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Antifungal activity against *Botrytis cinerea* of 2,6-dimethoxy-4-(phenylimino)benzenone derivatives

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Abstract: In this work the enzyme laccase from *Trametes versicolor* was used to synthetize 2,6-dimethoxy-4-(phenylimino)benzenone derivatives. Four products with different substitutions in the aromatic ring were synthetized, characterized using ^{1}H and ^{13}C NMR, IR, and mass spectrometry. The *p*-chlorinated compound showed higher antifungal activity against the phytopathogen *Botrytis cinerea*, while the *p*-methoxylated compound had the lowest activity; however, the antifungal activity of the products was higher than the substrates of the reaction. All the compounds retarded the conidial germination of the fungus. Finally, the observations suggested that these compounds produced damage in the fungal cell wall.

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

1. Introduction

Botrytis cinerea is a phytopathogenic fungus promoted by the presence of free surface water or high relative humidity and causing significant crop losses in a wide variety of plant species [1]. Regarding the control, different cultural methods aiming to reduce humidity can be combined to contribute to disease suppression, in addition to chemical botryticides or biocontrol treatments [1]. Chemical control based on the application of mostly synthetic compounds is the most common way to manage *B. cinerea* on many crops [1]. The restriction of this type of control becomes necessary to reduce the impact on the environment [2] and to avoid the acquired resistance to botrycides [3–7]. For this reason, the development of new antifungal compounds is essential. Natural products can be a good alternative to commercial fungicides [8,9]. For instance, terpenoids, phenolic compounds, nitrogen-containing compounds, and aliphatic compounds isolated from plants have shown antifungal properties [10–12]. Additionally, new antifungal compounds against B. *cinerea* derived of natural products have been synthesized, such as derivatives of natural stilbene resveratrol [13], chlorophenyl derivatives [14] or different clovanes [15].

Several phenolic compounds found in grape pomace have shown to have a low antifungal activity against the fungus *B. cinerea* [16], therefore, it is possible to increase the biological activity of phenolic compounds using the enzyme laccase [17]. These enzymes (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the oxidase group, and they are also useful for cleaner industrial application [18]. Laccases are also called multicopper oxidases, belong to the family of copper-containing phenol oxidases [19] and can oxidize a variety of compounds, e.g., phenolic and non-phenolic compounds [18]. Aromatic compounds can generate reactive radical intermediates, which undergo self-coupling reactions, thereby forming different dimers and trimers [20–24]. This enzyme has been previously used to improve the activity of antibiotics [25,26]. On the other hand,

the synthesis of a heterodimeric compound (2,6-dimethoxy-4-(phenylimino)benzenone) by the laccase-mediated coupling reaction between syringic acid and aniline was reported, this compound showed an antifungal effect against *B. cinerea* with an EC50 value of 0.14mM and delayed 2 hours the conidial germination of this fungus [27].

Antifungal compounds have shown several inhibition mechanisms related to the molecular structure. For instance, the resveratrol derivative (E)-3,5-dimethoxy-β-(2-furyl)-styrene cause cell membrane damage against *B. cinerea* [13]. Phenylpyrroles induce morphological alterations of germ tubes [28]. Fungicides such as dinocap and fuazinam have been described as uncouplers of oxidative phosphorylation [29,30] and fungicides like dicloran, cloroneb and etazol affect cell wall synthesis [28].

This work aimed to determine the antifungal activity against *B. cinerea* and the effect on its cell wall integrity of four derivatives obtained by reaction of syringic acid with substituted anilines. To analyze the effect of the carboxylic group in these laccase-catalyzed reactions, syringaldehyde was used instead syringic acid and the reaction product was characterized.

2. Results and Discussion

2.1 Laccase-mediated synthesis of 2,6-dimethoxy-4-(phenylimino)benzenone derivatives

In this work, laccase catalyzed reactions using syringic acid and four substituted anilines (4-chloroaniline, 3-chloroaniline, 4-methoxyaniline, and 3-methoxyaniline) were carried out. It has been previously reported that laccase-catalyzed reactions among phenolic compounds and anilines, heterodimeric compounds are formed [17,25,31,32] similarly found in this work (Figure 1).

To determine the yields in the formation of the cross-coupling products heterodimer, different reaction conditions, as substrate ratio and amount of enzyme were analyzed. The substrate ratio which better yields were obtained was not the same in all reactions (Table 1). For instance, with methoxyanilines lowest yield were obtained due to a high production of secondary products (results not shown), and the highest yield was obtained in the reaction using 3-chloroaniline (Table 1). This higher yield could be explained because the oxidation of 3-chloroaniline does not occur [33], which allows that all substrate is available for cross-coupling reaction.

