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

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Running title: Fungal associated to *Prays oleae*

Submission date: 28-05-2011

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ABSTRACT

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

Keywords: Olive tree; *Prays oleae*; fungal diversity; moth life cycle generation

Introduction

The olive tree is an important crop for Mediterranean basin countries including Portugal. Extensive areas occupied by olive groves in Trás-os-Montes region (Northeast of Portugal), not only have a significant economic impact, but also exhibit a social, environmental and landscape significance. The olive moth, *Prays oleae* Bern., is one of the major pests on these olive orchards, being responsible for high losses in the olive yield as much as 40% [33]. This lepidopteran presents three generations per year that damage the olive tree in different organs. The antophagous generation, occurring from April to June, causes damages to the olive tree flowers; the carpophagous generation, which usually appears from July to September, attacks the fruits leading to their premature fall; and the phyllophagous generation, present from October to March of the following year, damages the leaves [33]. Due to the growing awareness of detrimental effects of pesticides to the ecosystems [6], agricultural practices in these orchards have been changing to biological agriculture. Therefore, the search for methods to control pests and diseases has acquired a new motivation. One of the promising methods to control pests can be the use of entomopathogenic fungi, which are known by their ability to infect and kill several insect species [25].

Entomopathogenic fungi comprise a large group of pathogens that includes approximately 700 species in almost 85 genera [5]. Due to their large host range, entomopathogenic fungi could be potentially useful as control agents against different insect orders. When compared to conventional chemical pesticides, the use of insect pathogens presents many advantages, such as the safety for humans and other non-target organisms, environmental reduction of pesticides residues and a smaller effect on the natural biodiversity [15]. However, pest management using entomopathogenic fungi has been difficult to achieve. The ecological and environmental variations within agro-

ecosystems turn the formulation and application of this kind of biocontrol agent difficult to manage [42]. The isolation of native fungi could provide a collection of isolates, for the development of potential control agents already adapted and suited to a particular habitat. In this work, the fungal diversity encountered on *P. oleae* population was evaluated in olive groves from Trás-os-Montes and will be discussed taking into account the olive moth generation where it appears. From this survey, potential entomopathogenic fungi for future biocontrol strategies will be selected.

Methods

Study area

Larvae (mostly in the fourth and fifth instars of development) and pupae of *Prays oleae* were collected in six olive groves located in Mirandela – Bragança region, Northeast of Portugal (GPS coordinates: 41º34'03.77''N; 7º05'39.21''W; 41º33'53.29N, 7º05'40.23''W; 41º33'52.51''N, 7º05'30.59W; 41º33'33.11N, 7º05'35.62W; 41º33'08.02''N, 7º07'24.87''W; 41º32'35.20''N, 7º07'26.27''W). This region comprises an olive tree growing area around 65.000 ha, corresponding to 22% of the olive tree covered area in Portugal. It contains 20% of Portuguese olive trees, which altogether are responsible for 39% of national olive oil production [31]. The topography of this region is mountainous, with altitudes ranging between 300 and 500 m. The prevailing climate is Mediterranean, with cold and rainy winters and long, hot and dry summers. The average annual rainfall ranged from 600 to 800 mm, occurring mainly between October and February, and the annual mean temperature ranged from 9 to 20ºC. For this study, the selected orchards have been managed through organic [11] or integrated production guidelines [18] and display high levels of olive moth infestations, as observed by delta traps baited with pheromone. Although two different management

93 systems are referred, the studied olive groves are very similar, with low-external inputs.
94 The groves area ranged from 0.4 to 1.5 ha. Trees are of medium size with a planting
95 density of 7 x 7 m. Pruning was made every 2-3 years. No irrigation was done, and only
96 copper based products were used in the beginning of autumn for fungal diseases control
97 and no other pesticides were used in the last five years. The differences between the two
98 management systems are related with the products allowed in soil fertilization. The
99 most important cultivars were Cobrançosa and Verdeal Transmontana.
100
101 Experimental design and collection of plant material
102 The collection of larvae and pupae was performed in 15 randomly chosen trees in each
103 of the selected orchards for the three annual generations of *P. oleae*. Sampling dates
104 were variable according to the life stages of the pest. For the phyllophagous generation
105 the collection was conducted between 17th and 27th March of 2009, for the antophagous
106 generation between 24th May and 5th June and for the carpophagous generation between
107 1st and 3rd of September of the same year. For the first two generations, sampling of
108 leaves (phyllophagous generation) and flower clusters (antophagous generation), in
109 which larvae and pupae were present, was performed. For the carpophagous generation,
110 the collection of whole fruits was randomly performed, due to the presence of larvae
111 and pupae within olives. The plant material (leaves and flower clusters) and olives were
112 collected individually to sterile bags, and processed within a few hours after sampling.
113
114 Isolation of fungi from mycosed larvae and pupae of *P. oleae*
115 In the laboratory, the plant material was further examined for the presence of olive moth
116 larvae and pupae. Since carpophagous larvae develop inside the olive stone, the stone
117 was smoothly broken to expose larvae. Whenever present, the larvae and pupae were

