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Application of Bioactive Coatings Based on Chitosan and Propolis for *Pinus* spp. Protection against *Fusarium circinatum*

Iosody Silva-Castro [1,*], Julio Javier Diez [2,3], Pablo Martín-Ramos [4], Glória Pinto [5], Artur Alves [5], Jesús Martín-Gil [1] and Jorge Martín-García [2,5]

1 Department of Agricultural and Forestry Engineering, ETSIIAA, University of Valladolid, Avenida de Madrid 44, 34004 Palencia, Spain; mgil@iaf.uva.es
2 Sustainable Forest Management Research Institute, University of Valladolid—INIA, Avenida de Madrid 57, 34004 Palencia, Spain; jdcasero@pvs.uva.es (J.J.D.); jorge.martin@ua.pt (J.M.-G.)
3 Department of Plant Production and Forestry Resources, ETSIIAA, University of Valladolid, Avenida de Madrid 44, 34004 Palencia, Spain
4 Department of Agricultural and Environmental Sciences, EPS, Instituto Universitario de Investigación en Ciencias Ambientales de Aragón (IUCA), Universidad de Zaragoza, Carretera de Cuarte s/n, 22071 Huesca, Spain; pmr@unizar.es
5 Department of Biology & CESAM (Centre for Environmental and Marine Studies), University of Aveiro, Campus Universitario de Santiago, 3810-193 Aveiro, Portugal; gpinto@ua.pt (G.P.); artur.alves@ua.pt (A.A.)
* Correspondence: iosody.silva@alumnos.uva.es

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Abstract: Pine pitch canker (PPC) is a major threat to pine forests worldwide because of the extensive tree deaths, reduced growth, and degradation of timber quality caused by it. Furthermore, the aggressive fungus responsible for this disease (*Fusarium circinatum*) can also infect pine seeds, causing damping-off in young seedlings. This study proposes an approach based on coating treatments consisting of natural products to ensure seed protection. Seeds from two pine species (the most sensitive to this disease, *Pinus radiata* D. Don, and a more resistant one, *Pinus sylvestris* L.) were coated with single and binary mixtures of low and medium molecular weight chitosan and/or ethanolic-propolis extract. The germination rate, pre- and post-emergence mortality, total phenolic content, and radical scavenging activity were assessed. All treatments, and especially the one based on chitosan oligomers, had a beneficial impact on *P. sylvestris* seedlings, significantly enhancing survival rates and displaying a positive influence on the total phenolic content and on the seedlings' radical scavenging activity. Conversely, non-significant negative effects on germination percentages were observed in the case of *P. radiata* seeds. The proposed treatments show promise for the protection of *P. sylvestris* seedlings against PPC.

Keywords: antifungal; antioxidant; natural coating; seed protection; total phenolic content

1. Introduction

Fusarium circinatum Nirenberg & O'Donnell is a quarantine fungus according to the European and Mediterranean Plant Protection Organization (EPPO) [1] that causes pine pitch canker (PPC) and which has been deemed as one of the most damaging pathogens for *Pinus* spp. throughout the world [2]. In forest nurseries, *F. circinatum* causes pre- and post-emergence damping-off, wilting of seedlings, shoot and tip dieback, and it finally leads to the death of the infected seedlings [3]. *F. circinatum* can be found in nurseries of North and South America, South Africa, Asia, and Southern Europe [4]. The use of seeds from orchards poses a serious threat of spread of this fungus to nurseries

worldwide [5]. At present, there are no effective means of controlling PPC in nurseries and forest plantations. An integrated management plan should therefore include both adequate quarantine measures and appropriate nursery and silvicultural management strategies. Thus, the implementation of seed protection in nursery health practices would be of paramount importance.

Seed-coating technology may act not only as a phytosanitary against pests and diseases but may also enhance germination rates and crop yield [6]. Chemicals such as imidacloprid and tebuconazole [7,8] have been extensively used as coatings for seeds, but nowadays their use in forests is highly restricted (Directive 2009/128/EC). Consequently, natural substances and biological agents—such as starch [9] and *Trichoderma* spp. [10,11], respectively—are receiving increasing attention as environmentally friendly alternatives [12,13].

Amongst these natural products, chitosan and propolis have shown great promise for plant protection purposes, and, in particular, against *F. circinatum* [14–16]. Chitosan, obtained from chitin's deacetylation, is an organic polymer with a cationic character, which confers numerous physicochemical and biological properties, such as copolymerization, filmogenicity, biocompatibility, biodegradability, and also antibiotic properties [17–20]. In turn, propolis is a chemically very complex resinous bee product with many biological properties [21]. Due to its composition, mainly flavonoids and phenolic acids, it is able to alter membrane permeability and inhibit protein synthesis in microorganisms [22].

For agricultural applications, chitosan is applied as an elicitor (inducer of plant resistance) and antifungal product because of its ability to induce the synthesis of phenolic compounds [23], which are involved in tolerance mechanisms against biotic or abiotic stressors [24]. Among the large number of phenolic antioxidants, flavonoids, for instance, can directly inhibit microbial enzymes production [25]. Other specific flavonoids, such as anthocyanins, are known to increase the antioxidant activity, reducing the susceptibility to fungi [26]. The total phenolic content (TPC) and/or the radical scavenging activity (RSA) are properties commonly analyzed in order to identify responses caused by elicitors in plants [27,28].

The molecular weight of chitosan plays a key role in its fungicide properties [29], in such a way that low molecular weight chitosan (i.e., oligomers) is more effective at inducing a set of plant defense responses than its higher molecular weight counterpart (i.e., polymers) [30]. In spite of the fact that chitosan oligomers feature better antimicrobial activity than high molecular weight chitosan [31,32], the later has a higher viscosity [33], which explains why it is more frequently used as a coating [34,35].

In view of the chemical affinity and well-established synergies between chitosan and propolis [36–38], the main aim of the work reported here was to evaluate the protection conferred by bioactive seed coatings based on chitosan with two different molecular weights—medium (CM_{MW}) and low (CL_{MW})—and propolis ethanolic extract (PEE) composites against *F. circinatum* in the relatively resistant *Pinus sylvestris* L. and in the highly susceptible *Pinus radiata* D. Don.

2. Materials and Methods

2.1. Fungal and Plant Materials

The *Fusarium circinatum* isolate FcCa6 used in this study was obtained from the collection of the Forest Entomology and Pathology Laboratory at the University of Valladolid, Spain [39–43]. Plant material consisted of seeds of *P. radiata* and *P. sylvestris* (see provenance in Table 1).

Table 1. Provenance of plan material.

Seed Species	Provenance	Provided by
Pinus radiata (Monterey pine)	"Galicia montañas meseta Interior" (Spain)	Consellería do Medio Rural (Xunta de Galicia, Spain)
Pinus sylvestris (Scots pine)	"Sierra de Guadarrama" (Spain)	El Serranillo Nursery (Ministry of Agriculture and Environment, Spain)

2.2. Seed-Coating Preparation

2.2.1. Reagents

In order to obtain the coating material, medium molecular weight chitosan powder, purchased from Hangzhou Simit Chemical Technology Co. (Hangzhou, China), and propolis with a content of poly-phenols and flavonoids of ca. 10% (*w*/*v*) from Burgos (Spain) were used. High specific surface (70–85 m^2/g) halloysite in powder form, from Dunino mine, with reduced iron content (ca. 5%), was purchased from Intermark (Gliwice, Poland). All other reagents (viz., acetic acid, hydrochloric acid, hydrogen peroxide, ethanol, Tween 80, Folin–Ciocalteu reagent, 2,2-diphenyl-picrylhydrazyl, etc.) were of analytical grade and were purchased from Sigma-Aldrich Química S.L. (Madrid, Spain).

