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RESEARCH ARTICLE

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Role of the arbuscular mycorrhizal symbiosis in tolerance response against *Armillaria mellea* in lavender

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Abstract

Lavender species form the arbuscular mycorrhizal symbiosis and are at the same time highly susceptible to white root rot. In an attempt to evaluate the response of mycorrhizal *Lavandula angustifolia* L. to *Armillaria mellea* (Vahl:Fr) P. Kumm in a greenhouse experiment, plants were previously inoculated with an isolate of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (former *Glomus intraradices* BEG 72) and the influence of the pH growing medium on the plant-symbiont-pathogen interaction was tested in gnotobiotic autotrophic growth systems in which mycorrhizal inoculum was obtained from root organ cultures. After ten months growth dual-inoculated lavender plants grown in containers with a pasteurized substrate mixture produced a similar number of spikes than healthy plants and achieved equivalent plant diameter coverage. When the growing medium in the autotrophic systems was supplemented with calcium carbonate, the inoculation of lavender plantlets with *R. irregularis* at higher pH (7.0 and 8.5) media caused a significant decrease of *A. mellea* presence in plant roots, as detected by qPCR. Moreover, the observation of internal root mycorrhizal infection showed that the extent of mycorrhizal colonization increased in plant roots grown at higher pH, indicating that tolerance to white root rot in lavender plants inoculated with *R. irregularis* could be associated to mycorrhizal establishment.

Additional key words: white root rot**;** control strategies; *Glomus intraradices*; *Lavandula angustifolia*; pH; *Rhizophagus irregularis*.

Abbreviations used: AMF (arbuscular mycorrhizal fungi); MSR (Strullu-Romand medium); qPCR (real-time PCR); ROC (root organ culture).

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Introduction

Lavender species native of the western Mediterranean are the most economically important among herb crops due to their versatile use in phytochemical industries. They are commercially grown for essential oil extraction and as perennial drought-tolerant woody shrubs they are also used in restoration and sustainable ornamental landscaping activities. Lavender forms the arbuscular mycorrhizal symbiosis, and benefits of plant inoculation with selected fungi have been reported by several authors (Azcón & Barea, 1997; Bakkali-Yakhlef *et al*., 2011; Karagiannidis *et al.*, 2012).

Arbuscular mycorrhizal fungi (AMF), present in natural soils and undisturbed ecosystems, develop their life cycle in association with the roots of most terrestrial plant species, being the most common symbiosis between plant and fungi. The positive effects of AMF on host plant development and health have been extensively documented, with a special emphasis on improvement of plant nutrition (Jeffries & Barea, 2001), on adaptation to extreme environmental conditions (Bashan *et al.,* 2012), and on interactions with plant pathogens (Azcón-Aguilar *et al*., 2002; Pozo & Azcón-Aguilar, 2007).

As most woody shrubs, lavender is highly susceptible to white root rot, a fungal disease caused by *Armillaria* spp. that seriously damages agricultural production, especially in replant soils (Lis-Balchin, 2012). The ba-

sidiomycete genus *Armillaria* is recognized as the first cause of the replant syndrome in dry calcareous Mediterranean soils, infecting woody crops, such as fruit trees, grapevines and olive trees, leading to high economic losses (Gur & Cohen, 1989; Westphal *et al.,* 2002; Vossen, 2007). *Armillaria* species colonize living host roots during a parasitic phase, but can survive in dead wood tissue in their saprophytic phase, which can last for decades. This explains why soil infestation is extremely difficult to eradicate, while effective control measures are lacking (Rizzo *et al*., 1998). The fungal mycelium destroys the vascular tissue of primary roots and root crowns, causing poor nutrient uptake, decreased vigor, often leading to plant death (Rishbeth, 1985).

