IMPROVEMENT OF THE ANTIFUNGAL ACTIVITY AGAINST *BOTRYTIS CINEREA* OF SYRINGIC ACID, A PHENOLIC ACID FROM GRAPE POMACE

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ABSTRACT

The aim of this study was to improve the antifungal activity against *Botrytis cinerea* of syringic acid by using the enzyme laccase to synthesize a heterodimeric compound by a coupling reaction with aniline. The synthesized heterodimer is a quinone-imine like compound (2,6-dimethoxy-4-(phenylimino)benzenone), which was characterized by using ^{1}H and ^{13}C NMR, IR, and mass spectrometry. The antifungal activity of the heterodimeric compound against *B. cinerea* was determined in vitro and showed a higher antifungal effect than the substrates (syringic acid and aniline), inhibiting the mycelial growth with an IC_{50} value of 0.14mM and delayed 2 hours the conidial germination. Also, using the fluorochrome calcofluor white, cell wall damage was observed when *B. cinerea* was incubated with the compound. This is the first report on antifungal activity of this type of compound against *B. cinerea*.

Keywords: *Botrytis cinerea*; antifungal activity; laccase; 2,6-dimethoxy-4-(phenylimino)benzenone.

1. INTRODUCTION

Botrytis cinerea is a ubiquitous fungus which causes Grey Rot disease on many different economically important crops like tomato, cucumber, rose, grapevine, strawberry, etc. This pathogen attacks many organs, stems, fruits and flowers causing great economic losses especially in post-harvest period [1].

B. cinerea is mainly managed with synthetic fungicide. These fungicides are becoming less accepted due to the increase of resistant strains and environmental pollution [1,2]. Therefore, natural products isolated from plants are an alternative to synthetic fungicides. Terpenoids, phenolic compounds, nitrogen-containing compounds, and aliphatic compounds isolated from plants have shown antifungal properties [3–5]. Grape pomaces are an important source of phenolic compounds [6]. It has been reported that extracts obtained from grape pomaces present antifungal activity against *B. cinerea*. However, pure compounds identified in these extracts showed low antifungal effect [7]. Numerous authors have reported that the biological activity of phenolic compounds can be improved modifying phenolic molecules by using the enzyme laccase (EC 1.10.3.2) a polyphenol oxidase [8]. Antioxidant, antitumor, and anti-depressive compounds have been synthesized using this enzyme [8–11]. Additionally, this enzyme has been recently used for the synthesis of antifungal compounds [12].

Laccase is a copper-containing oxidase that catalyzes reduction of molecular oxygen to water and the oxidation of a phenolic compound [13]. Also, dimerization, oligomerization, and polymerization of phenolic compounds can be obtained [8]. This enzyme has been used to synthesize hydroxybiphenyl dimers [14], isoeugenol dimers [15], and ferulic acid dimers [16]. Laccase is also able to couple a laccase substrate with a non-laccase substrate (known as a mediator) to create new molecules (heterodimers) [8,17]. Niedermeyer et al. reported the heterodimeric synthesis among p-hydroquinones and primary aromatic amines using laccase [10]. Another example is the coupling between p-hydroquinones with p-aminobenzoic acid [18]. On the other hand, modification of antibiotics using laccase coupling reactions produced compounds with lower activities[19], however, using nitrogen compounds to modify the antibiotics, the synthesized products showed higher antibacterial activities [20,21]. The anticancer activity of naphtohydroquinones has been improved by nuclear monoamination with anilines using laccase [22]. These results indicate that the laccase-mediated amination of a phenolic compound could increase the biological activity.

The aim of this work was to improve the antifungal activity against *B. cinerea* of one phenolic acid found in grape pomace (syringic acid) through the laccase–catalyzed synthesis of a heterodimeric compound with aniline. In addition, the effect of synthetized product on the cell wall and plasmatic membrane integrity of *B. cinerea* was analyzed.

