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Research article

The king oyster mushroom *Pleurotus eryngii* behaves as a necrotrophic pathogen of *Eryngium campestre*

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Abstract
Under natural conditions in Spain, the fruiting bodies of *Pleurotus eryngii* var. *eryngii*, a basidomycete of great economic and gastronomic importance, appears at the base of the stem of (mainly) *Eryngium campestre*, surrounded by the plant's dried-out basal leaves. Traditionally collected in the wild of Mediterranean areas, this fungus is now cultivated all over the world for its culinary and even medicinal properties. However, controversy exists regarding its lifestyle. Some authors indicate it is a saprotroph, while others suggest it is a weak or even virulent parasite that causes male sterility in *E. campestre*. The present work aims to clarify whether *P. eryngii* behaves as a saprotroph, a weak or virulent parasite to *E. campestre*. The soil beside the roots of naturally growing *E. campestre* plants was inoculated with a commercial strain of *P. eryngii*, and the trophic mode of the fungus recorded. Soil and root samples were subjected to molecular analysis to confirm the absence/presence of the fungus. The death of the plants and the molecular analysis showed *P. eryngii* is a facultative necrotrophic parasite of *E. campestre* in natural conditions and a virulent parasite in greenhouse.

Keywords
Apiaceae, basidiomycetes, trophic mode, molecular analysis

Introduction

Pleurotus eryngii (DC.) Quél. – known as the king oyster mushroom in the USA and UK, "*seta de cardo"* in Spain, "*pleurote du panicaut"* in France, "*fungo cardoncello"* in Italy, and "*Braune Kräuter-Seitling"* in Germany – grows in association with different members of the family *Apiaceae* (Umbelliferae) when humidity and temperature conditions are favourable. In Spain, *P. eryngii* var. *eryngii* (DC.) Quél. is commonly seen in association with *Eryngium campestre*, a plant found across Central and Western Europe, North Africa, the Middle East and the Caucasus, as well as in North America and Australia, where it is an introduced species. Between spring and summer, *E. campestre* is encountered in flat, arid areas, along roadsides, in meadows and on uncultivated land, from sea level up to 1,900 m asl. As perennial hemicryptophyte, its aerial part usually detaches after fruit formation, facilitating the dispersal of the seeds as it is blown around by the wind. However, the storage root remains anchored in the ground where, at its top, the buds are protected by leaf bases, from which new aerial parts grow in the following year.

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The relationship between *P. eryngii* and *E. campestre* and other umbellifers is controversial. Some authors indicate the fungus to be a saprotroph, while others suggest it is a weak or even virulent parasite that causes male sterility in *E. campestre*. For example, Hilber (1982) described *P. eryngii* as a parasite of the roots of members of the family Apiaceae and demonstrated its pathogenicity – at least in the laboratory – through mycelium inoculations of the roots of different wild and cultivated umbellifers. Urbanelli et al. (2002) found *P. eryngii* and *P. eryngii* var. *ferulae* (Lanzi) Sacc. to be weak parasites, although they indicate the former to be largely saprophytic and the latter to be a pathogen. Zervakis and Balis (1996) described *P. eryngii* as a weak parasite affecting the roots of umbellifers in the northern hemisphere. Johansson et al. (1999) studied the presence of laccases in putatively pathogenic *P. eryngii*, as did Punelli et al. (2009). Serino et al. (2013) proposed that the species shows facultative biotrophic growth. Following laboratory-based inoculations into *E. campestre*, Lanau-Galceran (2014) described it as a parasite of the roots of members of the family Apiaceae. Similarly, Cailleux and Joly (1987) indicated *P. eryngii* to be a parasite of the roots of *E. campestre* and to be associated over time with "cinéique épidémiologique des ronds de sorcières", "fairy rings" or "false mycorrhizal rings". Joly et al. (1990) found *P. eryngii* to cause male sterility in host plants and proposed a model in which the introduction of *P. eryngii* to a population of *E. campestre* leads to the systematic destruction of susceptible plants. The present work aims to clarify whether *P. eryngii* var. *eryngii* behaves as a saprotroph, a weak or virulent parasite to *E. campestre* under natural conditions, in greenhouse and *in vitro* culture.

