF *B. bassiana*. As shown for other EPN-EPF combinations without adjuvant combination (Wu et al., 2014), the combination of both entomopathogens resulted in an additive effect, independently of the adjuvant tested. Previous studies have shown that the nature of their interaction (additive, synergic, or antagonistic) is species specific, but also the concentration and the timing of the applications affect it. For example, Ansari et al. (2008) reported that the combined applying of *Metarhizium anisopliae* with different EPN species resulted in synergistic effects. In the case of *H. bacteriophora,* this synergic effect was observed independently of the timing of the application, whereas for *S. kraussei* and *S. feltiae* it was synergistic only when applied simultaneously or after 1 to 2 weeks of fungal inoculation, respectively. Wu et al. (2014) reported additive interactions when *H. bacteriophora* and *H. megidis* were simultaneously applied with *B. bassiana* and *M. anisopliae.* Finally, other studies found that, except for a few exceptions, many EPN - EPF interactions resulted in antagonistic interactions (Shapiro-Ilan et al., 2004; Acevedo et al., 2007).

We conclude that this study supports the use of coconut and olive oils as adjuvants, with certain shelf-life properties depending on the time and temperature. This can be of interest for local EPN producers that might need to store and ship their IJs under certain

conditions. Moreover, we consider the additive effect of the simultaneous combination of EPN-EPF, and the lack of impact of the plant-based oil tested in their performance to be very promising. This approach could reduce the number of applications in the field and, consequently, the cost for growers. Further studies are required to confirm its feasibility. Additionally, the fact that the combination of EPN-EPF with olive oil resulted in larval mortality of 88% instead of ~50% recorded for the single EPN applications, but at similar times (~3 days), could be of interest when field treatments may require high mortalities at a short time. However, further studies are needed to deeper investigate the practical use of EPNs, single applied or combined with EPF, mixed with oils used herein or other plant-based oils, and at higher concentrations for biocontrol targeting aerial pests.

4.6 Acknowledgments

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V
DISCUSSION

5 DISCUSSION

In this thesis, we expanded the fundamental knowledge on the distribution and biodiversity of entomopathogenic fungi (EPF) in Portugal, investigating their possible association with selected habitats and biotic/abiotic factors. In addition, we explored, for the first time, whether the EPF biocontrol activity can be altered in the presence of other soil organisms such as entomopathogenic nematodes (EPN) and nematophagous fungi (NF). Finally, from an applied perspective, we also investigated whether EPF can be combined with EPN in plant-based oils formulations in search to enhence their efficiency as biocontrol agents. In addition, we evaluated the impact of temperature and time of storage in the EPN-plant-oil formulated eficacy. Hence, altogether, this thesis comprises the first aproach of the EPF in Algarve, with some novel insigns about the multitrophic interactions in the soil and their potential as biocontrol agents. These new data will serve as baseline to built up new basic and applied knowledge on the EPF group.

5.1 Entomopathogenic fungi and their prevalence in soils from the Algarve (Southern Portugal)

For the first time, the EPF distribution in Algarve region was evaluated, accounting for their spatial presence, biodiversity, soil factors driven their isolation, and association with other beneficial soil organisms. The study comprised the comprehensive analysis of soil samples retrieved from 50 sites located across the Algarve, representing four main habitats in the region. By combining three isolation methods, we were able to detect EPF in 68% of the sites, values that were in agreement with previous observations in surveys accounting for various habitats in the Iberian Peninsula (Quesada-Moraga et al., 2007), but far if compared with values of 20-30% in other regions such as UK and Mexico (Sánchez-Peña et al., 2011; Chandler et al., 1998). One possible explanation is the effort of sampling (number of samples taken per site, and number of total sites) and the effort of the method (number of insect-bait per sample). In this regard, Chandler et al. (1998) employed one larva per sample, while Quesada-Moraga et al. (2007) used 10 larvae per subsample, with 5 replications per site. Our study was closer to Quesada-Moraga et al. (2007) than to Chandler et al. (1998), because we used 10 larvae per sample, but repeating the process at different times and with two real samples per site. In addition, we combined