2. EXPERIMENTAL

2.1. General experimental procedures.

The NMR spectra were acquired using a Bruker Avance 400 MHz spectrometer (400,133 MHz for ^{1}H, 100.624 MHz for ^{13}C). All measurements were performed in $CDCl_3$ to 300 K. Chemical shifts (in ppm) for ^{1}H and ^{13}C spectra, were calibrated to solvent signal, $CHCl_3$ 7.26 ppm (residual signal solvent) and 77.16 ppm, respectively, and reported relative to Me_4Si. Thin-layer chromatography was performed on Merck Kiesegel 60 F254, 0.2 mm thick and semi-preparative thin layer chromatography on Merck Kieselgel 60 F_{254} 20x 20cmx 0.25 mm. The mass spectrum of the compound was acquired using a mass spectrometer Shimadzu-GCMS-Q5050 instrument using direct inlet system. The scan covered the mass range (m/z) from 150 to 350 m/z 243 $[M^+]$ $C_{14}H_{13}NO_3$.

2.2. Chemicals reagents and compounds used in this study.

Technical grade fungicide iprodione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] was provided from INIA (Santiago, Chile).

Syringic acid, aniline, and the enzyme laccase used in this study were obtained commercially, Sigma Chemical Co. (St. Louis, MO)

2.3. *Laccase-mediated synthesis of 2,6-dimethoxy-4-(phenylimino) benzenone (compound 1)*

Three different syringic acid:aniline ratios (1:1, 1:2 and 2:1) and three different enzyme amounts (2.25, 4.5 and 9U) were tested in order to increase the quantity of the heterodimeric compound synthesized. For the 1:1 syringic acid:aniline ratio, both compounds were dissolved in 1 mL ethyl acetate (0.05mM each), and the enzyme was dissolved in 1 mL sodium acetate buffer (50mM) at pH 4.5. Both solutions were mixed and the reaction mixture was stirred at 100 rpm during 180 minutes at 22°C. After this time, the solvent was evaporated using a rotary evaporator at 40 °C. The heterodimeric compound was purified from the reaction mixture that also contained secondary products, substrates, and the enzyme, by using semi-preparative thin layer chromatography (TLC silica gel 60 F_{254} glassplates 20x20cm) with hexane:ethyl acetate (1:1) as an eluent system. Compound 1 showed a Rf of 0.3. Since compound 1 has a yellow color, no revelation of the TLC was needed.

Compound **1** Yield 67.8%, IR (KBr) *v* 1635 (C=N) cm^{-1}, ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance RW- 400 spectrometer, deuterated acetone (CD_3COCD_3) was used as solvent.^{1}H NMR (CD_3COCD_3, 400 MHz) δ 3.624 (s, 3H, Hg), 3.872 (s, 3H, Hf), 6.158 (d, 1H, He), 6.423 (d, 1H, Hd), 6.898 (d, 2H, Hc), 7.407 (t, 2H, Hb), 7.177 (t, 1H, Ha); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ 57.076 (CH3), 57.351(CH3), 177.436 (C1), 157.51 (C2), 156.716 (C6), 100.234 (C3), 113.326 (C5), 158.862 (C4), 152.615 (C1'), 122.339 (C2'), 130.802 (C3'), 126.458 (C4').

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2.4. Fungal strain and culture conditions

The strain G29 of *B. cinerea* was used; it was isolated originally from grapes (*Vitis vinifera*) by the Instituto de Investigaciones Agropecuarias La Platina, Chile and is genetically characterized [23]. It was maintained on malt-yeast extract agar slants with (2% (w/v) malt extract, 0.2% (w/v) yeast extract and 1.5% (w/v) agar) at 4 °C. In studies on the effect of the compound on the cell wall and the plasmatic membrane integrity, liquid minimum medium was used, composed by KH_2PO_4 (1 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ x $7H_2O$ (0.5 g/L), KCl (0.5 g/L), $FeSO_4$ x $7H_2O$ (0.01 g/L) pH 6.5, 25 mol/L ammonium tartrate as a nitrogen source, and 1% (w/v) glucose as carbon source.