Woody shrubs infected by *Armillaria* develop root and crown damage identical to those occurring in fruit trees and grapevines (Lis-Balchin, 2012). However, lavender plants are more easily managed, and for this reason, *L. angustifolia* was chosen as a mycorrhizal model plant for autotrophic culture systems. Such systems allow to study interactions between both fungi, symbiont and pathogen, and to perform molecular assessment of *A. mellea* in inoculated roots.

The objective of this work was to test the early establishment of the symbiosis in lavender plants through inoculation with selected AMF as a control strategy against *Armillaria* by evaluating the response of mycorrhizal *Lavandula angustifolia* L. to a strongly virulent *Armillaria mellea* (Vahl: Fr) P. Kumm isolate in two different experiments. Plants were inoculated with both types of fungi, mutualistic and pathogenic, under controlled conditions in a greenhouse, in order to record growth parameters and fungal colonization *in vivo*. Additionally, the influence of increasing medium pH through $CaCO_3$ supplies on the plant-symbiont-pathogen interaction was evaluated in a gnotobiotic autotrophic growth system that allows quantifying the effects of external factors.

Material and methods

Greenhouse experiment

The first experiment was set up in a greenhouse under controlled conditions (23±5ºC) with 32 plants equally distributed in four treatments in a 2 × 2 factorial design, consisting of non mycorrhizal plants inoculated or not with *A. mellea* and mycorrhizal plants inoculated or not with *A. mellea*. *Lavandula angustifolia* plants (10±2 cm height) obtained from rooted cuttings were inoculated with *Rhizophagus irregularis* (former *Glomus intraradices* BEG 72) [(Blaszk., Wubet, Renker & Buscot) C Walker & A Shüssler comb nov], a native AMF from Spain originally isolated from *Citrus* (Camprubi & Calvet, 1996) with proven effectiveness in different agricultural systems involving root pathogens infestations (Calvet *et al.,* 2001; Camprubi *et al.,* 2008). Plants were placed in 2-liter containers filled with a mixture of pasteurized sandy soil, sphagnum peat and quartz sand (3:2:1; v/v/v) of pH 7.7. Each container in the mycorrhizal treatments received 10 mL of *R. irregularis* bulk inoculum obtained from leek (*Allium porrum* L.) cultures, containing spores (at least 1000 spores in 10 mL) and colonized root fragments. The inoculum was placed below the plant root systems at planting.

The *Armillaria* isolate was obtained from a heavily infested peach (*Prunus persica* L.) replant orchard. It was molecularly identified as *A. mellea* (Mansilla *et al.*, 2000), grown in pure cultures on malt agar, and used to inoculate autoclaved chestnuts (*Castanea sativa* L) in sterile malt agar medium (Difco®) supplemented with 0.2 g/L streptomycine sulphate and 10 mL/L benomyl. After a 5-month incubation period at 25ºC in the dark, infected chestnuts were used as individual sources of pathogenic inoculum.

One month after the mycorrhizal inoculation, lavender plants were placed in an open-air shade house and each container of the pathogen-inoculated treatments received one infected chestnut, placed at 10-cm depth and 10-cm distance from the plant stem. Fertilization was applied every two weeks (10 mL Hoagland's nutrient solution per plant). Plants were harvested after ten months, when decay symptoms were apparent on the plants. Plants were cut to measure shoot and root weights, largest plant diameter and number of spikes. The extent of mycorrhizal root colonization was measured in plants inoculated with *R. irregularis* and *A. mellea* damage was estimated in the root system of pathogen-inoculated plants. In order to assess *R. irregularis* internal infection, root samples from each inoculated plant were clarified and stained (Phillips & Hayman, 1980) and the percentage of root colonized by the mycorrhizal fungus was measured with the gridline intersect method (Giovannetti & Mosse, 1980). Disease symptoms caused by *A. mellea* were recorded in plants that had received the infected chestnuts. Two levels of root necrosis and crown necrosis were determined: "High" (infection ranking from 25% to 100% of the root system length) and "Low" (infected root below 25%) for root necrosis; "High" (necrotic tissue with presence of white mycelium) and "Low" (necrotic tissue with no mycelium) for crown necrosis.

