Potential control of forest diseases by solutions of chitosan oligomers, propolis and nanosilver

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Abstract

There is a growing necessity to replace chemical agents with ecofriendly materials, arising from their impact on the environment and/or human health, which calls for the design of new broad-spectrum fungicides. In this work, chitosan oligomers (COs), propolis (Ps) and silver nanoparticles (AgNPs) mixtures in solution were assessed to control the growth of different phytopathogenic fungi and oomycetes *in vitro*. Binary solutions of COs-Ps and COs-AgNPs evinced the highest antifungal effect against *Fusarium circinatum* and *Diplodia pinea* fungi, respectively, with a *ca*. 80% reduction in their mycelial growth. The COs solution by itself also proved to be greatly effective against *Gremmeniella abietina*, *Cryphonectria parasitica* and *Heterobasidion annosum* fungi, causing a reduction of 78%, 86% and 93% in their growth rate, respectively. Likewise, COs also attained a 100% growth inhibition on the oomycete *Phytophthora cambivora*. On the other hand, Ps inhibited totally the growth of *Phytophthora ×alni* and *Phytophthora plurivora*. The application of AgNPs reduced the mycelial growth of *F. circinatum* and *D. pinea*. However, the AgNPs in some binary and ternary mixtures had a counter-productive effect on the anti-fungal/oomycete activity. In spite of the fact that the anti-fungal/oomycete activity of the different treatments showed a dependence on the particular type of microorganism, these solutions based on natural compounds can be deemed as a promising tool for control of tree diseases.

Keywords: anti-fungal; anti-oomycetes; forest pathogens; natural compounds.

1. Introduction

Phytopathogenic microorganisms are responsible for major economic losses and ecological impacts, affecting from seedling nurseries to mature trees in plantations, seed orchards, landscape plantings, or native forests (Hirooka and Ishii 2013; Gordon et al. 2015). All over the world, several species of conifers are affected by common ascomycete fungi, such as *Fusarium circinatum* Nirenberg & O'Donnell, responsible for pitch canker disease (Wingfield et al. 2008); *Diplodia pinea* (Desmaz.) J. Kickx fil. (= *Sphaeropsis sapinea* (Fr.) Dyko & Sutton), which causes *Diplodia* tip blight and stem canker disease (Gibson 1979; Adamson et al. 2015); and *Gremmeniella abietina* (Lagerberg) Morelet (anamorph: *Brunchorstia pinea* (P. Karsten) Höhnel) that produces shoots dieback and cankers on stems and trunks (Kaitera and Jalkanen 1992; Romeralo et al. 2015), causing the death of conifers including spruce, fir, larch, pine and juniper. In the same way, another of the most important pathogens in coniferous forests is *Heterobasidion annosum* (Fr.) Bref. (= *Fomes annosus* (Fr.) Cooke) basidiomycete, which causes root and butt rot (Asiegbu et al. 2005; Garbelotto and Gonthier 2013).

Other main forest pathogens include *Cryphonectria parasitica*, one of the most undesirable introduced plant pathogens, which causes chestnut blight on species in the genus *Castanea* (Heiniger and Rigling 1994; González-Varela et al. 2011); and oomycetes species such as *Phytophthora*. These latter comprise *P. cambivora* (Petri) Buisman, also a common pathogen of *Castanea*, *Fagus* and other hardwoods (Jung et al. 2005); *P. ×alni* (Brasier & S.A. Kirk) Husson, Ioos & Marçais, nothosp. nov., which cause alder decline by dieback, small sparse and yellowish leaves, excessive fructification, and tarry and rusty exudates (Husson et al. 2015); and *P. plurivora* T. Jung and T.I. Burgess, which causes aerial canker and collar rot in several species, including beech, oaks and alders (Jung and Burgess 2009; Haque et al. 2014; Haque et al. 2015).

To date, control of plant diseases has typically been performed by application of high toxic chemicals, whose excessive use has occasioned undesired impacts on the environment and on human health (Hirooka and Ishii 2013). Moreover, regulations are increasingly limiting the utilization of high toxic chemicals and promoting the use of integrated pest management and non-chemical alternatives to pesticides (Directive 2009/128/EC).