the detection of three independent isolation methods. This strategy allowed us to explore the EPF in different phases. The emply of selective media might retrieve EPF that tend to be in the saprophytic phase (Meyling, 2007), while the baits retrive those fungi that are actively acting as entomopathogens (Zimmerman, 1986). Despite our effort to detect as much as possible the presence of EPF, one of the limitations of our study was that the survey in the Algarve was performed during springtime. As suggested by Meyling and Eilenberg (2006), taking samples at different times at the same place can contribute to extend the biodiversity detected. Also, the use of other insects as host might enhance the isolation of EPF that are not attacking the lepidopter *G. mellonella*. This strategy has been succesfully employed in other studies (Sánchez-Peña et al., 2011; Meyling et al., 2012; Sharma et al., 2018). Indeed, the use of a target insect to be managed with EPF can lead to the selection of the best candidates for that target pest, as suggested by Shapiro-Ilan et al. (2003). This approach can be followed in future studies.

All the isolation method combined provided a broad picture of presence and activity in the Algarve, with the recovery of 5 species, with *F. solani* and *F. oxysporum* detected as EPF for the first time in the Iberian Peninsula. Combining traditional with molecular methods to identify the EPF derived from the three methods of isolation was critical to disantangle the biodiversity. Some species are very close related, and the use of only traditional methods can prevent the identification of certain cryptic species (Glare, 2004; Schneider et al., 2011; Campos-Herrera and Lacey, 2018). This seems to be the case of species in the genera *Beauveria* and *Metarhizium*, where the use of traditional methods showed only the presence of *B. bassiana* and *M. anisopliae* in Spain (Quesada-Moraga et al., 2007), while the introduction of molecular markers has allowed the detection of a higher richness in the Iberian Peninsula. For example, Garrido-Jurado et al. (2015) detected four species in the *Beauveria* genus (*B. bassiana*, *B.* amorpha, *B.* pseudo*bassiana*, and *B.* varroae) and three species in the *Metarhizium* genus (*M. guizouhense*, *M. robertsii* and *M. brunneum*) by using the sequences of EF-1α region as molecular marker. Similarly, Sharma et al. (2018) detected three *Beauveria* species (*B. bassiana*, *B. pseudobassiana* and *B.* varroae) and two *Metarhizium* species (*M. robertsii* and *M. guizhouense*) by using the sequence of ITS regions as molecular marker. In our study, although we only detected one species of each of these two genus, we detected other three species that rarely behave as EPF, *F. solani*, *F. oxysporum*, and *P. lilacinum*. This provides more evidences to the necessity to combine traditional and molecular tools

for the identification of EPF (Campos-Herrera and Lacey, 2018). As described above, the use of other insect as baits, or expanding the sampling effort in multiples ways might contribute to detect additional species of EPF in the Algarve region.

Finally, none of the two hypothesis regarding the association of EPF with certain habitats or ecoregions were supported by our data, results that are in agreement with Quesada-Moraga et al. (2007) with soil samples from Iberian Peninsula. However, Sánchez-Peña et al. (2011) detected patterns of occurrence depending on the habitat in mexican soils. Despite the obvious difference of the geographical location, variables such as sampling and baiting effort, variability on the habitat, and differences in the soil physical-chemical properties might affect the final detection, and hence, extending the study of natural EPF occurrence to other botanical groups and regions might provide some light in this controversial issue. Finally, certain soil properties (soil pH, OM and Mg content) contributed to explain the EPF assemblage, which were congruent with some properties associated with other entomopathogens as the EPN (Campos-Herrera et al., 2019). This is one of the few insigns on the habitat share of EPF and EPN. It is notheworthly that despite the entomopathogens share the same resource, the host, most of the surveys focus their effort in one single entomopathogenic group (i.e. virus, bacteria, fungi, nematode), so little is known on the ecological drivers of this gild in a wide context. In this regard, Shapiro-Ilan et al. (2003) observed that EPF were retrieved in almost the double of samples and sites than the EPN in their survey in southeastern pecan orchards in USA, values that are similar to those observed if compared the mortality caused by EPF and EPN in the untreated soil samples analysis (Campos-Herrera et al., 2019). Advancing on the knowledge on the natural occurrence of entomopathogens, EPN and EPF, in the same sample, can provide key information on the best ecological scenarios for the prevalence and activity, and how they can managed to enhance their contribution to the ecosystem service of biological control.

