UNIVERSITY OF VALLADOLID

SUSTAINABLE FOREST MANAGEMENT RESEARCH INSTITUTE

Department of Plant Production and Forest Resources

Erasmus Mundus Master in Mediterranean Forestry and Natural Resource Management (MEDfOR)

Susceptibility of broadleaved and crop species to *Fusarium circinatum* and application of endophytes, chitosan and propolis to reduce the severity of the pitch canker disease

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July 2017

Acknowledgement

If I had to put three names to which I am grateful for having reached this moment of culmination with the work, I would have chosen it before a blink of my eyelids without a second thought. Professor **Dr. Julio Javier Diez Casero**, **Dr. Pablo Martínez Álvarez** and **Dr. Jorge Martín García** has been not only an outstanding experienced and master supervisors, but also an amicable, motivating, patient, enthusiastic and philosophic mentor for me. They provided mind-boggling knowledge on the respective field always kept me in pace of working with the topic of interest.

My special appreciation goes to the staffs and colleagues of SUSTAINABLE FOREST MANAGEMENT RESEARCH INSTITUTE; DEPARTMENT OF PLANT PRODUCTION and FOREST RESOURCES especially to **Mr. E. Jordán Muñoz** and **Mr. Mariano Rodriguez Rey** for laboratory assistance.

In addition, I am quite grateful to the superb scholars of University of Padova, Italy and the fruitful teaching of the faculty members of University of Valladolid, Palencia, Spain, that hosted me for MEDfOR specialization. I am thankful especially to professor **Dr. Andrea Battisti** and professor **Dr. Alejandro Solla** for I learned from them on my subject during their wonderful course in Padova. In addition to other co-coordinators, I convey my sincere acknowledgement to professor **Dr. Davide Pettenella** and professor **Dr. Felipe Bravo** for their kind coordination and all professors of MEDfOR**.**

European union granted me with two-year **Erasmus Mundus scholarship** without which it could have been impossible for me to attend the program. A worm thanks goes to **MEDfOR** Secretariat **Dr. Catarina Tavares,** who has been very well timed to cooperate in every aspects throughout the program.

Table of Contents

Abstract

Fusarium circinatum is the causal agent of the pitch canker disease, which affects and causes the death of *Pinus* spp. and *Pseudotsuga menziesii* worldwide. It has been present in Europe since at least 2004 when it was first found causing damage in nurseries and pine plantations in northern Spain. The spread of this pathogen to nurseries and plantations constitute a risk to forest production. Most of the pine species have been found to be susceptible to the pathogen under nursery conditions. Although Monterey pine (*Pinus radiata*) is the most frequently infected host, there is no information about how the fungus affects the broadleaved and crop species. On the other hand, the current restriction on the use of chemicals with strict quarantine measures, alternative approaches for disease control are necessary. Biological control using endophytes is considered an alternative and eco-friendly method to deal with plant diseases. Therefore, the aim of the present study was to identify the susceptibility of broadleaved and crop species to pine pitch canker, to know the effectiveness of two fungal endophytes against *F. circinatum* and to shed light on the efficiency of chitosan and propolis to control pitch canker. Two different experiments, both in laboratory conditions, were carried out. In general, conifer species were affected by the pathogen but no clear symptoms were recorded in the case of the broadleaved and crop species. At the same time, fungal endophytes *Chaetomium aureum* and the unidentified endophyte named 20.1 were used to evaluate against the *F. circinatum* isolates. At the end of the experiment, seedlings were cut and the necrosis length of each plant measured. The endophytes *Chaetomium aureum* and 20.1 together with the chitosan and propolis were not able to reduce the area under disease progress curve (AUDPC) for *P. radiata* seedlings, being necessary the study of new endophytes to fight against the pitch canker disease.

Keywords: pitch canker disease, susceptibility, biological control, chitosam, propolis.

1 Introduction

Fusarium circinatum Nirenberg & O'Donnell is a highly virulent pathogenic fungus, causing the Pine Pitch Canker (PPC) disease (Martínez-Álvarez et al., 2012) which affects pine species (Coutinho et al., 2007; Quesada et al., 2010) and Douglas fir (*Pseudotsuga menziesii* Mirb) (Gordon et al., 2006). *Fusarium circinatum*, belonging to the phylum Ascomycota, is the asexual reproductive stage of the fungus. The sexual reproductive stage, i.e. the teleomorph, is named as *Gibberella circinata.* Numerous plant pathologists have distinguished *F. circinatum* as a serious threat to the pine tree species. Due to the high tree mortality rate, reduced growth, and degradation of wood quality, this disease has important economic and ecological consequences (Forest commission, 2016). Among the affected pine species, Monterrey pine (*Pinus radiata* D. Don) is considered one of the most susceptible to this pathogen (Gordon *et al*., 2001).

The disease was first reported in 1946 in the south-eastern United States of America (Hepting and Roth, 1946) as causing damage to *Pinus virginiana* Mill. In California, the pitch canker pathogen was first recorded in 1986, being Santa Cruz Country the most severely affected area (Wingfield et al., 2008). It was later found in Haiti (Hepting and Roth, 1953) and Mexico (Guerra-Santos, 1998). Due to globalization, the pathogen arrived to other countries far from its origin. Therefore now, it has been detected in South Africa (Viljoen et al., 1994), Japan (Kobayashi, 2007), Chile (Wingfield et al., 2002), Korea (Cho and Shin, 2004), France (EPPO, 2006), Spain (Landeras et al., 2005), Italy (Carlucci et al., 2007), Portugal (Bragança et al., 2009), Uruguay (Alonso and Bettucci, 2009), Colombia (Steenkamp et al., 2012), Brazil (Pfenning et al., 2014). Spain was the first country in Europe reporting the PPC disease. Here, *F. circinatum* affects *P. radiata, P. pinaster* in forest plantation and some other pine species in nurseries (Pérez-Sierra et al, 2007). In the last decades, the European and Mediterranean Plant Protection Organization (EPPO) region, pitch canker appeared on the list of pests (A2) recommended for regulation as quarantine pests (EPPO, 2005).

Fusarium circinatum infects the pine trees and create the most common symptom of the disease. i.e. a bleeding, resinous canker on the trunk, terminals and/or large branches (Hepting and Roth, 1946). The pathogen also causes shoot die-back in adult trees (Correll et

al., 1991). In seedlings, this ascomycete fungus causes damping off, shoot and tip die-back and finally the death of the seedlings (Viljoen et al., 1994). It can also infect seeds, cones and roots in host trees of any age, causing pre and post-emergence damping-off in seedlings with mortality rates up to 90%, particularly in *P. radiata* (Muñoz-Adalia et al., 2016).

