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Characterization of the Antifungal Activity on *Botrytis cinerea* of the Natural Diterpenoids Kaurenoic Acid and 3*β*-Hydroxy-kaurenoic Acid

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The antifungal activity on *Botrytis cinerea* of the diterpenoids 3*β*-hydroxy-kaurenoic acid and kaurenoic acid, obtained from the resinous exudates of *Pseudognaphalium vira vira*, was determined. 3*β*-Hydroxy-kaurenoic acid reduced the mycelial growth of *B. cinerea* in solid and liquid media. Additionally, the damage produced by the fungus on the surface of tomato leaves in the presence of the diterpenoids was evaluated. A higher protective effect was observed in the presence of the hydroxylated diterpene. On the other hand, the effect of the diterpenoids on the production of enzymes that participate in the plant infection by *B. cinerea* was analyzed. *p*-Nitrophenylbutyrate esterase production was induced by both diterpenoids, whereas laccase production was only induced by the hydroxylated diterpene. In the study of the mechanism of action of these compounds, it was determined that 3*β*-hydroxy-kaurenoic acid would produce permeabilization of the cell membrane of *B. cinerea*.

KEYWORDS: ***Botrytis cinerea*; antifungal diterpenoids; kaurenoic acid; 3*β*-hydroxy-kaurenoic acid; *Pseudognaphalium vira vira***

INTRODUCTION

Botrytis cinerea, the agent of gray mold, is a facultative phytopathogenic fungus that attacks flowers, fruits, leaves, and stems of more than 200 plant species (*1*). Chilean climatic conditions such as high relative humidity and low temperatures lead to a high incidence of this disease. Diseases caused by this fungus produce considerable losses to crops in the field and during storage (*2*, *3*). In Chile, *B. cinerea* has traditionally been controlled by commercial fungicides (dicarboximides and benzimidazoles). The use of these fungicides has caused serious problems such as the appearance of highly resistant strains and the contamination of soil and water (*4*). The natural products isolated from plants are an alternative to chemical fungicide. Terpenoids, aromatic compounds, nitrogen-containing compounds, and aliphatic compounds isolated from plants show antifungal properties (*5*−*7*).

Some of these natural compounds present activity against *B. cinerea*. Germination of conidia of *B. cinerea* was inhibited by sakuranetin, a flavonoid isolated from the surface of *Ribes nigrum* (*5*). Resveratrol, a stilbene produced by grapes (*Vitis* spp.), inhibits the spread of *B. cinerea* infection (*8*). This compound produces cytological changes in *B. cinerea* such as production of additional germ tubes in conidia, cytoplasmic granulations, retractation of cytoplasm in hypha tip, and curved germ tubes (*9*). Laccase produced by this fungus is assumed to detoxify resveratrol (*10*). In recent studies, it was shown that a specific laccase of *B. cinerea* does not detoxify resveratrol but converts it into compounds more toxic for the fungus (*11*).

Besides, α-tomatine, a saponin produced by tomato (*Lycopersicon esculentum*), affected the growth of this fungus (*12*, *13*). The toxic action of saponins to fungi is associated with the ability of these compounds to complex with membrane sterols and cause pore formation (*6*, *14*).

The tetracyclic triterpenoids cucurbitacins protect cucumber tissue against infection by *B. cinerea* and inhibit the laccase production by the fungus (*15*). On the other hand, the compounds 5,7-dihydroxy-3,8-dimethoxyflavone and 3*β*-hydroxy-kaurenoic acid showed fungitoxic activity against *B. cinerea* (*16*). These compounds were isolated from resinous exudates of *Pseudognaphalium robustum* and *Pseudognaphalium vira vira,* respectively.

P. vira vira (Mol.) A. Anderb. is a perennial grass showing a characteristic combination of glandular and nonglandular trichomes with the production of resinous exudates in twigs and leaves (*17*). These exudates present antibacterial and antifungal properties and mainly contain the diterpenoids kaurenoic acid and 3*β*-hydroxy-kaurenoic acid (*16*, *18*).

The aim of the present study was to evaluate and to characterize the fungitoxic effect on *B. cinerea* of the diterpenoids kaurenoic acid and 3*β*-hydroxy-kaurenoic acid isolated from resinous exudates from *P. vira vira*.

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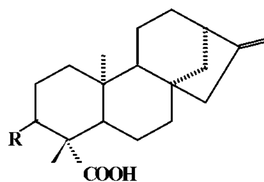

R= H, kaurenoic acid; R= OH, 3β-hydroxy-kaurenoic acid

Figure 1. Structure of diterpenoids.