Chitosan is a natural polymer composed of randomly distributed β-(1-4) D-glucosamine and N-acetyl-D-glucosamine units. It can be found in the form of chitin in the shells of crustaceans and in

the cell walls of fungi (Jayakumar et al. 2011). This cationic biopolymer is characterized by being biocompatible, biodegradable, non-toxic and features antimicrobial, antiviral and antifungal properties (Ngo et al. 2015). Indeed, all fungi are expected to be vulnerable to chitosan, except those containing chitosan as a major wall compound (i.e. zygomycetes) (Leuba & Stössel, 1986, cited in Laflamme et al. (2000)).

Another natural compound that has been widely used for its antiseptic properties, mainly in traditional medicine, but also in plant protection (Özcan et al. 2004), is propolis. It is a resinous material collected by bees from different parts of plants, buds and exudates, which –once mixed with their own enzymes– is used as a void sealant or as a sanitization agent in the hive (Marcucci 1995). Propolis is rich in flavonoids, polyphenols, steroids, aldehydes, amino acids and quinones, which account for its strong antimicrobial power (Farooqui 2012; Mărghitaş et al. 2013).

Regarding silver nanoparticles, they have gained attention in the past decade as a very promising bactericidal and antifungal agent, with a much higher activity than silver ions (Kashyap et al. 2012). Silver nanoparticles have the ability to destroy the cellular walls and interfere with bacterial DNA replication and protein production processes (Wei et al. 2009).

Natural alternatives based on chitosan products have been widely studied against plant diseases, meanly in the crop protection, such as rice (Boonlertnirun et al. 2008), soybean (Zeng et al. 2012) and potatoes (Kurzawińska and Mazur 2006), to name a few. So that, the development of application strategies such as seeds coating, foliar treatment and soil amendment is very broad (El Hadrami et al. 2010). Nonetheless, against forest diseases there are very few reports regard of chitosan uses (e.g. Reglinski et al. 2004; Fitza et al. 2013).

In this work, the anti-fungal/oomycete activity of chitosan oligomers (COs), propolis (Ps) and silver nanoparticles (AgNPs) and their binary and ternary combinations in solution has been assessed against eight forest pathogens through an *in vitro* study. The information on their effectiveness against each of the pathogens could pave the way for the development of novel natural compound-based antifungals, useful in an integrated management approach.

2. Materials and methods

2.1. *Fungal material and reagents*

To assay *in vitro* the effects of the different mixtures, eight species –five fungi and three oomycetes– were chosen. All these pathogens were isolated in natural areas in the North-West of

Spain (see Table 1). The pathogens were kept in dark at 25 ºC in Potato Dextrose Agar (PDA) culture medium in order to preserve the standard mycelial growth before the treatments.

Table 1. Assayed fungi and oomycetes, isolated in previous studies.

Species	Isolate	Host tree	Origin	Isolation year	References
Fungi					
Fusarium circinatum	FcCa1	*Pinus radiata*	Cantabria	2009	(Martínez-Álvarez et al. 2012)
Diplodia pinea	HP154	*Pinus radiata*	Cantabria	2009	(Martínez-Álvarez et al. 2016)
Gremmeniella abietina	VAI-13	*Pinus halepensis*	Valladolid	2003	(Botella et al. 2010)
Cryphonectria parasitica	EU1	*Castanea sativa*	Zamora	2005	(Zamora et al. 2012)
Heterobasidion annosum	A14009-AFZAPR001	*Pinus pinaster*	Zamora	2014	
Oomycetes					
Phytophthora cambivora	PH14012-LR2-2	*Quercus ilex*	Segovia	2014	
Phytophthora ×alni	PA02	*Alnus glutinosa*	Zamora	2012	(Zamora-Ballesteros et al. 2016)
Phytophthora plurivora	SORLDD4	*Alnus glutinosa*	Soria	2012	(Haque et al. 2014; Zamora-Ballesteros et al. 2016)

Unless otherwise stated, all chemicals and reagents were supplied by Sigma-Aldrich Química S.A. (Tres Cantos, Madrid) and were used without further purification. Chitosan with medium molar mass was purchased from Hangzhou Simit Chemical Technology Co. (Hangzhou, China). Propolis with a content of polyphenols and flavonoids of *ca.* 10% (w/v) came from Burgos (Spain).

