



In Vitro Antifungal Activity of Chitosan-Polyphenol Conjugates against *Phytophthora cinnamomi*

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Abstract: *Phytophthora cinnamomi* is responsible for radical rot in a wide range of hosts, resulting in large economic and ecological losses worldwide. In Spain, it is responsible for diseases such as the oak decline or the chestnut blight. In this study, different polyphenol-stevioside inclusion compounds dispersed in a hydroalcoholic solution of chitosan oligomers have been investigated, with a view to their application as natural bioactive complexes to replace conventional systemic fungicides against this fungus. The polyphenols tested in vitro were curcumin, ferulic acid, gallic acid and silymarin. Three concentrations (125, 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$) were assayed, with and without silver nanoparticles (AgNPs), and notable differences were found in the inhibition of mycelium growth, with EC_{50} and EC_{90} values ranging from 171 to 373.6 $\mu\text{g}\cdot\text{mL}^{-1}$, and from 446.2 to 963.7 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The results obtained showed that the addition of AgNPs, despite their antimicrobial activity, did not always lead to synergies. In the case of *P. cinnamomi*, an unexpected antagonistic behavior was found for two of the polyphenols (curcumin and silymarin), while an additive behavior for ferulic acid and a synergistic behavior for gallic acid were attained. In view of their inhibitory power, the preparations based on ferulic acid with AgNPs and on silymarin without AgNPs are proposed for applications in crop and forests protection against *P. cinnamomi*.

Keywords: chitosan oligomers; fungicide; phenolic compounds; silver nanoparticles; synergism

1. Introduction

Phytophthora cinnamomi is an oomycete that lives on the ground nourishing itself thanks to decomposing matter. This pathogen, responsible for “root rot” or “regressive death”, is one of the most invasive species worldwide. It infects close to 5000 species of plants [1], affecting a variety of plant families: conifers, grasses, ferns, ornamental plants and food crops such as pineapple or avocado [2]. Its expansion has been attributed to the phenomenon of climate change, given that the potential disease range is influenced by winter temperature (disease development is strongly hampered by cold winters) and other climatic variables, such as summer temperatures and hydrologic variables. For instance, in Mediterranean forests, the initiation of the disease at the root

level occurs during temporal waterlogging, caused by the higher frequency of extreme rain events [3]. Symptoms of *P. cinnamomi* infection include: wilting, reduced fruit size, death of young shoots, chlorosis of leaves and stem cankers [4].

In the Iberian Peninsula, *P. cinnamomi* is responsible for diseases such as the oak decline, with an enormous impact on the *dehesa* or *montado* ecosystem (a “man-made” ecosystem characterized by a savannah-like physiognomy that occupies extensive areas in Southern Spain and Portugal), or of chestnut blight [5].

At present there is no treatment able to eradicate regressive death by *P. cinnamomi*, although the stem injection of phosphites (which are elicitors that indirectly behave as fungicides) [6] and foliar spraying with metalaxyl + mancozeb (fungicides with contact and systemic activity) have been tested.

As an alternative to these conventional treatment agents, it is possible to use natural bioactive complexes extracted from plants, such as phenolic acids, flavonoids, tannins or lignans. In particular, polyphenols have been shown to feature a remarkable antimicrobial activity [7–10]. However, their applicability is limited by their low solubility and bioavailability [11], which may be improved through the formation of inclusion compounds with, for instance, terpene glycosides [12]. In this study, polyphenol-stevioside inclusion compounds dispersed in a hydroalcoholic solution of chitosan oligomers have been assayed. The polyphenols selected for the *in vitro* tests were curcumin, ferulic acid, gallic acid and silymarin, at different concentrations, with and without silver nanoparticles (AgNPs). The aim of the research has been to investigate the activity of the different chitosan-polyphenol conjugates against *P. cinnamomi* and to study synergistic effects upon addition of AgNPs to the conjugates.

2. Materials and Methods

2.1. Reagents and Preparation of the Bioactive Conjugates

Curcumin (CAS No. 458-37-7), ferulic acid (CAS No. 1135-24-6), gallic acid (CAS No. 149-91-7) and silymarin (MDL number MFCD01776359) were supplied by Sigma-Aldrich (Merck, Darmstadt, Germany); stevioside (CAS No. 57817-89-7) was supplied by Wako (Osaka, Japan); and medium molecular weight chitosan (MMWC) was purchased from Hangzhou Simit Chemical Technology Co. Ltd. (Hangzhou, China).