2.2.2. Preparation of the Seed Coating Solutions

Due to differences in viscosity, and therefore in the adhesion to the seed surface, chitosan with two different molecular weights was assessed. The medium molecular weight chitosan (CM_{MW} 60–130 kDa) was prepared by dissolving 2 g of commercial chitosan in 100 mL of acetic acid solution (1% *v*/*v*) under constant stirring at 60 °C for 2 h until its complete dissolution. To obtain the low molecular weight chitosan (CL_{MW} 20 kDa), it was necessary to add hydrogen peroxide (0.3 M) to the chitosan solution obtained in the previous step, keeping the same conditions until a brown and less viscose solution was obtained after 1 h [44]. Propolis ethanolic extract (PEE) composites were prepared by introducing the finely grinded resin into a hydroalcoholic solution (7:3 *v*/*v*). After stirring for 72 h at room temperature, the insoluble particles were filtered [45].

To obtain the first composite (CM_{MW}-PEE), Tween 80 was added dropwise to a 10 $mg \cdot mL^{-1}$ chitosan solution, followed by the addition of 1 $mg \cdot mL^{-1}$ of propolis solution. The mixture was sonicated with a probe-type UIP1000hdT ultrasonicator (Hielscher, Teltow, Germany; 1000 W, 20 kHz) for 3 min in cycles of 1 min with sonication and 1 min without sonication to keep the temperature below 40 °C [37]. To obtain the second composite (CL_{MW}-PEE), a similar process was followed, albeit replacing Tween 80 with halloysite, a natural clay innocuous to seeds and fungus. Halloysite was added to the less viscous solutions (CL_{MW}, PEE and CL_{MW}-PEE) in order to improve their adherence to the surface of the seeds.

2.2.3. Seed Coating Application

Prior to coating application, seeds underwent the following pre-germination procedure according to Martín-García et al. [40]: they were initially soaked in water for 24 h (renewing the water after 12 h), followed by soaking in hydrogen peroxide (3%) for 15 min, triple-washing with sterile distilled water, and an immersion in sterile distilled water for another 30 min (in order to clear away any remaining hydrogen peroxide). Subsequently, the seeds were placed in a laminar flow hood in order to dry them.

Six treatments were applied: (1) Control (sterile water), (2) CM_{MW}, (3) CL_{MW}, (4) PEE, (5) CM_{MW}-PEE, and (6) CL_{MW}-PEE. Halloysite (1 g to 100 mL of solution) was applied in treatments 3, 4, and 6. Then, the seeds were dried again to form a film on their surface (Figure 1) and were kept in sterile flasks until sowing. Seventy seeds (replicates) of each pine species were prepared per treatment (i.e., a total of 840 seeds).

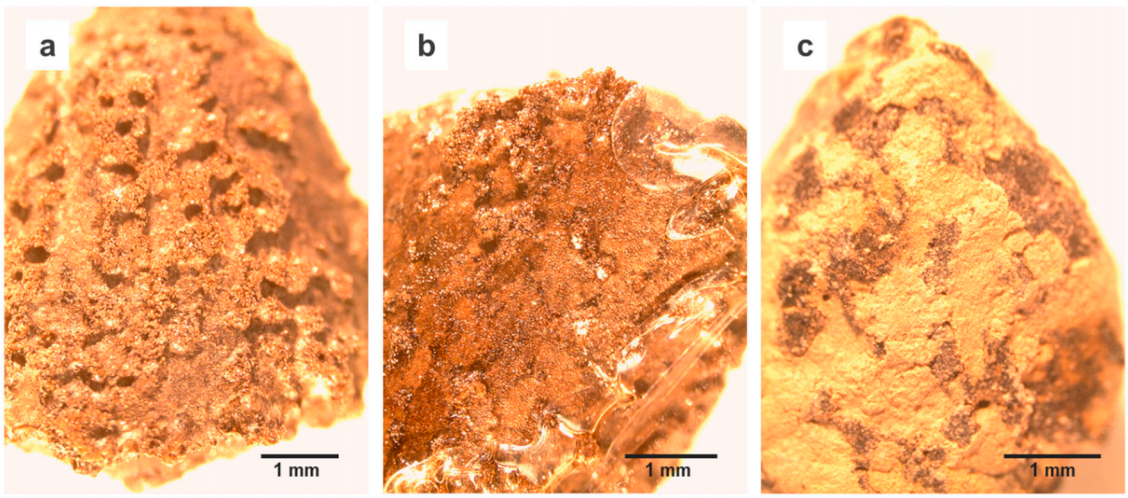

Figure 1. Film formation on seed surface of *P. radiata* seeds: (**a**) control (sterile water), (**b**) coating based on medium molecular weight chitosan (CM_{MW}) and (**c**) coating based on low molecular weight chitosan (CL_{MW}) with halloysite.

2.3. Pathogenicity Tests

Following the procedure presented in Martín-García et al. [41], a spore suspension of *F. circinatum* (Fc) was cultured on potato dextrose broth (PDB). Five mycelial agar plugs (5 mm in diameter) were added to 1 L of PDB and were placed on an orbital shaker at 180 cycles for 24 h at 25 °C. After that, the liquid medium was filtered twice through sterile cheesecloth to remove hyphae and the spore concentration was adjusted to 1×10^3 spores·mL^{-1} with a Neubauer hemocytometer.

Seventy seeds per each type of coating (plus 70 without coating) and per pine species were individually sown in germination trays (96 mL) containing a twice-autoclaved (105 kPa, 120 °C, 30 min) mixture of peat and vermiculite (1:1, *v*/*v*). For half of the seeds with each type of coating, the spore suspension of *F. circinatum* was added to the substrate when the seeds were sown. The other half of the seeds were mock-inoculated with sterile distilled water. Thus, the experimental design consisted of twelve treatments: (*i*) control, (*ii*) CM_{MW}, (*iii*) CL_{MW}, (*iv*) PEE, (*v*) CM_{MW}-PEE, (*vi*) CL_{MW}-PEE, (*vii*) Fc, (*viii*) CM_{MW}-Fc, (*ix*) CL_{MW}-Fc, (*x*) PEE-Fc, (*xi*) CM_{MW}-PEE-Fc, and (*xii*) CL_{MW}-PEE-Fc.

Incubation of the germination trays was conducted in a growth chamber under controlled conditions (temperature: 21.5 °C; photoperiod: 16/8 h light/dark). They were watered every two days, with equal water doses, all over the period of study. Seed germination and subsequent seedling mortality were monitored on a daily basis.

2.4. Determination of Total Phenolic Content (TPC) and Radical Scavenging Activity (RSA)

The extracts were obtained according to the following process: four asymptomatic seedlings were collected from each of the trays 30 days after the sowing date, including control (*i*) and inoculated treatments (*vii*) to (*xii*). These seedlings were dried (40 °C, 7 days) and grinded. Then, 20 µg of each sample in powder form was added to 2 mL of methanol (70%, vol.) acidified with a few drops of HCl (1 M). The mixture (methanol and sample) was kept in shaking condition for 2 h and filtered to get the extracts [27] that were used in both analyses (TPC and RSA).