Materials and Methods

Area of study

The location chosen to carry out the field experiments is the External Campus of the University of Alcala, on the outskirts of the city of Alcala de Henares (Madrid, Spain) between two alluvial terraces of the Henares River. On these terraces, there are meadows and pastures from the degradation of the original vegetation of the area. Nitrophilous plants abound (graminoids and hemicryptophyte vegetation), among them a high abundance of *E. campestre* and *Foeniculum vulgare*. The first terrace produces *P. eryngii*. In contrast, the second terrace, which is located 10 meters above and is less humid, is characterized by the absence of *P. eryngii* and it is considered a *P. eryngii*-free field plot.

Pleurotus eryngii *strain*

Three strains were used in this study. Two strains were isolated from commercial basidiocarps bought in Mercamadrid (Madrid, Spain) and labelled as "R. Álvarez (León)" and "Laumount"; another strain was isolated from the fruiting body harvested in the study area and labelled as "wild strain". In 1998, *in vitro* cultures were performed in malt extract agar and maintained in culture on the same substrate until the experiments were carried out. The mycelium for inoculation was cultured and expanded as grain spawn on sterilized (120 ^{o}C, 90 min) hydrated barley grain following previously described methods (Pérez et al., 1996; Manjón et al., 2004).

Field experiments

To examine the trophic mode of *P. eryngii* in *E. campestre*, 30 plants growing naturally in the *P. eryngii*-free field plot were selected, and their positions marked with a numbered metal tag during March 2005 (Supplementary Fig. S1). Fifteen of these plants, growing in one area of this plot, were chosen for inoculation (Supplementary Fig. S2) with *P. eryngii* (test plants); the remaining 15, in another area of the field plot, were not inoculated (control plants). A 20 cm deep hole was dug next to all 30 plants using a sterilized auger. The holes next to the test plants were inoculated with the grain spawn colonized by the isolated strain of *P. eryngii* (R. Álvarez, León) before being refilled

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with part of the same extracted soil. An approximate volume of 7 × 7 × 14 cm of grain spawn colonized by the fungal mycelium of *P. eryngii* (Supplementary Fig. S3) was used to inoculate each plant (Supplementary Fig. S4). The holes near the control plants received no such inoculum or any additional substrate; the extracted soil was simply replaced to simulate stress conditions (possibility of breakage of lateral thin roots) to the inoculated plants. All plants were monitored through to the following spring for signs of fungal infection, including the production of fruiting bodies, and the appearance of symptoms such as chlorosis, root degradation and lack of regrowth of the aerial parts after the appearance of fruit bodies.

The experiment was replicated in the spring of 2013, involving 20 *E. campestre* plants – 10 test plants and 10 controls – and again in 2014, involving five test plants that served as control plants in the 2013 round, plus five again used as controls. Fruiting bodies of the inoculated strain and of the wild strains were harvested in terrace 1.

An ITS molecular analysis (see below) of soil and roots samples was carried out to confirm the absence/presence of *P. eryngii* before and after the inoculation treatments. In addition, in order to confirm that the inoculated plants were effectively infected with the inoculated strains and not by a wild strain, subregion 2 of the intergenic spacer (IGS2), (comparing DNA sequences and by RFLP analysis), the most polymorphic region of *P. eryngii* ribosomal DNA, was examined (Martin et al., 1999; Zhang et al., 2006) (see below*).*

Pleurotus eryngii *mycelial development in soil*

The mycelial distribution of *P. eryngii* around *E. campestre* plants and its involvement in formations like “fairy rings” was examined in natural populations of *E. campestre* in 2015-2016 in the *P. eryngii* field plot of the External Campus of the University of Alcala. Soil samples (250 g; n = 38) were taken using a sterilized auger (Supplementary Fig. S5) at a depth of 20 cm from inside and outside of a random “fairy ring” during the spring of 2015, at flowering and when the soil was damp. All samples were stored separately, transported to the laboratory, and dried at 30 ºC overnight before storing at -20 ºC until ITS analysis.

Greenhouse experiments

Adult plant inoculation

Taproots from adult plants of *E. campestre* in vegetative standstill were collected under natural conditions in March 2018 (Supplementary Fig. S6). The terminal root apex was paraffin-embedded, and the taproots were kept at 4 ºC until the next day, when they were planted in raised pots with legs (2,200 ml capacity) to avoid potential contamination (Supplementary Fig. S7). The substrate used was sterilized soil (autoclaved at 121 ºC for 60 min) from the *P. eryngii*-free field plot. The taproots were cultivated for one month, until the plants generated new well-developed basal leaves. Then 21 plants were inoculated with *P. eryngii* “Laumount” strain during April 2018. The equal number of uninoculated taproots was chosen as control plants. The pots were placed in the greenhouse of the University of Alcalá.