In summary, this study concluded that:

- In the Algarve region, EPF activity was not driven by botanical group or habitat nor by ecoregion (calcareous *versus* non-calcareous).
- The use of selective media resulted in higher recovery of EPF that any of the insect bait techniques.
- Combination of traditional and molecular methods were required to gather information on EPF presence and diversity.
- Soil pH, OM and Mg content explained most of the variability of the EPF community, providing evidences of congruence of soil properties that support other entomopathogens such as EPN.

5.2 Outlook chapter II.

Because this study was performed with samples retrieved in spring, it remains to be investigated whether this spatial pattern, biodiversity and association with other soil organisms remain the same at different seasons (summer, autumn, and winter) and even at different years, where different climatic and anthropic forces might induce some changes. The use of other insect as host in the bait and selective media of different composition could contribute to increase both the frequency of EPF detection and expand the biodiversity. In addition, it would be interesting to evaluate other habitats, such as annual crops and grasslands in the Algarve, as well as to expand the study to the whole territory of Portugal. This ambitious study would cover also more soil ecoregions and could serve to disentangle which are the driven forces that modulate the EPF natural occurrence. This knowledge is critical in the context of global warming. We need to know the current distribution and factors affecting the activity of EPF so we can forecast them to identify possible constrains or reservoirs for the future situations.

5.2.1 Multitrophic interactions in the soil modulate the biocontrol of entomopathogenic fungi

Little is known about the complex interaction occurring in the soil involving three or more trophic guild, such as EPF, EPN and NF, and whether their presence can impact on the EPF biological control of insect pests. This study provided the first evidences on the modulation of the EPF biocontrol in various scenarios of co-occurrence with other beneficial soil organisms. We observed that the final interaction (synergic, additive or antagonistic) depended on the type of expossure, concentration and species. Overall, the nature of the interaction between EPN and other organisms such as EPN has provided contrasting results. For example, Shapiro-Ilan et al. (2004) observed that most of the interactions resulted in antagonistic effect, but also observed some additive effect that warrant further reseach, such as the combination of *H. indica* and *M. anisopliae* and also *S. carpocapsae* and *B. bassiana* and the entomopathogen bacteria *Serratia marcescens*. On the contrary, Acevedo et al. (2007) observed that the combination of EPN *H. bacteriophora* and two strains of the EPF. *M. anisopliae* resulted in a positive interaction, probably additive effect, reducing the time to kill the insect, *Diatraea saccharalis* (Lepidoptera: Pyralidae). However, they also registered some trade-off in the combination, represented by a significant reduction on the nematode progeny in the dual infections as well as the reduction of the conidia production in the EPF (Acevedo et al., 2007). In their study, Wu et al. (2014) did not observed any trade-off on the EPN reproduction when combined with *B. bassiana* or *M. anisopliae*. As showed in these previous studies, we obtained contrasting results depending on the type of expossure. Then, when the fungi were present as mycelia, most of the interactions were additive. When the host was immersed in the conidia suspension, some antagogistic and synergistic effect were observed in the presence of *B. bassiana*, while when the fungi were injected, triple interactions were all synergistic as well as other double interactions involving *B. bassiana*. However, the fact that we did not account for the reproduction of EPF (number of conidia) or EPN (number of IJ) limit our observation to the biocontrol potential of these organisms when combined two by two, or the three at once. It is possible that some trade-off effect might exist in the further reproduction or viability of EPF and EPF as biocontrol agents. These data were not established in our study. These results illustrate the complexity of the interactions that can occur in the soil that can affect the biocontrol potential of the EPF. In addition, it is well-known the ecological plasticity of the EPF *B. bassiana* that depending on the pressures of the environment, can change from

antagonistic interaction to synergism one, but also, can act as entomopathogen, endophyte, interact in the phylloplane and even as plant growth promoter fungi in the rizhosphere (Meyling and Eilenberg, 2006; 2007; Keyser et al., 2015; Vega et al., 2009; 2012; Ownley et al., 2010; Quesada-Moraga et al., 2014). This ecological plasticity opens its use in novel plant protection approaches such as enhancing the plant protection against phytopathogens and the nutrients availability in the plant (Ownley et al., 2008, 2010; Behie et al. 2012; Sánchez-Rodríguez et al., 2015, 2016). However, more studies are needed to unravel under which ecological scenarios the EPF are more likely to act with one funtion or the other and whether the presence of other soil organisms, both beneficial or detrimental, can modulate the final role taken by the EPF.