Most infections are produced by macroconidia or microconidia, being necessary a wound in the bark for the infection. Wounds may appear in the trees caused by storms or human activities. The pathogen spreads via the movement of contaminated material; seeds, wood, nursery seedlings, etc. On the other hand, it also spread via air or by insect vectors (Bezos et al., 2015). Several studies show an important association between insects and pitch canker (Blakeslee and Foltz, 1981). For example, wounds caused by *Contarinia* sp. (needle midge) are common on pine in seed orchards and plantations, and are often colonised by the pitch canker pathogen (Dwinell *et al*. 1985).

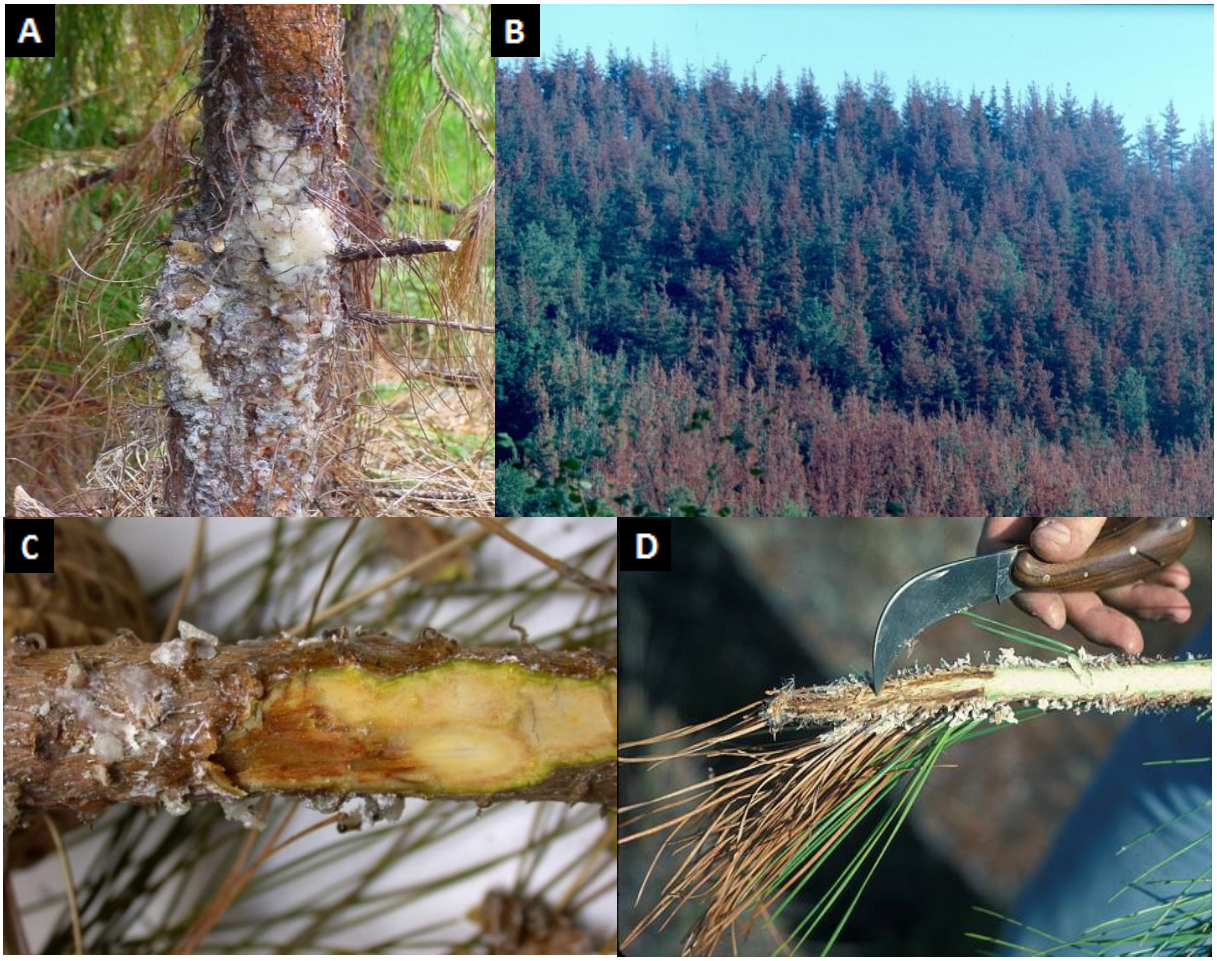

Fig. 1. Symptom of PPC. A: Resin extraction. B: Flag effects of pitch canker in mature *Pinus radiata* forest. C: Necrosis and detail of resin surrounding in the point of infection. D: Dead seedling are showing the infection of tip and needles.

Cone and seed feeding insects have also been identified, which may contribute to pitch canker dissemination and infection. These include *Leptoglossus corculus* (leaf footed pine seed bugs), *Tetrya bipunktulata* (seed bugs), *Laspreyresia* spp. (seed worms) and *Megastionus atedius* (seed chalcids) (Dwinell *et al*. 1985).

At present, there are no effective means of controlling the disease caused by this fungal pathogen both in seedlings in the nurseries and/or in adult trees in forest plantations. Good forest management such as planting less susceptible pine species, rapid movement of infected trees, quarantine measures (particularly restrictions on importation of wood products and asymptomatic seedlings) are the specific mechanisms that reduce the spread of *F. circinatum* (Iturritxa et al., 2017). Beside these activities some other methods to control the disease have been suggested such as application of adaptive silviculture programmes (Muñoz-Adalia et al., 2016), selection of particular species, treatment of seeds with hot water (Berbegal et al, 2015), addition of hydrogen peroxide to irrigation water (Dwinell and Fraedrich, 1999) and biocontrol techniques involving bacteria or other fungal species (Martínez-Álvarez et al., 2016; Soria et al, 2012).

In point of fact, the biological techniques have been used in agricultural cultivation practices over the last century as alternatives to chemical pesticides. In contrast, very few investigation of the potential of the biological agents has been done in forest sector particularly, against pitch canker pathogen. Some other biological methods, such as methyl jasmonate were tested against this disease, but it failed to protect pine seedlings from disease caused by *F. circinatum* and other fungi (Vivas et al., 2012). On the other hand, several essential oil such as cinnamon, oregano, thyme, lavender, tea tree, Japanese mint, clove, rose geranium and lemongrass were revealed to inhibit the growth of *F. circinatum* in vitro, although these products have a toxic effect on *P. radiata* trees (Iturritxa et al., 2017). Nevertheless, some of these techniques are potentially useful but recently, the new methods of biological control particularly to use fungal endophytes focused on nursery and forest application has been tested frequently. Currently, the use of endophytes is showing promising results against the fungal diseases not only in forest but also in nursery. For example, *Trichoderma harzianum* Rifai and *T. viride* were observed to be effective against *F. circinatum* in vitro but they did not reduce the growth of the pathogen on *P. radiata* seeds

or seedlings (Iturritxa et al, 2011; Martínez-Álvarez et al., 2012). Fungal endophytes can infect their hosts without causing visible symptoms of disease are used successfully as biological control agent (BCAs) in the fight against some fungal diseases (Arnold et al., 2003). According to the effects of endophytes on pathogen it may classified into three groups: direct, indirect and ecological effects (Martínez-Álvarez et al., 2016). Endophytes can be extracted from the same ecosystem in which they will be used as BCAs, so that there will be no biological impact in the same environment. Contrary to the use of chemicals, pathogens do not become resistant to endophytes. Moreover, they may have positive impact on plants, such as enhanced growth, resistance to drought stress, tolerance to unsuitable soil conditions, protection against herbivores and against important pests (Vega et al., 2008).

