Extract of *Mimosa tenuiflora* and *Quercus robur* as potential eco-friendly management tool against *Sclerotinia sclerotiorum* in *Lactuca sativa* enhancing the natural plant defences

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Abstract

The effectiveness of some plant extracts to protect against pests enhancing the natural defensive responses of the plant has been reported. Plant extract of *Mimosa tenuiflora* and *Quercus robur* has promising potential to reduce the incidence of a wide range of phytopathogenic fungi due to its antimicrobial compounds. In this study, we aimed to elucidate the effectiveness and mode of action of this mix extract in *Lactuca sativa* against *Sclerotinia sclerotiorum*. To achieve this objective, 4 weeks old lettuce plants of cv. Romana were treated with 2 cc l^{-1} of the plant extract either by soil drench or foliar applications 72 hours before the inoculation. The treatments were able to significantly reduce the progression of the pathogen, decreasing the diameter of the infection by 32% and 17% in foliar and soil drench application, respectively. Moreover, the results showed significantly higher levels of hydrogen peroxide (H_2O_2) as well as callose deposition in plants treated and inoculated compared with non-treated plants. However, no direct effect on the fungus growth was observed *in vitro* suggesting that foliar and root treatments with *M. tenuiflora* and *Q. robur* extract significantly reduce the infection of *S. sclerotiorum* in leaves of lettuce, through the strengthening of the wall mediated by the deposition of callose and the release of H_2O_2. The fact that the treatment enhances different processes involved in plant innate defense indicates that this treatment is acting as a resistant inducer, and could be effective against different microorganisms.

Keywords *Lactuca sativa* • Natural extract • Plant immune system • *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum ((Lib.) W. Phillips), the causal agent of white mold (Sclerotinia), is a necrotrophic fungus considered as one of the most harmful pathogens in plants. This fungus can attack 400 plant species including many economically important crops either woody or herbaceous plants (Azevedo et al. 2016). *Sclerotinia scleriotorum* is present in more than 90 countries all over the world producing damages worth several hundred million dollars (Saharan and Mehta 2008a). This pathogen can be disseminated through the adhesion to seeds, agricultural machinery and animals or humans (Saharan 2008). Thanks to its capacity to form aggregations of fungal tissue called sclerotia, which are responsible for the long-term survival of the pathogen, are able to produce viable inoculum to infect after years (Azevedo et al. 2016).

Sclerotia are able to germinate in two ways, by producing mycelium or apothecia, a structure formed by spore reproductive cells (Saharan and Mehta 2008b). The ability of *S. sclerotiorum* to penetrate a host depends on factors such as the type of inoculum, the nutritional status of the fungus, the type of host and the environment. The ascospores, released by the apothecium developed from sclerotia of the soil, can be disseminated by the wind and are capable of infecting the crops through the leaves (Purdy 1979).

Ascospores of *S. sclerotiorum* infect upper parts of plants through the cuticle and open stomata, developing a mycelium or penetrating directly into the host causing infection. Moreover, the fungus is able to release substances such as oxalic acid, which destabilize the cells and facilitate the infection (Davidson et al. 2016). Lesions appear as water-soaked spots that grow irregularly thought the leaves provoking damages similar to desiccated plant tissues (Heffer 2007). On the other hand, *S. sclerotiorum* is also able to infect plants through the stem. The sclerotia produce hyphae which grow through the organic matter present in the soil and invading the plants present on it (Purdy 1979). This type of infection can lead diseases such as the ‘drop’ disease of lettuce caused by *S. minor.*

After the infection of the roots, the fungus attacks the crown and the stem; plants show a soft watery decay, which produces a collapse of the leaves and finally provokes the plant death (Heffer 2007). Occasionally, the infection is missed until the stem and roots have been completely invaded.

Nowadays, there are no widespread resistant lettuce cultivars, for this reason, the control of Sclerotinia disease depends mainly on fungicides, which target the airborne ascospores (Clarkson et al. 2014). However, the efficacy of chemical treatments to control the fungus has decreased over the years due to the development of fungicide-insensitive strains and the rapid degradation of fungicides in the soil (Klose et al. 2010). Moreover, some authors reported that the degradation of the fungicides increased with time and application frequency, suggesting that the repeated application would benefit the populations of degrading microorganisms in the soils (Klose et al. 2010; McDonald et al. 2006). For these reasons, it is necessary the development of new approaches for the control of Sclerotinia based on the sustainable management of crops and environmental friendly compounds.