In addition, we showed that the co-occurence of EPF and NF inhibited the growth of the fungi by contact when expossed to the same resource. This inhibition depended on the media, with the richest media (PDA) showing higher inhibition rates than when the fungi were expossed in the limiting media (CMA ¼). In addition, although the inhibition was bidireccional, the EPF action was stronger than any of the NF. This result suggests that the EPF *B. bassiana* is ready to display more compounds to compete for the resources and the ecological niche that the NF, such as the production of secondary metabolites with antifungal activity (Strasser et al., 2000; Ownley et al., 2008; Sahab, 2012). It is known that the NF can also live as saprobionts, living from organic matter, and hence, they can ocupy other niches than the EPF. Hence, it is possible that their competition abilities against other fungi can be less than those from EPF. Expanding the research to other EPF and NF species is required to understand to which extend the EPF are stronger competitors than the NF under various conditions.

Because the application of beneficial soil organisms is arising as one of the strategies compatible with the novel approach of sustainable agriculture, learning about the complex interactions in the soil is urgent to avoid competition among agents. In this regard, recent studies provided evidences on the compatibility of simultaneous application of beneficial soil organisms such as EPN, Pseudomonads bacteria and arbuscular mycorrhizal fungi (AMF) (Imperiali et al., 2017; Chiriboga et al., 2017; Jaffuel et al., 2019). The combination with EPF can be also a strategy to evaluate. However, some limitations were also detected. In this regard, the selection of the species with the best attributes that match the target pest or present higher prevalence in the environemnt are critical. For example,

the EPN species *S. feltiae* showed the highest survival in a wheat field than *H. megidis*, but unfortunately, the combined treatment with *Pseudomonads* bacteria, and AMF was only perfomed with the later one (Imperiali et al., 2017; Chiriboga et al., 2017). Hence, it is unknown if the combined treatment would have been with *S. feltiae*, maybe a stronger protective values could have been recorded. Similarly, Jaffuel et al. (2019) combined the EPN species *H. bacteriophora* and *S. feltiae* in the same treatment (EPN) that were applied single of combined with Pseudomonads and AMF. Therefore, additional studies are required to investigate the interactions occurring in the soil and how to improve the use of the EPF in scenarios of combination with other beneficial soil organisms.

In summary, our study concluded that:

- EPF limited NF growth and *viceversa*.
- The EPF *Beauveria bassiana* dominated triple interaction when mycelia was exposed.
- The EPN *Steinernema feltiae* dominated triple interaction in immersion exposure.
- The NF *Arthrobotrys musiformis* caused larval mortality if vectored inside the host.

5.3 Outlook chapter III.

This study was the first time that the triple interaction among EPF, EPN and NF was investigated. We only explored the interaction of one EPF species, one EPN species and two NF. It would be interesting to investigate the outcome by using other species, with different potential action, such as *M. anisopliae* or *F. solani* as EPF, or *H. bacteriophora* in the case of EPN. Also, it remains to be investigated whether these interactions are modulated by the time of exposure. Here, we simulated different time of arrival by exposing to mycelia (same time), immersion in the fungal suspension (preference to the EPF or NF), and injection in the host body (first arrival inside the host for EPF and NF). The time of exposure can be extended so, one target organisms is applied after 24 or 48h

presence/arrival of the other(s). Also, the use of other insect as host can provide additional information on the competition for the resource. Different insects have different immune system, and how they face the infection of one of other pathogen can lead to a final result in the competition. Finally, this study was performed under laboratory conditions, and additional studies are required in more naturalized systems to unravel the impact in the biocontrol activity when these organisms are combined.