The preparation of chitosan oligomers from MMWC was carried out according to the methodology reported by Sun et al. [13]. Microwave-assisted aqueous biphasic system separation was used to prepare the polyphenol inclusion compounds. The conjugates with chitosan oligomers in hydroalcoholic solution medium were prepared according to the procedure previously reported in Matei et al. [14], and were characterized by infrared spectroscopy (FTIR), SEM and TEM microscopy techniques to ensure the reproducibility of the results presented in patent P201731489 [15].

It should be clarified that the antifungal efficacy assays reported herein and those reported in Matei et al. [14] (focused on the comparison between two dispersion media and on differences between polyphenols, but with AgNPs in all cases) were run in parallel. Since they were conducted at the same time and in the same conditions, the impact of physiological changes of the fungus or differences in abiotic conditions may be excluded.

2.2. Fungal Isolate, Growth Conditions and *In Vitro* Tests of Mycelial Growth Inhibition

The fungal isolate of *Phytophthora cinnamomi* used for the *in vitro* sensitivity assays (MYC43) was provided by ICMC-IPROCOR (CICYTEX, Mérida, Spain) and was maintained in potato-dextrose-agar (PDA) culture medium at 4 °C. Fresh subcultures to obtain the inoculum for the tests were obtained by transferring hyphae plugs to Petri dishes containing PDA medium, which were incubated at 25 °C in the dark for 7 days.

To test the antifungal activity of the conjugates, the agar dilution method was used. Final concentrations of 125, 250 and 500 µg·mL⁻¹ were obtained by adding aliquots of the stock solutions discussed in previous subsection to the PDA medium. Eight-mm mycelial disks from the margins of

these fresh subcultures were then placed in these PDA plates. Pure PDA culture medium was used as the control. Three experiments, with three replicates per experiment, were carried out.

After 7 days of incubation at 25 °C in the dark, mycelial growth inhibition for each treatment and concentration was determined according to the formula: $((d_c - d_t)/d_c) \times 100$, where d_c and d_t are the average diameters of the control and treated fungal colonies, respectively [16].

The concentrations that reduced mycelial growth by 50% and 90% (EC₅₀ and EC₉₀, respectively) were determined by regressing the values of radial growth inhibition (%) against the log₁₀ values of the concentration of the conjugates.

To assess the joint action of the bioactive products in mixtures, Wadley's method was used. In this method, if the synergy factor (SF) is 1, the hypothesis of similar joint action (additivity) can be accepted; if SF > 1, there is synergistic action; and if SF < 1 there is antagonistic action between the bioactive products [17].

2.3. Statistical Analyses

Data from the results obtained herein and from those reported in a previous study with AgNPs [14] were subjected to analysis of variance (ANOVA), followed by post hoc comparison of means through Tukey's HSD (honest significant difference) test at $p < 0.05$. SPSS Statistics v.25 software (IBM; Armonk, NY, USA) was used.

3. Results and Discussion

The radial growth of the mycelium was monitored to study the in vitro activity of the different treatments. The results of the sensitivity assays are shown Figure S1, in which one may observe that a reduction in the radial growth of the mycelium was attained in all cases when the concentration of the conjugates was increased from 125 to 500 µg·mL⁻¹.

It is worth noting that the addition of AgNPs only resulted in a noticeable enhancement of activity for the conjugates based on gallic acid. For the conjugates based on ferulic acid the improvement was statistically significant only at the lowest dose; and for the conjugates based on curcumin or silymarin, the addition of AgNPs led to a lower performance (particularly evident for the conjugate with silymarin at 500 µg·mL⁻¹).

The results from the factorial ANOVA (Table S1) indicated statistically significant one-way, two-way and three-way interaction effects. A classification of the treatments, both with and without AgNPs, according to Tukey's HSD test is shown in Table S2.

The effective concentrations EC₅₀ and EC₉₀ for each treatment are summarized in Table 1. The presence/absence of AgNPs in the conjugates and the phenolic compound had a noticeable influence on the sensitivity of the isolate. While the presence of AgNPs barely modified the EC₉₀ values for curcumin and ferulic acid, it significantly improved those of gallic acid conjugates (by 34% and 112% for EC₅₀ and EC₉₀, respectively).

In relation to the influence of the polyphenol, for the treatments without AgNPs, both of on the basis of the least square means and the EC₅₀ values, the efficacy would follow the sequence: silymarin > ferulic acid > curcumin > gallic acid. On the basis of the EC₉₀ values, the sensitivities of *P. cinnamomi* to curcumin, ferulic acid and silymarin would be similar, and gallic acid would be the least preferred choice.