In the recent years, the use of alternative control methods such as Bioactive plant compounds or resistance inducers, either synthetic chemicals or natural compounds, have been studied with promising results (Llorens et al. 2017). Many plant extracts have the potential for use in pest management since they are biodegradable, they possess a broad spectrum of activity and there is no resistance in pathogens (da Cruz Cabral et al. 2013). The main ability of the plant extracts to protect against pathogens due to its antimicrobial capacity provided by the composition of secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, and terpenoids (Gahukar 2012; Peter Gillitzer et al. 2012; Sales et al. 2016). However, several plant extracts have been reported as inducers of plant defense protecting crops from pests, displaying low or no toxicity against the

pathogens (Burketova et al. 2015). These extracts have the ability to induce a response on the plants that results in an effective tolerance against the infection. The response can include several physical and biochemical changes such as callose deposition, activation of salicylic (SA) and/or jasmonic acid (JA) pathways or synthesis of defence-related enzymes. The ability of the plant to protect itself against the attack relies on the effective activation of these defensive mechanisms (Llorens et al. 2017). Previous studies showed that extracts of *Azadirachta indica* leaves are effective as an antimicrobial compound against bacterial and fungal pathogens such as against *Pyricularia oryzae* in rice (Amadioha 2000) or against *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper (Abbasi et al. 2003). Moreover, *A. indica* possess effectivity as a resistance-inducing compound which provides control in barley against *Drechslera graminea* related to elevated activities of phenylalanine ammonia lyase and accumulation of phenolic compounds (Paul and Sharma 2002).

Mimosa tenuiflora and *Quercus robur* extracts have been previously displayed strong antimicrobial activity *in vitro* and against human pathogens (Andrenšek et al. 2004; de Souza Araújo et al. 2018; Padilha et al. 2010). Due to this fact, the search for new compounds based on *M. tenuiflora* and *Q. robur* extracts and its possible effect as plant resistance inductor would provide a useful alternative to control pathogens such as *Sclerotinia*. However, the possible application against plant pathogens as well as its compatibility and lack of phytotoxicity in plants have not been studied yet. For these reasons, the main goal of this study was to elucidate the efficacy of *M. tenuiflora* and *Q. robur* extracts in lettuce (*Lactuca sativa*) against *S. sclerotiorum* and its possible role as an inducer of plant immune system. In order to ascertain the mechanism of action of the treatment, infection development, physiological parameters, and several parameters involved in plant immune system were monitored.

Materials and Methods

Plant extract and fungal isolate

Formulation of plant extract mix of *Mimosa tenuiflora* and *Quercus robur* was provided by the company Idai Nature S.L. (La Pobla de Vallbona, Valencia, Spain). *Sclerotinia sclerotiorum* isolate SsUJI-1-2016 obtained from lettuces showing characteristic symptoms of the disease were used in this study. Prior to use., the specie of the isolate was characterized morphologically with a microscope, using the dichotomous keys determined by Kohn (1979) for sclerotia-producing cultures grown in PDA. This fungal isolate is maintained in the collection of the Department of Agricultural and Environmental Sciences of Universitat Jaume I (UJI) of Castellon (Spain).

Effect of the *Mimosa tenuiflora* and *Quercus robur* extract on mycelial growth and sclerotia germination

In order to analyse the direct effect of the treatment on the pathogen, *in vitro* tests were conducted. *Sclerotinia sclerotiorum* isolate SsUJI-1-2016 was grown in potato dextrose agar (PDA; Scharlau Microbiology, Sentmenat, Spain) for 3 days at 25ºC in darkness. Four mycelial plugs (4-mm in diameter) obtained from the margin of actively growing colonies were used to inoculate 100 ml Erlenmeyer flask containing 25 ml of sterile Potato Dextrose Broth (PDB, Scharlab S.L, Barcelona, Spain). Inoculated flasks were incubated at 25°C and shaken at 180 rpm (AG-200-A; JP Selecta) for 24 h. Subsequently, the plant extract mix was added at 0.14µl ml^{-1} (C1) following manufacturer's recommendation for field applications. Additionally, a lesser dose of the plant extract (0.07 µl ml^{-1}; C2) was also evaluated for comparative purposes. Flasks filled only with PDB and inoculated with *S. sclerotiorum* isolate SsUJI-1-2016 as described above were used as control (Ctr). Control flasks were incubated and shaked as described above. After

inoculation, flasks were incubated for 96 h at the same temperature and shaking conditions described above. After then, developed mycelium was filtered through sterile laboratory filter paper and dried in oven (Digiheat; JP selecta) at 60ºC for 24h. Subsequently, dry mycelial weigh of each treatment was obtained. There were five replicated PDB flasks per treatment (C1, C2 and control), and the experiment was conducted three times.