MATERIALS AND METHODS

Fungal Isolate and Culture Conditions. In this study, the strain G29 of *B. cinerea* was used. This strain was originally isolated from a naturally infected grape (*Vitis vinifera*) (*19*). It was maintained on malt−yeast extract agar slants (2% malt extract, 0.2% yeast extract, and 1.5% agar) at 4 °C. The fungus was grown in the dark in the following culture media.

(a) Solid Media. Two solid media were used as follows: malt−yeast extract agar medium and soft agar medium (2% malt extract, 0.2% yeast extract, and 0.6% agar).

(b) Liquid Media. The fungus was cultured in malt−yeast extract medium (2% malt extract and 0.2% yeast extract). It was also grown in liquid minimum medium, which contained KH_2PO_4 (1 g), K_2HPO_4 (0.5 g), $MgSO_4{\cdot}7H_2O$ (0.5 g), KCl (0.5 g), and $FeSO_4{\cdot}7H_2O$ (0.01 g) per liter. Ammoniun tartrate (25 mM) was used as a nitrogen source, and 1% (w/v) glucose, 1% (w/v) cutin, or 1% (w/v) pectin was added as carbon source. The pH was adjusted to 6.5. Tomato cutin was purified as described by Cordero and Cotoras (*20*).

Isolation of Diterpenoids Kaurenoic Acid and 3*β*-Hydroxy-kaurenoic Acid from *P. vira vira*. The diterpenoids used in this study are shown in **Figure 1**. These compounds were purified from the resinous exudate of *P. vira vira* (Mol.) A. Anderb. as has been described (*21*, *22*). Briefly, the plants were collected during the flowering season (October). The resinous exudates were obtained by dipping the fresh plant material in cold CH_2Cl_2 for 15−20 s. The extracts were concentrated by evaporation of the solvent on a rotary evaporator to a sticky residue that was fractionated by column chromatography on silica gel, using hexane with increasing amounts of ethyl acetate. The isolated diterpenoids were identified by comparison of their physical and spectroscopic (UV−vis, ^1H and ^{13}C NMR, and EIMS) data with literature (*21*, *22*).

Fungitoxicity Assay of Kaurenoic Acid and 3*β*-Hydroxy-kaurenoic Acid on *B. cinerea*. *(a) Effect on Mycelial Growth.* Fungitoxicity of diterpenoid was assessed using the radial growth test on malt−yeast extract agar (*23*). These compounds were dissolved in methanol to different final concentrations. At all concentrations tested, diterpenoids were completely soluble. Aliquots of these solutions (100 μL) were added to 5 mL of soft agar medium (final concentrations of diterpenoids of 40, 60, 120, and 160 μg/mL). After the diterpenoids dissolved in methanol were added to the soft agar medium, a precipitate was not observed. The final methanol concentration was identical in control and treatment assays. The medium with or without test substances was poured into 9 cm diameter Petri dishes containing malt−yeast extract agar. Dishes were left open in a laminar-flow hood for 30 min to remove methanol. This method allows a homogeneous distribution of the compounds on the agar surface. After evaporation of the solvent, the Petri dishes were inoculated with 0.5 cm agar disks with thin mycelium of *B. cinerea*. Cultures were incubated in the dark at 22 °C for 9 days. Mycelial growth diameters were measured daily.

Fungitoxicity of diterpenoid was also assessed in liquid media. Diterpenoids at a final concentration of 40 μg/mL or methanol were added to Erlenmeyer flasks containing malt−yeast extract medium or liquid minimum medium with glucose 1% as a carbon source. The final methanol concentration was identical in control and treatment assays. Methanol was allowed to evaporate prior to inoculation. Flasks were inoculated with conidia to a final concentration of 3×10^5 conidia/mL. Cultures were incubated in the dark at 22 °C. After 5 days of incubation, the wet weight of the mycelia was determined.

(b) Effect on the Ability of B. cinerea to Colonize Tomato Leaves. Detached tomato (*Lycopersicon esculentum* cv. Roma) leaves were disinfected with 10% sodium hypochlorite, washed three times with sterile deionized water, and placed in Petri dishes containing water−agar (1.5% w/v agar). The methanol solution or the diterpenoids at 40 μg/mL were spread on the surface of the leaves with a paintbrush. This procedure was repeated three times. A suspension of conidia was made in Gamborg's B5 medium (Duchefa BV, Haarlem, The Netherlands), supplemented with 10 mM sucrose and adjusted to 10 mM potassium phosphate (pH 6). A 5 μL amount of this conidia suspension (10^5 conidia/mL) was inoculated on the upper side of the tomato leaves. Petri dishes were incubated at 22 °C. After 4 days of incubation, the lesion area on tomato leaves was measured.