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

Extraction of fungal DNA

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

143 temperature and re-suspended in 50 μL of ultra pure water. DNA was stored at -20ºC
144 until use.
145
146 Molecular identification of fungal isolates
147 Molecular identification was achieved by amplification of the internal transcribed
148 spacer region (ITS), using the universal primers *ITS1* and *ITS4* [44]. PCR reactions
149 (50 μl) comprised 50 ng of genomic DNA, 0.2 μM of each primer (*ITS1* and *ITS4*), 1x
150 GoTaq® Flexi buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.2 μM dNTP Mix
151 (Fermentas) and 1 U GoTaq® DNA polymerase (Promega). Amplifications were
152 carried out in the thermocycler Biometra UNO II (Thermoblock, Biotron) using a
153 temperature gradient protocol as follows: initial denaturation at 94ºC for 3 min,
154 followed by 35 cycles of 0.5 min at 94ºC, 0.5 min at 55ºC, 1 min at 72ºC, and a final 10
155 min extension at 72ºC. PCR amplification products were analysed by electrophoresis
156 and those reactions that amplified a single PCR product were selected for purification
157 using the JETquick PCR product purification kit (Genomed). Amplified fragments were
158 sequenced using both *ITS1* and *ITS4* primers at the sequencing services of STAB Vida
159 (Oeiras, Portugal). DNA sequences were analysed with DNASTAR v.2.58 software,
160 and fungal identification was performed using the NCBI database
161 (http://www.ncbi.nlm.nih.gov) and BLAST algorithm.
162 The ecological classification of identified species was only based on the impact they can
163 have on olive orchards regarding their potential role on pests and diseases control. Even
164 though many of the identified fungi are also considered general saprophytes, they were
165 only classified into three ecological roles: phytopathogenic, antagonistic and
166 entomopathogenic. The classification given to each fungal species was based on
167 previously described characteristics.

Data Analysis

For each *P. oleae* generation, the species richness, *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes, total and relative abundances were estimated. Calculations of *Simpson* and *Shannon-Wiener* diversity indexes were done using the software *Species Diversity and Richness* (v. 3.0). Total abundance (N) was estimated as the number of isolates per fungal taxa, whereas the proportion of isolates from each fungal taxa in relation to the total number of fungal isolates was considered as the relative abundance of a certain taxa. Principal component analysis (PCA) was applied to access the relationship between fungal taxa and the three *P. oleae* generations. PCA was performed using the SPSS software, version 17.0 (SPSS, Inc.). It was applied as an unsupervised approach for reducing the number of variables (43, corresponding to the number of identified fungal species) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information. This analysis will define which fungal species are correlated with each olive moth generation. PCA analysis also allowed the recognition of patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). The aim of the PCA is to produce components suitable to be used as predictors or response variables in subsequent analysis. The number of factors to keep in data treatment was evaluated by the Scree plot, taking into account the eigenvalues and the internal consistency by means of αCronbach's value [21; 34].

Results

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

From the 3828 larvae and pupae (2552 larvae and 1276 pupae), collected in all generations of *P. oleae*, an insect emergence percentage of 38.6% was observed (Table 1). Of the total number of dead larvae and pupae (1477), 40.6% (599) exhibited the growth of a fungal agent on their surface (Table 1). From those, the isolation of fungi allowed the identification of 43 species, belonging to 24 genera and 14 families (Table 2). The families comprising more diversity were Pleosporaceae and Quambalariaceae (8 species each) and Mucoraceae and Nectriaceae (5 species each). These families accounted for as much as 62% of the total identified species. The greatest number of taxa belonged to the genera *Penicillium* (7), *Arthrinium*, *Mucor*, *Fusarium* and *Alternaria* (all with 4 taxa). Concerning abundance, 166 different isolates were obtained in this work (Table 2). The most common taxa were *Beauveria bassiana* (N=50) and *Fusarium oxysporum* (N=25), representing together 45.2% of the total identified isolates.