Excepting the reaction 1, all reactions reached the higher yield with the acid and the anilines in the ratio 1:1, when aniline was used as substrate, the same result was reported [27], indicating that the increase of the concentration of one of them decrease the yield of the analyzed compounds.

On the other hand, the yield reaction did not increase when the enzyme concentration was increased (Results not shown). Bollag et al. [31] showed that the prolonged incubation or higher enzyme concentration caused further polymerization reaction decreasing cross-coupling formation. Furthermore, Itoh et al. [34] concluded that reactivity of the laccase mediated reaction between phenolic acids and chlorophenols is due to the substrate specificity of the laccase rather than the chemical property of the substrates, which could explain the lack of relations among electron donating and withdrawing groups and yield of the reactions.

Table 1. Percentage yields of products at different reactant ratios

Reaction/Compound	Substrate Ratio (syringic acid : aniline)		
	1:2	**1:1**	**2:1**
1	10.4	36.9	55.5
2	38.1	72.0	24.1
3	15.5	26.7	ND[1]
4	8.2	24.2	4.1

[1]ND: Not determined.

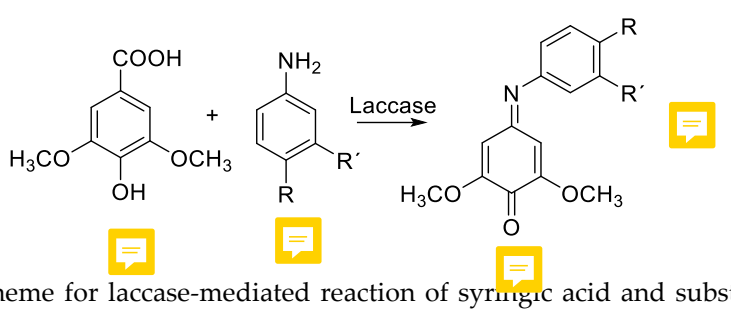

Figure 1. Reaction scheme for laccase-mediated reaction of syringic acid and substituted anilines. Reaction 1 R=Cl, R′=H (4-chloroaniline), reaction 2: R=H, R′=Cl (3-chloroaniline), reaction 3: R=OCH_3, R′=H (4-methoxyaniline), and reaction 4: R=H, R′=OCH_3 (3-methoxyaniline).

The four synthetized compounds were purified using semi-preparative chromatography and were identified using ^{1}H NMR and ^{13}C NMR spectra and mass spectrometry. Compound **2** showed two aliphatic proton signals [δ 3.670 (s, 3H, H8) and δ 3.874 (s, 3H, H7)] and two aliphatic carbon signals (δ 56.212 and δ 56.321) that determined the presence of two methoxy moieties. Two olefin hydrogen signals at higher fields [δ 6.010 (d, 1H, H3 *J*=1.9 Hz) and δ 6.368 (d, 1H, H5 *J*=1.9 Hz)], the olefin carbon signals [δ 98.583 (C3) and δ 111.717 (C5)] and one carbon signal at δ 176.633 (C1) indicated the quinonoid character of the products (Figure 2). Table 3 presents the NMR data (chemical shift assignments for short and long-range hetero-nuclear coupling) of compound **2**.