2.2. *Synthesis of chitosan-based mixtures in solution*

The synthesis of the solutions based on COs with Ps and AgNPs was conducted according to the procedure described by Matei et al. (2015), with some modifications. COs aqueous solutions were prepared from medium molecular weight commercial chitosan (140000–300000 g/mol) in AcOH 2% at pH 4–6, after neutralization with KOH. However, when the final pH of the substrate was close to 6, there was no influence on the growth of the pathogen (Jönsson-Belyazio and Rosengren 2006). Then, 0.3 M H_2O_2 was added to obtain 2000 g/mol oligomers. Ps extraction was carried out by grinding raw propolis to fine powder and by maceration in a hydroalcoholic solution 7:3 (v/v), which was subsequently percolated (1 L/min) and filtrated with a stainless steel 220 mesh to remove any residues. AgNPs were prepared with the procedure described by Venkatesham et al. (2012), where the nanoparticles from an aqueous solution of $AgNO_3$ (50 mM) were obtained with chitosan acting as both reducing and stabilizing agent without using any toxic chemicals. The reaction was carried

out in an autoclave at 120 °C for 15 min to obtain a clear yellow color indicating the formation of silver nanoparticles.

COs-Ps, COs-AgNPs, Ps-AgNPs binary and COs-Ps-AgNPs ternary solutions were prepared by mixing –under vigorous stirring– the necessary volumes of each solution in order to obtain a concentration of 10 mg/mL of COs, 1 mg/mL of Ps and 10 µg/mL of AgNPs in every solution. The AgNPs content was kept to a minimum to preserve the stability of the nanoparticles.

In order to characterize the mixtures and identify the interaction of the chemical functional groups, the samples in solution were freeze-dried (lyophilized) for 24 hours and their infrared spectra in the 400-4000 cm^{-1} spectral range was measured using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FT-IR Spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system.

2.3. *In vitro experiments*

To assay the anti-fungal/oomycete activity, a typical *in vitro* mycelial growth inhibition test was performed. Indeed, the experimental design consisted of a factorial scheme with three factors: (1) COs (presence/absence), (2) Ps (presence/absence) and (3) AgNPs (presence/absence). So, the anti-fungal/oomycete activity of the three compounds separately and their binary and ternary combinations was analyzed for each pathogen (Figure 1). Each solution was uniformly incorporated at a ratio of 1:10 (v/v) into PDA after its sterilization for 20 min at 121 °C, as described by Wang et al. (2014), obtaining a final concentration of 1 mg/mL of COs, 0.1 mg/mL of P and 1 µg/mL of AgNPs in every treatment. These concentrations correspond to the minimum inhibitory concentrations used in other similar studies (Yoksan and Chirachanchai 2010; Torlak and Sert 2013; Olicón-Hernández et al. 2015). 20 mL of the mixtures were spread in Petri dishes (9 cm in diameter) setting four replicates for each treatment.

Once the culture medium had solidified, an inoculum of every pathogen (a 5×5 mm^2 plug cut from the margins) was placed at the center of the Petri dish. Then, Petri dishes were sealed and incubated at 25 °C in the dark. The mycelial growth (g) was measured on a daily basis until the day in which the dishes of the control treatment were fully covered with mycelium (n).

The radial growth rate was calculated according to the following equation:

$$\text{Radial growth rate} = \frac{\sum_{i=1}^{n} g_i}{n} \qquad \text{Eq. (1)}$$

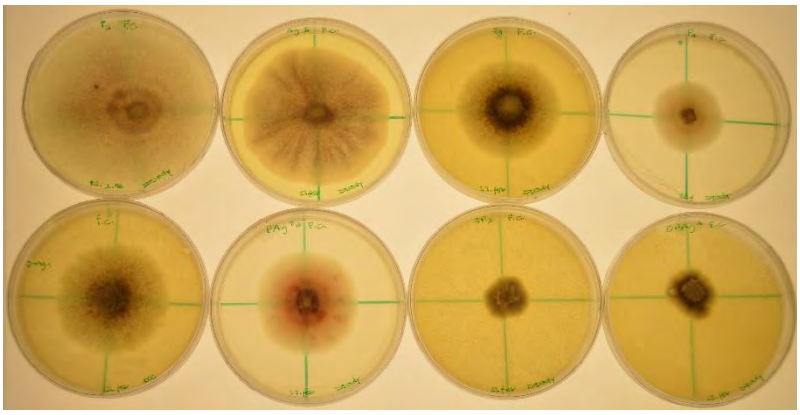

Figure 1. *In vitro* growth inhibition test for *Fusarium circinatum* (day 7). Control and treatments with AgNPs, COs, Ps, COs-AgNPs, Ps-AgPNs, COs-Ps, COs-Ps-AgNPs (*left-to-right, top-to-bottom*).