The number of fungal taxa identified in the present study varied between the three generations of *P. oleae* (Table 2). In the phyllophagous generation, where a total of 70 fungal isolates were obtained, 16 different species were identified, belonging to 13 genera and 9 families. The most representative family was Quambalariaceae, comprising 25% of the identified species in this generation, being the genus *Penicillium* the most represented (3 species). In the antophagous generation, 52 isolates were obtained belonging to 13 species, 8 genera and 8 families. The families Nectriaceae and Apiosporaceae include the majority of the identified taxa (54%). The genus that included more taxa (4) was *Fusarium* (Nectriaceae), which contained 31% of the fungal species surveyed in this generation. Concerning the carpophagous generation, 44 fungal isolates were obtained being identified 21 species, belonging to 14 genera and 10 families. Pleosporaceae was the family representing more species (33%), and the genera

217 *Alternaria* (Pleosporaceae) and *Penicillium* (Quambalariaceae), both with 4 species
218 each, comprised 38% of the total fungal taxa found in this generation of *P. oleae*.
219 The most abundant species also differed between generations. For the phyllophagous
220 generation, the most abundant one was *B. bassiana* (N=49), which represented 70% of
221 the relative abundance in this generation. For the antophagous generation, *F. oxysporum*
222 was the most abundant (N=23), corresponding to a relative abundance of 44.2%. In the
223 carpophagous generation, several taxa presented comparable relative abundances, being
224 *P. pinophilum* the most frequent (N=7, corresponding to a relative abundance of
225 15.9%), followed by *Alternaria sp.1* and *A. ustus*, both with N=6 corresponding to
226 13.6% of relative abundance.
227 In order to provide more information about fungal community composition in each
228 *P. oleae* generation, the *Simpson* (*D*) and *Shannon-Wiener* (*H*) diversity indexes were
229 determined (Table 3). In the present work, both diversity indexes differed between the
230 three *P. oleae* generations. As verified for species richness, the highest fungal diversity
231 was verified in the carpophagous generation (*D*=0.09 and *H*=2.70). In fact, when using
232 larvae or pupae from this generation, the highest number of unique species (17) was
233 obtained. The number of exclusive species identified from phyllophagous and
234 antophagous generations was only 11 and 9, respectively. The lowest value of species
235 diversity (*D*=0.5 and *H*=1.39) was detected in the phyllophagous generation.
236 When performing a principal component analysis (PCA) using the total fungal taxa,
237 according to the moth generation from which they were isolated, it was possible to
238 separate the samples in eight groups (Fig 1). The phyllophagous generation is clearly
239 related to groups 4 and 7, the antophagous generation is linked to groups 3 and 6, and
240 the carpophagous generation is associated to groups 1 and 2. The two remaining groups
241 (5 and 8) do not appear associated to a specific generation. Group 5 is mainly correlated

to the antophagous generation, although presenting some relation to the phyllophagous generation. Group 8 is equally correlated to both phyllophagous and carpophagous generations.

Fungal ecological roles

Taking into account the potential use for the biological control of pests and diseases, 45% of the isolates found in this work have been described in the literature as presenting the useful features of antagonism and/or entomopathogenecity (Fig 2A). The remaining 55% of the isolates presented phytopathogenic features, displaying also in large extent (41%) antagonistic and/or entomopathogenic characteristics. When comparing the number of identified species, the majority (75%) has been described as phytopathogenic fungi (Fig 2B). While the mentioned attribute was present alone in 28% of the identified species, the remaining 47% also displayed antagonistic and/or entomopathogenic characteristics. From those species described as non-phytopathogenic (23%), the majority are defined as antagonistic (17%).