5.3.1 Advances toward the enhancement of entomopathogenic fungi formulation for local productions

The succesful application of the biological control agents requires a suitable formulation and adapted equipment (Lacey et al., 2015). The selection of the best approach will depend on the target species ecology and behaviour, and the location of the desired control, above or belowground (Hiltpold, 2015). Compared with soil application, aerial formulation and application system are critical because require the adaptation to harrasing conditions (extreme temperature, UV radiation, extreme dry), so the biocontrol agent are still viable before reaching the host (Brusselman et al., 2012; Shapiro-Ilan and Dolinski, 2015). In this regard, oils formulationsare a promising alternative for the application of EPF. For example, the use of various vegetable oils to formulate *M. anisopliae* var. *acridum* allowed to fight against locusts and grasshoppers even in the harassing conditions of dry and wild associated with the areas of distribution (Bateman, 2004; Moore, 2008). Similarly, *Zoophthora radicans* was combined with selected low-cost oil based products to fight against *Plutella xylostella*, resulting in a significant increment of the efficacy of the EPF (Batta et al., 2010). Our study showed, for the first time, the compatibility of the EPF and EPN combination with two easy to reach plant-based oils: coconut and olive oils. The combination not only registered additive effect, increasing from 50% to 80% the mortality, but also reduced the time to kill the insects. These results provide new opportunities for the formulation of entomopathogens, in particular, in areas of reduced incomes where the plant-based oils associated with food industry are cheap and accesible. In addition, the combined application can be seen as a saving cost strategy for the growers. Further investigation is needed to optimize their application, in terms of EPF and EPF species combined, concentration, oil type and target insect.

The time of storage and temperatura were also evaluated for the EPN species *S. colombiense*, alone or combined with the two plant-based oils. Overall, the formulation was not better than the water treatment, with some exceptions. For example, when the IJ of this nematode were combined with coconut oil, they survived better at 4ºC up to 7 days, whereas the IJs combined with olive oil resulted in higher larval mortality at extreme temperatures and 14 days. In any case, these two plant-based oils maintained viable the IJs at moderate temperatues (8-20ºC) up to 7-14 days. Hence, if adherence to the leafs and the viability under aboveground conditions are also increased, then, the formulation would be suitable for aerial applications. Further studies are required to perform a comprehensive characterization of this formulation to fight against aerial pests.

The study concluded that:

- The combination of EPN+EPF showed an additive effect compared to EPN and EPF single treatments.
- EPN survival was higher in coconut than olive oil and water mixtures up to 7 days at 4 ºC.
- Olive oil supported higher larval mortality caused by EPN than coconut oil at 4-20 ºC and 14 days.

5.4 Outlook chapter IV.

Exploring new adjuvants that can enhance the biocontrol attributes (survival, infectivity, virulence) of certain entomopathogens such as EPF and EPN applied in the aerial plant part is a fundamental step to advance toward the combined use of beneficial organisms for sustainable agriculture strategies. The use of the plant-based oils derived from coconut and olive products has shown some promising results, with additive effect when EPF and EPN are combined but shortening the time to kill the insects. However, our results are limited to laboratory conditions and the use of the model insect *G. mellonella*. It remains to be investigated whether this effect is also observed when this formulation is applied in the aerial part of the plant against other insects. In this regard, it

would be interesting to describe the survival of both entomopathogens once applied in the surface of a leaf. Garriga et al. (2019) observed that when the EPN *S. carpocapsae* was applied in combination with the oil-adjuvant Addit (Koppert), the survival decreased below 50% of the IJ after 6 hours. Hence, it is recommended that before using the plant-based oil tested here, coconut and olive oil, the study of their survival is performed. Also, a good retention in the leaf is desired. Platt et al. (2019) observed that when the EPN *S. yirgalemense* was applied in combination with two adjuvants (Zeba and Nu-Film-P), the deposition of the EPN can increase to almost double than in applications in water. In this line, testing other co-adjuvants, either from commercial sources or from basic market such as other oils (canola, sunflower, etc.) can expand the knowledge on the performance of combined EPF-EPN formulation. Finally, the formulation of beneficial soil organisms can enhance the entomopathogen activity/survival, but also can be linked to enhance the attraction of the pest. Recent studies combined the encapsulation of EPN in beads with plant-based wireworm attractions, resulting in a significantly higher mortality of the pest. This approach can be also explored in future research on the formulation and aerial application of the EPF+EPN together.