Data obtained for plant growth were analyzed in a multifactorial ANOVA and means were compared by Tukey's test when there was no interaction between factors: pathogen and mycorrhiza.

In vitro experiment

In order to evaluate the interaction between *R. irregularis* and *A. mellea*, in medium with different calcium (Ca) levels, *L. angustifolia* plantlets were used to establish mycorrhizal autotrophic culture systems described by Voets *et al.* (2005). In such systems, the plant leaves perform photosynthesis while roots grow axenically inside a petri plate filled with modified Strullu-Romand medium (MSR) with no sugar or vitamins (Voets *et al.,* 2005) and 7 g agar/L. Under such conditions, the root can be colonized by an AMF monoxenically grown on *Agrobacterium rhizogenes*-transformed roots in a root organ culture (ROC) (Declerck *et al.*, 1996). In our experiment, the procedure followed to obtain the tripartite association (lavender plant-AMF-root rot fungus) was basically the same described by Nogales *et al.* (2010) for grapevine plants. Since the amount of growing medium in a 100 mm petri plate was insufficient to sustain grapevine growth longer than a week, requiring the system to be refilled periodically, which increased the risk of contaminations, we used 350-mL autoclaved plastic containers, in order to avoid unnecessary manipulation (Lovato *et al*., 2014). *Lavandula angustifolia* seeds were surface-sterilized in 32% sodium hypochlorite for 30 min and thoroughly washed with sterile distilled water three consecutive times for 10 min. The seeds were germinated on sterilized filter paper in petri plates at 25ºC in the dark, and plantlets with incipient roots were transferred to sterilized glass pots (Le pratique®) containing 150 mL of Murashige Skoog (MS) (Murashige & Skoog, 1962) medium with vitamins (Duchefa Biochemie) and supplemented with 30 g/L saccharose and 7 g/L agar (Difco). Plants were grown for 10 weeks in a growth chamber at 25ºC and 16 h photoperiod (200 µmol/m^2/s).

The same four treatments described in the greenhouse experiment were adopted for the *in vitro* experiment: non mycorrhizal plants inoculated or not with *A. mellea* and mycorrhizal plants inoculated or not with *A. mellea*. The containers not inoculated with *A. mellea* were directly filled with 350 mL of modified MSR medium.

In *A. mellea* treatments, 80 mL of MSR medium were first poured into the containers, cooled and solidified (Lovato *et al*., 2014). Two 7-mm diameter plugs of *A. mellea* mycelium from a pure culture were inserted in the medium after removing agar plugs which were replaced afterwards. Each container received then 270 mL of the same modified MSR medium, previously cooled at 40ºC. An incision was made on the lid of the container, through which a lavender plant was inserted, in order to keep the shoot outside the container and the root inside the growing medium. At the same time, the inoculation with the AMF in mycorrhizal treatments was performed using a six-month old ROC culture in a bi-compartmented dish of *R. irregularis* (Nogales *et al*., 2010). Two 1-cm^2 plugs containing 100 to 150 mycorrhizal spores were excised from the compartment lacking roots and introduced next to the root tips, following the same procedure used for *A. mellea* plugs. Once both inoculations were completed, the container was carefully closed, the space around the stem in the incision hole was sealed with sterile silicone (Panreac) and parafilm was wrapped around the lid.

In order to evaluate the effects of pH, the growing medium in the container was supplemented with increasing levels of $CaCO_3$ (Panreac): 0, 100 mg/L and 3000 mg/L, which resulted in different pH values of 5.5, 7.0 and 8.5, respectively. There were eight replicates for each pH treatment, and lavender autotrophic systems were placed in a growth chamber at 25ºC.

After nine weeks plants were harvested, shoot dry weight and root fresh weight were recorded, and morphological symptoms of *A. mellea* infection were observed. Root samples were excised in order to estimate mycorrhizal colonization after clarifying and staining (see the greenhouse experiment) and to quantify *A. mellea* by real-time PCR (qPCR).