2.5. Antifungal Assay

2.5.1. Effect on mycelial growth.

The effect of compound **1** and the substrates (syringic acid and aniline) on mycelial growth of *B. cinerea* was assessed in vitro using the radial growth test on malt-yeast extract agar [24]. Synthesized compound, syringic acid, and aniline dissolved in acetone at different concentrations (0.1mM to 0.4 mM for compound 1 and 1mM to 5mM for syringic acid and aniline) were added to Petri dishes containing a malt-yeast extract agar medium. The final acetone concentration was identical in the control and treatment assays. Commercial fungicide iprodione was used as a control. After evaporation of the acetone in a laminar flow cabinet, the culture medium was inoculated with 0.5 cm agar disks from an actively growing culture of *B. cinerea*. Cultures were incubated in the dark at 22 °C for seven days. Mycelial growth diameters were measured daily. Results were expressed as IC_{50} (the concentration that reduced mycelial growth by 50%), determined by the inhibition of radial growth against compound concentrations. Each experiment was done at least three times.

2.5.2. Effect on conidial germination.

Conidial germination assay was carried out as described by Cotoras et al [25] using the synthesized compound, syringic acid, or aniline dissolved in acetone at a final concentration of 0.16mM. The final acetone concentration was identical in the control and treatment assays.

2.6. Effect on B. **cinerea** *cell wall integrity*

The effect of compound **1** on the cell wall integrity was determined using fluorochrome calcofluor white. *B. cinerea* conidia at a final concentration of 1 x 10^5 conidia mL^{-1} were inoculated in 24-well plates (lined with 12-mm glass coverslips) containing 1 mL of liquid minimum medium. Cultures were incubated at 22°C for 15 h to permit the germination of the conidia. After this time, liquid medium was removed. Afterwards, liquid medium containing: lysing enzymes (positive control), 10% (v/v) acetone (negative control), and compound **1** (0.16mM) were added to each well. The mixtures were incubated at 22°C for six hours. *B. cinerea* hyphae adhered to glass coverslips were washed three times with liquid minimum medium and stained with fluorochrome calcofluor white. After 10 min of incubation, the hyphae were washed with minimum medium and glass coverslips containing hyphae were mounted in slides. For the assembly of the samples in the slides, 15 µL of DABCO (1,4-diazabicyclo[2.2.2]octane) was used. The fluorescence of *B. cinerea* hyphae stained with fluorochrome calcofluor white was observed under a fluorescence microscope, Blue/Violet (110033V2) filter was used. These experiments were done at least in triplicate.

2.7. Effect on the plasmatic membrane integrity of B. **cinerea.**

This was determined using the SYTOX Green uptake assay [26]. *B. cinerea* conidia at a final concentration of 1 x 10^5 conidia mL^{-1} were inoculated in 24-well plates (lined with 12-mm glass coverslips) containing 1 mL of liquid minimum medium. Cultures were incubated at 22°C for 15 h to permit the germination of the conidia. After this time, liquid medium was removed. Afterwards, liquid medium containing: 70% (v/v) ethanol (positive control), 10% (v/v) acetone (negative control), and compound **1** (0.16mM) were added to each well. The mixtures were incubated at 22°C for six hours. *B. cinerea* hyphae adhered to glass coverslips were washed three times with liquid minimum medium and stained with 50 nmol L^{-1} SYTOX Green. After 10 min of incubation, the hyphae were washed with minimum medium and glass coverslips containing hyphae were mounted in slides. For the assembly of the samples in the slides, 15 µL of DABCO was used. The fluorescence of *B. cinerea* hyphae stained with SYTOX Green was observed under a confocal microscope (Carl Zeiss LSM 510) at an excitation wavelength of 488 nm and an emission wavelength of 540 nm. Each experiment was done at least three times.

2.8. Experimental design and statistical analyses.

The antifungal activity of the different compounds against *B. cinerea* was analyzed with a one-way analysis of variance (Prism 5.01). Means were separated with the least significant difference test ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Synthesis of heterodimeric compound

The reaction catalyzed by the laccase from *T. versicolor* among syringic acid and aniline revealed the formation of a main product (compound 1) and a secondary product. Therefore, this laccase was able to mediate the coupling of syringic acid with aniline, similar to the reactions of other anilines, such as 4-chroloaniline [27] and 4-aminobenzoic acid [18].