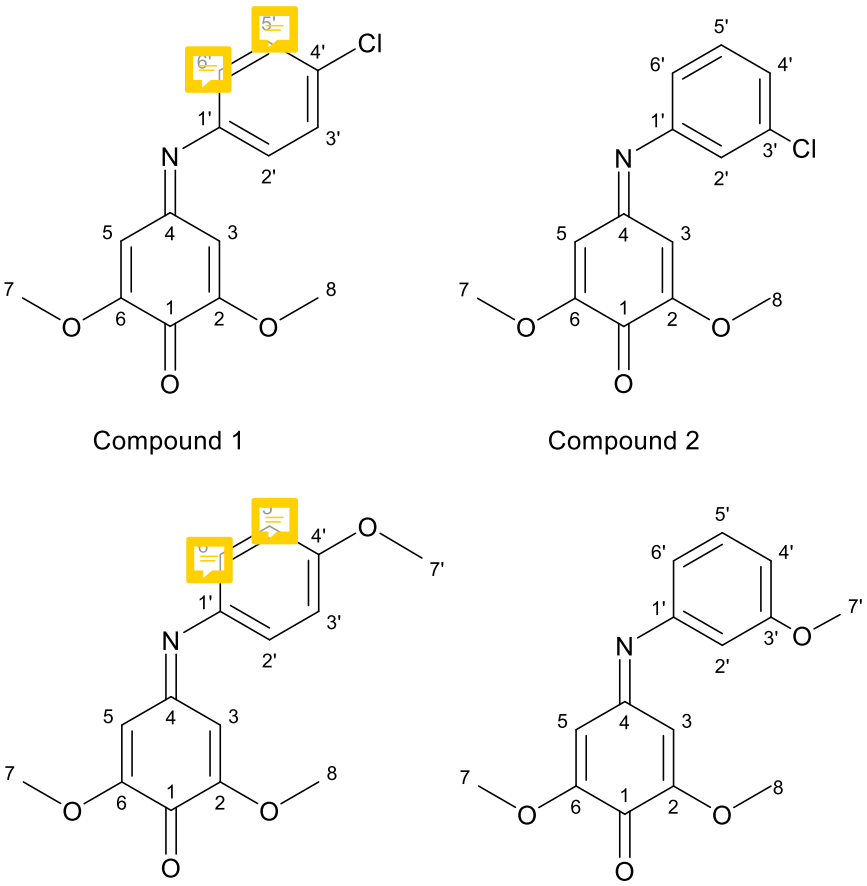

Figure 2. Structure of synthetized compounds Compound 1 (2,6-dimethoxy-4-(4′-chlorophenylimino)benzenone), compound 2 (2,6-dimethoxy-4-(3′-chlorophenylimino)benzenone), compound 3 (2,6-dimethoxy-4-(4′-methoxyphenylimino)benzenone) and compound 4 (2,6-dimethoxy-4-(3′-methoxyphenylimino)benzenone)

The only difference in spectrum signals among compound 1 and 2 was in the aromatic zone of the spectrum. Compound 2 showed four aromatic proton signals δ 6.737 (d, 1H, H6′, *J*=8.0 Hz), δ 6.888 (s, 1H, H2′), δ 7.159 (d, 1H, H4′, *J*=8.0 Hz) and δ 7.316 (t, 1H, H5′, *J*=8.0 Hz) that indicated the existence of a *m*-substituted aromatic fragment. The assignation of the entire molecule was achieved by using two-dimensional NMR analysis. Therefore, identifying this compound as 2,6-dimethoxy-4-(3′-chlorophenylimino)benzenone (Figure 2). Compound 1 was previously synthesized [31

Table 3. ^{1}H and ^{13}C chemical shifts assignments for compound 2. Information form HSQC and HMBC experiments are also provided.

	^{13}C	^{1}H	HMBC	HSQC
1	176,633		5, 3	
4	157,816			
2	155.741		8	
6	154.881		7	
1′	151.483		5′	
3′	134.928		5′	
5′	130.247	7.316 (t, J 8.0 Hz, 1H)		5′
4′	125.023	7.159 (d, J 8.0 Hz, 1H)	6′, 2′	4′
2′	120.635	6.888 (s, 1H)	6′, 4′	2′
6′	118.705	6.737 (d, J 7.9 Hz, 1H)	4′, 2′	6′
5	111.717	6.368 (d, J 1.9 Hz, 1H)	3	5
3	98.583	6.010 (d, J 1.9 Hz, 1H)	5	3
7	56.321	3.874 (s, 3H)		7
8	56.212	3.670 (s, 3H)		8

The only structural difference between compound 2 and 3 was the methoxyl group on the aromatic ring. Compound 3 also showed one set of aromatic proton signals [δ 6.903 (d, 2H, Hb, *J*=8.9 Hz) and δ 6.956 (d, 2H, Ha, *J*=8.9 Hz)] that indicated the existence of a *p*-substituted aromatic fragment. The other difference was a new proton signal [δ 3.846 (s, 3H, Hf)] and a new carbon signal (δ 55.656), indicating a third methoxy moiety in the structure. The assignation of the whole molecule was achieved by using two-dimensional NMR analysis (results not shown). Compound 3 was identified as 2,6-dimethoxy-4-(4′-methoxyphenylimino)benzenone (Figure 2).

The last product, compound 4, was synthesized using syringic acid and 3-methoxyaniline. This molecule showed a set of four aromatic protons [δ 6.429 (d, 1H, Hd, J= 7.7 Hz), δ 6.452 (s, 1H, Hc), δ 6.736 (d, 1H, Hb, J= 8.3 Hz) and δ 7.281 (t, 1H, Ha, J= 8.0 Hz) that indicated the existence of a *m*-substituted aromatic fragment, also a third proton signal [δ 3.819 (s, 3H, Hh)] and a carbon signal (δ 55.476) in the aliphatic zone, indicated the third methoxy moiety. The assignation of the whole molecule was confirmed by using two-dimensional NMR analyses (results not shown). Compound 4 was identified as 2,6-dimethoxy-4-(3′-methoxyphenylimino)benzenone (Figure 2).