2.4. *Statistical analyses*

Analyses of variance (ANOVAs) and multiple comparison procedures were performed to test the effect of three different anti-fungal/oomycete agents (chitosan, propolis and nanosilver) and their combinations on the mycelia growth of the eight forest pathogens. As the raw data violated two ANOVA assumptions (normality and homogeneity of variances), robust methods were applied (García Pérez 2011). In particular, three-way fixed factor ANOVAs were performed under non-normality and inequality of variances, using the generalized Welch procedure, a 0.2-trimmed mean transformation and alpha value of 0.05. ANOVAs were carried out using the "Wilcox' Robust Statistics (WRS2)" package, in particular the functions "t3way" and "lincon" (see Wilcox (2016)), implemented in the R software environment (R Development Core Team 2016).

3. Results

3.1. *Aqueous solutions characterization*

Insight into the interaction of COs with the functional groups (phenolic and acids) from Ps and into the chelation of AgNPs in the binary and ternary aqueous solutions was gained by attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy. The vibrational spectra of the COs, COs-Ps binary and COs-Ps-AgNPs ternary mixtures were depicted in Figure 2.

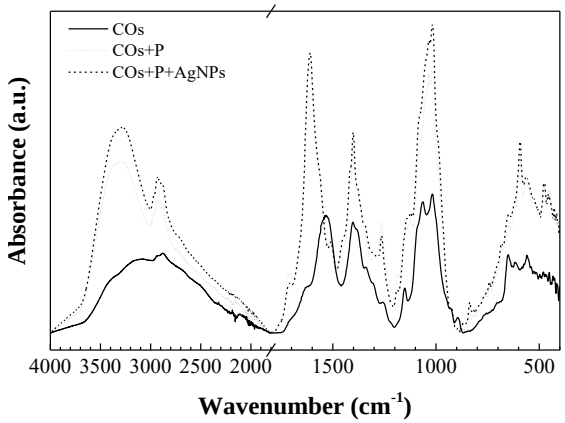

Figure 2. ATR-FTIR spectra of chitosan oligomers (COs), binary solution of chitosan oligomers and propolis (COs-Ps) and ternary solution of chitosan oligomers, propolis and silver nanoparticles (COs-Ps-AgNPs). A break has been inserted in the *x*-axis at 1800 cm^{-1} to allow a clearer representation of the fingerprint region.

The COs spectrum (solid line in Figure 2) showed the characteristic absorption peaks of chitosan at 3256 cm^{-1} (stretching vibration of the O-H and N-H bonds); at 1633 and 1550 cm^{-1} (amide I (C=O stretching) and to N-H (amine) vibration overlapped to amide II (N-H vibration), respectively); at 1152 cm^{-1} (C–O in oxygen bridges resulting from deacetylation of chitosan); and at 1065 and 1018 cm^{-1} (C-O-C and C-O vibrations).

The spectrum of the binary mixture of COs-Ps (dotted line in Figure 2) sensitized the interaction between the two components by significant changes *vs*. the COs spectrum, caused by the bonded Ps components (mainly flavonoids and lipids). An increase in the intensity of the bands at 1165 cm^{-1} (C–O and C–OH vibration), 1434 cm^{-1} (C–H vibration), 1508 and 1610 cm^{-1} (aromatic ring deformations), and 1681 cm^{-1} (C = O stretching) took place. Another important difference between the COs-Ps and COs spectra was a shift of the band associated to $\nu(C_{\Phi}\text{-}O)$ from 1257 cm^{-1} to 1263 cm^{-1}, which occurs when hydrogen bonding between COs and phenolic groups from Ps components takes place.

The lyophilizate of the ternary mixture COs-Ps-AgNPs (dashed line in Figure 2) showed a very similar pattern to the infrared spectrum of the COs-Ps binary mixture, albeit with a decrease in intensity for the bands at 1721, 1271 and 1130 cm^{-1}. This change, unaccompanied by a shift in the bands, suggests weak bonding of NH_2-AgNPs.