Effect of Diterpenoids on the Production of *p*-Nitrophenylbutyrate Esterases and Laccases by *B. cinerea*. To determine enzymatic activities in liquid media, the mycelium was pregrown in liquid malt−yeast medium for 3 days at 22 °C. After this time, the mycelium was ground with glass beads and inoculated in liquid minimum media containing 1% cutin or 1% pectin as a carbon source. Diterpenoids were added to the cultures at a final concentration of 40 μg/mL. Cultures were incubated at 22 °C.

p-Nitrophenylbutyrate esterase and laccase activities were measured in the culture filtrates of the fungus during 30 days. Culture filtrates were collected by passage through Whatman No. 4 paper. *p*-Nitrophenylbutyrate esterase activity was determined spectrophotometrically at 405 nm using *p*-nitrophenylbutyrate as the substrate, as described previously (*24*). The reaction was run in 3 mL of 100 mM sodium phosphate buffer (pH 8.0) containing 0.62 mM *p*-nitrophenylbutyrate, 11.2 mg of Triton X-100, and appropriate amounts of culture filtrates at 22 °C for 10 min. One unit per mL of esterase (U/mL) corresponds to the amount of enzyme that releases 1 μmol of *p*-nitrophenol in 1 min.

To evaluate laccase activity, syringaldazine was used as the substrate (*25*). Enzyme activity was determined spectrophotometrically by monitoring the absorbance at 530 nm. The reaction mixture (1 mL) contained 30 μL of culture filtrates and 0.1 mM syringaldazine in 50 mM sodium phosphate buffer (pH 6.0) and was incubated at 22 °C for 15 min. One unit per mL of laccase (U/mL) corresponds to the amount of enzyme that increases the absorbance in 0.1 in 1 min.

Determination of Mechanism of Action of the Active Diterpene. To analyze if 3*β*-hydroxy-kaurenoic acid produces alteration of the permeability of *B. cinerea* cytoplasmic membrane, the efflux of phosphorus from treated mycelium with diterpenoids was measured. Pregrown mycelia were incubated in 5 mM Tris-HCl buffer (pH 7.0) with the diterpenoids at a final concentration of 100 μg/mL for 6 or 24 h at 22 °C. The concentration of phosphorus was determined in the bathing medium, using an inductively coupled plasma optical emission spectrometry (ICP-OES) (Optimo 200 DV ICP-OES, Perkin-Elmer). In controls, pregrown mycelia were incubated in 5 mM Tris-HCl buffer (pH 7.0) with methanol in the same concentration.

Mycelium incubated with saponins purified from *Quillaja saponaria* (Quillaja Ultra Powder QP UF 1000, Desert King Chile Ltd.) was used as a positive control. Pregrown mycelia of *B. cinerea* were incubated in 10 mM sodium acetate buffer (pH 4.5) with the saponins at a final concentration of 100 mg/mL for 6 h at 22 °C. Phosphorus efflux from the mycelium was determined as above.

Experimental Design and Statistical Analyses. The effect of diterpenoids on the enzymatic activity and cellular leakage was evaluated using a one way analysis of variance (Genstat 5, Realease 4.1). Means were separated using the least significant difference test ($P \leq 0.05$).

RESULTS

Antifungal Activity of Diterpenoids on *B. cinerea*. To determine the antifungal activity of the diterpenoids, the effect of these compounds on mycelial growth in solid and liquid media and on the ability to colonize tomato leaves was evaluated.