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

In the antophagous generation, a large fraction of isolates (73%) has been described as displaying phytopathogenic characteristics, of which 71% also displays antagonistic

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

Discussion

In the present work, the diversity of fungi isolated from dead larvae and pupae of a major olive pest (*P. oleae*) was evaluated for the first time. Following molecular identification, it was possible to identify 43 fungal species, belonging to 24 genera and 14 families. The identification of such a high number of fungal taxa described as presenting antagonistic, entomopathogenic or phytopathogenic features was only possible because the studied olive groves were maintained under organic or integrated production guidelines. If sampling had been performed on a conventional orchard the expected fungal diversity would have been lower. Organic and integrated production management creates a healthier and safer environment with higher biological diversity [19]. Similar diversity levels (46 fungal species and 27 genera) were obtained when

292 studying insect-associated fungi isolated from soil samples of different field crops
293 (wheat/maize, corn and soybean) and orchards (peach and apple) [38].
294
295 Fungal diversity and abundance
296 Concerning genera diversity, three main patterns were detected: (i) species-rich and
297 highly abundant genera, e.g. *Fusarium*, *Alternaria* and *Penicillium* (about 35% of the
298 taxonomic diversity and 42% of the total of isolates), (ii) species-rich genera, but
299 displaying low abundance, e.g. *Mucor* and *Arthrinium* (about 19% of the taxonomic
300 diversity and 5% of the total of isolates); and (iii) species-poor but highly abundant
301 genera, e.g. *Beauveria* and *Trichoderma* (about 5% of the taxonomic diversity and 35%
302 of the total of isolates). Among the 43 species recorded during the present study, the
303 genera *Alternaria, Arthrinium, Fusarium, Mucor* and *Penicillium* were the most
304 represented in terms of taxa number. These genera include some of the most ubiquitous
305 fungal species in nature, which have been found in soils, plants and agricultural
306 communities. The wide presence of such microorganisms has been related to the broad
307 ecological roles they play, either as saprophyts, phytopathogens or biocontrol agents
308 [8].
309 The genera that contributed most to species diversity depended on which *P. oleae*
310 generation was used for fungal isolation. While in phyllophagous generation
311 *Penicillium* represented the genus with highest taxonomic diversity (18%), in
312 antophagous generation that genus was *Fusarium* (31%) and in carpophagous
313 generation were *Alternaria* and *Penicillium* (representing together 39%). The
314 composition of fungal community was also inferred by the *Simpson* (*D*) and *Shannon-*
315 *Wiener* (*H*) diversity indexes, which offer valuable information about rarity and
316 frequency of species in a community. Both indexes provide more information than

317 simply species richness, since they also take the relative abundances of different species
318 into account. While the carpophagous generation presented the highest fungal diversity
319 (displaying the lowest *D* and highest *H* values), the phyllophagous generation displayed
320 the lowest diversity (presenting the highest *D* and lowest *H* values). This difference
321 between moth generations could be related to two main factors: climatic conditions
322 during larvae and pupae collection and the moth life cycle.
323 Climate conditions, in particular relative humidity and temperature, are known to affect
324 both conidia dispersion and germination [39]. The collection of larvae and pupae from
325 phyllophagous generation (March) matched with the time of year where the temperature
326 is low, decreasing the ability of spore germination and thus reducing fungal diversity.
327 Furthermore, the moth life cycle also seems to strongly affect the fungal diversity
328 observed in this generation, as a large part of phyllophagous moth development occurs
329 in the leaves as miner larvae. Therefore, the reduced fungal diversity observed in this
330 generation can be associated to the low chances of larvae contact with fungal spores.
331 The high occurrence of the entomopathogenic *B. bassiana* in the phyllophagous
332 generation (70% of the isolates) could be related with an epizootic outbreak that might
333 have occurred during the time of larvae and pupae sampling. These epizootics are
334 described as being dependent on host population dynamics, the number and viability of
335 infective stages in the pathogen population, infection efficiency and development, in
336 addition to a complex set of environmental factors and timing [25]. Although dispersal
337 of *B. bassiana* conidia by larvae could have been limited, due to their small
338 displacement in trees, the wind and rain may have caused the spread of those infectious
339 structures. Also, the high number of infected *P. oleae* individuals could have functioned
340 as sources of infective conidia, thus contributing for the abundance of *B. bassiana* in
341 this generation.