3.2. *Anti-fungal activity*

All individual agents and some mixtures demonstrated the ability to reduce the mycelial growth of fungi (Figure 3), in particular those with COs, however, their antifungal activity was dependent on the particular type of pathogen assayed. With regard to *F. circinatum* fungi (Figure 3*a*), an interaction

amongst the three agents was observed in the Post-hoc analysis (F=354.6, p<0.001). Nevertheless, the binary mixture of COs-Ps showed the best antifungal effect, where the radial growth rate (1.4 mm·day^{-1}) was 5.6 times lower than of the control treatment (8.0 mm·day^{-1}), corresponding to 82% of inhibition. In this case, the addition of AgNPs did not increase the effectiveness of the COs-Ps binary solution, whereas it reduced the effect of Ps and COs as individual compounds, which presented a high capacity to inhibit the mycelial growth, with a 68% and 53% of inhibition, respectively. However, AgNPs by itself, although in a lesser extent, also inhibited the mycelial growth regarding the control (27% of inhibitions).

The treatments against *D. pinea* (Figure 3*b*) also showed an interaction amongst the three agents in the Post-hoc analysis (F=7.4, p=0.02). The binary mixture COs-AgNPs showed the best antifungal effect, with a radial growth rate of 0.9 mm·day^{-1}, over 4.3 times lower than the control treatment (3.9 mm·day^{-1}), that is equivalent to 77% of inhibition. No significant differences were found considering the addition of Ps in the ternary mixture (COs-Ps-AgNPs). Nonetheless, the binary mixture of COs-Ps showed a 69% of inhibition. The separate application of COs, Ps and AgNPs also evinced some antifungal activity, with lower radial growth rates of 1.7, 2.4 and 3.2 mm·day^{-1}, i.e., 55, 37 and 18% of inhibition, respectively.

With regard to *G. abietina* (Figure 3*c*), there was an interaction between COs and AgNPs (F=6.6, p=0.03). The best antifungal effect was associated to COs, which caused a 78% reduction of the growth rate. On the contrary, the addition of AgNPs to COs not only did not improve the antifungal activity, but had a counter-productive effect. No significant differences were found with AgNPs solution in comparison to the control.

In relation to *C. parasitica* ascomycete (Figure 3*d*), the results showed an interaction between COs and Ps (F=371, p=0.001), and the effectiveness of COs both with and without Ps was around 93%. This study seems to point out that there is no advantage in adding Ps to the COs, in spite of that the inhibition percentage of the individual Ps solution was also high (2.35 mm day^{-1}, 64% inhibition).

The treatments on *H. annosum* basidiomycete (Figure 3*e*) showed a high antifungal activity of individual COs solution (86% of inhibition), without any interactions amongst the three elements, so it may be inferred that the use of Ps and AgNPs, by themselves, or in addition to COs did not significantly increase the inhibitory effect.