Figure 2 shows the effect of different concentrations of the diterpenoids on the mycelium growth of *B. cinerea* in solid medium. 3*β*-Hydroxy-kaurenoic acid presented a higher inhibi-

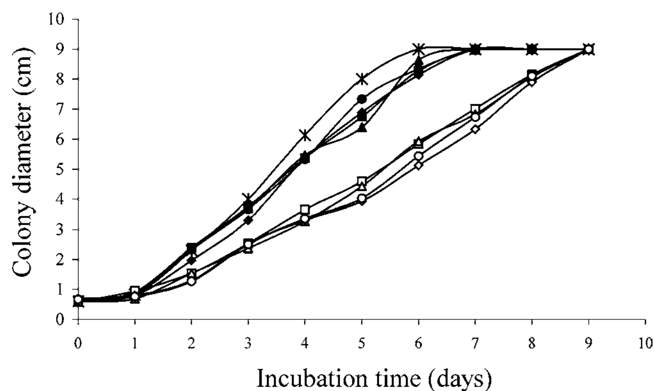

Figure 2. Effect of different concentrations of the kaurenoic acid (dark symbols) and 3β-hydroxy-kaurenoic acid (open symbols) on mycelial growth of *B. cinerea* in solid medium. Diterpenoids dissolved in methanol were added at a final concentration of 40 (□), 60 (△), 120 (◇), or 160 (○) μg/mL. The control contained methanol at the same concentration as treatment assays (*). Each point represents the mean of at least five independent experiments.

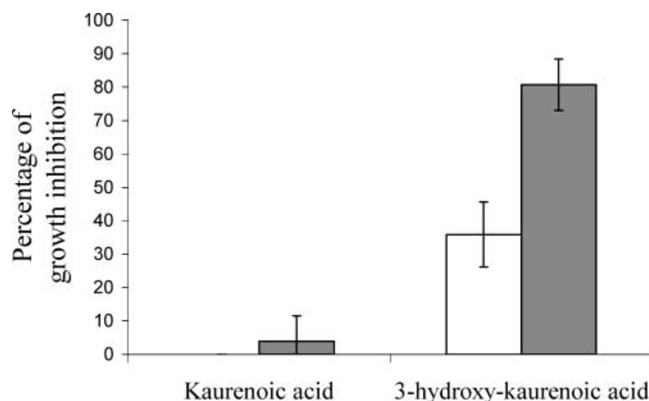

Figure 3. Effect of kaurenoic acid and 3β-hydroxy-kaurenoic acid on the mycelial growth of *B. cinerea* in liquid medium. Liquid media were used as follows: malt–yeast extract medium (□) or minimum medium with 1% (w/v) glucose as the carbon source (■). Diterpenoids dissolved in methanol were added at a final concentration of 40 μg/mL. The control contained methanol at the same concentration as treatments. After 5 days of incubation, the wet weight of the mycelia was determined and the percentages of inhibition relative to the control with methanol were calculated. Each bar represents the mean of at least five independent experiments ± standard deviation.

tory effect than kaurenoic acid. In the presence of methanol, mycelium growth reached a maximum after 6 days of incubation. Instead, in the presence of kaurenoic acid and 3β-hydroxy-kaurenoic acid, maximum growth was reached after 7 and 9 days, respectively. In the control, the mycelium growth rate during the exponential phase was 1.82 cm/day. In the presence of the kaurenoic acid and 3β-hydroxy-kaurenoic acid, this rate was 1.49 and 1.06 cm/day, respectively. The growth rate did not decrease at higher concentrations of the diterpenoids.

In liquid media, the effect of the diterpenoids on growth was determined by the wet weight of the mycelium (**Figure 3**). At 40 μg/mL, in malt−yeast extract medium or liquid minimum medium with glucose as the carbon source, only 3β-hydroxy-kaurenoic acid produced inhibition of mycelium growth. In malt−yeast extract medium, the kaurenoic acid did not inhibit mycelium growth. In minimum medium, the hydroxylated diterpene inhibited mycelium growth by 80.8%. Instead, in malt−yeast extract medium, the percentage of inhibition was 36%.

Table 1. Effect of the Diterpenoids, Kaurenoic Acid, and 3β-Hydroxy-kaurenoic Acid, on the Ability of *B. cinerea* to Colonize Tomato Leaves[a]

treatments	lesion area (mm^2)
control	9.7 ± 1.8
control with methanol	8.3 ± 2.1
kaurenoic acid	7.5 ± 1.0
3β-hydroxy-kaurenoic acid	4.8 ± 0.8

[a] The results represent means of 12 determinations ± standard deviations.

Finally, the effect of the diterpenoids on the ability of *B. cinerea* to colonize tomato leaves was analyzed (**Table 1**). Control leaves and leaves spread with kaurenoic acid presented larger lesions than 3β-hydroxy-kaurenoic acid-treated leaves. Therefore, hydroxylated diterpene reduced the infection by *B. cinerea*.