342 In antophagous and carpophagous generations, the most abundant taxa were *Fusarium*
343 *oxysporum* (44% of the total isolates) and *Penicillium pinophilum* (15.9% of the total
344 isolates), respectively. As referred, these two taxa are very frequent in nature, being
345 present in almost all environments. Furthermore, the amount of spores of *Fusarium*
346 species is known to increase from April to July [39] and those from *Penicillium* species
347 rise between August and October [23]. These periods are coincident with the sampling
348 dates of larvae and pupae from antophagous (May-June) and carpophagous (September)
349 generations, explaining in some degree the high abundance of *Fusarium* and
350 *Penicillium* genera in antophagous and carpophagous generations, respectively.
351 The association of climate and life cycle negatively affect the fungal diversity when
352 using antophagous larvae and pupae. In the region where the study was conducted,
353 temperature rapidly increases during May and June, leading to an extremely fast larvae
354 development. Therefore, larvae and pupae stay a short period exposed to the
355 surrounding environment, reducing the possibility of fungal infection. In contrast,
356 carpophagous larvae are exposed to fungal spores for a longer period. After ecloding
357 from the egg, larvae must travel from the oviposition site in the fruits to the petiole and
358 bore down into the stone, where they usually feed for several weeks. After completing
359 their development, larvae re-emerge from the fruit. The chance of infection is still
360 enhanced by the extension of carpophagous generation which, together with the
361 favourable climatic conditions, allows the development of more fungal taxa.
362 Accordingly, it was in this moth generation that higher species diversity was found.
363
364 Fungal ecological roles
365 The relation between the amount of dead larvae and pupae displaying fungal growth and
366 the ecological role of the identified fungal taxa provides interesting data. The higher

367 amount of infected dead larvae and pupae was observed in the phyllophagous
368 generation (61.5%). It was also from this generation that the most isolates exhibiting
369 entomopathogenic features (76%) were obtained. As the presence of fungal growth on
370 dead larvae and pupae decreases (from 61.5% to 32.7% and finally 23.0%, in the
371 phyllophagous, antophagous and carpophagous generations, respectively), the
372 abundance of fungi exhibiting entomopathogenic characteristics also decreases (76%,
373 50% and 30%, respectively). These results seem to suggest that the infection with
374 entomopathogenic fungi could have led to larvae and pupae death. Most of the fungal
375 isolates (86%) and species (70%) identified in this work has been described as
376 antagonistic and/or entomopathogenic, although some of them have also been
377 considered as displaying phytopathogenic features. Because only the non-
378 phytopathogenic fungi displaying antagonistic and/or entomopathogenic features could
379 be explored for limiting fungal diseases and/or pests, the percentage of identified fungal
380 species that might have a future application as biocontrol agents is reduced to 23%.
381 From identified taxa in this work, *B. bassiana* seems to be the most conspicuous among
382 the entomopathogenic/antagonistic species. The natural occurrence of this fungus in
383 over 700 insect hosts from almost all taxonomic orders is well documented [25].
384 However, there are still some reservations about the host range of this fungus. Some
385 authors claim that *B. bassiana* is a "species complex", referring that different isolates
386 have a restricted host, while others point out that this fungus has no host specificity
387 [29]. Accordingly, several pests are susceptible to the entomopathogenic aptitude of this
388 fungus, like *Alphitobius diaperinus* [36], whiteflies *Bemisia tabaci* and *Trialeurodes*
389 *vaporariorum* [32], *Capnodis tenebrionis* [20], *Lutzomyia longipalpis* [2],
390 *Callosobruchus maculates* [26], and *Tetranychus urticae* [10]. Some studies also
391 indicate the presence of this fungus associated to several lepidopterans [2, 3, 7, 13, 22,

32, 36]. In addition, natural occurrence of mycoses caused by *B. bassiana* is reported in lepidopteran pests such as *Helicoverpa armigera* (Hubner) and *Spodoptera litura* (Fab.) [9], *Argyresthia conjugella* [41], and *Plutella xylostella* [37]. As far as we know this is the first report of *B. bassiana* related to *P. oleae*, where a large amount of isolates were obtained from larvae and pupae of the phyllophagous generation. The presence of this fungus in olive grove soils has already been reported [20, 32], as well as its ability to control pests in this crop, such as *Bactrocera oleae* [14, 17]. All these evidences may suggest that this fungus could be effective in controlling *P. oleae* in olive orchards, although experiments to evaluate its infecting ability towards *P. oleae* have to be performed. The natural occurrence of this species in the studied olive groves guarantees an already adapted and suited strain to be used as a control agent in this particular ecosystem.