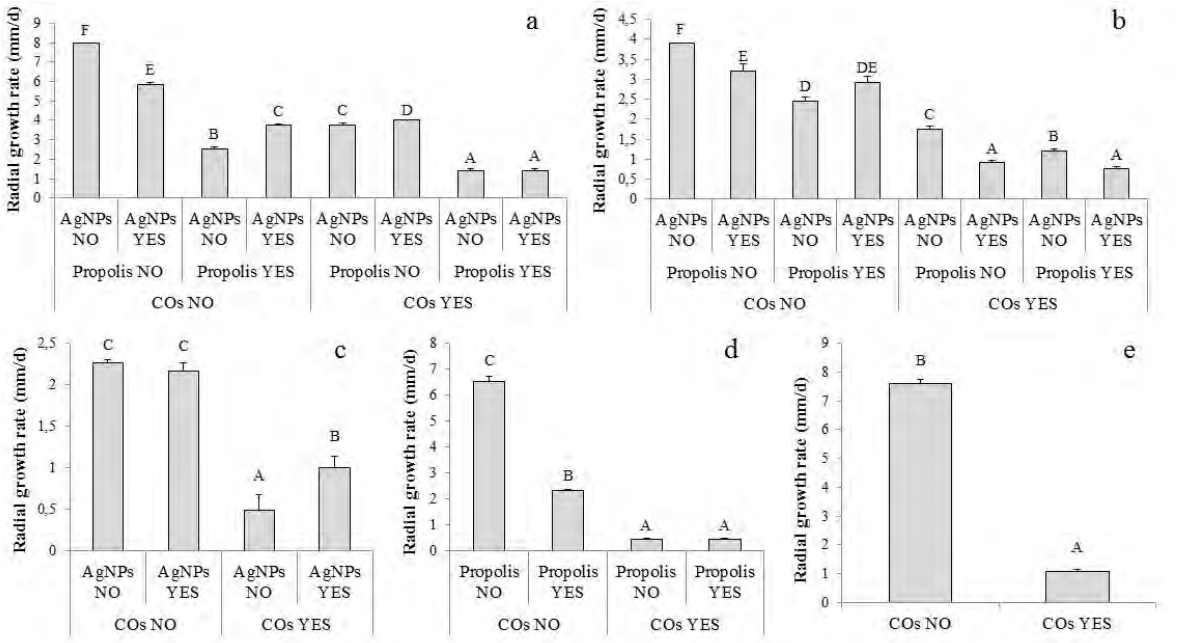

Figure 3. Radial growth rate and interaction among treatments based on chitosan oligomers (COs), Propolis and silver nanoparticles (AgNPs) against (**a**) *F. circinatum*; (**b**) *D. pinea*; (**c**) *G. abietina;* (**d**) *C. parasitica*; and (**e**) *H. annosum* fungi. Different letters above bars indicate significantly different means (generalized Welch procedure 0.2 trimmed means, a = 0.05). Error bars show the standard deviation. Note: Only significant interactions from the Post hoc analyses are shown.

3.3. *Anti-oomycete activity*

As regards the assays conducted with oomycetes (Figure 4), a remarkable inhibitory activity was attained for COs and Ps. Treatments against *P. cambivora* (Figure 4*a*) evidenced an interaction among the three agents (F=64.1, p=0.001), but all treatments with COs (individual, binary and ternary mixtures) presented 100% of growth inhibition. The treatment with the individual Ps solution also showed growth inhibition (43%), but AgNPs and Ps-AgNPs treatments did not exhibit any significant differences *vs*. the control.

On the other hand, an interaction between COs and Ps was found in treatments against *P. ×alni* and *P. plurivora* (F=23, p=0.002 and F=722.8, p=0.001, respectively). While the application of COs and Ps (individual or mixed) resulted in a similar growth inhibition for *P. ×alni*, the addition of Ps played a leading role in the growth inhibition for *P. plurivora* (Figure 4*b* and Figure 4*c*).