Seedling inoculation

Seeds were collected from random specimens of *E. campestre* from *P. eryngii*-free field plot between April and June 2018. These seeds were observed under a magnifying glass to discard those that presented deterioration or incomplete development. For *in vitro* culture, the seeds were surface-sterilized, soaked for 5 min in an aqueous solution of 0.5% sodium hypochlorite, and then rinsed three times with sterile distilled water and air-dried.

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Eryngium campestre seeds were grown in containers (26 × 26 × 8 cm). These seeds were sown semi-concentrically with sterilized substrate from the *P. eryngii* free field plot and cultivated under natural conditions. Subsequently, they were inoculated with the wild strain of *P. eryngii*. Similarly, a container with control seeds was prepared (Supplementary Fig. S8).

In vitro experiments

Twenty *E. campestre* seeds were transferred into 20 mL test tubes, where they were grown under aseptic conditions in MS liquid medium (Murashige-Skoog, 1962) and vermiculite. These seeds were grown in controlled conditions until cotyledon development. Once this development was achieved, the seedlings were transferred into a Petri dish with MS agar. *Pleurotus eryngii* mycelium of the strain was placed in the middle of the Petri dish, placing the *E. campestre* seedling at one end and a drop of sterile distilled water at the other end as a negative control (Supplementary Fig. S9). All seedlings were monitored for 15 days for signs of fungal infection, including symptoms such as chlorosis, root degradation and a lack of regrowth of the aerial parts.

Molecular analyses

Samples (100 mg) of roots, soil and mushroom tissues (taken in the region of the stipe) were collected and preserved at -20ºC until analysis. DNA extraction was carried out using the PowerSoil DNA Isolation Kit (DNeasy) following the manufacturer's recommendations with all the above sample types. PCR was then performed in 25 µL volume using the Illustra™puReTaqReady-To-Go™ PCR Beads Kit (GE Healthcare Life Sciences), a MultiGene Optimex Thermal Cycler (Labnet International). Primers used in this study belonged to the ITS and IGS2 regions of ribosomal DNA.

ITS region in soil samples was amplified using the primers ITS1F and ITS4 firstly, and in a second PCR with PeryITSF and PeryITSR specific primers (Supplementary Table S1). Regarding the PCR program used, the initial denaturing step involved a temperature of 94 ºC for 4 min; this was followed by 35 cycles at 94 ºC for 30 s, Tm for 30 s and 72 ºC for 90 s, and a final extension step at 72 ºC for 10 min. IGS2 analysis in basidiocarp samples was amplified using the primers 5SRNAR and invSR1R and the IGS1_PeF and IGS2_PeR specific primers. The PCR reaction conditions were as follows: an initial denaturing cycle at 94 ºC for 4 min, followed by 40 cycles at 94 ºC for 1 min, Tm for 1 min, and 72 ºC for 3 min, with a final extension step at 72 ºC for 7 min.

For confirmation of the identity of the infecting fungal strain, the IGS2 amplicons obtained were subjected to restriction length fragment polymorphism (RLFP) analysis by digesting them with the restriction enzyme BtgI (New England Biolabs), following the supplier's instructions. Amplicons were then visualized in 1% agar gels stained with GelRed (Biotum) and purified using the UltraClean PCR Clean-up DNA Purification Kit (MoBio Laboratories Inc). Finally, they were sequenced by the Molecular Biology Unit of the University of Alcalá. Each DNA sequence was assembled and edited manually as required. Sequence alignments were performed using the MUSCLE algorithm in Mega 7.0. (Kumar et al., 2016). IGS2 sequences derived from this study were assigned to the following GenBank accession numbers: MF498584, MF498585, MF498586, MF498587, MF498588 and MF498589.

Results

Field experiments

After setting the first experiment at the external campus of the Alcala University, the inoculated plants became chlorotic and died after two weeks, showing a mortality rate of 100% for the inoculated plants and 6.7% for the control plants. Fruiting bodies of *P. eryngii* were collected from all the inoculated

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plants during the following autumn, in November 2005. The basidiocarps were always found on the inoculated roots (Table 1).