Another potential entomopathogen identified in the present study that could be able to control *P. oleae* larvae is *Cordyceps sinensis*. Although no literature is available for its ability to infect *P. oleae*, the capacity of infecting other lepidopterans larvae was already described, such as those of *Hepialus armoricanus* [30]. Nevertheless, these fungal taxa described as entomopathogenic cannot be definitely linked to the cause of death of *P. oleae* larvae and pupae. Assays confirming the infection ability and virulence of such fungi must be performed, in order to confirm their entomopathogenic potential.

The identification of fungi that could limit the growth of other infectious fungi by their antagonistic properties may also be important for designing future biocontrol strategies for restricting fungal diseases in olive groves. The most abundant taxa with antagonistic characteristics identified in the present study were *Penicillium pinophilum* and *Trichoderma gamsii*. *P. pinophilum* is one of the most important antagonists of

417 *Rhizoctonia solani*, a fungal pathogen of tobacco [1]. Although scarce information is
418 available about *T. gamsii*, antagonistic properties of *Trichoderma* species against a great
419 number of fungal species have already been described, including *Verticillium dahliae*
420 that causes one of the most severe diseases affecting olive (Verticillium wilt) [27, 43].
421 The identification of several isolates with antagonistic features (44%), some of them
422 against fungi that cause olive diseases, opens up the possibility of further research on
423 those antagonistic species for limiting the occurrence of such phytopathogenic fungi in
424 olive orchards.
425 In this work, besides the identification of potential biocontrol agents for limiting pests
426 and fungal diseases in olive groves, many other fungi were identified that could play a
427 role in olive grove ecosystems. One of the most common genus found in this work was
428 *Alternaria* that comprise species that have already been reported to cause spoilage of
429 olives [35] and cause a disease on olive shoots grown under greenhouse conditions [4].
430 The most frequent *Alternaria* species isolated in the present work was *A. tenuissima*,
431 which has been associated to late blight of pistachio and black point of small-grain
432 cereals [16], among other crops. One of the most abundant fungal taxa identified in this
433 study was *Fusarium oxysporum* that exhibits antagonistic, entomopathogenic and
434 phytopathogenic properties. This species has been mainly described as phytopathogenic,
435 causing vascular wilts or rot and crown rots in a large number of crops, including tree
436 crops [12]. This species also presents antagonistic features against *Colletotrichum*
437 *gloeosporioides* and *Pestalotia psidii* [28], and most important, against *Verticillium*
438 *dahliae* [24]. Furthermore, *F. oxysporum* has also been described as an opportunistic
439 insect-pathogen [38]. Accordingly, it has been isolated from several insect hosts, such
440 as those from Homoptera and Coleoptera orders, being able to parasite the greenhouse
441 whitefly, *Trialeurodes vaporariorum* [40].

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

Conclusion

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

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

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

467 well be investigated, in order to control this pathogen that is spreading throughout olive
468 orchards.
469
470 **Acknowledgements**
471 This work was supported by Science and Technology Foundation (Fundação para a
472 Ciência e Tecnologia – FCT) project PTDC/AGR-AAM/102600/2008
473 "Entomopathogenic fungi associated to olive pests: isolation, characterization and
474 selection for biological control". The first author is grateful to the Science and
475 Technology Foundation for the PhD grant SFRH/BD/44265/2008.
476

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Figure captions

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

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

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

Generation	Nº of collected larvae and pupae	Insect emergence	Cadavers presenting surface fungal growth
Phyllophagous	1246	30.1%	61.5%
Antophagous	1745	53.9%	32.7%
Carpophagous	837	19.5%	23.0%
Total	3828	38.6%	40.6%

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643 **Table 2** - Total abundance (N) and relative percentage (%) of each fungal taxa isolated from dead *P. oleae* larvae and pupae in relation to the
644 total number of identified fungi. Values are presented for all the three moth generations. The ecological role (ER) of each fungus is also
645 presented (P – phytopathogenic, A – antagonistic, E – entomopathogenic).
646