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ANNEXES

Annex 1

Annex 1.A. Pearson correlations (*P* < 0.05) to compare the recovery occurrence and larval mortality percentages among different isolation methods of entomopathogenic fungi (EPF). Codes: n.s., no significant; n.d., no data

	Recovery occurrence %		
	Fresh soil	Pre-dried soil	Selective media
Untreated soil bait	-	n.s.	n.s.
Pre-dried soil bait		-	n.s.
Selective medium			-
	Larval mortality %		
Untreated soil bait	-	n.s.	n.d.
Pre-dried soil bait		-	n.d.
Selective medium			-

Annex 1.B. Statistical analysis (One-way ANOVA and T-test, *P* < 0.05) for the occurrence of fungal that confirmed Koch´s postulates accordingly the variables isolation method, vegetation type, and soil eco-region. Code: n.s., no significant.

EPF species	EPN isolation method F (*P*)	Vegetation type F (*P*)	Ecoregion t (*P*)
B. bassiana	2.206 (n.s.)	0.402 (n.s.)	0.769 (n.s.)
F. solani	1.000 (n.s.)	0.544 (n.s.)	1.302 (n.s.)
F. oxysporum	2.478 (n.s.)	0.569 (n.s.)	0.478 (n.s.)
P. lilacinum	2.972 (0.054)	n.d.	n.d.
M. anisopliae	n.d.	n.d.	n.d.

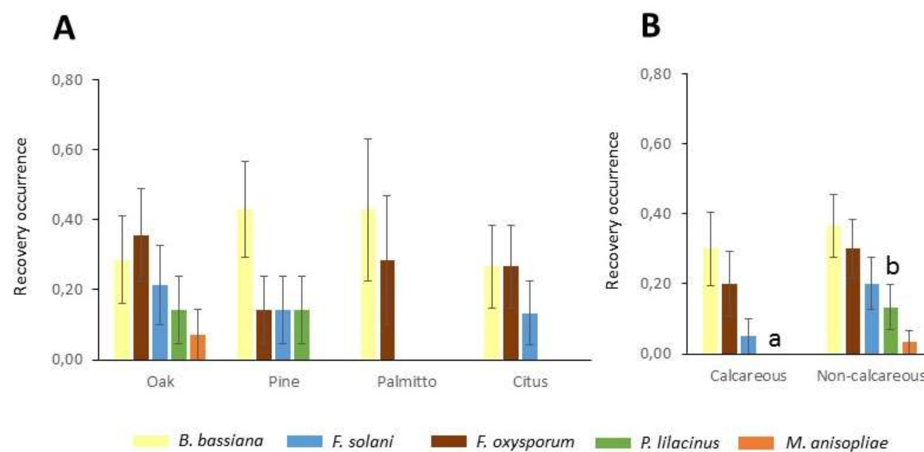
Annex 1.C. Statistical analysis (One-way ANOVA and T-test, *P* < 0.05) of the impact of the variables vegetation type or soil ecoregion on the occurrence of entomopathogenic fungi (EPF) and larval mortality recorded for each of EPF isolation method. Codes: n.s., no significant; n.d., no data

	EPF occurrence %		Larval mortality %	
EPF isolation method	Vegetation type F (*P*)	Ecoregion t (*P*)	Vegetation type F (*P*)	Ecoregion t (*P*)
Untreated soil bait	0.862 (n.s.)	2.362 (0.022)	0.642 (n.s.)	2.527 (0.016)
Pre-dried soil bait	0.197 (n.s.)	1.006 (n.s.)	1.165 (n.s.)	1.799 (n.s.)
Selective medium	0.494 (n.s.)	0.226 (n.s.)	n.d.	n.d.
All methods combined	0.196 (n.s.)	0.958 (n.s.)	-	-

Annex 1.D. Statistical analysis (One-way ANOVA, $P < 0.05$) of the efficiency among isolation methods for the occurrence of entomopathogenic fungi (EPF) depending on the factors vegetation type or ecoregion. Code: n.s., no significant.

Ecological drivers	EPF occurrence % F (*P*)
Vegetation type	
Oaks	0.100 (n.s.)
Pines	2.256 (n.s.)
Palmetto	1.500 (n.s.)
Citrus	2.492 (n.s.)
Ecoregion	
Calcareous	5.791 (0.005)
No-calcareous	1.286 (n.s.)