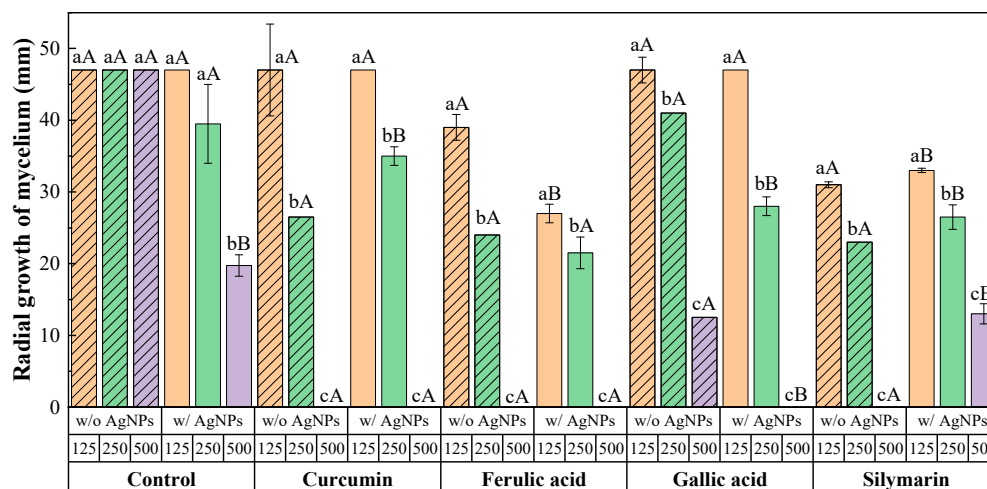


Figure 1. Radial growth values of *P. cinnamomi* mycelium in the presence of the conjugates, composed of various polyphenol inclusion compounds with (w/) or without (w/o) silver nanoparticles (AgNPs) [14] at different concentrations (in µg·mL⁻¹). For each treatment, concentrations labelled with the different lowercase letters are significantly different at $p < 0.05$ by Tukey’s test. For treatments with the same polyphenol and at the same dose, different uppercase letters indicate that the absence/presence of AgNPs resulted in significant differences at $p < 0.05$ by Tukey’s test. Mean values of three experiments with three replicates are presented, with error bars representing the standard deviation.

Table 1. Effective concentrations of the conjugates that inhibited mycelial growth by 50% and 90% (EC₅₀ and EC₉₀, respectively).

Treatment	EC ₅₀ (µg·mL ⁻¹)		EC ₉₀ (µg·mL ⁻¹)	
	w/o AgNPs	w/AgNPs	w/o AgNPs	w/AgNPs
Control	--	458.4	--	1192.8
Curcumin	257.5	279.9	448.3	487.4
Ferulic acid	228.7	171.6	446.2	450.4
Gallic acid	373.6	261.3	795.3	455.6
Silymarin	195.5	261.8	453.1	963.7

According to Wadley’s method [17] for quantification of the level of interaction, a synergy factor $FS = 1.7$ was obtained for gallic acid, indicative of a synergistic interaction with AgNPs; for ferulic acid, $FS = 1.0$ was found, indicative of an additive behavior between the two antifungal products; and for silymarin and curcumin, FS values of 0.5 and 0.9 were calculated, respectively, indicative of an antagonistic behavior with the AgNPs.

Although rare in the literature, it should be clarified that, for example, cases of antagonistic behavior of AgNPs with amoxicillin versus methicillin-resistant *Staphylococcus aureus* (MRSA) have been reported [18]. These authors, in trials with 7 organisms and 19 antibiotics, completed 96 tests, finding 5 combinations with synergistic behavior, 89 with additive behavior and 2 with antagonistic behavior. This work would provide evidence of the existence of analogous complex interactions with AgNPs in the case of phenolic complexes.

5. Conclusions

The in vitro tests led to notable differences in the inhibition of mycelium growth of *P. cinnamomi*, observing a superior performance of the preparations based on ferulic acid with AgNPs and silymarin without AgNPs (thus evidencing that the addition of AgNPs, in spite of their antimicrobial activity, does not always result in synergies). In fact, an unexpected antagonistic behavior was detected for two of the polyphenols (curcumin and silymarin), an additive behavior was observed

for ferulic acid and a synergistic behavior was found for gallic acid. In any case, in view of the EC₅₀ and EC₉₀ concentrations, these preparations can be promising bioactive products for protection applications of crops and forests against *P. cinnamomi*.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Example of sensitivity test. Radial growth of mycelium for the control and treatments with curcumin, ferulic acid, gallic acid and silymarin; Table S1: Test of between-subjects effects; Table S2: Categories as a function of radial growth values for each polyphenol*dose*AgNPs combination, with a confidence interval of 95%, by Tukey's HSD test.

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