For quantification of the pathogenic fungus 1.0 g root samples were separated and ground in mortar and pestle with liquid nitrogen. Genomic DNA was extracted from a 70-100-mg (fresh weight) sample of ground roots with phytoplasma grinding buffer (PGB) (Arhens & Seemüller, 1992). Specific primers designed by Baumgartner *et al*. (2010) to amplify a fungal gene for the nuclear elongation factor subunit 1-alpha, EF1a-F1 (5GGATGGCACGGTGATAACAT) and EF1a-R1 5AGTCTTGCCCTTGACGACAC), were used to amplify a 150-bp section of the EF1a gene.

Real-time PCR was carried out using the Step One™ Instrument (Life Technologies) and the SYBR® Premier Ex Taq™ kit (Takara, Shiga, Japan). We used a 25 µL reaction volume containing the following components: 12.5 µL SYBR green Super mix, 1 µL of each forward/reverse (F/R) primers (10 µM), 2 µL DNA templates (20 ng/L of DNA), 8 µL sterile HPLC water and 0.5 µL ROX reference dye. Triplicate reactions were routinely used for each sample and each set included controls containing water to check for contamination in the reaction components. The qPCR cycling program was: 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 45 s, and a dissociation stage with 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. The qPCR signal was calibrated by making a standard curve with DNA extracted by the previously described method

from known amounts of mycelium from a pure *A. mellea* culture. The cycle threshold (Ct) was calculated for all the standards and samples to indicate the number of cycles required for the fluorescence signal to cross the threshold above background during the exponential PCR amplification phase. DNA quantities were adjusted for the amount of root material used.

Results

Greenhouse experiment

The *A. mellea* isolate used was highly virulent: five out of eight non-mycorrhizal plants infected by *A. mellea* were dead at harvest, while all mycorrhizal plants survived and showed less severe disease symptoms (Table 1). None of the mycorrhizal lavender plants showed a high level of necrotic root tissue and only one among them attained a high level of crown necrosis. The presence of *A. mellea* in *R. irregularis*-inoculated roots had no significant effect on the root system mycorrhizal colonization extent, that reached mean values of 36±14 % in plants inoculated only with the mutualistic fungus and 29±12% in plants with the dual inoculation. Growth parameters measured at harvest (Fig. 1) indicated that both the AM fungus and the pathogen had significant effects on lavender growth. The effect of mycorrhizal inoculation was highly significant at increasing shoot biomass and plant shoot diameter. Infection by *A. mellea* lowered shoot biomass and the number of spikes produced at flowering. However, dual inoculated lavender plants were capable to