Effect of the Diterpenoids on the Production of *p*-Nitrophenyl Butyrate Esterases and Laccases by *B. cinerea*. To study the effect of diterpenoids on the production by *B. cinerea* of some enzymes that may facilitate penetration of the plant tissues, the production of *p*-nitrophenyl butyrate esterases and laccases by *B. cinerea* was measured. *p*-Nitrophenyl butyrate esterases activity was measured because it has been confirmed previously that hydrolysis of *p*-nitrophenyl butyrate provides an accurate and simple measurement of cutinase activity that correlated with hydrolysis of ^{3}H-cutin (*26*, *27*).

To quantify the production of *p*-nitrophenyl butyrate esterases or laccases by *B. cinerea*, the fungus was grown in minimum media in the presence of diterpenoids with cutin or pectin, respectively. The maximum enzymatic production was reached after 10 days for laccase and 20 days for *p*-nitrophenyl butyrate esterase (data not shown).

Because the addition of diterpenoids to the culture produced a difference in the mycelium growth, the enzymatic activity was expressed as U/mL and per mg wet mycelium. In the presence of kaurenoic acid or 3β-hydroxy-kaurenoic acid, the production of *p*-nitrophenyl butyrate esterases was increased (**Figure 4A**). The activity of this enzyme, produced in the presence of both diterpenoids, was similar, and it was twice the enzymatic activity produced by the controls. Laccase production was induced only by the hydroxylated diterpene (**Figure 4B**). In the presence of kaurenoic acid, production of laccase was similar to the controls. Instead, in the presence of 3β-hydroxy-kaurenoic acid, the laccase activity was three times higher than the controls.

Effect of the Diterpenoids on Membrane Leakage. It has been reported that the antifungal diterpene (*E*)-8β,17-epoxylabd-12-ene-15,16-dial would produce changes in the membrane permeability of *Candida albicans* (*28*). A similar mechanism could utilize the diterpene 3β-hydroxy-kaurenoic acid on *B. cinerea*. To determine the effect of this compound on the permeability of the *B. cinerea* cell membrane, the efflux of phosphorus from the mycelium was determined by ICP-OES. Saponins were used as a positive control because it has been reported that these compounds interact with cell membrane sterols and produce membrane disruption (*18*). A buffer at pH 4.5 was used because it was previously determined that these saponins were more active at acidic pH (data not shown). The phosphorus concentration was increased in the extracellular medium of *B. cinerea* mycelium incubated with the saponins (**Figure 5A**). Therefore, this technique was adequate to evaluate changes in the permeability of the cell membrane from *B. cinerea*.

Figure 5B shows the effect of the diterpenoids on the efflux of phosphorus from the mycelium of *B. cinerea*. Cellular leakage

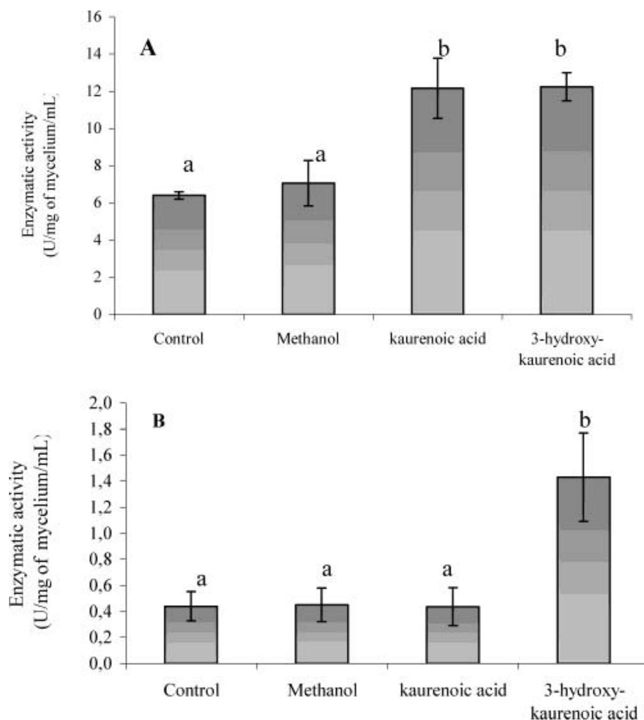

Figure 4. Effect of the diterpenoids on the enzymatic production by *B. cinerea*. (**A**) *p*-Nitrophenylbutyrate esterase activity produced in minimum media with cutin as the carbon source after 20 days of incubation. (**B**) Laccase activity produced in minimum media with pectin as the carbon source after 10 days of incubation. Each bar represents the mean of at least five independent experiments ± standard deviation. Different letters indicate that the means are significantly different at $P \leq 0.05$.