To increase yield in the production of compound 1, two variables were considered: substrate ratio (syringic acid:aniline) (Table 1) and the amount of the enzyme (Table 2). For substrate ratios 1:1 and 1:2, no difference in the yield was observed. But when ratio was 2:1, the yield dropped to about half.

Table 1. Purification yields of products at different reactant ratios.

Substrate ratio (syringic acid:aniline)	Purification yield (%)
1:1	41.3
1:2	39.2
2:1	18.5

On the other, the yield in compound 1 production increased when the amount of the enzyme was increased. Consequently, a better yield for compound 1 production was obtained using a reactant ratio of 1:1 and 9U of laccase. In this reaction conditions, the laccase-mediated reaction among syringic acid and aniline formed the product with a yield of 67.8%.

Bollag et al. [28] synthesized the dimer N-(2,6-diethylphenyl)-2,6-dimethoxy-p-benzoquinone imine with a higher yield (around 85%) using the enzyme laccase isolated from the fungus *Rhizoctonia praticola* as catalyzer and syringic acid and 2,6-diethylaniline as substrates. They observed that yield increased with the concentration of syringic acid [28]. On the contrary, in this work, it can be observed that the yield dropped to 18.5% when the concentration of syringic acid was increased. The different yields in dimer production could be attributed to differences in reaction conditions and also in the laccase used. It has been reported that these enzymes have different optimal pH, and the products formed may vary using a different pH in the reaction [29].

Table 2. Purification yields of products at different enzyme amounts.

Amount of laccase (U)	Purification yield (%)
2.25	35.1
4.5	41.3
9	67.8

After purification by semi-preparative thin layer chromatography, compound 1 was analyzed by ^1H and ^{13}C NMR spectroscopy. The recorded ^1H NMR spectrum showed differences with the spectra of the precursor molecules. Aniline spectrum displayed three aromatic signals (δ 7.16 t (*J*= 7.8 Hz), 6.76 t (*J*= 7.4 Hz), and 6.69 d (*J*= 7.6 Hz)), the product has the same aromatic signals with identical multiplicity of the aniline δ 7.41 t (*J*= 7.8 Hz), δ 7.18 t (*J*= 7.4 Hz), and δ 6.9 d (*J*= 7.4 Hz). However, it presented different chemical shifts, due to a different chemical environment produced by the quinone ring. The spectrum of the other precursor molecule (syringic acid) showed only one aromatic signal (δ 7.35 s), but in the synthesized product, this signal disappeared and two new signals appeared (δ 6.158 d (*J*=2.0 Hz) and 6.423 d (*J*=2.0 Hz)) this coupling constant suggests a 4-bond coupling [30]. These signals belong to the two protons of the quinone-imine ring. The spectral data agree with a similar compound previously reported in the literature [28]. Therefore, the two protons have different chemical environments and this was also observed for the methoxyl groups. The ^{13}C NMR spectrum exhibited twelve signals: aromatic signals (between δ 115 and 160), C=C signals (between δ 100 and 140), methoxyl signals δ 57.076 and 57.351 and finally, and a carbonilic carbon signal (δ 177).

In order to determine the molecular structure of the synthesized product and to assign all the ^1H and ^{13}C signals, ^1H–^{13}C HSQC and ^1H–^{13}C HMBC experiments were performed (results now shown). All these findings suggested that they belong to a six carbon quinone-ring. Mass spectral analysis of the compound showed a molecular ion peak (M^+ m/z=243) corresponding to

the compound shown in figure 1 according to formula $C_{14}H_{13}NO_3$. Therefore, compound 1 was identified as 2,6-dimethoxy-4-(phenylimino)benzenone (Figure 1), this quinone-imine like compound has not been reported previously in the literature. In a previous work, the laccase-mediated reaction between syringic acid and p-chloroaniline was described [27]. These authors suggested that the mechanism of the reaction was through a nucleophilic addition to quinone structures [27]. On the other hand, Mikolasch et al. [8] presumed that the laccase reactions proceed by formation of a radical cation, following by a deprotonation of the hydroxyl group to give the radical. Further investigation is needed to describe the reaction mechanism in the synthesis of compound 1.