In 2013 and 2014, the experiment was repeated. In 2013, the inoculated plants quickly showed signs of chlorosis and died within 15 days. The control plants grew normally. The mortality rate was 100% for the inoculated plants and 10% for the control plants. The autumn of 2013 was hot and dry, and the inoculated plants produced no mushrooms during that season; however, they did produce them in the spring of 2014. Dead control plants never produced basidiocarps. In the 2014, the inoculated plants became chlorotic at 12 days and died 15 days later. The controls remained asymptomatic. Mortality rate in inoculated plants was 100%, and in control plants was 20%. Mushrooms were collected from the test plants in October and November 2014 (Table 1).

Table 1 – Results obtained during these inoculations.

Year	**Number of plants examined per year**		**Mortality rates**			
	Inoculated	**Control**	**Inoculated**	**%**	**Control**	**%**
2005	15	15	15	100	1	6.7
2013	10	10	10	100	1	10
2014	5	5	5	100	1	20
Total	**30**	**30**	**30**	**100**	**3**	**10**

The results of the ITS analysis of soil samples confirmed the absence of the fungus before introducing the *P. eryngii* inoculum in the plants selected for the 2013 and 2014 experiments. Subsequently, the ITS analysis detected the presence of the fungus in soil and root samples once the plants were inoculated and reconfirmed its absence in control plants by ITS analysis.

The IGS2 analysis results confirmed that the treated plants had all been infected by the inoculated commercial *P. eryngii* strain (R. Álvarez, León). A band revealing a length of 1,400 bp was always obtained for this commercial strain, being absent in the wild strain. Wild fruiting bodies samples provided IGS2 lengths of 2,000-4,000 bp (Supplementary Fig. S10a). In one sample (line 8), an extra band revealing a domain about 3,000 bp long was recorded. The RFLP profiles for the inoculated plants produced a different profile to the wild fungal samples (Fig. S10b), with fragments about 700 and 200 bp (lines 2-6), instead of 750 (line 7), and 650, 500 and 200 bp (line 8). Finally, the IGS2 sequences from fruit bodies of inoculated plants were compared with the original inoculum. All the sequences were 100% identical.

Pleurotus eryngii *mycelial development in soil*

To confirm the "fairy ring" formation of *P. eryngii* origin, the distribution of its mycelium in the soil was investigated. Figure 1a shows the distribution of seven *E. campestre* plants that would be involved in a "fairy ring", while the results of the soil samples that were positive and negative for the presence of *P. eryngii* appear in Figure 1b. Positive soil samples were detected within the circle and around *E. campestre* plants, but their distribution was not homogeneous; a bias was seen towards the center and right of the circle (the positive samples recorded at the three sampling points outside the top left of the ring probably represent the front of a different mycelium). During the spring of 2016, it was detected that plants number 5 and 6 died due to *P. eryngii*.

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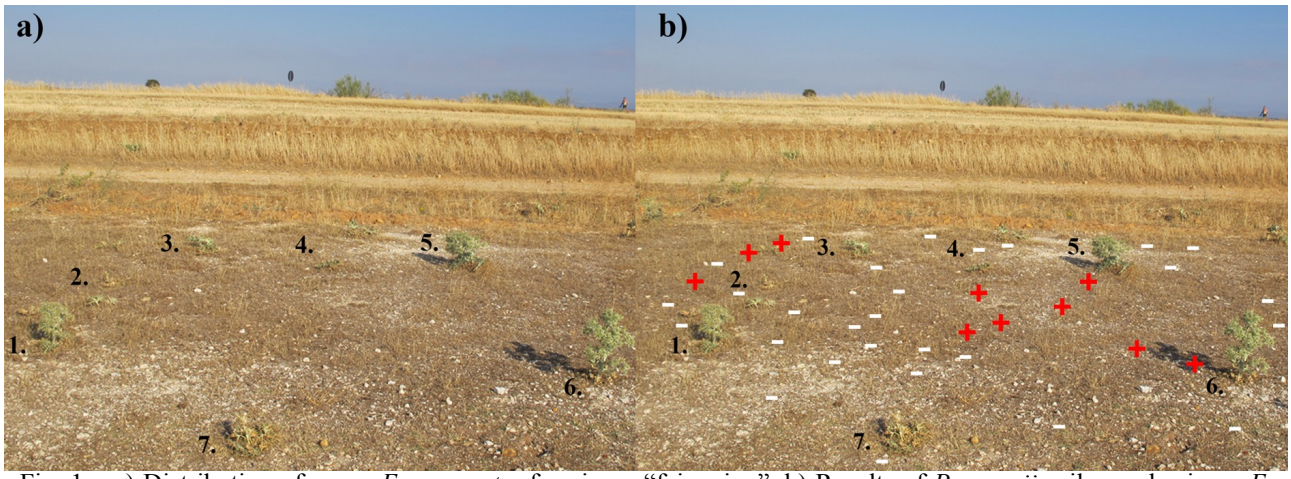

Fig. 1 – a) Distribution of seven *E. campestre* forming a "fairy ring". b) Results of *P. eryngii* soil samples in an *E. campestre*-involving fairy ring. Red = positive; White = negative.