In Spain, several conifer species particularly, *Pinus radiata, Pinus nigra, Pinus sylvestris, Pinus pinaster, Pinus uncinata,* etc have been tested for their susceptibility to *F. circinatum*. But the effect of the pitch canker pathogen on some of the native or introduced broad leaved species such as *Quercus pyrenaica* and *Acer pseudoplatanus*, or even crops, has not yet been studied in Spain to date. Moreover, some species specially conifers were tested for susceptibility to *F. circinatum* but broadleaved species have not yet been tested in the present environmental conditions. Although many forest pathologists have mainly focused their efforts on *Pinus* spp., further research is needed to detect other possible resistant species. Therefore, the aims of the present study were a) to evaluate the susceptibility of broadleaved and conifers species and agricultural crops to Pitch canker disease. b) to know the effectiveness of two fungal endophytes against *Fusarium circinatum*. c) to test the efficiency of chitosan and propolis to control Pine Pitch canker disease.

2 Materials and Methods

2.1 Host and non-host interactions in Pine Pitch Canker disease

Seeds of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* (L.) M.Bieb.) were used for this experiment. They were collected from Palencia being the provenance Castilla y Leon, Spain. The variety of wheat, barley and rye were Rimbaud, Ainsa and Gigauton respectively. All collected seeds were immersed into normal water for 24 hours. One day later, seeds were again immersed into 3 % Hydrogen Peroxide solution for 20 minutes and washed about 3 times with sterile distilled water (SDW) to remove the remaining hydrogen peroxide. After that, all the seeds were dried as spreading it on white paper before sow them. Finally, the seeds were sowed in peat and vermiculate (1:1, v:v) with each pot containing ten seeds (Shin et al., 2014). Before inoculation the seedlings were grown in open exposed area with adequate sunlight for 15 days.

To prepare the inoculum, four pieces of mycelium were added to 500 ml potato dextrose broth (PDB). Spore production was induced in an orbital shaker for 24 h and spores were recovered from the culture by filtration through cheese cloth to remove mycelium from the suspension. The concentration of the spores was determined with a haemocytometer, and was adjusted to 10^6 spores ml^{-1} with SDW.

Each seedling was inoculated at one to two centimetres above the root collar with 10 µl of *F. circinatum* spore suspension (10^6 spores ml^{-1} of SDW) through injection needle. The inoculated seedlings were put into the plant growth chamber for at least 15 days at 20 ^oC temperature with a 16/8 h photoperiods. The seedlings were watered twice a week throughout the study period, with equal amounts (2 litters) of sterile distilled water.

One-year-old seedlings were directly collected from a nursery. The provenances of all of the seedlings were different: *Pinus radiata* from Cantabria, Spain; *Pseudotsuga menziesii* from New Mexico, USA; *Acer pseudoplatanus* from Alps Juva, Switzerland and *Quercus pyrenaica* from Valladolid, Spain. Height of the seedlings and collar diameter were measured at the time of inoculation. Once inoculated, plants were moved to a growth chamber under control temperature at 20 ^oC and humidity of 69% with a 16/8 h photoperiod and left to acclimatize for a month (Martin-Rodrigues et al., 2015). The mycelium of the fungi *F.*

circinatum (isolate FcCa6), was cultured in PDA (Potato Dextrose Agar). The isolate of the pathogen *F. circinatum* has been used in previous pathogenicity studies causing mortality in seeds and seedlings (Martínez-Álvarez et al., 2012; Martínez-Álvarez, et al., 2014; Cerqueira et al., 2017). The isolate was grown at 25 ^{o}C in the dark for seven days. Five PDA mycelial plugs (diameter 5 mm) were cut and placed in Erlenmeyer flasks containing PDB. To prepare the suspension, samples were kept in an orbital shaker at 180 cycles per minute during 24 hours. The resulting suspension was filtered through sterile gauze and/or cheese cloth and the spore suspension was adjusted with a haemocytometer at 10^6 spores ml^{-1}. Control treatment only contained with SDW.

To test *in vivo* the ability of *F. circinatum* to cause disease (pathogenicity) 120 individuals about one-year-old nursery seedlings of *P. radiata*, *P. menziesii, A. pseudoplatanus* and *Q. pyrenaica* were used. Fifteen seedlings per species were inoculated with the pathogen and fifteen were used as control and therefore they were mock-inoculated. A small incision was made using a scalpel, two centimetres above the root collar and 10 µl of spore suspension (10^6 spores ml^{-1} of distilled water) was placed there. In control seedlings, an incision was made in the same way, but only distilled water was inoculated into the wound. After inoculation, the wound area was covered with Parafilm® for two weeks. The treated and control seedlings were held separately in plant growth chambers under controlled conditions of temperature (25 ^{o}C) and photoperiods (light/darkness 16/8 hours). The seedlings were watered three times a week throughout the study period, with equal amounts of SDW. After one week, the visual severity of symptoms in each plant were assessed every two days during a period of 45 days, according to the following scale: 0 = healthy plant, 1 = necrosis only at the point of inoculation and healthy foliage, 2 = necrosis > 2 cm beyond the point of inoculation, 3 = needles wilting and appreciable dieback and 4 = dead plant (Correll et al., 1991) (Fig. 2). Finally, the area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids as previously described (Martinez-Alvarez et al., 2014).