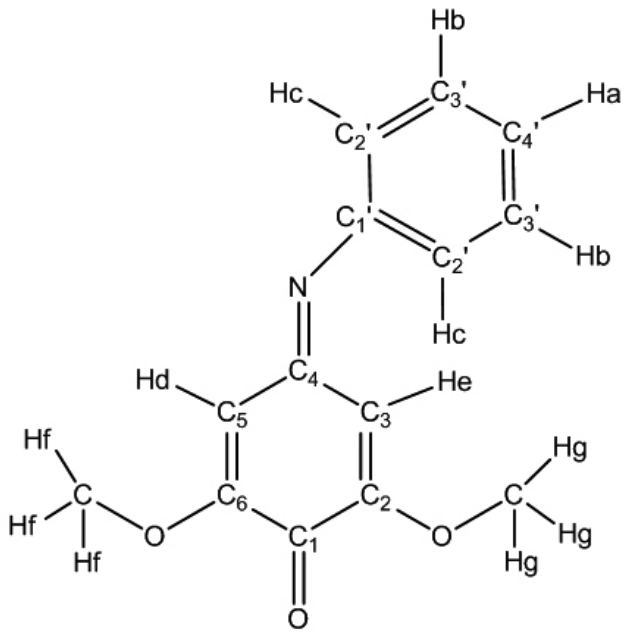

Figure 1. Structure of compound **1** (2,6-dimethoxy-4-(phenylimino) benzenone)

3.2. Antifungal activity of the compound 1, the syringic acid and the aniline

In order to determine the antifungal activity of compound 1, syringic acid, and aniline against *B. cinerea*, the effect on mycelial growth and conidial germination was evaluated in solid medium.

Compound 1 showed a higher antifungal effect on the mycelial growth of *B. cinerea* than the substrates (Table 3), these differences are statistically significant ($P < 0.05$). This compound inhibited mycelial growth, with an IC_{50} value of 0.14mM. The commercial fungicide iprodione was also tested; the results showed that this fungicide had a higher antifungal activity than compound 1.

Table 3. Effect of compound **1**, syringic acid, and aniline on the mycelial growth of *B. cinerea* in a solid medium.

Compound	IC_{50} (mM)*
Syringic acid	3.97±0.52 a
Aniline	2.14±0.31 b
Compound **1**	0.14±0.02 c
Iprodione	0.015±0.003 d

*IC_{50} was determined after 4 days of incubation. Data represents mean ± standard deviation of three independent experiments. Different letters indicate that the means are significantly different at $P < 0.05$.

Figure 2 shows the effect of compound 1 on the conidial germination of *B. cinerea*. At a concentration of 0.16mM, compound 1 delayed the beginning of the conidial germination by two hours compared to the control.

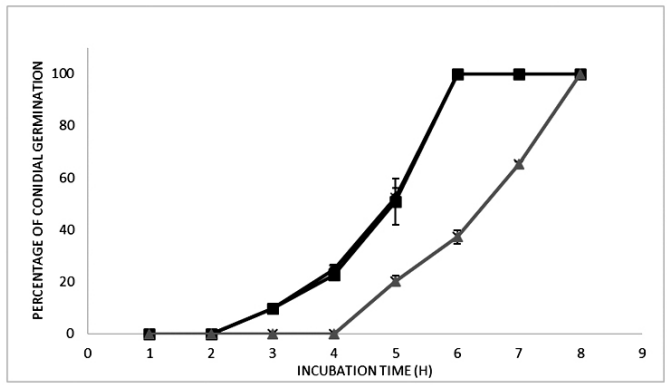

Figure 2. Effect of compound **1** on the conidial germination of *B. cinerea.* Compound **1**, dissolved in acetone, was added at final concentration of 0.16mM (▲). Control with (■) and without (■) acetone. Each value corresponds to the average of two independent experiment ± standard deviations.