The TPC was evaluated with a modified Folin–Ciocalteu procedure [46]: firstly, 100 µL of the sample methanolic extract was mixed with 450 µL of distilled water and 50 µL of Folin–Ciocalteu reagent. After 10 min, 400 µL of Na_2CO_3 was added and the samples were kept in dark for 90 min. The absorbance was measured at 765 nm using a Thermo Scientific Multiscan Go Microplate Spectrophotometer (Waltham, MA, USA). A calibration curve was prepared with standard gallic acid ($y = 0.0407x - 0.0595$; $r^2 = 0.99$) and used to express the results as gallic acid equivalents (GAE, in mg of gallic acid per mL of extract).

The RSA was determined by the 2,2-diphenyl-picrylhydrazyl (DPPH) method, according to the procedure described by Chiang et al. [47], with some modifications. Briefly, 50 µL DPPH radical solution (1×10^{-4} M) was added to 450 µL of the sample methanolic extract, and the reaction mixtures were kept at room temperature for 30 min. Absorbance was recorded at 517 nm. The radical scavenging activity was expressed as a percentage (RSA%) relative to the control, using the following equation:

$$RSA\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \quad (1)$$

where A_{blank} is the absorbance of the blank (distilled water) and A_{sample} is the absorbance of the sample extracts.

2.5. Statistical Analyses

All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria). Chi-square tests (χ^2) were carried out using the mock-inoculated treatments—(*i*) to (*vi*)—to test the effect of the coatings on germinative capacity. Likewise, chi-square tests (χ^2) were carried out using the control (*i*) and inoculated treatments—(*vii*) to (*xii*)—to test the protective effect of seed coatings on the pre-emergence mortality caused by *F. circinatum*. To prevent overestimation of statistical significance for small data, Yates' correction for continuity was applied for counts smaller than 5. To test the post-emergence mortality up to the end of the experiment (when no seedling from Fc treatment (*vii*) was alive, 40 and 30 days after sowing for *P. sylvestris* and *P. radiata*, respectively; in the case of *P. radiata*, the last four living seedlings were removed to carry out the TPC and RSA analyses), a survival analysis based on the Kaplan–Meier non-parametric estimator [48] was carried out using "Survival" package [49]. "Sirvfit" and "Survdiff" functions, also available in the same package, were used to create survival curves and to analyze the differences between the curves, respectively. Analyses of variance (ANOVAs) and Tukey's HSD (honestly significant difference) post-hoc test were carried out to assess the effect on TPC and RSA of seedling from control (*i*) and inoculated with *F. circinatum* treatments—(*vii*) to (*xii*)—as a function of the seed coatings at 30 days after sowing. These analyses were only performed in the pine species (*P. sylvestris*) in which changes on mortality rates as a result of seed coating were demonstrated in the previous step. All analyses were performed using R software environment (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Germination Test

Data on the germination percentage (GP) for each of the species and coatings is shown in Figure 2. The highest GP (85.7%) for *P. sylvestris* was attained for the control and PEE treatments. The use of chitosan, either individually or in combination with PEE, did not enhance the GP. In fact, CM_{MW} and CM_{MW}-PEE treatments led to lower GP values (60% and 62.9%, respectively) than that of the control treatment (Figure 2a). In the case of *P. radiata* seeds, the use of chitosan and propolis did not enhance the GP either, and the binary CL_{MW}-PEE composite decreased the GP vs. the control treatment (Figure 2b). No significant differences in terms of GP were found between Monterey pine and Scots pine ($\chi^2 = 1.43$, $p = 0.23$).

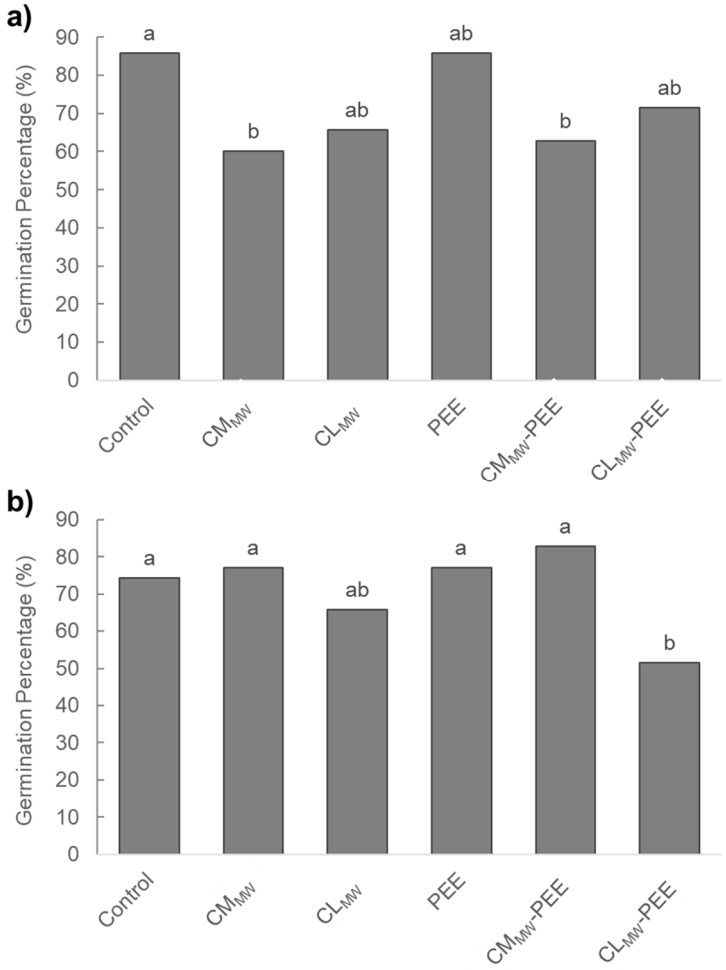

Figure 2. Germination percentage (GP) of (**a**) *P. sylvestris* and (**b**) *P. radiata* seeds coated with medium and low molecular weight chitosan (CM_{MW} and CL_{MW}, respectively) and/or with propolis ethanolic extract (PEE) at 40 and 30 days after sowing, respectively. Averages with the same letter were not significantly different according to the Chi-square test (χ^2) ($\alpha \leq 0.05$).

3.2. Pathogenicity Test

Inoculations with *F. circinatum* did not cause pre-emergence mortality for either *P. sylvestris* ($\chi^2 = 0.85$, $p = 0.36$) or for *P. radiata* ($\chi^2 = 0.85$, $p = 0.36$). Nonetheless, survival analyses demonstrated significant differences in post-emergence mortality among treatments in *P. sylvestris* ($\chi^2 = 49.7$, $p < 0.001$). No seedlings of the Fc treatment survived beyond 40 days after inoculation (dai), whereas no mortality was recorded in the control seedlings. All coating treatments improved the survival of *P. sylvestris* inoculated seedlings, resulting in survival rates of over 50% for the CM_{MW}-Fc, CL_{MW}-Fc, and CM_{MW}-PEE-Fc treatments. Chitosan-only treatments (CL_{MW}-Fc and CM_{MW}-Fc) showed similar protection efficacies as PEE-Fc ($\chi^2 = 1.4$, $p = 0.23$; and $\chi^2 = 1.3$, $p = 0.26$, respectively). However, in the CM_{MW}-PEE-Fc and CL_{MW}-PEE-Fc binary composites, the addition of PEE did not improve the survival rate in comparison to CM_{MW}-Fc and CL_{MW}-Fc ($\chi^2 < 0.001$, $p = 0.88$; and $\chi^2 = 3.2$, $p = 0.07$, respectively) (Figure 3a).