Concerning the study on the effect of the treatments on sclerotia germination, the experiment was conducted following the protocol described by Ortega-Aguilar et al. (2011). To this end, 10 sclerotia collected from 1-month-old colonies of *S. sclerotiorum* isolate SsUJI-1-2016 grown on PDA at 25ºC in darkness were treated by dipping for 30 min in a plant extract solution at 0.14µl ml^{-1} of plant extract (C1 treatment) in sterile distilled water (SDW). 10 additional sclerotia were treated by dipping them in SDW as control treatment. After dipping, sclerotia were removed from the solution treatment, plated onto PDA and incubated at 25ºC in darkness for 72 h. The experiment was repeated three times.

The effect of plant extract on sclerotia germination was assesses analysing the growth of the mycelium from the sclerotia on PDA. The largest and smallest diameters of the colonies developed from sclerotia were measured using a digital scale ruler and averaged. According to this parameter, the percentage of sclerotia germination for each treatment (C1 and control) was estimated according to the following rating scale: 0 = No germination; 1 = Germination lower than 0.5 mm; 2 = Germination between 0.5 and 1 mm; 3 = Germination between 1 and 2 mm; 4 = Germination around 2 mm with development of mycelia; and 5 = massive germination.

166 Effect of *Mimosa tenuiflora* and *Quercus robur* extract on plant growth and plant immune
167 system
168 *Plant material, treatments, and fungal inoculation*
169 Four weeks-old lettuce plants of cv. Romana obtained from a commercial nursery were
170 transplanted into polypropylene pots filled with a mix of sterilized peat moss and perlite
171 (4:1, vol:vol). The plants were kept in a greenhouse at an average temperature of 27 and
172 19ºC along day and night periods, respectively, and relative humidity (RH) around 70%.
173 These same conditions were subsequently maintained along the trial.

174 The following six treatments were evaluated: untreated and non-inoculated
175 control (Negative control, Ctr); untreated and inoculated control (Positive control, Ctr
176 inoc); soil drench treatment with extract of *M. tenuiflora* and *Q. robur* and non- inoculated
177 (Soil treat); soil drench treatment with extract of *M. tenuiflora* and *Q. robur* and
178 inoculated (Soil treat/inoc); foliar treatment with extract of *M. tenuiflora* and *Q. robur*
179 and non-inoculation (Fol. treat); and foliar treatment with extract of *M. tenuiflora* and *Q.*
180 *robur* and inoculated (Fol. treat/inoc). Treatments were applied following the
181 manufacturer's recommendations for field applications (0.14µl ml^{-1} for foliar or soil
182 drench treatments). Foliar application was done by spraying both sides of the leaves until
183 leaves were absolutely soaked. Soil drench application was applied by watering until the
184 field capacity of the substrate was achieved. Once treated, plants were placed in plastic
185 boxes in order to maintain 100% RH. There were 24 replicated plants per treatment (6
186 treatments), 144 plants in total.

187 In order to assess the effect of treatments in the plant growth development, three lots of
188 24 plants each were treated as described above for Ctr, Soil treat and Foliar treat (72 plants
189 in total). These three lots were kept in the same conditions described above for a month,

and the treatment was repeated once (15 days after the first application) following manufacturer's recommendation. All the experiments were conducted three times.

For fungal inoculation, inoculum of *S. sclerotiorum* isolate SsUJI-1-2016 was prepared on PDA as described above. Mycelial plugs of 4-mm in diameter were obtained from the margin of 3-days old actively growing colonies. Three leaves per plant were inoculated 72 h after treatment with the plant extract mix by deposition of three mycelial plugs in each one, placing them with the mycelium in contact with the leaf surface. Plants belonging Ctr, Soil treat and Foliar treat (non-inoculated treatments) were treated with three 4-mm in diameter PDA sterile plugs.