Interesting, the compound 1 was also obtained using syringaldehyde instead of syringic acid in the reaction with 4-chloroaniline. This could be explained with an extra step when using syringaldehyde, an oxidation of the aldehyde to a carboxylic acid, similar oxidations has been previously described using several aromatic aldehydes with laccase, yielding carboxylic acids. [35]. Hence, syringaldehyde is oxidized to syringic acid and then the same product (compound 1) could be found in both reactions, starting with syringaldehyde or with syringic acid.

2.2 Antifungal activity

Antifungal activity of substrates and compounds 1-4 against *B. cinerea* was measured both on mycelial growth and conidial germination in solid media, and the EC_{50} calculated using the mycelial growth (Table 4). The most active compound was the *p*-chloro-substituted product (compound 1), while the lowest activity was the compound 3. It has been reported that the substituent affects the antifungal activity of a molecule[36], for instance, the position of the chlorine atom in the aromatic ring is important for the antifungal activity against *B. cinerea* since *para*-substituted compound was

157 more active than the *meta*-substituted compound and unsubstituted compound, while activity of
158 *meta*-substituted compound (compound 2) and unsubstituted compound are similar.

159 Additionally, the methoxy derivative compounds (3 and 4) were less active against the fungus
160 than the chlorinated or the non-substituted compounds [27], same effect observed for aspirin
161 derivatives, where the methoxy *para*-substituted derivative showed almost 30% less antifungal
162 activity against *B. cinerea* than the chlorinated para-substituted compound [37]. Similar behavior was
163 previously reported for oxadiazole derivatives when tested the activity of the methoxy *meta*-
164 substituted oxadiazole derivative against *B. cinerea*, and its activity was less than half compared to
165 the non-substituted compound [38]. Usually, the chloro-substituted compounds have higher
166 antifungal activity in commercial fungicides, for example, chlorine compounds like boscalid,
167 chlorothalonil and iprodione have been used to control *B. cinerea* [6].

168 **Table 4.** Effect of the four compounds on the mycelial growth of *B. cinerea* in solid medium.

Compound	EC_{50} (mM)
1	0.065 ± 0.003
2	0.15 ± 0.01
3	0.54± 0.06
4	0.39 ± 0.02

169 Furthermore, all the substrates used in the reactions showed lower antifungal activity than the
170 products (Table 5). According to the results, the antifungal activity of this type of molecules is highly
171 affected by the substituent and the position on the aromatic ring.

172 **Table 5**. Effect of the substrates on the mycelial growth of *B. cinerea* in a solid medium.

Compound	EC_{50} (mM)
Syringic acid	>1.51
***p*-chloroaniline**	0.71 ± 0.08
***m*-chloroaniline**	0.59 ± 0.04
***p*-methoxyaniline**	>3.00
***m*-methoxyaniline**	>3.00

173 Regarding conidial germination, Figure 3 shows that all the compounds delayed the beginning
174 of the conidial germination for about two hours compared to the control. These molecules showed a
175 similar effect to the non-substituted molecule [27].