Mortality of inoculated *P. radiata* seedlings was faster than that of *P. sylvestris* seedlings. In fact, just four seedlings from the non-coated seeds inoculated with *F. circinatum* survived until 30 dai. Significant differences in post-emergence mortality were found between control treatment and inoculated seedlings, regardless of the coating treatment ($\chi^2 = 67.7$, $p < 0.001$). However, none of the coating treatments was able to significantly improve the survival of the inoculated seedlings, neither the individual components CM_{MW}-Fc, CL_{MW}-Fc, and PEE-Fc ($\chi^2 = 1.7$, $p = 0.19$; $\chi^2 = 0.2$, $p = 0.64$; and

$\chi^2 = 0.2$, $p = 0.66$, respectively) nor the CM_{MW}-PEE-Fc and CL_{MW}-PEE-Fc binary composites ($\chi^2 = 2.5$, $p = 0.11$; and $\chi^2 = 0.2$, $p = 0.69$, respectively) (Figure 3b).

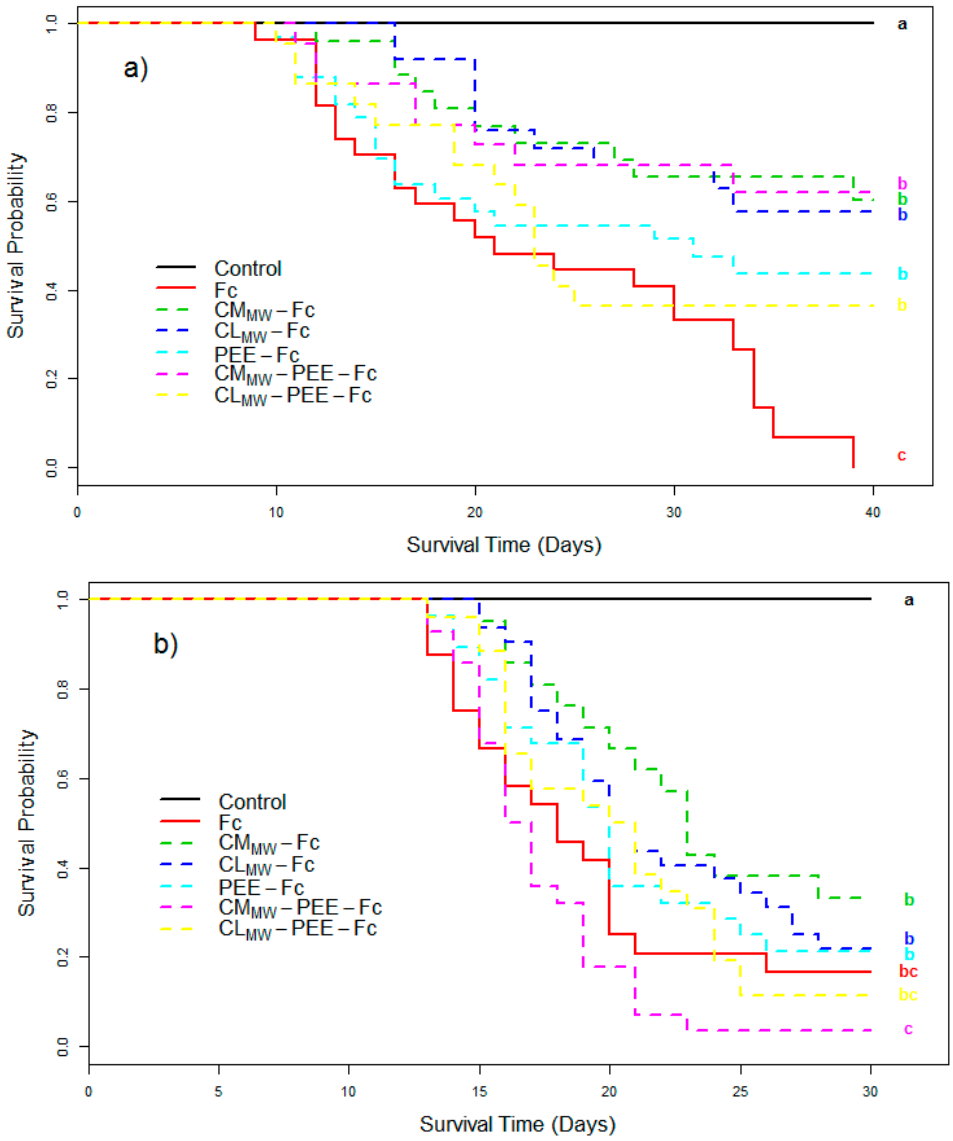

Figure 3. Plot of survival probability, determined using the Kaplan-Meier estimate of the survival function, for (**a**) *P. sylvestris* and (**b**) *P. radiata* seedlings inoculated with *Fusarium circinatum* (Fc) as a function of the seed coating treatments. CM_{MW} and CL_{MW} stand for medium and low molecular weight chitosan, respectively, PEE stands for propolis ethanolic extract. No mortality was registered for mock-inoculated and coating treatments (i.e., (*ii*) to (*vi*)). These curves were not shown to avoid multi-overlaps with the curve of the control treatment. Averages with the same letter were not significantly different according to the Kaplan–Meier estimator ($\alpha \leq 0.05$).

3.3. Total Phenolic Content and Radical Scavenging Activity

The TPC varied significantly in *P. sylvestris* seedlings as a function of the coating treatment ($F = 4.99$, $p < 0.01$). The mean TPC of the control treatment was 6.8 $mg \cdot mL^{-1}$ of GAE, which was significantly higher than the value obtained in the Fc treatment (2.7 $mg \cdot mL^{-1}$), evidencing a significant TPC reduction caused by the pathogen (Figure 4a). TPC values did not change in inoculated-coated seeds in comparison to the control treatment, with the exception of the CM_{MW}-Fc treatment. In particular, the CL_{MW}-Fc treatment led to a TPC value comparable to that of the control treatment.