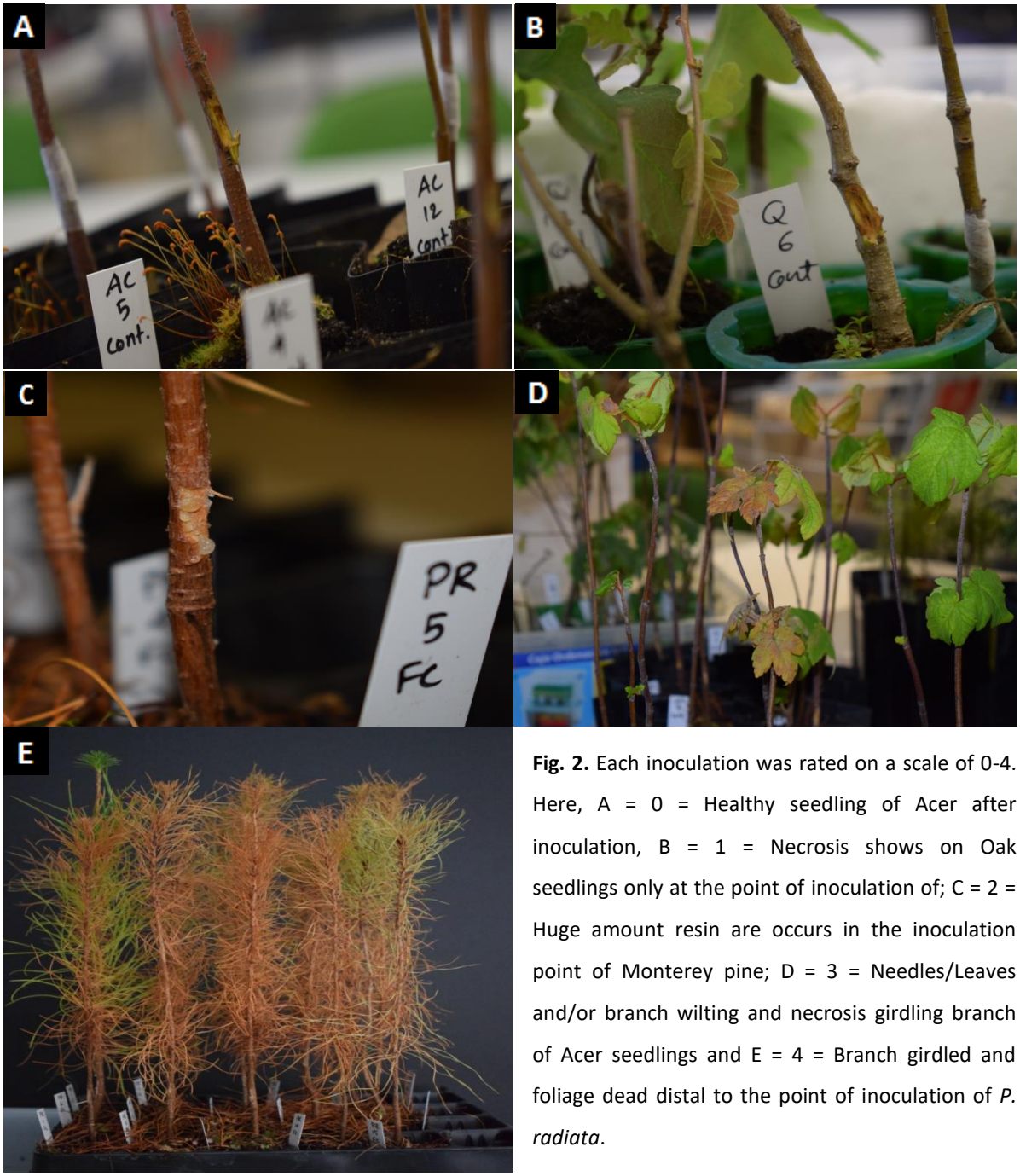

Fig. 2. Each inoculation was rated on a scale of 0-4. Here, A = 0 = Healthy seedling of Acer after inoculation, B = 1 = Necrosis shows on Oak seedlings only at the point of inoculation of; C = 2 = Huge amount resin are occurs in the inoculation point of Monterey pine; D = 3 = Needles/Leaves and/or branch wilting and necrosis girdling branch of Acer seedlings and E = 4 = Branch girdled and foliage dead distal to the point of inoculation of *P. radiata*.

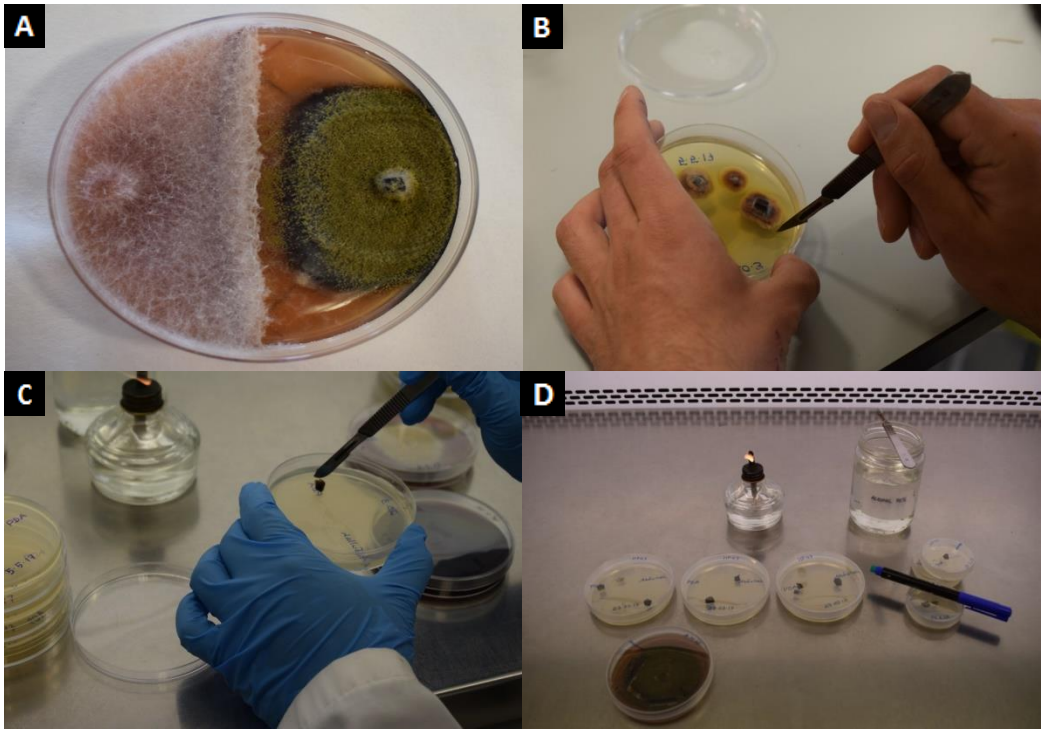

Fig. 3. Culture of Pathogen and Endophytes. A; a) *F. circinatum* & b) *Chaetomium aureum*, B; Unidentified endophytes 20.1, C; Putting a part of 4x4 mm endophytes on the PDA, D; Pathogen and Endophytes (*Chaetomium aureum* and 20.1) are ready for further culture.

After the collection of the data, five seedlings per treatment were selected randomly to reisolate the pathogen. The seedlings were cut in seven pieces to know the progress of the pathogen inside the seedling. The parts of the seedling selected for the reisolation were: point of inoculation, two and four cm below this point of inoculation and two and four cm over it, five cm below the tip, and two cm below the collar root (root sample). These samples were plated in PDA and the colonies appearing transferring to new PDA plates and SNA (Spezieller Nährstoffarmer Agar) to identify the pathogen by its morphological characteristics.