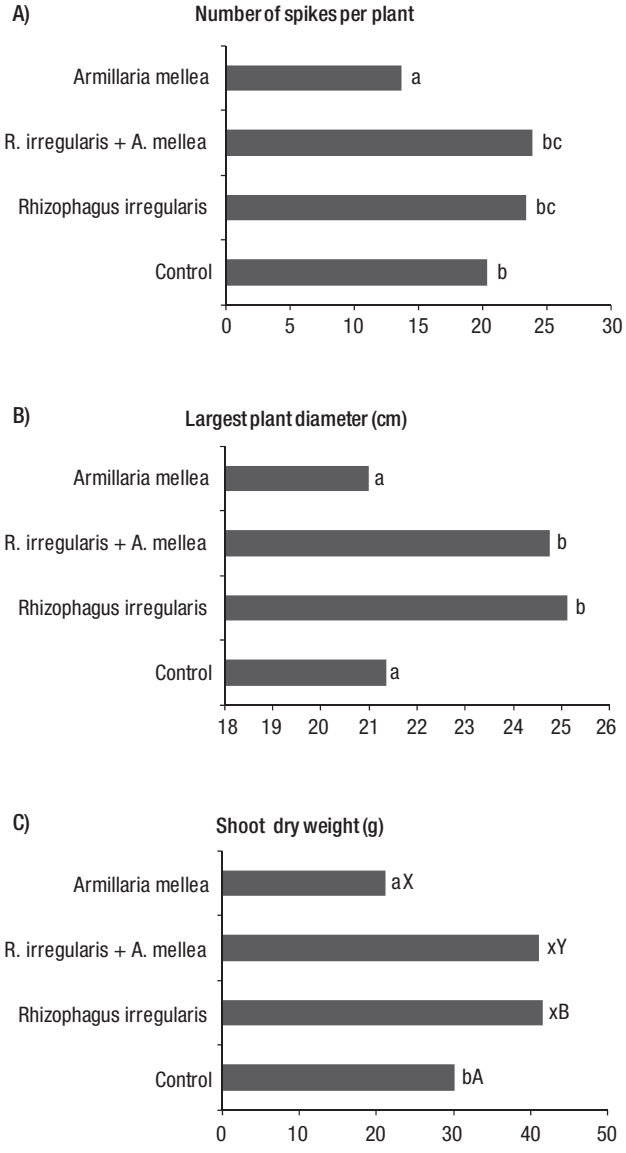

Figure 1. Plant growth parameters: number of spikes per plant (A), largest plant diameter (B) and shoot dry weight (C) of *Lavandula angustifolia* inoculated and non-inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and the root rot fungus *Armillaria mellea,* eleven and ten months after inoculation with the symbiont and the pathogen respectively. Data are means of eight replicates per treatment. They were analyzed with a multifactorial ANOVA at 95% confidence level. Different letters next to bars indicate significant differences ($p \leq 0.05$) between treatments after Tukey's test. Both factors considered, pathogen and mycorrhiza, and their interaction had a significant effect on shoot dry weight, therefore factors were analyzed separately with a one way ANOVA and Tukey's test: lower case a and b indicate differences between non-mycorrhizal plants inoculated or not with *A. mellea*; lower case x indicates no differences between mycorrhizal plants inoculated or not with *A. mellea*; capital A and B indicate differences between mycorrhizal and non-mycorrhizal plants in the absence of *A. mellea*; capital X and Y indicate differences between mycorrhizal and non-mycorrhizal plants inoculated with *A. mellea.*

Table 1. Estimation of white root rot *Armillaria mellea* disease symptoms at harvest in *Lavandula angustifolia* plants inoculated and non-inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* ten months after inoculation with the pathogen

Disease symptoms	*A. mellea*	*A. mellea* + *R. irregularis*
Mortality	5/8[a]	0/8[a]
High level root necrosis (25-100% infected root)	4/8	0/8
Low level root necrosis (≤ 25%infected root)	1/8	4/8
High level crown necrosis (necrotic tissue + mycelium)	3/8	1/8
Low level crown necrosis (necrotic tissue)	2/8	4/8

[a] Data are numbers of plants per treatment showing the disease symptoms described.

overcome root-rot infection, as there were no significant differences in the number of spikes produced by mycorrhizal plants inoculated with the pathogen (Fig. 1) and by healthy plants. Additionally, they reached the same plant diameter coverage than mycorrhizal plants not infected with the pathogen.

In vitro experiment

Armillaria mellea was detected by qPCR only in part of the plants inoculated with *R. irregularis*. For instance, the pathogenic fungus was detected only in one out of eight mycorrhizal plants grown in pH 7.0 medium and in none of the mycorrhizal plants grown in pH 8.5 medium. Consequently, the quantification analysis was discarded and a contingency analysis of chi-square values followed by Pearson's test (JMP® 8.0.1 SAS) was used to analyze the effect of mycorrhizal inoculation with *R. irregularis* on the detection of *A. mellea* in lavender roots grown in autotrophic systems.