Plant growth and disease severity

The effect of the treatment on plant growth development was assessed measuring the dry weight of the leaves and root one month after the first application in the additional lots of plants of Ctr., Soil treat and Foliar treat (non-inoculated treatments). To this end, leaves and roots of each plant were carefully separated and introduced in oven at 60ºC for 72 h. After then, all samples were weighed.

Disease severity (DS) was assessed measuring the two-perpendicular axis of the lesion developed in each inoculation point by a digital scale ruler as described by Pane et al. (2017). Mean data were converted to radial growth rate (millimeters per day). Due to the fast development of the fungus on inoculated fresh leaves of lettuce, DS was evaluated just 72 h after inoculation since DS reached more than 50% severity in the untreated and non-inoculated control plants.

Determination and quantification of hydrogen peroxide (H_2O_2)

213 Samples of 6 leaves per treatment were collected 72 hours after inoculation. Collected
214 leaves were immersed immediately in 1 mg ml^{-1} of 3′,3-diaminobenzidine (DAB) at pH
215 <3 for 24 h in darkness. Subsequently, the leaves were discoloured in 96% ethanol and
216 rehydrated in distilled water for 30 min. Formation of brown precipitates were quantified
217 in micrographies and expressed as the number of dark-brown DAB pixels in relation to
218 the total pixels corresponding to plant material using the GIMP program (version 2.6.12)
219 (Llorens et al. 2016)

220 *Callose deposition*

221 Six leaves per treatment were collected at 72 hours after inoculation and incubated in 95%
222 ethanol at room temperature. Discoloured leaves were rehydrated in 0.07 M phosphate
223 buffer (pH = 7), incubated for 24h in 0.5% aniline blue (0.07 mM phosphate buffer) at
224 room temperature. Observations were performed with an epifluorescence microscope.
225 Fluorescence emitted by stained callose was observed under UV light microscope and
226 quantified in micrographies. Results were expressed as the number of pixels in relation
227 to the total pixels corresponding to plant material using the GIMP program (version
228 2.6.12)

229 *Evaluation of hormones related to plant defence by chromatographic analysis*

230 Six leaves per treatment were collected at 72 hours after inoculation and this fresh
231 material was immediately frozen in liquid N, ground, and lyophilized. Dry tissue (0.03 g)
232 was immediately homogenized in 2.5 ml of ultrapure water, and 100µL of internal
233 standard composed of deuterated abscisic acid ($[^2H_6]$ ABA), deuterated SA ($[_2H_4]$),
234 dihydrojasmonic acid (dhJA) and propylparaben} at 100 ng ml^{-1} each. After extraction,
235 a 20 µl aliquot was injected directly into the ultrahigh-performance liquid
236 chromatography (UPLC) system. Analyses were carried out using a Waters Alliance 2690

HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 mm × 2mm, i.d. 5 μm; Scharlab, Barcelona, Spain; http://www.scharlab.com). The chromatographic system was interfaced to a Quatro LC (quadrupole–hexapole–quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). The MASSLYNX NT software version 4.1 (Micromass) was used to process the quantitative data from calibration standards and plant samples (Llorens et al. 2016).

Data analysis

All experiments were conducted at least three times. Data from different repetitions was analysed together due to the fact that analysis of variance (ANOVA) did not show significant differences ($P > 0.05$) between repetitions in each experiment. Subsequently, for each assessment, ANOVA was performed with leaf and root dry weigh, lesion size, H_2O_2 quantification, callose deposition and hormone levels as dependent variables and treatment (plants treated or non-treated with extracts of *M. tenuiflora* and *Q. robur* and/or inoculated or non-inoculated with *S. sclerotiorum*) as the independent variable. All data of this study were tested for normality, homogeneity of variances, and residual patterns. When ANOVA showed significant differences between variables, mean values were compared using the Fisher's protected least significant difference (LSD) test at $P = 0.05$(Steel and Torrie 1986). Statistical analyses were performed using the software Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA, USA).

Results

Effect of the *Mimosa tenuiflora* and *Quercus robur* extract on mycelial growth and sclerotia germination

When ANOVA was conducted to evaluate the effect of the extract of *M. tenuiflora* and *Q, robur* on mycelial growth development, the analysis showed no significant differences on mycelial growth development between amended or non-amended PDA with the botanical extract (*data not shown*).