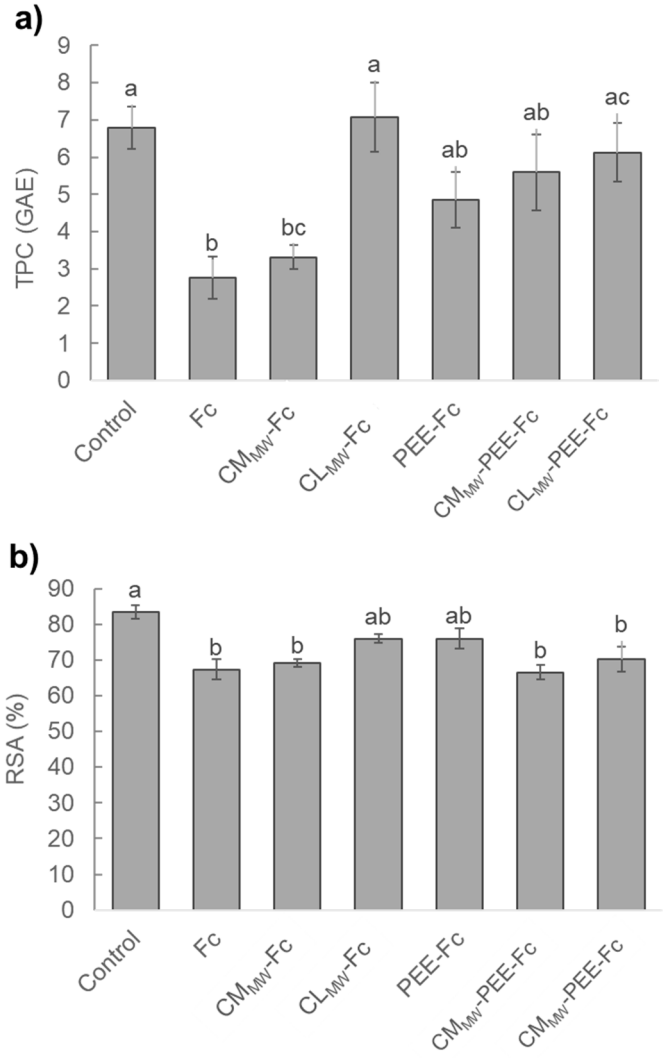

Figure 4. (**a**) Total phenolic contents (TPC) in gallic acid equivalents (GAE, mg·mL^{-1}) and (**b**) percentage of radical scavenging activity (RSA%) of *P. sylvestris* seedlings from control (*i*) and inoculated with *Fusarium circinatum* (Fc) treatments—(*vii*) to (*xii*)—as a function of the seed coatings at 30 days after sowing. CM_{MW} and CL_{MW} stand for medium and low molecular weight chitosan, respectively, PEE stands for propolis ethanolic extract. Averages with the same letter were not significantly different according to the Tukey's HSD test ($\alpha \leq 0.05$).

The negative effect caused by the pathogen was also evidenced in the low antioxidant capacity of inoculated seedlings in comparison to the high antioxidant activity shown by the control treatment (83.5%). This RSA value was just preserved by the CL_{MW}-Fc and PEE-Fc treatments, which did not vary significantly in comparison with the control treatment (Figure 4b). However, CM_{MW} and the binary composites did not succeed in reverting the aforementioned negative effect exerted by the pathogen.

4. Discussion

At least 60 species of *Pinus* along with *Pseudotsuga menziesii* (Mirb.) Franco are known to be susceptible to PPC. Amongst them, *P. radiata* is recognized as the most susceptible [3], while *P. sylvestris* has also been reported to present a high susceptibility [41,43,50–53]. In this study, seed coatings based on chitosan—with two different molecular weights—and propolis were applied in order to test if these bioactive products were able to confer resistance against *F. circinatum* in the two species mentioned above.

It is well-known that chitosan application on seeds is beneficial in order to enhance the germination rate and/or yield in many agricultural crops, such as maize [54], wheat [6], soybean [55], canola [10], artichoke [35], or ajowan [56]. However, the application of chitosan and propolis did not enhance the germination rates in the present study. In fact, germination decreased with CM_{MW} and CM_{MW}-PEE in *P. sylvestris* and with CL_{MW}-PEE in *P. radiata* seeds. This discrepancy may actually be due to the type of species/provenance, since a previous study on *P. sylvestris* seeds treated with a commercial chitosan product did not result in an increase in seedling emergence percentage either [57]. Likewise, in an in vitro test on orchid *Dendrobium bigibbum* seeds with low viability (32.6%) that were treated with high and low MW chitosan, an improvement in seed germination with respect to the control did not occur either. There were no significant differences between polymers and oligomers of chitosan [23]. Differences among studies may also be ascribed to the coating process used, the type and concentration of chitosan [57], and the inoculum dose [16]. It should also be taken into consideration that, although chitosan's excellent filmogenic properties are well-established (it forms a semipermeable film on the seed surface which can maintain the seed humidity and absorb moisture from the soil [55]), the exact mechanism through which chitosan promotes germination is still unknown.

No pre-emergence mortality was observed in either *P. sylvestris* or in *P. radiata* seedlings as a result of *F. circinatum* inoculation. Conversely, Martínez-Álvarez et al. [43] reported lower emergence rates of both *P. sylvestris* and *P. radiata* when their substrate was inoculated with *F. circinatum* in comparison with controls. Differences on pre-emergence rates of seeds placed in infested substrates have been previously related to the genetic effect of pines [40]. However, in this case, the discrepancy may be due to the inoculum dose, since Martínez-Álvarez et al. [43] tested Scots pine seeds with the same provenance and *F. circinatum* isolate, but the inoculum dose was 1×10^6 spores·mL^{-1} instead of 1×10^3 spores·mL^{-1} applied in the present study. Thus, a higher inoculum dose may speed up the infection process, killing the germlings even before emergence.

Inoculations with *F. circinatum* caused post-emergence mortality in both *P. sylvestris* and *P. radiata*, which concurs with the results obtained by Martínez-Álvarez et al. [43]. Post-emergence mortality was reduced as a result of seed coating only in *P. sylvestris*, the species which featured a slightly lower susceptibility to *F. circinatum*, as noted above. Survival rates higher than 50% were found for seeds coated with chitosan (both CM_{MW} and CL_{MW}) at 40 dai, whereas all inoculated non-coated seedlings (Fc treatment) died. Although several studies have demonstrated that low molecular weight chitosan is more effective at inducing a set of defense responses and antifungal protection than higher molecular weight chitosan in crops [30,32], no significant differences as a function of the molecular weight were found in this study. This inconsistency could be due to the plant material, since conifers seem to show a different pattern: for instance, Fitza et al. [15], who evaluated chitin and chitosan as inducers of resistance to *F. circinatum* in *P. patula* seedlings, found that chitin—with higher molecular weight than chitosan—resulted in a higher percentage of healthy stems for lesion lengths caused by the artificial inoculation of the fungus. The survival rate also increased with propolis application. This antifungal effect of seed coatings based on propolis extracts has been already demonstrated for the postharvest treatment of papaya [45] and chilli [58] during storage. In vitro experiments demonstrated that the binary combination of chitosan and propolis was an efficient combination to deal with *F. circinatum* [14], *Diplodia seriata* [37], and *Hemileia vastatrix* [59]. Likewise, this binary combination has also been successfully used for the protection of food packaging materials [60,61] and against foodborne pathogens [36]. Nevertheless, in this study, the binary combinations did not improve the post-emergence mortality vs. the individual application of chitosan or propolis. It may be possible that the activity of propolis had been reduced due to the degradation of the extracts along the assay and that constant irrigation had washed the PEE coating. On the other hand, seed coatings based on chitosan and/or propolis failed to protect the *P. radiata* seedlings against *F. circinatum*. This seems to point out that the quick progression of the infection in this species (the most susceptible species to PPC [3]) may mean that the activation of induced resistances takes place too late.

In order to determine the influence of chitosan and propolis coatings on the host response mechanisms against the pathogen, TPC and RSA were measured on the seedlings at 30 dai. A decline in TPC and RSA was observed in the Scots pine seedlings infected by *F. circinatum*, which is consistent with the results obtained in *P. halepensis* seedlings inoculated with *Gremmeniella abietina* [27]. However, such an effect was not observed when the seeds were coated, except for the CM_{MW} treatment. This suggests that CL_{MW} and, to a lesser extent, PEE and the binary combinations would prevent phenolic compounds from decreasing when seedlings are infected by *F. circinatum*. Similarly, coatings based on chitosan showed an increase in TPC on tomato fruit infected by *Botrytis cinerea* and *Penicillium expansum* [62,63]. Although it is well known that phenolic content is an indicator of the activation of defense mechanisms in plants [28], there are other frequent responses that include increase of peroxidase, glucanase, and chitinase activity; higher lignin production; existence of toxic proteins and inhibitors of enzymes; among others [24]. Actually, chitosan has been reported as an elicitor of plants, with capacity to promote the production of phenolic compounds (e.g., the induction of phenylalanine ammonia-lyase) [23]. However, it has also been shown that chitosan affects many other plant responses, increasing the production of hydrogen peroxide, the activities of chitinase, the transcription of defense-related genes β-1,3-glucanase and chitinase, and the accumulation of pathogen-related protein (PR1) [30].