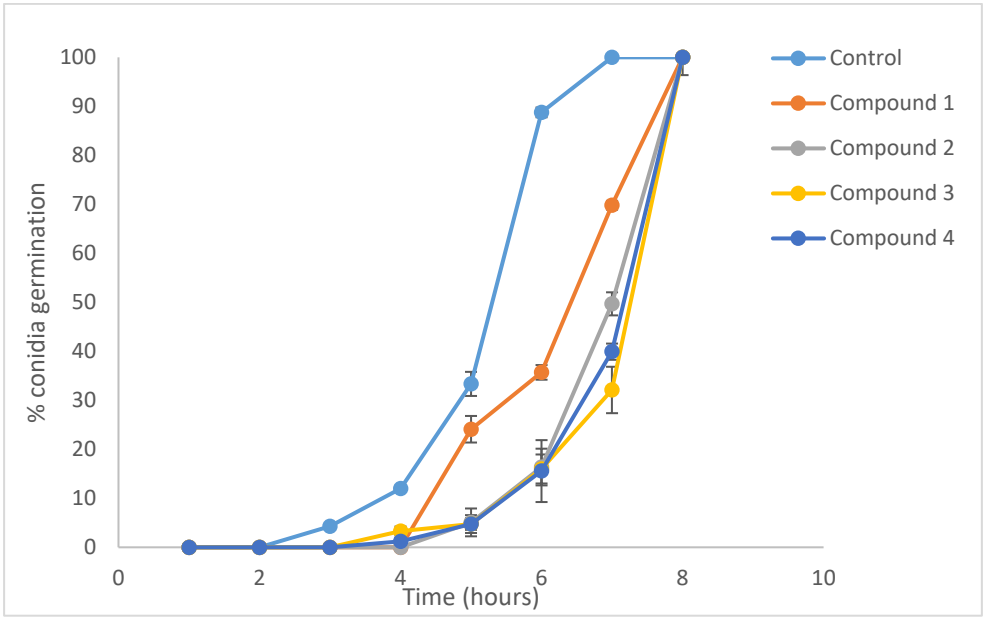

Figure 3. Effect of compounds 1, 2, 3 and 4 on the conidial germination of *B. cinerea.* Compounds, dissolved in acetone, were added at final concentration of 0.16mM. Each value corresponds to the average of two independent experiment ± standard deviations.

2.3 Effect on the cell wall integrity of B. cinerea

Figure 4 shows the effect of compound 1 on the cell wall of *B. cinerea.* CFW stain 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, the fluorescence of the hyphae could be observed [39]. Treatment with compound 1 (Figure 4) showed a lower fluorescence intensity than the negative control (acetone), indicating that this compound can damage the cell wall of this fungus. The same assay was performed using compounds 2, 3 and 4. The four synthesized compounds caused a decrease of the fluorescence intensity compared to the control; relative fluorescence intensity is observed in Figure 5. This result could be attributed to the toxicity of quinones, this is related to the production of reactive oxygen species (ROS) and can cause severe oxidative stress within cells oxidizing membrane lipids, proteins, and DNA [40]. Quinone derivative N-acetyl-p-benzoquinone imine (NAPQI) can react with nucleophiles such as thiol groups of proteins or glutathione [41,42]; this last molecule is an important antioxidant molecule in fungi [43]. Also, some aromatic antifungal compounds have shown effects on cell wall synthesis [6], inhibiting glucan and chitin synthases [44], enzymes that catalyze the main polymers of the cell wall in fungi.