2.2 Management of PPC with fungal endophytes and bio-rationale compounds

a. In Vitro Antagonism

The antagonism produced by *Chaetomium aureum* and the unidentified endophyte 20.1 against *F. circinatum* was studied on PDA. One representative isolate of the pathogen and one isolate of each one of endophytes were tested. A 4 mm square plug of growing mycelium taken from the pathogen was placed 10 mm from the edge of the plate. A similar

plug of the respective endophyte was placed in front of the pathogen and 10 mm from opposite edge of the plate. Five replicates were prepared per treatment (pathogen*endophyte) (Martínez-Álvarez et al., 2012). The plates were maintained under laboratory conditions (25^{o}) for 25 days and the increase in the mycelial growth of *F. circinatum* colonies was measured along three axes from the middle of the plug, one joining both fungal plugs and the other two axes forming an angle of 45° with the first one (Santamaría et al., 2007). The difference between the mean length of the lateral axes and the length of the middle axis was used as an indicator of the shape of the colony and therefore of the effect of *Chaetomium aureum* and 20.1 on the growth of *F. circinatum*.

b. In Vivo Antagonism

To test the effect of the endophytes *C. aureum* and 20.1, and the effectiveness of chitosan and propolis controlling the pitch canker disease, an in vivo experiment was performed. On it, a total of 250 seedlings distributed in ten different treatments were tested (Table 1). The inoculation of the endophytes was done two weeks before the inoculation of the pathogen. To perform the inoculation, a wound was done in the stem of the seedling, and an agar plug of the colony of the endophyte was placed in the mentioned wound. One week before the inoculation of *F. circinatum*, the chitosan and propolis was sprayed to the seedlings (2.5 ml per seedling). The concentration of the chitosan and the propolis in the final solution was 1 mg ml^{-1} and 0.1 mg ml^{-1} respectively. The pathogen was inoculated making a small incision with the scalpel, around two centimetres above the root collar and placing 10 µl of the spore suspension (10^{6} spores ml^{-1} of distilled water). One week later, another treatment with chitosan and propolis was done in the same conditions.

Table 1. treatments tested in the in vivo experiment. A.P.: agar plug, F.c.: *Fusarium circinatum*, HP47: *Chaetomium aureum*, SDW: sterile distilled water

Treatment	Endophytes/A.P.	Chito. + Profo./SDW	*F. circinatum*/SWD
1	A.P.	SDW	SDW
2	A.P.	SDW	F.c.
3	A.P.	Chitosan + Propolis	F.c.
4	HP47	SDW	F.c.
5	20.1	SDW	F.c.
6	HP47	Chitosan + Propolis.	F.c.
7	20.1	Chitosan + Propolis.	F.c.
8	20.1	SDW	SDW
9	HP47	SDW	SDW
10	A.P.	Chito. + Propo.	SDW

2.3 Statistical analyses

Survival analysis based on the nonparametric estimator Kaplan–Meier was carried out with the "Survival" package (Therneau, 2015) implemented in the R software environment (R Foundation for Statistical Computing, Vienna, Austria). Survival curves were created with the "Survfit" function and the differences between the curves were tested with the "Survdiff" function.

Analysis of variance (ANOVAs) and multiple comparison procedures were performed to test the effects of *F. circinatum* inoculations on necrosis length and AUDPC in in vivo experiments and on mycelial growth and mycelial shape in the in vitro assay. As the data violated two of the ANOVA assumptions (normality and homogeneity of variances), robust statistical methods were applied (García-Pérez, 2010). In particular, heteroscedastic one-way ANOVAs were performed using the generalized Welch procedure and a 0.2 trimmed mean transformation. The ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS2)" package (Mair et al., 2015) implemented in the R software environment. A Wilcoxon test was carried out to confirm the susceptibility of *P. radiata* seedlings to *F. circinatum*.

3 Results

3.1 Host and non-host interactions in Pine Pitch Canker disease

Regarding the experiment in which three different agricultural crops were inoculated with *F. circinatum*, we found that wheat and barley resulted less affected by the pathogen than ray (Fig. 4). However, in all species the statistical analyses showed significant differences between the inoculated and the control seedlings. Ray was the species most susceptible to *F. circinatum*, followed by wheat and barley (Fig. 5).

Fig. 4. Difference between wheat, barley and ray after inoculation with sterile distilled water (SDW) as control (A) and with *F. circinatum* (B) in the plant growth chamber.

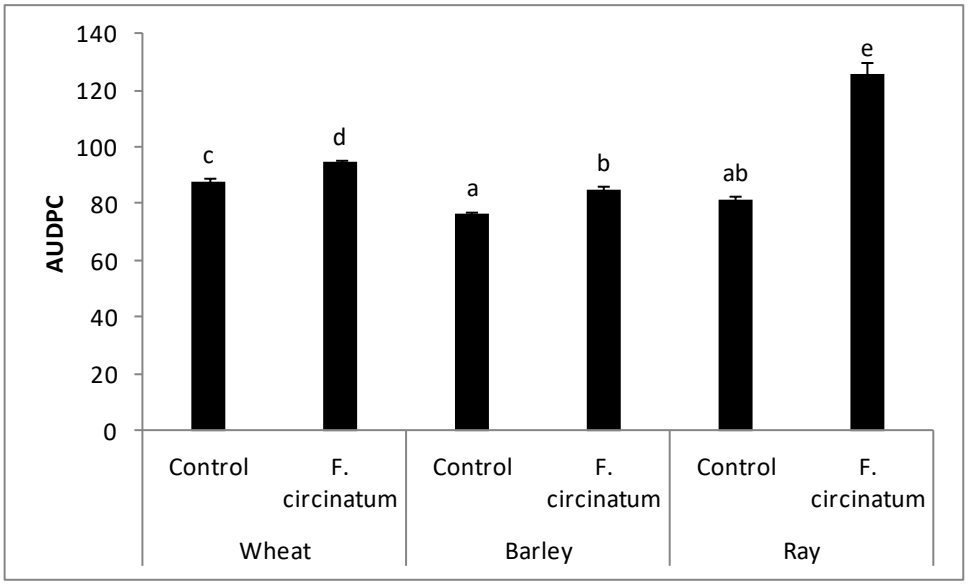

Fig. 5. Area Under Disease Progress Curve (AUDPC) of each one of the crop species tested in the assay, when they were inoculated with *F. circinatum* or in the control treatment. Letters (a-e) denote significant differences ($p < 0.05$) among all columns.

In case of forest species inoculation experiment, the first symptoms caused by *F. circinatum* appeared one week after the inoculation, mainly on *P. radiata*. Symptoms observed consisted of dried needles, wilting, discoloration, resin production, necrosis, small cankers and the death of some plants. The analysis of the dependent variable AUDPC revealed statistically significant differences among species (p-value=0.001) and treatments (inoculated with *F. circinatum* vs. control, p-value=0.031). The effect of the pathogen differs among species. While in the species *P. radiata* and *P. menziesii* the AUDPC was higher in the inoculated seedlings than in the control treatment, the opposite pattern was found for *A. pseudoplatanus* and *Q. pyrenaica* (Fig. 6).