There are no previous works on antifungal effect of quinone-imines against fungal species neither against *B. cinerea*; nevertheless, the cytotoxic activity of a similar quinone-imine has been extensively reported, mainly by its reaction with proteins [31,32]. However, antifungal activity of quinones has been analyzed; Mendoza et al. [33] reported the antifungal effect of various quinones on mycelial growth and conidia germination of *B. cinerea*. A similar effect was observed for the compound synthesized in this study. The IC_{50} values for the reported quinones were among 0.125 mM and 0.71mM, similar to the IC_{50} obtained for compound 1, 0.14 ± 0.02mM. In regard to conidial germination, both the reported quinones and compound 1 showed a delay at the beginning of the process.

Laccase-mediated modification of phenolic compounds has been previously reported with the objective of increase a higher biological activity. For instance, the ferulic acid was modified using laccase to produce two dimeric molecules with higher antioxidant capacity than the ferulic acid [16]. An additional example is the enzymatic modification of antibiotics using nitrogen compounds, the products showed higher antibiotic activity than the initial antibiotic [20,21]. Similarly, in this study, the enzymatic modification of syringic acid with aniline increased its antifungal activity.

***3.3. Effect on cell wall of B.* cinerea.**

The redox properties of some quinones have been described. These are able to generate reactive oxygen species causing damage to several macromolecules like membrane lipids, DNA, and proteins [34]. According to this, the effect of compound 1 on the membrane integrity was measured using SYTOX Green nucleic acid stain, fluorescence of the nuclei is only observed when the plasma membrane is damaged, and SYTOX is able to enter to the hypha [26], however, after the incubation with compound 1, no fluorescence was detected (results not shown).

Afterwards, the effect of compound 1 on the cell wall was tested using the fluorochrome calcofluor white which binds to β-1,3 and β-1,4 polysaccharides, such as chitin, one of the main components of the cell wall in fungi, and when this occurs, fluorescence of the hyphae could be observed [35]. In the negative control, (acetone), intense fluorescence was observed, indicating no damage of the cell wall (Figure 3, row A), when hyphae were treated with lysing enzymes (positive control) only a small amount of fluorescence was observed, indicating a damaged cell wall (Figure 3, row B). When hyphae were treated with compound 1, fluorescence was lower than the observed in the negative control (Figure 3, row C), indicating an alteration of *B. cinerea* cell wall.

Therefore, compound 1 would interact with cell wall of *B. cinerea* and it would not cause damage in the plasmatic membrane.

4. CONCLUSIONS

In this study, the antifungal activity of syringic acid was improved using the enzyme laccase to synthesize the compound 2,6-dimethoxy-4-(phenylimino)benzenone by the coupling reaction with aniline. This compound has not been reported previously. Subsequently, the laccase modification is a good alternative to improve the antifungal activity found in grape pomace. Cell wall damage was observed when the fungus was incubated with compound 1.

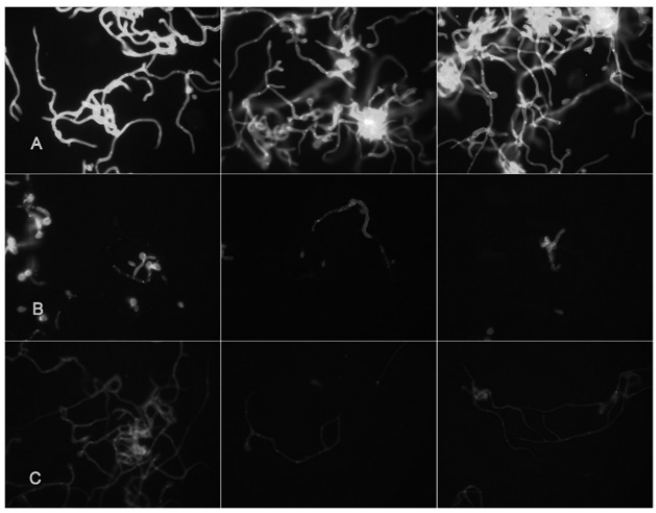

Figure 3. Effect of compound **1** on the cell wall integrity of *B. cinerea.* Conidia at a final concentration of 1 x 10^5 conidia mL^{-1} were inoculated in liquid minimum medium at 22°C for 15 h in the presence of 10% (v/v) acetone (row A), Lysing enzymes (row B), and 0.16mM of compound **1** (row C). Each row shows three different microscope fields of the same treatment.

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