There were significant differences in *Armillaria* detection due to AM fungal inoculation when substrate pH was increased by $CaCO_3$ addition (Table 2). At pH 5.5 *A. mellea* was detected in equal amounts in mycorrhizal and non-mycorrhizal plants, since more than 70% of the plants from both treatments were positive for the pathogen's presence. When plants grew in higher pH media, detection of *A. mellea* decreased only in mycorrhizal plants. The percentage of detection in dual-inoculated plants grown in pH 7.0 medium was only 17, and it was null in plants grown in medium with pH 8.5. Differences in *A. mellea* detection due to pH values were significant according to Pearson's test (p=0.0036) only for plants inoculated with both fungi, *R. irregularis* and the pathogen. On the other hand, growth medium pH in autotrophic systems had no significant influence on the detection of *A. mellea* in non-mycorrhizal plants (Table 2).

Mycorrhizal plants grown in pH 5.5 medium without *A. mellea* had only 34±3 % of the plant root system colonized by the mycorrhizal fungus (Table 3), while this percentage attained 61±3 % at pH 8.5. The value

Table 2. Detection of *Armillaria mellea* by qPCR in the roots of *Lavandula angustifolia* plants grown in autotrophic culture systems with three different medium pH values, inoculated or non-inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

	pH			Pearson test
	5.5	**7.0**	**8.5**	
A. mellea	71.43%	85.71%	57.14%	χ^2 1.4 p=0.4966
R. irregularis + *A. mellea*	75.0%	16.66%	0%	χ^2 11.244 p=0.0036*
Pearson test	χ^2 0.024 p= 0.8760	χ^2 6.198 p= 0.0128*	χ^2 6.234 p= 0.0125*	

Data are means of percentages of plants positive for *A. mellea* infection among eight replications per treatment. A statistical analysis (JMP®8.0.1, SAS Inst. Inc.) gave chi-square values after Pearson's test and indicate significant differences between inoculation treatments in columns for each pH value, and between pH values in rows for each inoculation treatment.

Table 3. Mycorrhizal colonization extent by *Rhizophagus irregularis* in roots of *Lavandula angustifolia* plants grown in autotrophic culture systems with three different medium pH values, inoculated or non-inoculated with *Armillaria mellea*

	pH		
	5.5	**7.0**	**8.5**
R. irregularis	33.92% a	46.65% b	60.6% b
R. irregularis + *A. mellea*	24.51% a	74.27% b	65.51% b
ANOVA factors analyzed pH S p=0.0029 *A. mellea* NS p=0.4594			

Data are means of percentages of eight root systems colonized by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. They were transformed Asin[sqrt(%mycorrhizal colonization/100)] and analyzed with a multifactorial analysis of variance. Different letters after figures in rows indicate significant differences (LSD, $p \leq 0.05$) between pH values of the growing medium for each inoculation treatment. S and NS: significant and not significant effect, respectively.

for dual inoculated plants was similar, as 25±12 % of the root system was mycorrhizal in plants grown in 5.5 pH medium, while in plants grown in pH 8.5 medium this index was 66±23 %. *Armillaria mellea* infection had no effect on mycorrhizal root colonization after 10 weeks. The percentages of mycorrhizal colonization in plants inoculated with the AMF or with both fungi were similar (34±3 *vs* 25±12) and (61±3 *vs* 66±23) at pH 5.5 and pH 8.5 respectively.

Discussion

Rhizophagus irregularis BEG 72 applied as bulk inoculum in container-grown lavender plants was very effective as plant growth stimulator, as reported before for other host plant species, both under controlled conditions and in field trials in which the fungus had been introduced in plant production systems (Estaún *et al*., 2003; Camprubi *et al.,* 2008; Nogales *et al.,* 2009). Moreover, results obtained in the greenhouse experiment showed that mycorrhizal early inoculation with this selected AMF isolated from a high-pH soil in southern Catalonia (Camprubi & Calvet*,* 1996) alleviated the deleterious effect of the root rot fungus on container-grown lavender plants. Despite *A. mellea* infection, mycorrhizal plants showed high flowering potential, as shown by higher number of spikes, and achieved biomass and plant diameter equivalent to those of mycorrhizal plants not challenged by the pathogen. Plant survival, flower production, and soil coverage surface are all important characteristics for crop production and for revegetation that benefit from early mycorrhizal inoculation, even when the growth substrate is infested by *A. mellea*. Those results suggest that higher level of mycorrhizal inoculation may be an advantage when using lavender plants, either for production or for revegetation, in *Armillaria*-infested soils.