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

Cordycipitaceae									
Beauveria									
B. bassiana (Bals.-Criv.) Vuill	A, E	49	70.0	0	0.0	1	2.3	50	30.1
Cordyceps									
C. sinensis (Berk.) Sacc.	E	1	1.4	0	0.0	0	0.0	1	0.6
Davidiellaceae									
Cladosporium									
C. cladosporioides (Fresen.) G.A. de Vries	P, A, E	1	1.4	0	0.0	1	2.3	2	1.2
Hypocreaceae									
Trichoderma									
T. gamsii Samuels & Druzhin.	A	0	0.0	8	15.4	0	0.0	8	4.8
Incertae sedis									
Septogloeum									
S. mori (Lév.) Briosi & Cavara	P	0	0.0	0	0.0	1	2.3	1	0.6
Trichothecium									
T. roseum (Pers.) Link	A	1	1.4	2	3.8	0	0.0	3	1.8
Mucoraceae									
Lichtheimia									
L. ramosa (Zopf) Vuill.	-	0	0.0	0	0.0	1	2.3	1	0.6
Mucor									
M. circinelloides Tiegh.	P, A, E	0	0.0	0	0.0	3	6.8	3	1.8
M. fragilis Bainier	P	1	1.4	0	0.0	0	0.0	1	0.6
M. hiemalis Wehmer	P, E	0	0.0	1	1.9	0	0.0	1	0.6
M. racemosus Bull.	P	1	1.4	0	0.0	0	0.0	1	0.6
Nectriaceae									
Fusarium									
F. equiseti (Corda) Sacc.	P, A,	0	0.0	8	15.4	0	0.0	8	4.8
F. oxysporum Schltdl.	P, A, E	2	2.9	23	44.2	0	0.0	25	15.1
F. solani (Mart.) Sacc.	P, E	0	0.0	1	1.9	0	0.0	1	0.6
Fusarium sp. 1	P, A, E	0	0.0	1	1.9	0	0.0	1	0.6

Gibberella									
Gibberella sp. 1	P	1	1.4	0	0.0	0	0.0	1	0.6
Pleosporaceae									
Alternaria									
Alternaria arborescens E. G. Simmons	P	0	0.0	0	0.0	1	2.3	1	0.6
Alternaria sp.1	P, A	3	4.3	3	5.8	6	13.6	12	7.2
Alternaria sp.2	P, A	0	0.0	0	0.0	2	4.5	2	1.2
Alternaria tenuissima (Kunze) Wiltshire	P	0	0.0	0	0.0	5	11.4	5	3.0
Embellisia									
Embellisia sp.	P	0	0.0	0	0.0	1	2.3	1	0.6
Epicoccum									
E. nigrum Link	P, A	1	1.4	0	0.0	0	0.0	1	0.6
Stemphylium									
S. solani G. F. Weber	P	0	0.0	0	0.0	1	2.3	1	0.6
S. vesicarium (Wallr.) E.G. Simmons	P	0	0.0	0	0.0	1	2.3	1	0.6
Quambalariaceae									
Quambalaria									
*Q. cyanescens (*de Hoog & G.A. de Vries) Z.W. Beer, Begerow & R. Bauer	P	3	4.3	0	0.0	0	0.0	3	1.8
Penicillium									
P. biourgeianum K.M. Zalessky	P	1	1.4	0	0.0	0	0.0	1	0.6
P. commune Thom	P	1	1.4	0	0.0	0	0.0	1	0.6
P. echinulatum Fassat.	P	1	1.4	0	0.0	0	0.0	1	0.6
P. italicum Wehmer	P	0	0.0	0	0.0	1	2.3	1	0.6
P. pinophilum Thom	A	0	0.0	1	1.9	7	15.9	8	4.8
Penicillium sp. 1	P, A, E	0	0.0	0	0.0	1	2.3	1	0.6
Penicillium sp. 2	P, A, E	0	0.0	0	0.0	1	2.3	1	0.6
Trichocomaceae									
Aspergillus									
A. ustus (Bainier) Thom & Church	P, A, E	0	0.0	0	0.0	6	13.6	6	3.6

Talaromyces									
T. flavus (Klöcker) Stolk & Samson	A	0	0.0	0	0.0	1	2.3	1	0.6
Total		70	100.0	52	100.0	44	100.0	166	100.0

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

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

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