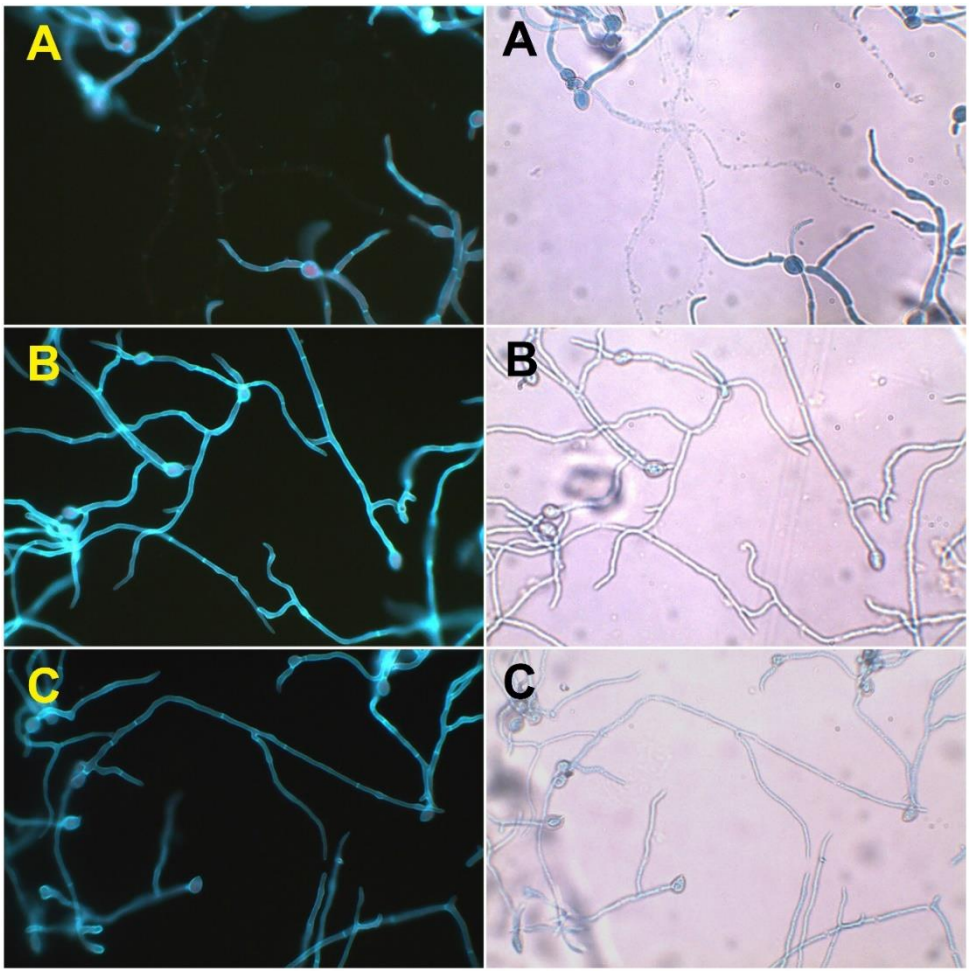

Figure 4. Effect of compound 1 on the cell wall of *B. cinerea*. Hyphae of *B. cinerea* incubated with PBS 10 mM buffer (pH 5.8) along with; A) lysing enzymes (positive control), B) acetone 5% (v/v) (negative control) and C) compound 1 at 40 µg/mL. *B. cinerea* hyphae were treated with CFW stain. Assays were carried out in triplicate.

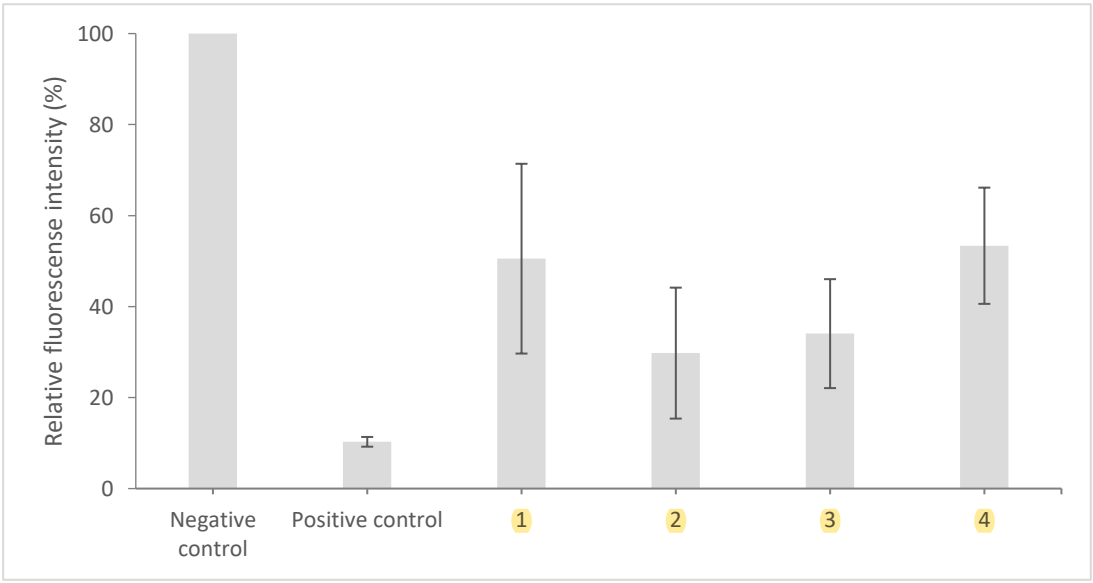

Figure 5. Effect of the compounds on the cell wall integrity of *B. cinerea*. The effect was measured as relative fluorescence intensity compared to maximum fluorescence (negative control).

3. Materials and Methods

3.1 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). Measurements were done 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 F_{254}, 0.2 mm thick and semi-preparative thin layer chromatography on Merck Kieselgel 60 F_{254} 20x20cmx0.25 mm. The mass spectra of these compounds were acquired using a mass spectrometer Shimadzu-GCMS-Q5050 instrument using the direct inlet system. The scan covered the mass range (m/z) from 150 to 350 m/z.

3.2 Chemicals reagents

Laccase from *Trametes versicolor* (EC 1.10.3.2), lysing enzymes from *Trichoderma harzianum*, Calcofluor white stain, 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid), 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde) and 4-chloroaniline were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 3-chloroaniline, 4-methoxyaniline, 3-methoxyaniline, organic solvents and salts were obtained from Merck (Hohenbrunn, Germany). Technical grade fungicide iprodione [3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] was provided from INIA (Santiago, Chile). Agar was obtained from Difco Laboratories (Detroit, MI, USA).

3.3 Laccase-mediated synthesis of 2,6-dimethoxy-4-(phenylimino)benzenone derivatives (compounds 1-4)

Syringic acid with an aniline derivative at different ratios (1:1, 1:2 and 2:1) (e.g. for ratio 1:1 means 0.1mmol for both syringic acid and the aniline derivative were used) and three different enzyme amounts (2.25, 4.5 and 9 U) were tested to increase the quantity of the synthesized compounds.