Greenhose experiments

Adult plant inoculation

To confirm the results obtained in natural conditions additional experiments were carried out on adult plants in greenhouse. Most of the plants inoculated with *P. eryngii* died (85.7% mortality), in a time interval between 1-2 months. The symptoms (chlorosis) appeared 15 days after inoculation, at the same time as the beginning of the fructifications (Supplementary Fig. S11). The secondary and tertiary roots generated from the taproot were completely invaded (Supplementary Fig. S12) by secondary (dikaryotic) white mycelium (Fig. 2a) of the inoculated strain. The absorbent roots had clear signs of infection and cortical cell penetration (Fig. 2b) and the accumulation of coloured gums (Fig. 2c). In addition, taproots from inoculated plants also showed the accumulation of coloured gums (Fig. 2d and Supplementary Fig. S13) and clear signs of fungal colonization and necrosis (Supplementary Fig. S14). These signs were absent in the control plants.

Seedling inoculation

One month after inoculation with *P. eryngii,* the seedlings showed 42% mortality, regardless of the distance from the inoculation point (Fig. 3a) with the control (Fig. 3b).

In vitro trials

The 30% of the *E. campestre* seeds inoculated with *P. eryngii* in sterile conditions showed signs of chlorosis 15 days after inoculation. However, none of the inoculated plants died during this experiment. Signs of root infection by the mycelium were observed in symptomatic seedlings (Supplementary Fig. S15). Chlorosis signs were absent in the control plants.

Discussion

The present results indicate that the tested strains of *P. eryngii* var. *eryngii* are necrotrophic fungal pathogens of *E. campestre*. In fact, in field conditions, the inoculated plants soon became chlorotic and died, giving rise to mushrooms at the base of the stem during the following autumn and spring when conditions were favourable. The molecular analyses confirmed that these symptoms were caused by the inoculated commercial strain. The analysis of the IGS2 sDNA region (i.e., IGS2 sequences and RFLP-IGS2 profiles) confirms that these plants had been infected only by the inoculated commercial strain and none had been infected by any wild strain. These results highlight the usefulness of IGS2