On the other hand, the antioxidant capacity of chitosan and its oligomers has been well-studied [44,64], as well as that of propolis [65], which is well-known in traditional medicine. This capacity to scavenge free radicals is related to the antimicrobial activity; for instance, anthocyanins are commonly induced under stress conditions and upon infection by pathogens in plants [66]. In this work, it was found that chitosan oligomers and propolis maintained the level of antioxidants in Scots pine seedlings under pathogen presence. Other works also found a high level of inhibition of free radicals in fruit exposed to fungus attack [26,34].

Future experiments should be carried out to elucidate the response mechanism that chitosan and propolis activate as seed protectors in the resistance to *F. circinatum* in pines, and to assess the effectiveness of these coatings in combination with other environmentally friendly methods used for seed protection, such as biological control agents (e.g., *Trichoderma* spp.) [40,67] and heat water treatments [68–70]. Other factors, such as the genetic resistance of plant material, including maternal effects and morphological traits of seeds, could also be studied [71,72].

5. Conclusions

The study presented herein demonstrated that the application of bioactive coatings based on chitosan and propolis could be helpful for protecting certain *Pinus* species' seeds against *F. circinatum*. In spite of the fact that their efficacy against pine pitch canker was limited in the case of *P. radiata*, which is recognized as the most susceptible species to this pathogen, the coatings significantly reduced the post-emergence mortality of *P. sylvestris* seedlings, resulting in survival rates higher than 50%, while all inoculated non-coated seedlings died at the end of the experiment. Seed coatings also had a positive influence on total phenolic content, leading to similar values to those found in non-inoculated seeds, and helped preserve the seedlings' radical scavenging activity. No significant differences in the germination percentages were observed. Among the various coating treatments proposed, the one based on low-molecular weight chitosan led to the best results, suggesting that more complex formulations including propolis would not be needed, and that medium molecular weight chitosan—despite its higher viscosity—would not be the preferred option. Notwithstanding these promising findings, further studies are needed to confirm the effectiveness of the seed coatings against *F. circinatum* in a wide range of environmental conditions (not only on sterilized substrate), their long-term persistence, and their potential use in combination with other environmentally friendly approaches.

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References

1. European and Mediterranean Plant Protection Organization (EPPO). PM 7/91(1): *Gibberella circinata*. *EPPO Bull.* **2009**, *39*, 298–309. [CrossRef]
2. Bezos, D.; Martínez-Álvarez, P.; Fernández, M.; Diez, J.J. Epidemiology and management of Pine Pitch Canker disease in Europe—A Review. *Balt. For. Int. Sci. J.* **2017**, *23*, 279–293.
3. Wingfield, M.J.; Hammerbacher, A.; Ganley, R.J.; Steenkamp, E.T.; Gordon, T.R.; Wingfield, B.D.; Coutinho, T.A. Pitch canker caused by *Fusarium circinatum*—A growing threat to pine plantations and forests worldwide. *Australas. Plant Pathol.* **2008**, *37*, 319–334. [CrossRef]
4. Gordon, T.R.; Swett, C.L.; Wingfield, M.J. Management of *Fusarium* diseases affecting conifers. *Crop Prot.* **2015**, *73*, 28–39. [CrossRef]
5. Monterey, N.; Nelson, R. The pitch canker epidemic in California. *Plant Dis.* **1085**, *85*, 1128–1139.
6. Zeng, D.; Luo, X. Physiological effects of chitosan coating on wheat growth and activities of protective enzyme with drought tolerance. *Open J. Soil Sci.* **2012**, *2*, 282–288. [CrossRef]
7. Ahmed, N.E.; Kanan, H.O.; Inanaga, S.; Ma, Y.Q.; Sugimoto, Y.S. Impact of pesticide seed treatments on aphid control and yield of wheat in the Sudan. *Crop Prot.* **2001**, *20*, 929–934. [CrossRef]
8. Yang, D.; Wang, N.; Yan, X.; Shi, J.; Zhang, M.; Wang, Z.; Yuan, H. Microencapsulation of seed-coating tebuconazole and its effects on physiology and biochemistry of maize seedlings. *Colloids Surf. B Biointerfaces* **2014**, *114*, 241–246. [CrossRef] [PubMed]
9. Accinelli, C.; Abbas, H.K.; Little, N.S.; Kotowicz, J.K.; Mencarelli, M.; Shier, W.T. A liquid bioplastic formulation for film coating of agronomic seeds. *Crop Prot.* **2016**, *89*, 123–128. [CrossRef]
10. Swaminathan, J.; Van Koten, C.; Henderson, H.V.; Jackson, T.A.; Wilson, M.J. Formulations for delivering *Trichoderma atroviridae* spores as seed coatings, effects of temperature and relative humidity on storage stability. *J. Appl. Microbiol.* **2015**, *120*, 425–431. [CrossRef] [PubMed]
11. Xue, A.G.; Guo, W.; Chen, Y.; Siddiqui, I.; Marchand, G.; Liu, J.; Ren, C. Effect of seed treatment with novel strains of *Trichoderma* spp. on establishment and yield of spring wheat. *Crop Prot.* **2017**, *96*, 97–102. [CrossRef]
12. Iturritxa, E.; Trask, T.; Mesanza, N.; Raposo, R.; Elvira-Recuenco, M.; Patten, C.L. Biocontrol of *Fusarium circinatum* infection of young *Pinus radiata* Trees. *Forests* **2017**, *8*, 32. [CrossRef]
13. Vivas, M.; Martín, J.A.; Gil, L.; Solla, A. Evaluating methyl jasmonate for induction of resistance to *Fusarium oxysporum*, *F. circinatum* and *Ophiostoma novo-ulmi*. *For. Syst.* **2012**, *21*, 289–299. [CrossRef]
14. Silva-Castro, I.; Martín-García, J.; Diez, J.J.; Flores-Pacheco, J.A.; Martín-Gil, J.; Martín-Ramos, P. Potential control of forest diseases by solutions of chitosan oligomers, propolis and nanosilver. *Eur. J. Plant Pathol.* **2017**, *150*, 401–411. [CrossRef]
15. Fitza, K.N.E.; Payn, K.G.; Steenkamp, E.T.; Myburg, A.A.; Naidoo, S. Chitosan application improves resistance to *Fusarium circinatum* in *Pinus patula*. *S. Afr. J. Bot.* **2013**, *85*, 70–78. [CrossRef]