AMF-induced tolerance to pathogens and pests has been observed in several plant species (Calvet *et al.,* 2001; Pozo *et al*., 2002; Elsharkawy *et al*., 2012; Schausberger *et al.,* 2012) and the protection mechanisms involved are subject of research (Pineda *et al.,* 2013; Torres-Vera *et al.,* 2014). Using an *in vitro* system to study the interaction between white root rot infection caused by *A. mellea* and *Glomus intraradices* in the grapevine rootstock 110 Richter, Nogales *et al.* (2010) showed a decrease in disease symptoms in mycorrhizal plants, confirming that the root colonization by a mycorrhizal fungus increased tolerance to white root rot, as reported in vineyard trials with mycorrhizal plants conducted by the same authors (Camprubi *et al*., 2008; Nogales *et al.,* 2009). The AMF *Glomus mosseae* lowered mortality by *Fusarium* in basil (*Ocimum basilicum* L.), a plant species from the same family as lavender, and that effect was not related to P contents, which were similar in non-mycorrhizal and mycorrhizal plants (Toussaint *et al.,* 2008). A meta-analysis work by Veresoglou *et al*. (2013) showed that N fertilization increases severity of pathogen damage, with no effect from P or K fertilization, but their publication sample does not include any study regarding effects of calcium or pH. Heyman *et al.* (2007) showed that increased Ca content, but not higher soil pH, increased prevalence and severity of *Aphanomyces* in pea. Calcium and pH had no effect on mortality caused by *Armillaria ostoyae* (Mallett & Maynard, 1998) and on the other hand higher soil pH increased the severity of *Armillaria* attacks on forest trees (McLaughlin *et al.,* 2011), but those works were performed with the ectomycorrhizal species red pine and lodgepole pine, respectively.

Lavender plants grew well in the autotrophic systems and colonization patterns by both *R. irregularis* and *A. mellea* were easily established *in vitro* using ROC mycorrhizal inoculum and pure cultures of the pathogenic isolate. When the effects of interaction between root-rot fungus and AMF were evaluated in autotrophic plant growing systems, the inoculation of lavender plantlets with *R. irregularis* at higher pH media caused a significant decrease of *A. mellea* presence in plant roots, as detected by qPCR. At the same time, the extent of mycorrhizal colonization increased when the medium was supplemented with $CaCO_3$, indicating that the growth medium pH affected the percentage of root colonized by *R. irregularis*. The growth conditions were more suitable for the AMF isolate according to the characteristics of the soil of origin (Camprubi & Calvet, 1996), as pointed out for the effect of soil pH on mycorrhizal fungi efficiency (Hayman & Tavares, 1985). In fact, *A. mellea* was detected in more than 70% of the plants, irrespectively of mycorrhizal inoculation, at pH 5.5, but only in non-mycorrhizal plants (almost 60%) in medium with pH 8.5, showing that *R. irregularis* protection effect against *A. mellea* was associated with mycorrhizal establishment.

Results obtained in the molecular detection of *A. mellea* in the autotrophic systems are in accordance with those obtained in the dual inoculated lavender plants tested *in vivo*. Detection by qPCR is a quick experimental test that proved suitable to evaluate the effect of plant mycorrhizal inoculation and the influence of an external factor, such as the pH of the growing medium modified by $CaCO_3$, on the interaction between both microorganisms. This methodology could be very useful when assessing the belowground infective potential of *A. mellea* in field grown lavender plants.

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