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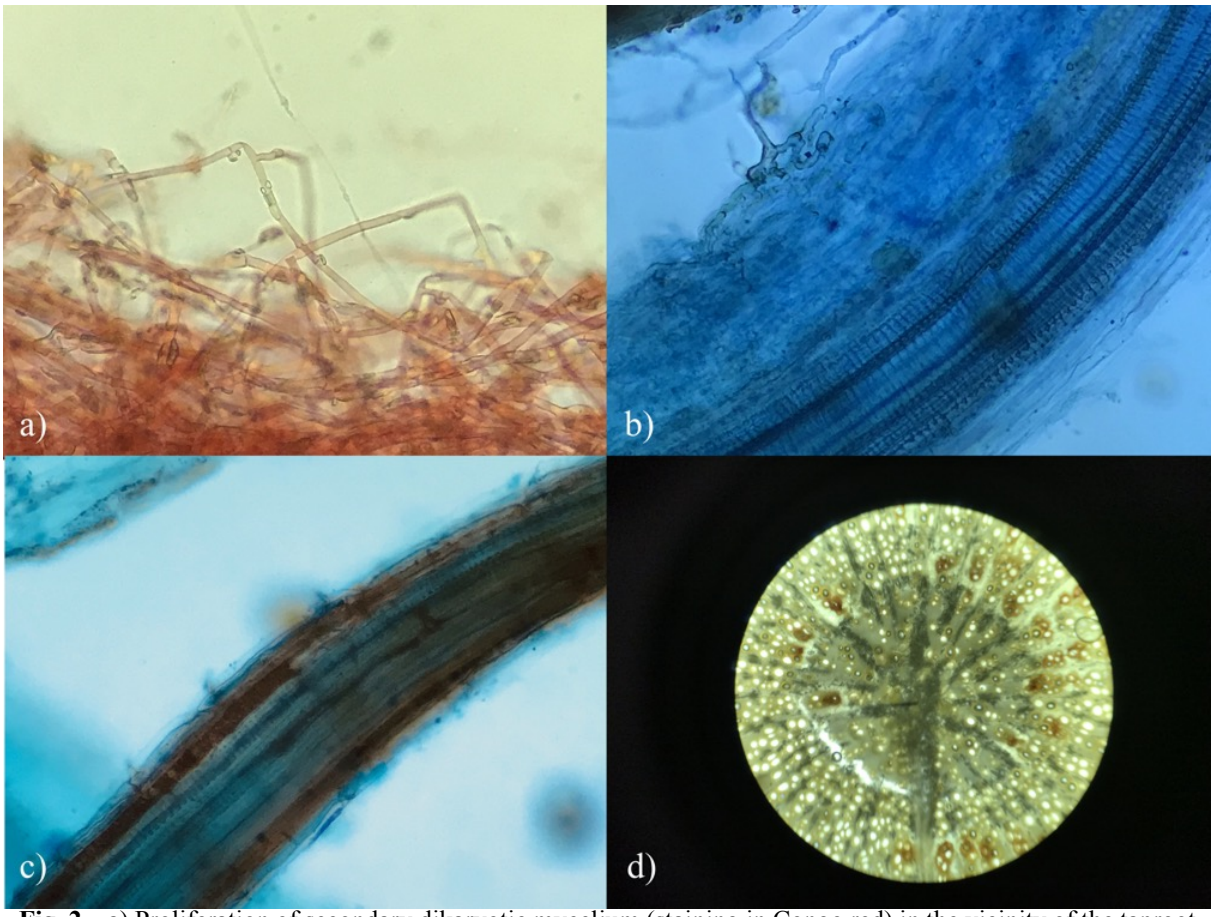

Fig. 2 – a) Proliferation of secondary dikaryotic mycelium (staining in Congo red) in the vicinity of the taproot of an adult plant of *Eryngium campestre.* b) Dikaryotic fungal hyphae penetration on the cortical cells absorbent root of an adult *Eryngium campestre* inoculated plant (staining in cotton blue). c) Proliferation of coloured gums in a longitudinal section of an absorbent root (staining in cotton blue) from an adult *Eryngium campestre* inoculated. d) Start of gum formation in a cross section of an adult *Eryngium campestre* taproot inoculated plant.

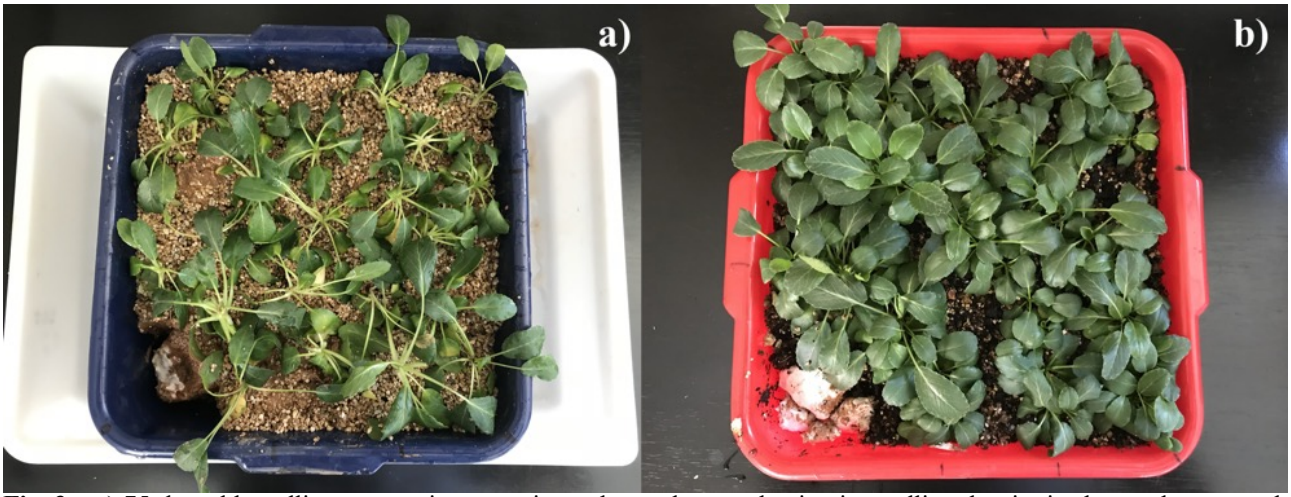

Fig. 3 – a) 75-day-old seedlings grown in a container where a large reduction in seedling density is observed one month after being inoculated with *Pleurotus eryngii*, compared to the control. **b)** *Eryngium campestre* control container with a high seedling density, compared to inoculated seedlings, just after being inoculated for the first time at 75 days of age.