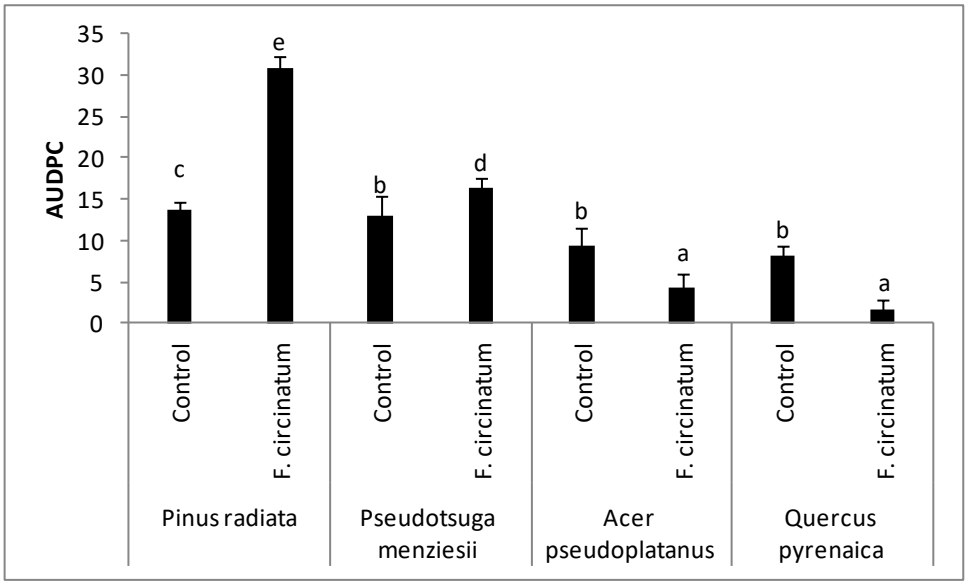

Fig. 6. Area Under Disease Progress Curve (AUDPC) of each one of the forest species tested in the assay, when they were inoculated with *F. circinatum* or in the control treatment. Letters (a-e) denote significant differences ($p < 0.05$) among all columns.

The AUDPC of the inoculated *P. radiata* seedlings was more than ten and six times larger than in the species *Q. pyrenaica* and *A. pseudoplatanus* respectively. The AUDPC for *Q. pyrenaica* and *A. pseudoplatanus* was very low since these seedlings only showed small necrosis in the inoculation point. *Pseudotsuga menziesii* resulted susceptible to the disease as differences between the two treatments were found in the statistical analysis. At the end of the assay, no seedlings of *Q. pyrenaica* and *A. pseudoplatanus* died. On the other hand, 60% of the inoculated *P. radiata* seedlings died throughout the experiment. In the case of the species *P. menziesii*, 6% of the inoculated seedlings and 13% of the control seedlings died (one inoculated and 2 of the control treatment) before the end of the experiment.

Regarding the statistical analysis of the variable necrosis length, differences were found between species (p-value=0.001). On the other hand, the effect of the pathogen varied depending on the species tested (Fig. 8). Thus, seedlings inoculated with the pathogen showed higher necrosis length than those used as controls in the species *P. radiata* (p-value=0.008), but in the rest of the species it was the opposite.

Fig. 7. Necrosis in a *P. radiata* seedling (A) and in a seedling of the species *P. menziesii.*(B)

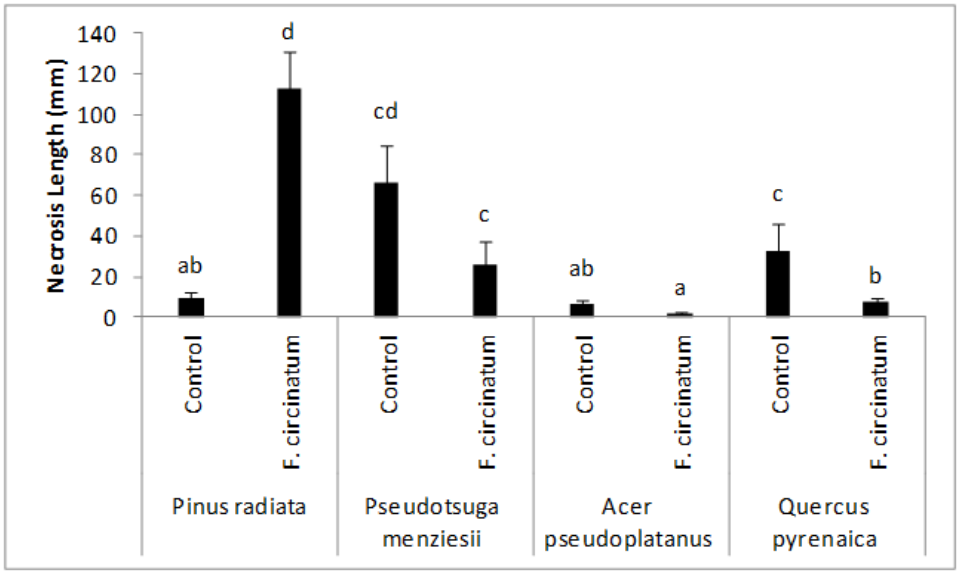

Fig. 8. Necrosis length found in seedlings after inoculation with *F. circinatum* and in the control treatment. Letters (a-d) denote significant differences ($p < 0.05$) among all columns. Bars represent standard error.

Regarding the reisolation of the pathogen, it was found in the point of inoculation of all the species. In the case of *P. radiata*, it was found even in the roots of the seedlings. For *P. menziesii*, the pathogen was reisolated two cm over the point of inoculation and two cm below it. In two seedlings of *Q. pyrenaica* the pathogen was found two cm below the point of inoculation. In the case of *A. pseudoplatanus*, the pathogen was detected only in the point of inoculation.

3.2 Management of PPC with fungal endophytes and bio-rationale compounds

a. In Vitro antagonism

The effect of two fungal endophytes (*Chaetomium aureum* and Endophyte 20.1) and two culture media (PDA and PDA amended with chitosan and propolis) on the mycelial growth of *F. circinatum* was studied in this experiment. *Fusarium circinatum* showed maximum growth on PDA medium but when the media was amended with chitosan and propolis the growth of the pathogen was reduced (Fig.9). The chitosan and propolis added to the media had also a significative effect on the shape indicator (p-value=0.043, Fig.10). Regarding the effect of the endophytes, the statistical analysis shows significant differences in the growth of the colony when the endophytes were plated in dual cultures with the pathogen compared to the control treatment (p-value=0.024). However, only *C. aureum* was able to reduce the growth of the colony of *F. circinatum* in PDA. No reduction was achieved by any of the endophytes when the media was amended with chitosan and propolis (Fig 9).