For the first reaction syringic acid and 4-chloroaniline, 3-chloroaniline, 4-methoxyaniline, or 3-methoxyaniline were dissolved in 1 mL ethyl acetate and the enzyme was dissolved in 1 mL sodium acetate buffer (20 mM, pH 4.5). Both solutions were mixed and stirred at 100 rpm for 180 minutes at 22°C. After this time, the solvent was evaporated using a rotary evaporator at 40 °C. The synthetized compounds were purified from the reaction mixture by using semi-preparative thin layer chromatography with hexane: ethyl acetate (1:1) as an eluent system. Percentage yield of products was calculated using the dry weight of the reaction mixture and the weight of the purified product.

3.4 Spectroscopic data

Compound 1 (2,6-dimethoxy-4-(4′-chlorophenylimino)benzenone) Yield 5[illegible].5 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.668 (s, 3H, H8), 3.870 (s, 3H, H7), 6.040 (d, 1H, J=1.9 Hz, H3), 6.377 (d, 1H, J=1.9 Hz, H5), 6.816 (d, 2H, J=8.5 Hz, H2′ and H6′), 7.361(d, 2H, J=8.5 Hz, H3′ and H5′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.188 (C8), 56.298 (C7), 98.529 (C3), 111.846 (C5), 122.040 (C2′ and C6′), 129.333 (C3′ and C5′), 130.749 (C4′), 148.777 (C1′), 154.813 (C6), 155.745 (C2), 157.642 (C4), 176.647 (C1). mp 208.0 – 209.1 °C. 277 [M^+] $C_{14}H_{12}ClNO_3$.

Compound 2 (2,6-dimethoxy-4-(3′-chlorophenylimino)benzenone) Yield 72.0 % ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance RW- 400 spectrometer, deuterated chloroform ($CDCl_3$) was used as solvent. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.670 (s, 3H, H8), 3.874 (s, 3H, H7), 6.010 (d, 1H, J=1.9 Hz, H3), 6.368 (d, 1H, J=1.9 Hz, H5), 6.737 (d, 1H, J=7.9 Hz, H6′), 6.888 (s, 1H, J=8.0 Hz, H2′), 7.159 (d, 1H, J=[illegible]4′), 7.316 (t, 1H, J=7.9 Hz, H5′); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ 56.212 (C8), 56.321 (C7), 98.583 (C3), 111.717 (C5), 118.705 (C6′), 120.635 (C2′), 125.023 (C4′), 130.247 (C5′), 134.928

(C3′), 151.483 (C1′), 154.881 (C6), 155.741 (C2), 157.816 (C4), 176.633 (C1).mp 155.7 – 156.0 °C. 277 [M+] $C_{14}H_{12}ClNO_3$.

Compound 3 (2,6-dimethoxy-4-(4′-methoxyphenylimino)benzenone) Yield 79.07 %, ^1H and ^{13}C NMR spectra were recorded using a Bruker Avance RW- 400 spectrometer, deuterated chloroform ($CDCl_3$) was used as solvent.^1H NMR ($CDCl_3$, 400 MHz) δ 3.691 (s, 3H, H8), 3.846 (s, 3H, H7′), 3.870 (s, 3H, H7), 6.242 (d, 1H, J=1.9 Hz, H3), 6.467 (d, 1H, J=1.9 Hz, H5), 6.903 (d, 2H, J=8.9 Hz, H2′ and H6′), 6.956 (d, 2H, J=8.9 Hz, H3′ and H5′); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ 55.656 (C7′), 56.104 (C8), 56.277 (C7), 98.966 (C3), 112.113 (C5), 114.594 (C3′ and C5′), 123.047 (C2′ and 6′), 143.196 (C1′), 154.661 (C6), 155.739 (C2), 156.809 (C4), 158.018 (C4′), 176.669 (C1). mp 111.7 – 112.3 °C. 273 [M+] $C_{15}H_{15}NO_4$.