The viability of the sclerotia was scored according to the rating scale described before (Fig. 1). The percentage obtained for both the control and the C1 was 20% of sclerotia rated as 3 or below, and 80% rated as 4 or higher. In both cases, the mycelium had a considerable size and consistency. Thus, the treatment does not seem to have an effect on the induction of sclerotia germination (Fig. 1).

Effect of Mimosa tenuiflora *and* Quercus robur *extract on plant growth and disease severity*

To check if the treatment affects the growth of the plant, the dry weight of the leaves and root of the lettuce plants was measured. Results showed that the treatment did not affect plant growth development in comparison with non-treated plants. (Fig. 2).

The analysis of the results showed that the application of *M. tenuiflora* and *Q. robur* extract either by soil drench or foliar spray is able to reduce the progress of infection in lettuce by 23.8% and 33.9%, respectively (Fig. 3). Regarding the effectiveness of the method of application, there were no significant differences between the foliar spray and soil drench.

Effect of the treatments inducing the formation of H_2O_2

Treatments with *M. tenuiflora* and *Q. robur* extract promoted the synthesis of H_2O_2 in plants subjected to challenge infection. Both foliar and radicular treatments acted in a positive way promoting the activation of oxidative burst, which is responsible for the

induction of the natural plant defences system. Both treatments resulted in significant higher accumulation compared with control plants. Plants treated by soil drench and inoculated showed an accumulation of H_2O_2 70% higher than observed in positive controls. On the other hand, in inoculated plants, the foliar application seems to be more effective regarding the induction of oxidative burst, showing levels of H_2O_2 two-fold higher than observed in positive controls (Fig. 4; Fig. 6a-d). No differences were observed in between negative controls and soil or foliar treated plants.

Effect of the treatments on callose deposition

Botanical extract treatments promoted the deposition of callose in both inoculated and non-inoculated lots of plants, which leads to the idea that it is activating the natural plant defence system. However, results observed are different depending on the type of applications. Regarding radicular treatments, a significant enhancement of callose deposition was observed only in inoculated plants, reaching levels 1.6-fold higher than those observed in positive controls (Fig. 5). On the other hand, the induction of callose deposition in plants treated by foliar spray was significantly higher in non-inoculated and inoculated plants in comparison with both positive and negative control treatments, with Fol treat/inoc treatment combination showing the highest level of callose deposition (19664.8 pixels per image) (Fig. 5; Fig. 6e-f). In the case of foliar treatments, the level callose deposition on non-inoculated plants was 9-fold higher than that observed in negative control, whereas the level of callose deposition observed in inoculated plants was 4.2-fold higher than in positive controls (Fig. 5).

It is interesting to note that during the observation of callose deposition in the microscope, it was seen that the treatment was affecting the development of the mycelium and producing a slight degradation. In Fig. 6g-i we can see the three types of treated and

306 untreated mycelia. In Fig. 6g (Ctr inoc) a homogeneous mycelium is observed following
307 the normal branching patterns in this pathogen. In Fig. 6h (soil treat/inoc), the spatial
308 structure of the hyphae is disordered and it seems degraded in various areas. Finally, in
309 Fig. 6i (foliar treat/inoc), degradation was observed in several parts of the mycelium
310 expansion.

311 *Effect of the treatment on the enhancement of hormones related to plant defence*

312 Hormonal analyses of the different treatments conducted in this study on lettuce are
313 shown in Table 1. The accumulation of ABA showed an enhancement in in positive
314 control compared with negative control, as well as in foliar treated plants regardless of
315 the inoculation. No significant differences were observed in soil treated.

316 Regarding SA levels, our results showed different responses depending on the
317 treatment and the inoculation. Positive control and soil treated plants showed a small
318 reduction of SA levels after the challenge inoculation, but no significant differences were
319 observed. However, plants treated by foliar spray showed a completely different pattern,
320 since the treatment reduces significantly the levels of SA in non-inoculated plants and,
321 the level of SA is significantly higher in inoculated plants.

322 Concerning JA levels, significant accumulation of this molecule was observed
323 only in lettuce leaves from positive control plants. Significant differences were observed
324 between positive control and soil drench treated plants, regardless the inoculation.
325 Moreover, no significant differences were observed between negative controls and plants
326 treated by soil drench or foliar spray. On the other hand, results obtained when analysing
327 the JA-Ile showed a strong increment in infected plants regardless of the treatment.
328 Finally, the OPDA, precursor of the JA, showed inconclusive results without any
329 significant differences either between treatments or challenge infections.