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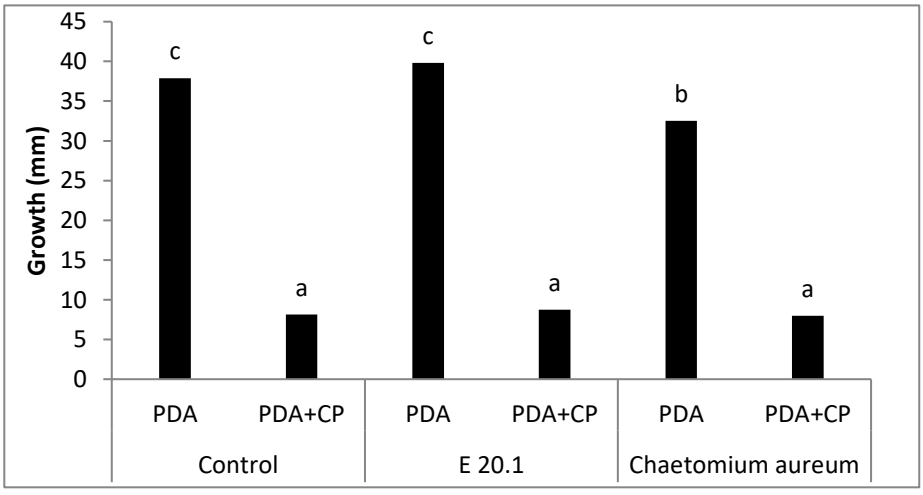

Fig. 9. Growth of the colony of *F. circinatum* in the two media cultures depending on the endophyte used in the confrontations. Letters (a–c) denote significant differences ($p < 0.05$) among all columns.

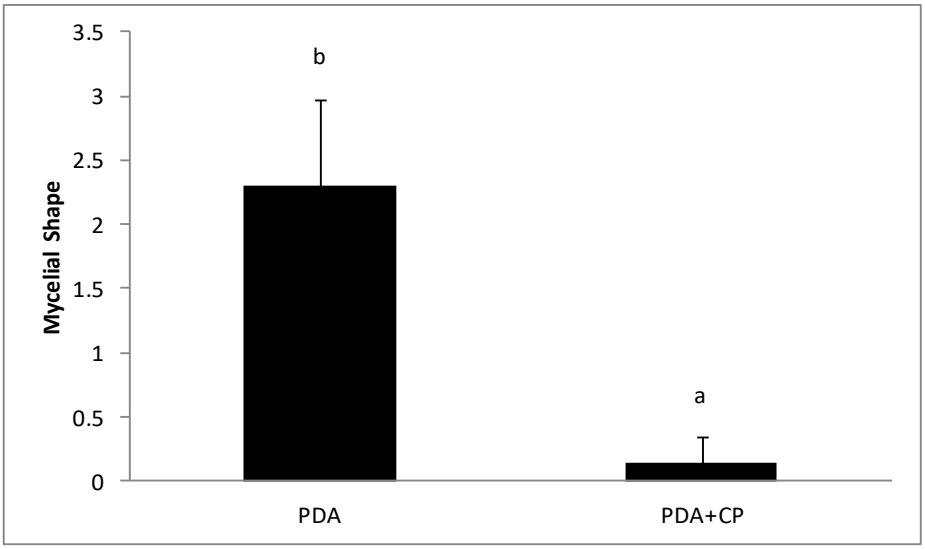

Fig. 10. Values of the mycelial shape indicator for the two media cultures tested Letters (a-b) denote significant differences ($p < 0.05$) among all columns.

b. In Vivo antagonism

The AUDPC was different between the inoculated and the control seedlings (p-value<0.001). The value of this variable was almost five times higher in the inoculated seedlings that in the control. The endophytes inoculated did not produce a significant effect on the progress of the disease, since the seedlings inoculated with the endophytes and the control seedlings (inoculated with *F. circinatum* but not with the endophytes) showed similar values (p-value=0.128). However, the treatment of the seedlings with the chitosan and propolis had a significant effect on the variable AUDPC. The seedlings treated with the mix showed more symptoms of the disease that the non-treated seedlings (p-value=0.005, Fig. 11).

Regarding the survival analyses performed, no clear effect of the endophytes or the chitosan-propolis on the survival of the seedlings were observed. Among the seedlings inoculated with *F. circinatum*, the lowest survival probability was obtained by the treatment in which the seedlings were treated with the endophyte HP47 (*C. aureum*) and with the chitosan-propolis, followed by the seedlings treated with only chitosan-propolis. On the other hand, when the seedlings were treated with *C. aureum* but not with chitosan-propolis, the survival probability was the highest among the *F. circinatum*-infected seedlings (Fig.12).

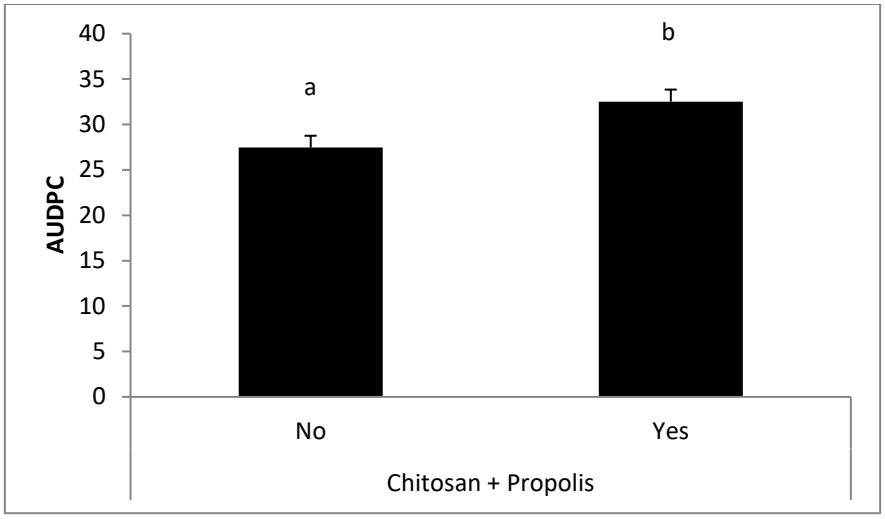

Fig. 11. Area Under Disease Progress Curve (AUDPC) of each treatment of the *P. radiata* seedlings. (P = 0.005).