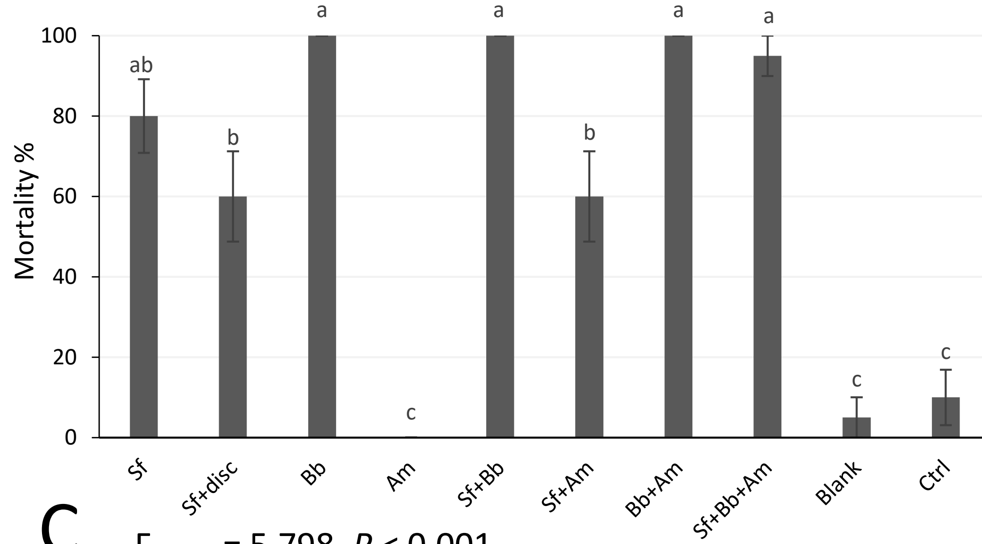
Annex 1.E. Comparison of entomopathogenic fungi (EPF) recovery frequency by species depending on two ecological drivers. **A.** Botanical habitats. **B**. Soil ecoregion. Different letters indicate significant differences in T-test ($P < 0.05$). Values are least-square means ± SE.