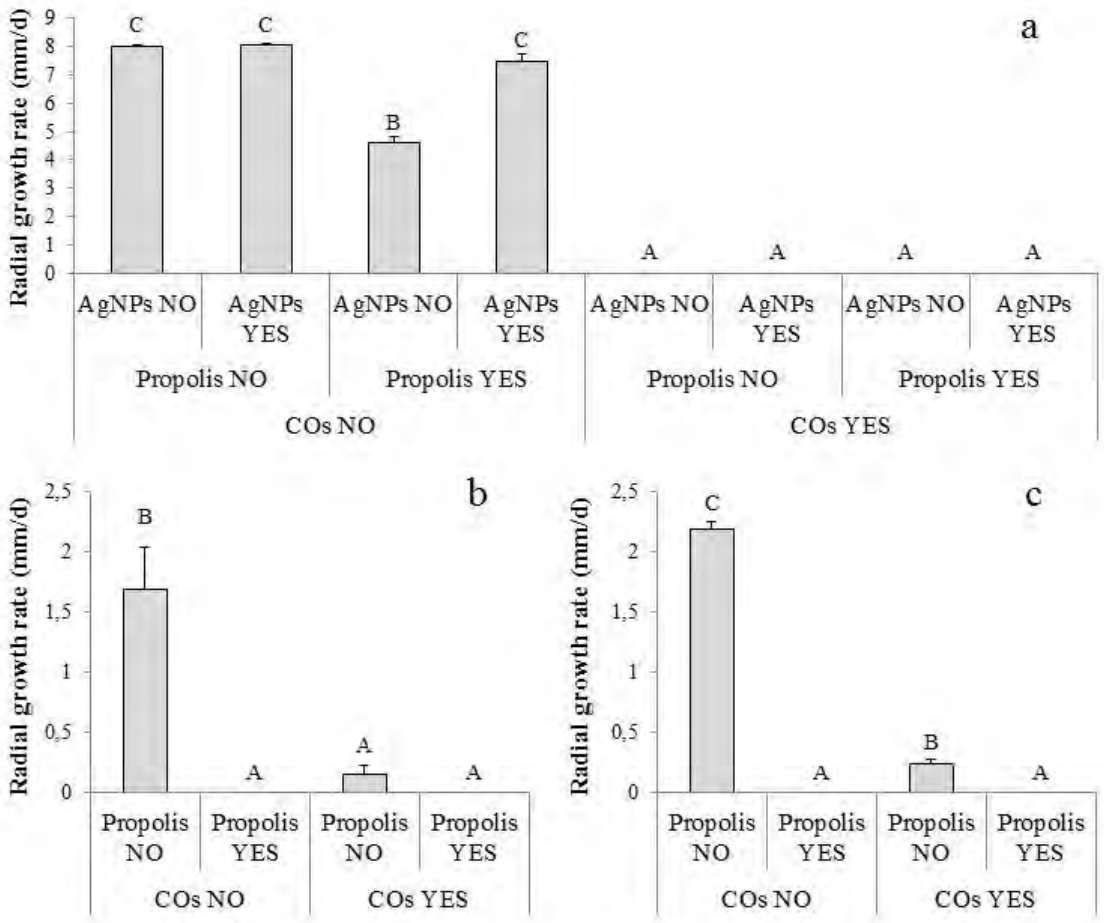

Figure 4. Radial growth rate and interaction among treatments based on chitosan oligomers (COs), Propolis and silver nanoparticles (AgNPs) against (**a**) *P. cambivora*; (**b**) *P. ×alni*; and (**c**) *P. plurivora* oomycetes**.** Different letters above bars indicate significantly different means (generalized Welch procedure 0.2 trimmed means, a = 0.05). Error bars show the standard deviation. Note: Only significant interactions from the Post hoc analyses are shown.

4. Discussion

The present study has demonstrated that the three compounds (COs, Ps and AgNPs) have an antifungal effect on different forest pathogens. COs by itself showed an inhibitory effect on the mycelial growth of all pathogens tested. Although the antifungal activity of chitosan polymer has been already reported by other authors both in *in vitro* and *in vivo* experiments, for example, chitosan applications to increase the resistance of pine seedlings to *F. circinatum* and *D. pinea* (Reglinski et al. 2004; Fitza et al. 2013), this study confirms the importance of the use of low molecular weight chitosan such as COs. It is worth noting that when chitosan with higher molecular weight than that used in this study (e.g., 50,000 Da instead 2,000 Da) are applied, a lower antifungal activity is attained, with 35% of reduction of mycelial growth of *D. pinea* in the first day as reported by Singh

et al. (2008). This is consistent with the results reported by Avelelas et al. (2014), Qiu et al. (2014) and Cobos et al. (2015), who demonstrated that chitosan antifungal activity increased in inverse proportion to its molecular weight. Consequently, COs of molecular weight under 2,000 Da, might be a preferable option as compared to commercial 'low molecular weight' chitosan (i.e., 50,000 to 190,000 Da, CAS Number 9012-76-4) in terms of its activity against *D. pinea*. On the other hand, in an *in vitro* study by Ziani et al. (2009), the use of chitosan solutions proved to be more effective against *Aspergillus niger*, *Alternaria alternata* and *Rhizopus oryzae* than the use of films, where presumably, the chitosan solution had positive charges on the quaternary amino groups that interacted with the fungal cell walls, while for the films a protonation loss occurred.

The inhibitory effect of Ps was demonstrated on most of the pathogens tested (*F. circinatum*, *D. pinea*, *C. pararisitica*, *P. cambivora*, *P. ×alni* and *P. plurivora*). The use of propolis has not been as well studied as chitosan, although its inhibition capacity against *F. circinatum* was already reported by Iturritxa et al. (2013). However, they reported a fungicidal effect, whereas in this study a growth inhibition effect was found.

The application of AgNPs by itself also reduced the mycelial growth of *F. circinatum* and *D. pinea*, which is consistent with the results reported by Narayanan and Park (2014), who observed slight to moderate inhibition against wood-degrading fungi when a low dose of AgNPs was used. Nevertheless, AgNPs had not a significant anti-oomycete activity on the species tested in this study, contrasting with Mahdizadeh et al. (2015), who found that another oomycete (*Pythium aphanidermatum* (Edson) Fitzp.) was the most sensitive pathogen to nanosilver among the six tested species.