16. Reglinski, T.; Taylor, J.T.; Dick, M.A. Chitosan induces resistance to pitch canker in *Pinus radiata*. *N. Z. J. For. Sci.* **2004**, *34*, 49–58.
17. Kong, M.; Chen, X.G.; Xing, K.; Park, H.J. Antimicrobial properties of chitosan and mode of action: A state of the art review. *Int. J. Food Microbiol.* **2010**, *144*, 51–63. [CrossRef] [PubMed]
18. Silva-Castro, I.; Martín-Ramos, P.; Matei, P.M.; Fernandes-Correa, M.; Hernánez-Navarro, S.; Martín-Gil, J. Eco-friendly nanocomposites of chitosan with natural extracts, antimicrobial agents, and nanometals. In *Handbook of Composites from Renewable Materials*; Thakur, V.K., Thakur, M.K., Kessler, M.R., Eds.; Scrivener Publishing LLC: Beverly, MA, USA, 2017; Volume 8, pp. 35–60. ISBN 978-1-119-22383-2.
19. Verlee, A.; Mincke, S.; Stevens, C.V. Recent developments in antibacterial and antifungal chitosan and its derivatives. *Carbohydr. Polym.* **2017**, *164*, 268–283. [CrossRef] [PubMed]
20. Nunes da Silva, M.; Cardoso, A.R.; Ferreira, D.; Brito, M.; Pintado, M.E.; Vasconcelos, M.W. Chitosan as a biocontrol agent against the pinewood nematode (*Bursaphelenchus xylophilus*). *For. Pathol.* **2014**, *44*, 420–423. [CrossRef]
21. Silva-Carvalho, R.; Baltazar, F.; Almeida-Aguiar, C. Propolis: A complex natural product with a plethora of biological activities that can be explored for drug development. *Evid.-Based Complement. Altern. Med.* **2015**, *2015*, 1–29. [CrossRef] [PubMed]
22. Marghitas, L.A.; Dezmirean, D.S.; Bobis, O. Important developments in recent propolis research. *Evid.-Based Complement. Altern.* **2013**, *2013*, 1–9. [CrossRef] [PubMed]
23. Kananont, N.; Pichyangkura, R.; Chanprame, S.; Chadchawan, S.; Limpanavech, P. Chitosan specificity for the *in vitro* seed germination of two *Dendrobium* orchids (Asparagales: Orchidaceae). *Sci. Hortic. (Amsterdam).* **2010**, *124*, 239–247. [CrossRef]
24. Lattanzio, V.; Lattanzio, V.M.T.; Cardinali, A. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In *Phytochemistry: Advances in Research*; Imperato, F., Ed.; Research Signpost: Kerala, India, 2006; pp. 23–67. ISBN 8130800349.
25. Treutter, D. Significance of flavonoids in plant resistance: A review. *Environ. Chem. Lett.* **2006**, *4*, 147–157. [CrossRef]
26. Zhang, Y.; Butelli, E.; de Stefano, R.; Schoonbeek, H.; Magusin, A.; Pagliarani, C.; Wellner, N.; Hill, L.; Orzaez, D.; Granell, A.; Jones, J.D.G.; Martin, C. Report anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. *Curr. Biol.* **2013**, *23*, 1094–1100. [CrossRef] [PubMed]
27. Romeralo, C.; Santamaría, O.; Pando, V.; Diez, J.J. Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings. *Biol. Control* **2015**, *80*, 30–39. [CrossRef]
28. Benhamou, N. Elicitor-induced plant defence pathways. *Trends Plant Sci.* **1996**, *1*, 233–240. [CrossRef]
29. Rahman, M.H.; Hjeljord, L.G.; Aam, B.B.; Sørlie, M.; Tronsmo, A. Antifungal effect of chito-oligosaccharides with different degrees of polymerization. *Eur. J. Plant Pathol.* **2015**, *141*, 147–158. [CrossRef]
30. Lin, W.; Hu, X.; Zhang, W.; Rogers, W.J.; Cai, W. Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice. *J. Plant Physiol.* **2005**, *162*, 937–944. [CrossRef] [PubMed]
31. Benhabiles, M.S.; Salah, R.; Lounici, H.; Drouiche, N.; Goosen, M.F.A.; Mameri, N. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocoll.* **2012**, *29*, 48–56. [CrossRef]
32. Chien, P.; Sheu, F.; Lin, H. Coating citrus (Murcott tangor) fruit with low molecular weight chitosan increases postharvest quality and shelf life. *Food Chem.* **2007**, *100*, 1160–1164. [CrossRef]
33. Hamdi, M.; Nasri, R.; Hajji, S.; Nigen, M.; Li, S.; Nasri, M. Acetylation degree, a key parameter modulating chitosan rheological, thermal and film-forming properties. *Food Hydrocoll.* **2018**, *87*, 48–60. [CrossRef]
34. Vieira, J.M.; Flores-López, M.L.; de Rodríguez, D.J.; Sousa, M.C.; Vicente, A.A.; Martins, J.T. Effect of chitosan–*Aloe vera* coating on postharvest quality of blueberry (*Vaccinium corymbosum*) fruit. *Postharvest Biol. Technol.* **2016**, *116*, 88–97. [CrossRef]
35. Ziani, K.; Ursúa, B.; Maté, J.I. Application of bioactive coatings based on chitosan for artichoke seed protection. *Crop Prot.* **2010**, *29*, 853–859. [CrossRef]
36. Torlak, E.; Sert, D. Antibacterial effectiveness of chitosan-propolis coated polypropylene films against foodborne pathogens. *Int. J. Biol. Macromol.* **2013**, *60*, 52–55. [CrossRef] [PubMed]