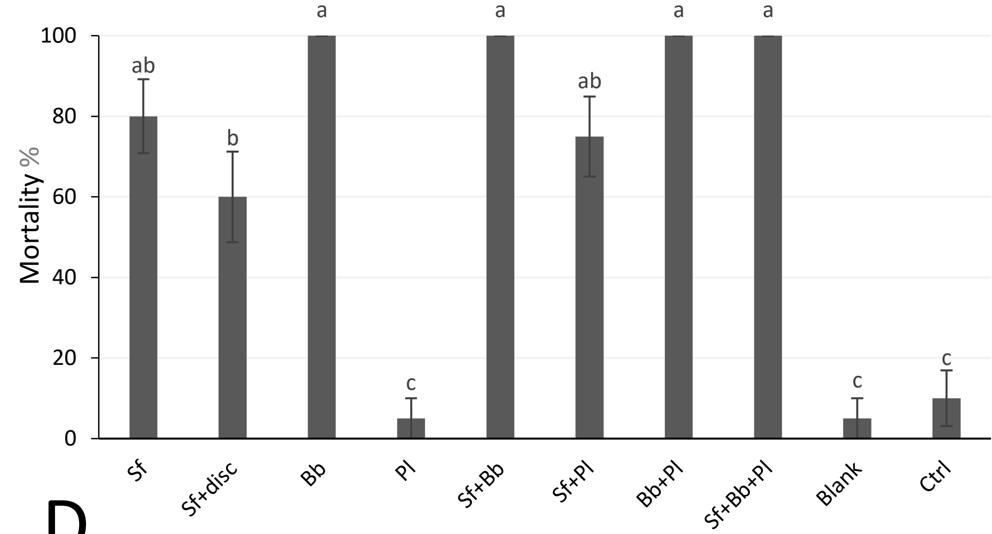


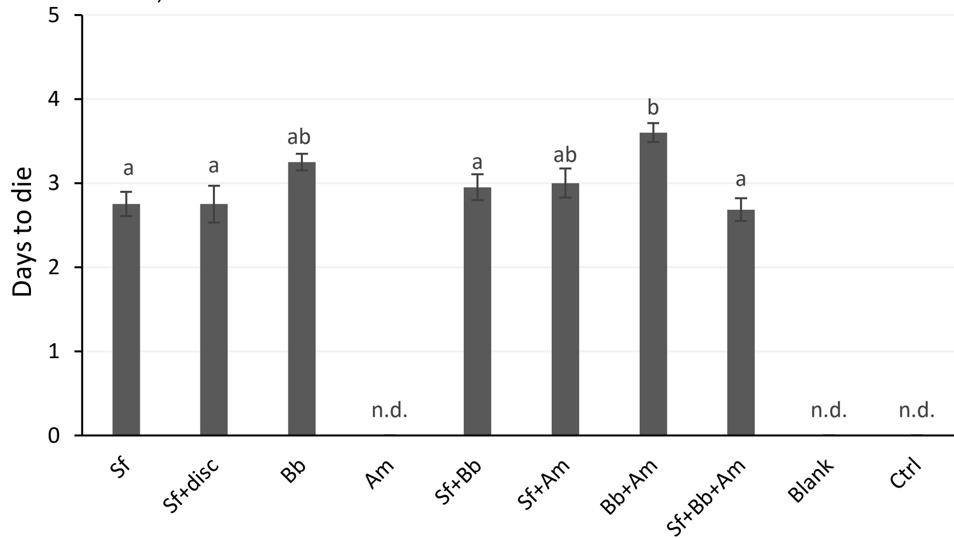
Annex 2

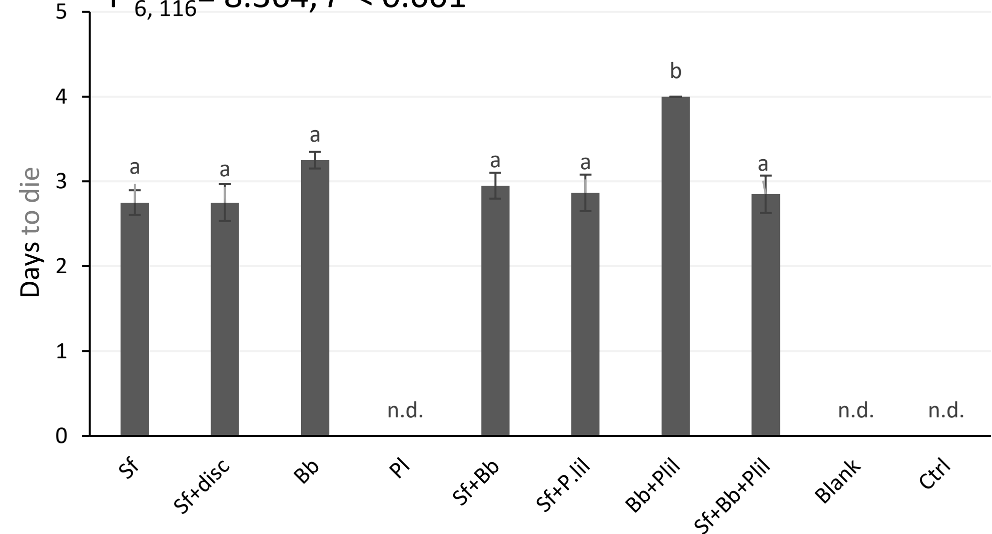
NF: *A. musiformis*

NF: *P. lilacinus*









Supplementary material 1. Figure S1. Evaluation of the interaction among nematophagous fungi (NF) and entomopathogens (nematode and fungi) in the direct-contact with mycelia-conidia experiment (trial 2). **A** and **B.** Larval mortality percentage. **C** and **D.** Number of days until larval death. Different letters above bars indicate statistical differences (one way ANOVA and Tukey test, $P < 0.05$). Treatments code: control (Ctrl, larvae exposed to two sterile disks), Blank (untreated larvae), *Steinernema feltiae* (Sf), *Beauveria bassiana,* (Bb), *Arthrobotrys musiformis* (Am), *Purpureocillium lilacinus* (Pl), and the double or triple combinations of these organisms; not data, n.d. Data are expressed as average ± SEM.

ENTOMOPATHOGENIC FUNGI FROM ALGARVE REGION (PORTUGAL): STUDIES ON THEIR NATURAL OCCURRENCE IN THE SOIL AND MULTITROPHIC INTERACTIONS THAT SHAPE THEIR BIOCONTROL POTENTIAL

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