The effect of the binary solutions of the compound tested varies according to the species. While the COs-Ps binary solution showed the highest antifungal effect against *F. circinatum*, the result of the application of AgNPs in the binary solutions varies according to the pathogen. Indeed, the binary solution COs-AgNPs recommended by Wang et al. (2015) was the most promising mixture in order to control *D. pinea*. Nevertheless, the use of COs-AgNPs and Ps-AgNPs solutions had a counter-productive effect on the anti-fungal/oomycete activity against *G. abietina* and *P. cambivora*, respectively. This is in contrast to other studies in which nanosilver was also incorporated into chitosan, although in higher doses. For example against ascomycete *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the mixture showed excellent results: the inhibitory action increased from 44% to 100% as the AgNPs concentration was increased from 0.1 up to 100.0 µg/mL (Chowdappa et al. 2014). It is also noteworthy that the solution consisting only of AgNPs did not show statistically significant difference *vs*. the control treatment, in contrast to the study by Narayanan and Park (2014),

who observed slight to moderate inhibition against wood-degrading fungi when a low dose of AgNPs was used. In the same vein, Saharan et al. (2013) and Saharan et al. (2015) found that the nanocopper-chitosan complex showed growth inhibition against other ascomycota such as *Fusarium oxysporum* and *A. alternata*. They suggested that addition of nanometals increased the surface charge density and provided more electrostatic interaction with fungal membrane.

Differences in the inhibitory behavior of the similar COs-Ps-AgNPs mixture have been reported for other fungal species and different application procedures: the COs-Ps-AgNPs ternary complex did not improve the antifungal/anti-oomycete activity compared to the binary solutions in this study, which contrasts with the complete inhibition obtained using similar COs-Ps-AgNPs mixtures applied to *D. seriata* and *Bipolaris oryzae* (Breda de Haan) Shoemaker (Matei et al. 2015; Araujo-Rufino et al. 2016). This discrepancy may be associated to that the gel phase used was ascribed to the higher concentrations of chitosan oligomers in the gel (20-25 mg/mL) *vs.* the aqueous solution of this study (1 mg/mL).

The bands in the ATR-FTIR spectrum of the COs-Ps-AgNPs composite evidenced a weak interaction among COs and AgNPs, even weaker than that reported for chitosan-AgNPs thin films and nanocomposites manufactured by spin-coating (Wei et al. 2009; Wang et al. 2015), whose infrared spectra showed shifts between 5 and 10 cm^{-1}. On the other hand, the spectrums of COs and COs-Ps showed very similar bands to those reported in other works for chitosan (Matei et al. 2015; Stroescu et al. 2015; Branca et al. 2016) and propolis extracts (Franca et al. (2014); Siripatrawan and Vitchayakitti (2016).

A differential feature of this investigation in comparison to the literature was that in the preparations described above Green Chemistry procedures were used, without need for the addition of chemical bond reinforcing agents, widely used in other works (Gu et al. 2014; Jemec et al. 2016). Accordingly, these eco-friendly compounds could be useful in management strategies based on integrated approach, for example in the use of appropriate nursery hygiene practices. Likewise, the application of chitosan had been suggested using the chitosan-based Biochikol 020 PC, a biological agent with fungicidal properties and resistance stimulator, in order to control *P. xalni* complex in forest nurseries (Oskazo 2007).

In conclusion, from the results of the *in vitro* growth inhibition experiments respect the anti-fungal/oomycete effect of individual, binary and ternary mixtures of COs, Ps and AgNPs, assayed against eight plant pathogens, it could be inferred that: (*i*) the inhibitory activity against fungi and oomycetes of the individual low molecular weight COs solutions was significantly high (reaching growth rate reductions of up to 78, 86, 93% and 100% against *G. abietina*, *C. parasitica, H. annosum*

and *P. cambivora*, respectively); (*ii*) the growth inhibition is enhanced by association of COs with Ps (e.g., *F. circinatum*) and COs with AgNPs (e.g. *D. pinea*); and (*iii*) the COs-P-AgNPs ternary complex did not improve the antifungal/anti-oomycete activity compared to the binary solutions. Thus, the weak interactions that appear in solution amongst the three components (evidenced by FTIR) suggested that strong interactions are necessary to achieve the desired anti-fungal/oomycete effect. Additionally, further studies are essential to determine the effect of the COs-Ps-AgNPs combinations on seeds, tree seedlings and mature trees infested by different pathogens, as an innovative application system useful in an integrated management approach.

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6. Compliance with Ethical Standards

The authors declare that they have no conflict of interest.

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