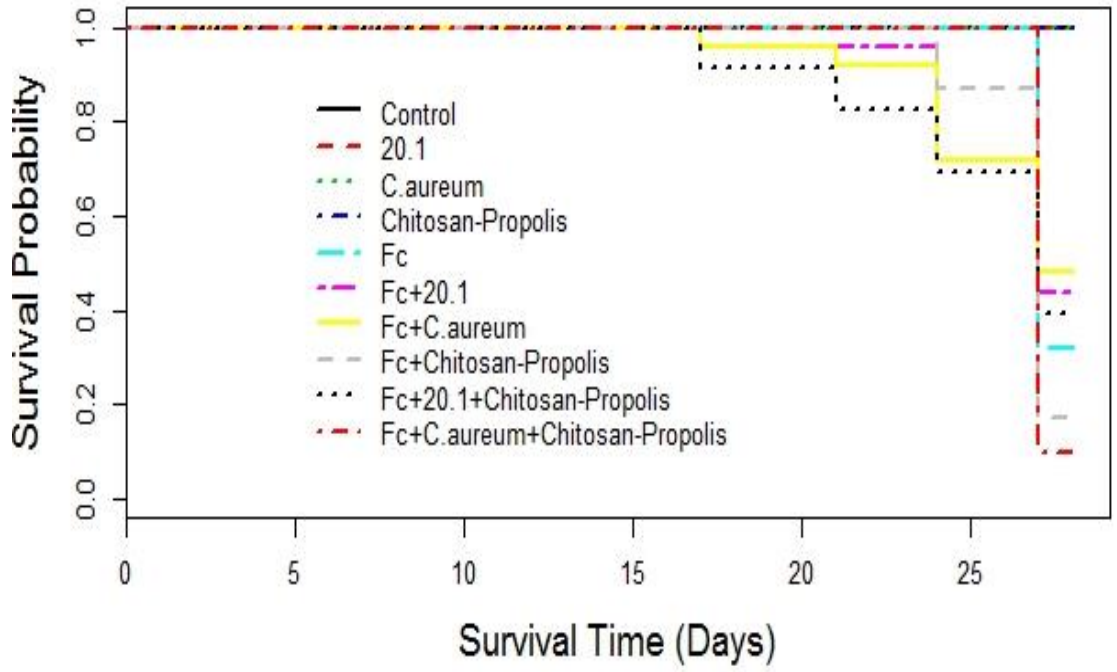

Fig. 12. Survival curves for the different treatments tested in the experiment..

4 Discussion

In Spain, after official detection of the pathogen *F. circinatum*, a royal declaration was announced to eradicate and control the pathogen. In this document it was stated that the planting of *Pinus* spp. and *Pseudotsuga menziesii* are forbidden in the affected areas (Ministerio de Agricultura Pesca y Alimentacion, 2006). Production of timber in some of the infected provinces has decreased significantly and nursery losses have also been substantial (Martínez-Álvarez et al., 2012). However, some nurseries in Spain continue with the production of Monterey pine seedlings despite the high risk of infection by *F. circinatum* and its devastating consequences if the pathogen turns up in the nurseries. In this circumstance, the forest owners are now asking for an alternative species to plant on their land (Martínez-Álvarez et al., 2014). As the environment plays an important role in the interaction between the pathogen and the plant, this study tested the susceptibility of broadleaved and conifer species using seedlings in controlled conditions in the laboratory. The results from the laboratory experiment confirmed that Monterey pine is highly susceptible to pitch canker disease, killing ca. 60% of the inoculated seedlings at the end of the assay. Similar results has been obtained and reported by other authors (McCain et al., 1987; Gordon et al., 1998; Landeras et al., 2005; Martínez-Álvarez et al., 2012).

Susceptibility of some other species such as *P. menziesii*, *Q. pyrenaica* or *A. pseudoplatanus* occasionally planted and naturally regenerated in northern Spain to produce timber, is unknown to date. The study of this kind of species, sometimes coexisting with pines, is important due to the possibility that the pathogen can use other non-conifer species as reservoir. Some studies in California and in South Africa indicate that *F. circinatum* is capable of infecting grasses as a symptomless endophyte (Swett & Gordon, 2012; Swett et al, 2014). Thus, to the authors' knowledge, this is the first time a trial has been carried out to test the effect of *F. circinatum* on *Q. pyrenaica* and *A. pseudoplatanus*. Regarding the species *P. menziesii*, symptoms of disease appeared, but the statistical analysis did not find significant differences between the inoculated and the control seedlings. The species *Q. pyrenaica* and *A. pseudoplatanus* did not show symptoms of the disease and therefore we can say they are not susceptible to *F. circinatum*. However, when the pathogen was reisolated from *A. pseudoplatanus* inoculated seedlings, it was found at two centimetres

beyond the inoculation point in two of the five analysed seedlings. This means that *F. circinatum* was able to live and even progress inside the tissues of the oak. In the case of the species *A. pseudoplatanus*, the pathogen only was reisolated from the inoculation point, so the only thing we can assure is that the inoculum of the pathogen is able to survive there, but the progress inside this species has not been proved.

This study explores the effectiveness of biological control methods against *F. circinatum* in vitro and in vivo, over Monterey pine seedlings. The effect of the endophyte *C. aureum* was promising in the in vitro experiment, reducing significantly the growth of the pathogen on PDA. Martínez-Álvarez et al (2016) reported similar effect over the colony of the pathogen in vitro. However the endophyte 20.1 did not exerted a significant effect on the growth or the shape of the colony of the pitch canker pathogen. On the other hand, the effect of the natural compounds chitosan and propolis was also tested in vitro. In this case, the growth of the colony of the pathogen was significantly reduced, and therefore the antifungal effect of the compounds ratified (Silva-Castro et al., 2017).

Regarding the experiment perfomed in vivo on the *P. radiata* seedlings, the results showed that the inoculation with the endophytes *C. aureum* and 20.1 did not produce a significant effect on the reduction of the symptoms caused by *F. circinatum*. This is the first time the endophyte 20.1 is tested against this pathogen, but promising results were obtained against *Gremmeniella abietina*, (Romeralo et al, 2015). On the other hand, the endophyte *C. aureum* was able to reduce the AUDPC of the *P. radiata* seedlings in an experiment performed in field conditions (Martínez-Álvarez et al, 2016). Nonetheless, promising results in the in vitro assay are not always good indicators of positive antagonistic effects in vivo. Further experiments are needed to clarify the effect of this endophyte against the pitch canker pathogen.

The effect of the chitosan and propolis in vivo was also tested. Contrary to the endophytes, a significant effect of these natural products was observed over the *P. radiata* seedlings. However, these compounds exerted an opposite effect to that expected.. Seedlings treated with the chitosan and propolis showed more symptoms and therefore higher value of the variable AUDPC than the non-treated. Besides, the probability of survival was lower when

we applied the products to the seedlings that in the case of the treatments without these products. On the contrary, other studies reported a resistance in pine species induced by chitosan (Reglinski et al, 2004; Fitza et al, 2013). However, this is the first time propolis has been used together with chitosan on seedlings against this pine disease. It would be interesting to perform new inoculation experiments to discover the unexpected results obtained here.

It should be borne in mind that biological control in forest diseases is increasingly important, because the use of chemicals to control forest diseases is often not allowed or is expected to be banned in the future Thus, biocontrol may be one of the best options for controlling pitch-canker in *P. radiata* and other tree diseases, e.g. as successfully achieved by the use of *Phlebiopsis gigantea* to control *Heterobasidion annosum* (Sun et al. 2009), in order to protect the health of forests and forest nurseries. Further assays with more isolates and endophyte species are underway in an attempt to discover a fungus that is able to reduce the effects of the pitch canker disease.

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