Discussion

Previous studies have tested the effectiveness of *Mimosa* extract as an antifungal compound, obtaining positive results. In fact, Ferreira et al. (2013) used the extract of *M. ophthalmocentra* and *M. tenuiflora* in the control of the candida fungus. Despite the pharmacological and therapeutically effects of *M. tenuiflora* extracts have been previously reported, few studies have focused on its potential use as phytosanitary in crop protection. Therefore, the main objective of the present work was the evaluation as resistance inducer of *M. tenuiflora* and *Q. robur* extract in *L. sativa* inoculated with *S. sclerotiorum* under greenhouse conditions. *Sclerotinia sclerotiorum* is a necrotrophic fungus that develops from the surface of the crown towards the roots and the aerial zone. Although this fungus can grow through the plant as an endophyte, usually develops its mycelium on the surface (Azevedo et al. 2016).

The first step of this study was to check whether the treatment has any fungicide effect and interfere with the growth of the fungi. Contrary to observed with *Azadirachta indica*, *Zingiber officinale* or *Salvia officinalis* extracts against Sclerotinia (Ojaghian et al. 2014; Pansera et al. 2008), or other plant pathogens (Dorantes et al. 2000), the mix extract of *M. tenuiflora* and *Q. robur* is unable to inhibit the mycelial growth *in vitro* or the germination of sclerotia. These results suggest that this extract is not toxic to the fungi, and its possible mode of action is not related to fungicidal or fungistatic effects. This lack of direct toxic effect is not surprising since several compounds have been previously reported as able to protect crops from pests, without displaying direct toxicity against the pathogens (Llorens et al. 2017), but inducing the innate plant defence. On the other hand, we also tested if the application of plant extracts could have a detrimental effect on the growth of *L. sativa*. Other studies conducted with botanical extracts from plants belonging

354 *Jatropha* genus, demonstrated an allelopathic effect on the growth of the aerial and
355 radicular parts of the lettuce (Sanderson et al. 2013). Our results showed that the treatment
356 does not interfere with the growth of the plant, being safe for its use in the field.

357 Plants possess a wide variety of innate mechanisms to cope with pathogen attacks
358 (Llorens et al. 2017). These mechanisms include several layers of defence that range from
359 the cell wall reinforcement, expression of defensive genes or accumulation of toxic
360 compounds. Moreover, plants are able to detect and distinguish between herbivory,
361 biotrophic or necrotrophic pathogens and activate the appropriate defensive mechanism.
362 For this reason, in order to colonize the hosts, pathogens must interfere with the activation
363 of plant defences. During the infection process, *S. sclerotiorum* releases oxalic acid (OA)
364 which has been described as necessary for the establishment of successful infection
365 (Davidson et al. 2016). The release of OA produces a modification of the redox state of
366 the plant, leading in a suppression of the plant innate defences such as callose deposition,
367 elicitation of a strong oxidative burst, induction of phenolic compounds (Williams et al.
368 2011). Within the plant defensive mechanisms, one of the fastest responses against
369 pathogens is the accumulation of reactive oxygen species (Wojtaszek 1997). Upon
370 pathogen infection levels of reactive oxygen species (ROS) increase rapidly. This
371 oxidative burst has two main roles, a direct toxicity against pathogens and acts as
372 extracellular and intracellular signalling (Mittler et al. 2011). It is well known that upon
373 the colonization of *S. slcerotiorum* the production of OA inhibits the oxidative burst,
374 whereas it has been observed that the mutants unable to produce OA failed to colonize
375 the host and induced a strong oxidative burst (Williams et al. 2011). In the same way, the
376 induction of a strong oxidative burst can be also promoted by the application of priming
377 agents. In monocots, Paulert et al. (2010) observed that a pre-treatment of plants with
378 ulvan, a sulfated heteropolysaccharide, significantly reduced the severity of *Blumeria*

graminis infection, and induced an oxidative burst up to 150 times higher than other elicitors. In tomato, Fernandez-Crespo et al. (Fernández-Crespo et al. 2015) observed that NH_4^+ pre-treated plants showed higher levels of hydrogen peroxide which were related with higher resistance against *Pseudomonas syringae*. Our results showed a high accumulation of H_2O_2 in plants treated by foliar or soil drench application of the *M. tenuiflora* and *Q. robur* extract compared with untreated controls. These results could indicate that the application of the compound induces the oxidative burst reinforcing the first layer of defence, impairing the pathogen mechanisms for a successful infection.