Compound 4 (2,6-dimethoxy-4-(3′-methoxyphenylimino)benzenone) Yield 24.24 %, ^1H and ^{13}C NMR spectra were recorded using a Bruker Avance RW- 400 spectrometer, deuterated chloroform ($CDCl_3$) was used as solvent.^1H NMR ($CDCl_3$, 400 MHz) δ 3.656 (s, 3H, H8), 3.819 (s, 3H, H7′), 3.868 (s, 3H, H7), 6.125 (d, 1H, J=2.0 Hz, H3), 6.392 (d, 1H, J=2.0 Hz, H5), 6.429 (d, 1H, J=7.7 Hz, H6′), 6.452 (s, 1H, H2′), 6.736 (d, 1H, J=8.1 Hz, H4′), 7.281 (t, 1H, J=8.0 Hz, H5′); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ 55.476 (C7′), 56.124 (C8), 56.248 (C7), 99.051 (C3), 106.387 (C2′), 110.942 (C4′), 112.023 (C5), 112.868 (C6′), 126.946 (C5′), 151.664 (C1′), 154.783 (C6), 155.569 (C2), 157.264 (C4), 160.402 (C3′), 176.785 (C1). mp 133.5 – 134.6 °C. 273 [M+] $C_{15}H_{15}NO_4$.

3.5 Laccase-mediated synthesis of 2,6-dimethoxy-4-(4′-chlorophenylimino) benzenone using syringaldehyde

For the synthesis 2 using syringaldehyde and 4-chloroaniline, same conditions were used as for syringic acid and 4-chloroaniline.

3.6 Fungal strain and culture conditions

The strain G29 of *B. cinerea* isolated from infected grapes (*Vitis vinifera*) by the Instituto de Investigaciones Agropecuarias La Platina, Chile and is genetically characterized was used in this study [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 compounds on the cell wall integrity, minimal 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 7H2O (0.01 g/L) pH 6.5, 4,6 g/L ammonium tartrate as a nitrogen source, and 1% (w/v) glucose as carbon source.

3.7 Antifungal Assay

3.7.1 Effect on mycelial growth

The antifungal activity of the compounds was assessed using the radial growth test on malt-yeast extract agar [13]. The compounds were dissolved in acetone and added to Petri dishes containing malt yeast agar medium. The final acetone concentration was identical in the control and treatment assays. After 72 hours of incubation, the inhibition percentages relative to the control with acetone were calculated. Results were expressed as the effective concentration that reduced mycelial growth by 50% (EC_{50}), determined by regressing the inhibition of radial growth values (percent control) against the values of compound concentration. These experiments were done in triplicate.

3.7.2 Effect on conidial germination

Conidial germination assays were carried out on microscope slides coated with soft agar medium (2 mm thickness). Pure compounds were added dissolved in acetone at a final concentration of 40 μg/mL. Acetone was evaporated before inoculation. The slides were inoculated with dry conidia obtained from sporulated mycelia (1-week-old), placed in a humid chamber (90% relative humidity), and incubated in the dark at 22 °C for 7 hours. Conidial germination was determined directly on the

slides at 1-hour intervals. The percentage of germination was estimated by counting the number of germinated conidia in five microscope fields each containing approximately 40 conidia. Conidia were judged to have germinated when the germ tube length was equal to or greater than the conidial diameter. Each experiment was done at least in triplicate.

3.8 Effect on the cell wall integrity of B. cinerea

B. cinerea conidia at a final concentration of 1 x 10^3 conidia mL^{-1} were inoculated in 24-well plates (lined with 12-mm glass coverslips) containing 1 mL of liquid minimal medium supplemented with glucose (10% p/v) and fructose (10 mM). Cultures were incubated at 22°C for 15 hours to permit the germination of the conidia. After this time, liquid medium was removed. Afterward, the liquid medium containing: lysing enzymes (positive control), 10% (v/v) acetone (negative control), and compounds 1-4 (0.16mM) were added to each well. The mixtures were incubated at 22°C for 6 hours. *B. cinerea* hyphae adhered to glass coverslips were washed three times with liquid minimal medium and stained with Calcofluor White (CFW). After 5 min of incubation, the hyphae were washed with minimal medium and glass coverslips containing hyphae were mounted on slides. For the assembly of the samples on the slides, 15 µL of DABCO was used. The fluorescence of *B. cinerea* hyphae stained with CFW was observed under a fluorescence microscope using a BL/VIO filter. Each experiment was done at least three times. To measure the effect of these compounds on the cell wall, fluorescence intensity was quantified using ImageJ (v1.80), an outline was drawn around each hypha, mean fluorescence measured, along with several adjacent background readings. Mean fluorescence was compared to the negative control (maximum fluorescence).

3.9 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 ($P < 0.05$).

4. Conclusions

Four compounds were synthetized, only one of them (compound 1) has been previously described. All the products showed a higher antifungal activity than the substrates. Chloro-substituted compounds showed a higher antifungal effect against *B. cinerea and* the *p*-chlorinated product was more active than *m*-chlorinated compound. Synthesis using syringic acid or syringaldehyde with 4-chloroaniline formed the same main product (compound 1). Finally, regarding the inhibition mechanism of these compounds, the results suggest damage to the cell wall components.

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