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polymorphism in the identification of strains belonging to members of the genus *Pleurotus* (Zhang et al., 2006). Most of the susceptible inoculated plants died in less than two weeks before their flowers could form. These findings corroborate Joly et al. (1990) hypothesis, since the adult inoculated plants died before flowering.

Detection and distribution of *P. eryngii* in soil samples, as well as the death of *E. campestre* plants, seem to confirm mycelial outgrowth in soil, where it would grow as a saprophytic fungus decomposing organic matter. However, once the mycelium reaches the roots of a *E. campestre* plant, the fungus will penetrate and infect them, as indicated by experiments carried out with inoculated plants. Within the root tissues, the fungus will obtain the ideal nutritional conditions to complete fruiting body development, as suggested by the collections of mushrooms on root remains in Zervakis et al. (2001) and Lewinsohn et al. (2002). From this infected plant, *P. eryngii* mycelium would grow as a saprophytic fungus again. The distribution and abundance of *E. campestre* plants could lead to formations similar to "fairy rings" over time. Although, the fairy rings are not well defined, the mycelium was found in a large area within a bare circle of *E. campestre* plants.

There are more than 50 species of basidiomycete fungi which cause fairy rings; some species grow saprotrophically on decaying organic matter, while other species also have a parasitic phase on plant roots (Terashima et al., 2004; Nelson, 2008; Bonanomi et al., 2011). Our study would support the hypothesis of Cailleux and Joly (1987), which stated that *P. eryngii.* is be associated over time with "fairy rings" or "false mycorrhizal rings". Furthermore, these formations would fall within the definition of "fairy ring" described by Kirk et al. (2011), who stated that fairy rings of fungal origin are also seen as bare circles of vegetation, as occurs under trees colonized by the black truffles *Tuber melanosporum* Vittad. The non-homogeneous distribution of *P. eryngii*-positive soil in the present ring might due to the more compact and stony soil in the left side of the sampled area, which may have hindered fungal development, or the distribution/abundance of *E. campestre* plants.

Due to the necrotrophic plant pathogen lifestyle of *P. eryngii,* it remains alive in the field, especially in the dry, steppe-like conditions of central Spain as saprothroph. The dikaryotic mycelium of *P. eryngii,* after having infected the host plants, takes refuge in the deep main roots of the killed plants until the mycelium begins to proliferate in the autumn when the rains rehydrate the soil. This growth might be encouraged by synergetic bacteria or their secondary metabolites (Clausen, 1996; Kim et al., 2008). The *P. eryngii* inoculation of *E. campestre* seedlings demonstrates a preference of the fungus for older tissue, compared to young tissue as shown by the highest mortality rate in the old inoculated plants. Differences in pathogen preferences by tissue age have been mentioned by Okubara and Paulitz (2003).

Although the high virulence of *P. eryngii* in our experiments can be explained by the wounds that are caused during the inoculation process which have facilitated host infection, the results obtained in this study seem to support the pathogenicity of *P. eryngii.* This confirms Hilber's conclusions (1982), who described *P. eryngii* as a parasite of the roots of members of the *Apiaceace* family (such as *E. campestre*, *Ferula communis*, etc.). For this reason, the Spanish administration should take the pertinent measures to avoid the sale of *P. eryngii* mycelium to inoculate adult *E. campestre* in the field, as certain companies indicate on their websites, to produce field basidiocarps. These practices with phytopathogenic fungi in natural conditions may generate environmental problems.

In conclusion, *P. eryngii* var. *eryngii* would appear to be a necrotrophic pathogen of *E. campestre,* and thus perhaps of other umbellifers. It infects its hosts as a virulent pathogen, later surviving saprotrophically on the roots after the aerial parts of the plant become detached, where the fungus fructifies from the taproots. However, to confirm its trophic mode and its formation of "fairy rings", additional research will be necessary, extending the study to different strains and sites.

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