Together with the oxidative burst, the accumulation of callose in the site of the infection has been often related to the resistance against *S. Sclerotiorum* (Liang et al. 2015; Williams et al. 2011). The presence of fungi and other pathogens, induce localized cell wall modifications, so-called papillae, at sites of attempted penetration. This papillae is mainly composed of (1,3)-β-glucan (Voigt 2016) and its accumulation upon pathogen infection has been directly correlated whit the increase of resistance. Ellinger et al. (2013) demonstrated in Arabidopsis that the deposition of elevated amounts of callose at early time points is related to resistance against *Golovinomyces cichoracearum* penetration. Similar than oxidative burst, callose deposition can be induced by priming agents. The application of resistance inducers such as beta-aminobutyric acid (BABA) or hexanoic acid primed deposition of callose, which has been associated with the resistance against several pathogens (Ji et al. 2015; Llorens et al. 2017; Vicedo et al. 2009), and thereby, this induction of resistance can be partially suppressed by the callose synthesis inhibitor 2-deoxy-D-glucose (Hamiduzzaman et al. 2005; Llorens et al. 2013). In our experiment, we observed a clear increase of callose deposition in treated and infected plants, especially after foliar treatment, which could be interfering with the pathogen penetration.

403 The treatment with *M. tenuiflora* and *Q. robur* extract not only induced changes
404 in the plant performance. The observation of the mycelium under the microscope showed
405 abnormal Sclerotinia hyphae in treated plants. In previous works, it has already been
406 observed that plant extracts of *Anethum graveolens* or *Platycladus orientalis* are able to
407 produce disorders in the normal morphology of fungal structures as detrimental and direct
408 effects of plant extracts (Tian et al. 2011; Wang et al. 2014). On the other hand, *Bacillus*
409 *thuringiensis* C25 is able to induced degeneration, distortion, and rupture of hyphae of *S.*
410 *minor* produced by a variety of cell wall degrading enzymes such as proteases, β-1,3-
411 glucanase, and chitinase (Shrestha et al. 2015). Since this result has been observed in
412 plants treated either by soil drench or foliar application, it seems indicate that treatment
413 *per se* does not produce hyphae degradation, but it could induce the expression of cell
414 wall degrading enzymes as part of the defensive response promoted by the treatment.

415 To define which defence systems were activated in the plant due to the treatment,
416 we analysed the hormonal levels in the leaves. The results obtained showed that the
417 treatments or the inoculation are able to modulate pathways of ABA and JA, and the foliar
418 treatment, which has proved to be the most effective, provoke changes both pathways.
419 Our result showed that the soil application of the plant extracts induces an enhancement
420 of ABA accumulation in non-inoculated plants, however, this increase in non-inoculated
421 plants was not observed in the case of foliar application. Between the defensive roles of
422 ABA, at the pre-invasive level, it is responsible for the PAMP-triggered defence by
423 promoting the stomatal closure to reduce the pathogen entry (C. Lim et al. 2015).
424 Moreover, this hormone has also been positively related to the deposition of callose (Oide
425 et al. 2013). However, previous studies have also suggested a negative relation between
426 ABA and SA induced resistance, which could impair the defence against biotrophic
427 pathogens such as *Pseudomonas syringae* (C. W. Lim et al. 2014). On the other hand, the

JA pathway is related to the plant defence against necrotrophic pathogens such as *Sclerotinia* (Llorens et al. 2017). Accordingly, our results showed a strong up-regulation of JA in positive controls. However, the treatment, despite being effective, did not show an enhancement of the JA pathway or the other metabolites analysed from this pathway. On the other hand, results observed in SA accumulation showed an induction only by soil treatment and inoculation. Despite this hormone is usually related in defence against biotrophic pathogens, recently Nováková et al., (2014) observed a possible implication of SA in *Brassica napus* against Sclerotinia indicating that all the main defence signalling pathways may respond to the attack by *S. sclerotiorum*. All these results indicate that there is not a clear relationship between the application of the compound and the activation of hormonal pathways, suggesting that the effectiveness of the plant extract does not require the activation of hormonal pathways but relies on previous defensive mechanisms induced by the treatment which could be sufficient to restrict the pathogen attack.