37. Matei, P.M.; Martín-Ramos, P.; Sánchez-Báscones, M.; Hernández-Navarro, S.; Correa-Guimaraes, A.; Navas-Gracia, L.M.; Rufino, C.A.; Ramos-Sánchez, M.C.; Martín-Gil, J. Synthesis of chitosan oligomers/propolis/silver nanoparticles composite systems and study of their activity against *Diplodia seriata*. *Int. J. Polym. Sci.* **2015**, *2015*, 1–11. [CrossRef]
38. Araujo-Rufino, C.; Fernandes-Vieira, J.; Martín-Ramos, P.; Silva-Castro, I.; Fernandes-Côrrea, M.; Matei Petruta, M.; Sánchez-Báscones, M.; Carmen, R.-S.M.; Martín-Gil, J. Synthesis of chitosan oligomers composite systems and study of their activity against *Bipolaris oryzae*. *J. Mater. Sci. Eng. Adv. Technol.* **2016**, *13*, 29–52.
39. Martínez-Álvarez, P.; Alves-Santos, F.M.; Diez, J.J. In vitro and in vivo interactions between *Trichoderma viride* and *Fusarium circinatum*. *Silva Fenn.* **2012**, *46*, 303–316. [CrossRef]
40. Martín-García, J.; Paraschiv, M.; Flores-Pacheco, J.A.; Chira, D.; Diez, J.J.; Fernández, M. Susceptibility of several northeastern conifers to *Fusarium circinatum* and strategies for biocontrol. *Forests* **2017**, *8*, 318. [CrossRef]
41. Martín-García, J.; Lukačevičová, A.; Flores-Pacheco, J.A.; Diez, J.J.; Dvořák, M. Evaluation of the susceptibility of several czech conifer provenances to Fusarium circinatum. *Forests* **2018**, *9*, 72. [CrossRef]
42. Cerqueira, A.; Alves, A.; Berenguer, H.; Correia, B.; Gómez-Cadenas, A.; Diez, J.J.; Monteiro, P.; Pinto, G. Phosphite shifts physiological and hormonal profile of Monterey pine and delays *Fusarium circinatum* progression. *Plant Physiol. Biochem.* **2017**, *114*, 88–99. [CrossRef] [PubMed]
43. Martínez-Álvarez, P.; Pando, V.; Diez, J.J. Alternative species to replace Monterey pine plantations affected by pitch canker caused by *Fusarium circinatum* in northern Spain. *Plant Pathol.* **2014**, *63*, 1086–1094. [CrossRef]
44. Sun, T.; Zhou, D.; Xie, J.; Mao, F. Preparation of chitosan oligomers and their antioxidant activity. *Eur. Food Res. Technol.* **2007**, *225*, 451–456. [CrossRef]
45. Barrera, E.; Gil, J.; Restrepo, A.; Mosquera, K.; Durango, D. A coating of chitosan and propolis extract for the postharvest treatment of papaya (*Carica papaya* L. cv. Hawaiiana). *Rev. Fac. Nac. Agron.* **2015**, *68*, 7667–7678. [CrossRef]
46. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with acid reagents. *Am. J. Enol. Vitic.* **1964**, *16*, 144–158.
47. Chiang, P.S.; Lee, D.J.; Whiteley, C.G.; Huang, C.Y. Extracting antioxidant phenolic compounds from compressional-puffing pretreated *Pinus morrisonicola*: Effects of operational parameters, kinetics and characterization. *J. Taiwan Inst. Chem. Eng.* **2017**, *75*, 70–76. [CrossRef]
48. Kaplan, E.L.; Meier, P. Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **1958**, *53*, 457–481. [CrossRef]
49. Therneau, T.M. A Package for Survival Analysis in S. version 2.38. Available online: https://cran.r-project.org/package=survival (accessed on 25 September 2018).
50. Mullett, M.; Pérez-Sierra, A.; Armengol, J.; Berbegal, M. Phenotypical and molecular characterisation of *Fusarium circinatum*: Correlation with virulence and fungicide sensitivity. *Forests* **2017**, *8*, 458. [CrossRef]
51. Pérez-Sierra, A.; Landeras, E.; León, M.; Berbegal, M.; García-Jiménez, J.; Armengol, J. Characterization of *Fusarium circinatum* from *Pinus* spp. in northern Spain. *Mycol. Res.* **2007**, *111*, 832–839. [CrossRef] [PubMed]
52. Iturritxa, B.E.; Ganley, R.J.; Raposo, R.; García-Serna, I.; Mesanza, N.; Kirkpatrick, S.C.; Gordon, T.R. Resistance levels of Spanish conifers against Fusarium circinatum and Diplodia pinea. *For. Pathol.* **2013**, *43*, 488–495. [CrossRef]
53. Iturritxa, E.; Mesanza, N.; Elvira-Recuenco, M.; Serrano, Y.; Quintana, E.; Raposo, R. Evaluation of genetic resistance in *Pinus* to pitch canker in Spain. *Australas. Plant Pathol.* **2012**, *41*, 601–607. [CrossRef]
54. Peña-Datoli, M.; Isabel Hidalgo-Moreno, C.M.; Arturo González-Hernández, V.; Gabriel Alcántar-González, E.; Dionisio Etchevers-Barra, J. Maize (*Zea mays* L.) seed coating with chitosan and sodium alginate and its effect on root development. *Agrociencia* **2016**, *50*, 1091–1106.
55. Zeng, D.; Luo, X.; Tu, R. Application of bioactive coatings based on chitosan for soybean seed protection. *Int. J. Carbohydr. Chem.* **2012**, *2012*, 1–5. [CrossRef]
56. Mahdavi, B.; Rahimi, A. Seed priming with chitosan improves the germination and growth performance of ajowan. *EurAsian J. Biosci.* **2013**, *76*, 69–76. [CrossRef]
57. Aleksandrowicz-trzcińska, M.; Bogusiewicz, A.; Szkop, M.; Drozdowski, S. Effect of chitosan on disease control and growth of Scots pine (*Pinus sylvestris* L.) in a forest nursery. *Forests* **2015**, *6*, 3165–3176. [CrossRef]
58. Ali, A.; Chow, W.L.; Zahid, N.; Ong, M.K. Efficacy of propolis and cinnamon oil coating in controlling post-harvest anthracnose and quality of chilli (*Capsicum annuum* L.) during cold storage. *Food Bioprocess Technol.* **2013**, *7*, 2742–2748. [CrossRef]

59. Silva-Castro, I.; Barreto, R.W.; Rodríguez, M.C.H.; Matei, P.M.; Martín-Gil, J. Control of Coffee Leaf Rust by chitosan oligomers and propolis. *Agric. Life Life Agric. Conf. Proc.* **2018**, *1*, 311–315. [CrossRef]
60. Rollini, M.; Mascheroni, E.; Capretti, G.; Coma, V.; Musatti, A.; Piergiovanni, L. Propolis and chitosan as antimicrobial and polyphenols retainer for the development of paper based active packaging materials. *Food Packag. Shelf Life* **2017**, *14*, 75–82. [CrossRef]
61. Siripatrawan, U.; Vitchayakitti, W. Improving functional properties of chitosan films to be used as active food packaging by incorporation with propolis. *Food Hydrocoll.* **2016**, *61*, 695–702. [CrossRef]
62. Badawy, M.E.I.; Rabea, E.I. Potential of the biopolymer chitosan with different molecular weights to control postharvest gray mold of tomato fruit. *Postharvest Biol. Technol.* **2009**, *51*, 110–117. [CrossRef]
63. Liu, J.; Tian, S.; Meng, X.; Xu, Y. Effects of chitosan on control of postharvest diseases and physiological responses of tomato fruit. *Postharvest Biol. Technol.* **2007**, *44*, 300–306. [CrossRef]
64. Ngo, D.-H.; Vo, T.-S.; Ngo, D.-N.; Kang, K.-H.; Je, J.-Y.; Pham, H.N.-D.; Byun, H.-G.; Kim, S.-K. Biological effects of chitosan and its derivatives. *Food Hydrocoll.* **2015**, *51*, 200–216. [CrossRef]
65. Zabaiou, N.; Fouache, A.; Trousson, A.; Baron, S.; Zellagui, A.; Lahouel, M.; Lobaccaro, J.M.A. Biological properties of propolis extracts: Something new from an ancient product. *Chem. Phys. Lipids* **2017**, *207*, 214–222. [CrossRef] [PubMed]
66. Landi, M.; Tattini, M.; Gould, K.S. Multiple functional roles of anthocyanins in plant-environment interactions. *Environ. Exp. Bot.* **2015**, *119*, 4–17. [CrossRef]
67. Morales-Rodríguez, C.; Bastianelli, G.; Aleandri, M.P.; Chilosi, G.; Vannini, A. Application of *Trichoderma* spp. complex and biofumigation to control damping-off of *Pinus radiata* D. Don caused by *Fusarium circinatum* Nirenberg and O'Donnell. *Forests* **2018**, *9*, 421. [CrossRef]
68. Agustí-Brisach, C.; Pérez-Sierra, A.; Armengol, J.; García-Jiménez, J.; Berbegal, M. Efficacy of hot water treatment to reduce the incidence of *Fusarium circinatum* on *Pinus radiata* seeds. *Forestry* **2012**, *85*, 629–635. [CrossRef]
69. Berbegal, M.; Landeras, E.; Sánchez, D.; Abad-Campos, P.; Pérez-Sierra, A.; Armengol, J. Evaluation of *Pinus radiata* seed treatments to control *Fusarium circinatum*: effects on seed emergence and disease incidence. *For. Pathol.* **2015**, 1–9. [CrossRef]
70. Iturritxa, E.; Desprez-Loustau, M.L.; García-Serna, I.; Quintana, E.; Mesanza, N.; Aitken, J. Effect of alternative disinfection treatments against fungal canker in seeds of *Pinus radiata*. *Seed Technol.* **2011**, *3*, 88–110.
71. Vivas, M.; Zas, R.; Solla, A. Screening of maritime pine (*Pinus pinaster*) for resistance to Fusarium circinatum, the causal agent of pitch canker disease. *Forestry* **2012**, *85*, 185–192. [CrossRef]
72. Vivas, M.; Zas, R.; Sampedro, L.; Solla, A. Environmental Maternal Effects Mediate the Resistance of Maritime Pine to Biotic Stress. *PLoS ONE* **2013**, *8*. [CrossRef] [PubMed]