In conclusion, in this work, it has been demonstrated that the mix extract of *M. tenuiflora* and *Q. robur* is able to induce plant innate defences applied either by soil drench or foliar spray. The lack of *in vitro* direct toxicity against *Sclerotinia* suggests that the treatment works by inducing the plant defence systems. The results observed in the induction of peroxide formation and the deposition of callose would be sufficient to counteract the infection process and avoid the *Sclerotinia* colonization. Moreover, the application of this extract could be impairing the hyphae development, but more studies would be necessary in order to clarify this point.

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Research involving human participants and/or animals This research is focused on the mechanism of action of botanical extracts against a plant pathogenic fungus. This article does not contain any experiments with human participants or animals.

Informed consent Please be informed that authors are satisfied to publish this work in European Journal of Plant Pathology.

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Fig. 1 Germination of sclerotia. Percentage of germination of sclerotia for the treatments Ctr: mycelium growth in PDB; C1: mycelium growth in PDB + 0.14 µL/ml of plant extract. Results are represented according to a rating from 1 to 5 where 0 = No germination; 1 = Germination lower than 0.5 mm; 2 = Germination between 0.5 and 1 mm; 3 = Germination between 1 and 2 mm; 4 = Germination around 2 mm with development of mycelia; and 5 = massive germination. For each treatment, data represent the means of three independent experiments of 10 sclerotia per repetition.

Fig. 2 Effect of *Mimosa tenuiflora* and *Quercus robur* extract on plant growth development. Dry weight (g) of the leaves and roots of treated plants of lettuce for: Positive control (non-treated and inoculated plants); Fol treat/inoc (foliar treated and inoculated plants), and Soil treat/inoc(soil drench treated and inoculated plants-). Columns represent the means of three independent experiments of 24 replicated plants per treatment, and repetition. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Fig. 3 Effect of *Mimosa tenuiflora* and *Quercus robur* extract on the resistance of lettuce plants against *S. sclerotiorum* 72 hours after inoculation. Diameter of lesion (mm) for the treatments Positive control (non-treated and inoculated plants); Fol treat/inoc (foliar treated and inoculated plants); and Soil treat/inoc (Soil drench treated and inoculated plants). Columns represent the means of three independent experiments of 24 replicated

plants per treatment, and repetition. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Fig. 4 Quantification of the accumulation of hydrogen peroxide (H_2O_2; ROS) represented as brown pixels per image in samples of lettuce leaves taken 72 hours after inoculation for the six treatments evaluated: untreated and non-inoculation control (negative control, Ctr); untreated and inoculated control (Positive control, Ctr inoc); soil drench treatment without inoculation (Soil treat); soil drench treatment and inoculated (Soil treat/inoc); foliar treatment without inoculation (Fol treat); and foliar treatment and inoculated (Fol treat/inoc). Columns represent the means of three independent experiments of 24 replicated plants per treatment, and repetition. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Fig. 5 Quantification of callose deposition represented as number of pixels in samples of lettuce leaves taken 72 hours after inoculation for the six treatments evaluated: : untreated and non-inoculation control (negative control, Ctr); untreated and inoculated control (Positive control, Ctr inoc); soil drench treatment without inoculation (Soil treat); soil drench treatment and inoculated (Soil treat/inoc); foliar treatment without inoculation (Fol treat); and foliar treatment and inoculated (Fol treat/inoc). Columns represent the means of three independent experiments of 24 replicated plants per treatment, and repetition. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

Fig. 6 Micrographies of the different analysis performed: (**a-d**) accumulation of hydrogen peroxide (H_2O_2; ROS) observed as brown precipitate for (**a**) Sample from negative control

leaf (**b**) Sample from Fol treat leaf (**c**) Sample from Positive control leaf (**d**) Sample from Fol treat/inoc leaf (**e-f**) Visualization of callose deposition for (**e**) Sample from positive control leaf (**f**) Sample from Fol treat/inoc leaf (**g-i**) Effect of treatment with extract of *M. tenuiflora* and *Q. robur* in the mycelial development of *S. sclerotiorum* in lettuce plants (**g**) Mycelium in leaf samples from negative control plants in which a homogeneous growth is observed and the structures of the hyphae are equivalent to each other (**h**) mycelium in leaves of plants treated via foliar (**i**) mycelium in leaves of plants treated via radicular. Arrows point segments where mycelium is abnormal.