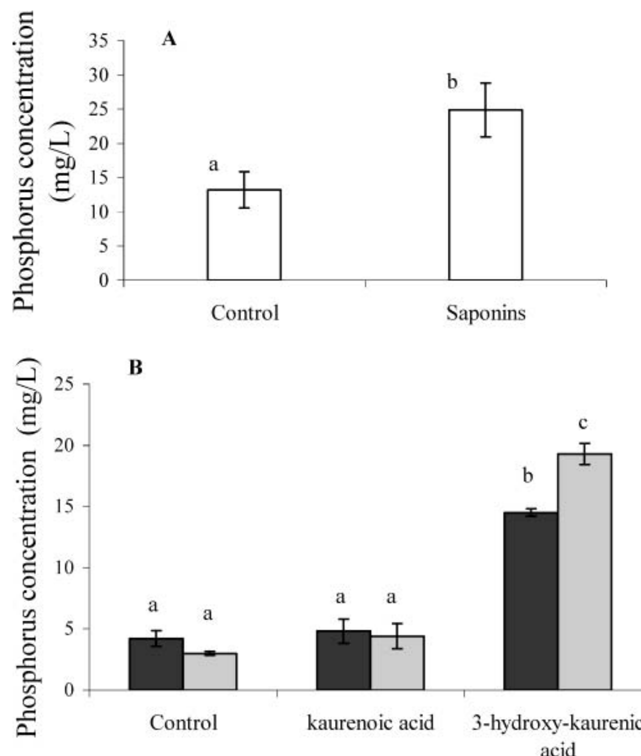

Figure 5. Effect of saponins (**A**) and diterpenoids (**B**) on the phosphorus concentration in the extracellular medium of *B. cinerea*. Pregrowth mycelia were incubated in 10 mM sodium acetate buffer (pH 4.5) with the saponins at a final concentration of 100 mg/mL for 6 h at 22 °C (□) or in 5 mM Tris-HCl buffer (pH 7.0) with the diterpenoids at a final concentration of 100 μg/mL at 22 °C during 6 (■) or 24 (□) h. In controls, fungus was maintained in the buffer. The phosphorus concentration was measured in the extracellular medium by ICP-OES. Each bar represents the mean of at least five independent experiments ± standard deviation. Different letters indicate that the means are significantly different at $P \leq 0.05$.

did not occur when the fungus was incubated with kaurenoic acid. Phosphorus concentration measured in the extracellular media was similar to the control. When fungus was treated with the hydroxylated diterpene, the phosphorus concentration was three times higher than in the control or fungus incubated with kaurenoic acid. This effect was still higher when the fungus was incubated with 3β-hydroxy-kaurenoic acid for a longer time period (24 h).

DISCUSSION

In this work, it was shown that the 3β-hydroxy-kaurenoic acid presented higher fungitoxic activity against *B. cinerea*. In a previous work, it was shown that kaurenoic acid did not affect the germination of *B. cinerea* conidia, but 3β-hydroxy-kaurenoic acid did retard the germination. In the presence of the hydroxylated diterpene, complete germination was attained 2 h later than in the controls (*16*).

These results suggest that the hydroxyl group at the C-3 position is important for the fungitoxic effect of the 3β-hydroxy-kaurenoic acid. It has been suggested that this hydroxyl group would increase the polarity of the rather lipophilic kaurenoic acid and that this characteristic might be important for the antifungal activity of this compound (*16*). Another explanation could be that the carboxyl group at C-19 might be protected by formation of an intramolecular hydrogen bond with the β-hydroxyl group at C-3, with the result that the carboxyl group at C-19 would be less available to detoxification reactions by the fungus (*16*, *29*).

The results of the present work, together with earlier results (*16*), showed that 3β-hydroxy-kaurenoic acid did not completely inhibit the mycelium growth and the germination of *B. cinerea*. This compound only retarded these processes. These results could be explained by the ability of *B. cinerea* to biotransform the hydroxylated diterpene to a less active metabolite. In fact, it has been reported that *B. cinerea* biotransforms compounds as terpenoids, flavonoids, saponins, etc. (*12*, *23*, *30*, *31*). Another possible explanation to these results could be that 3β-hydroxy-kaurenoic acid induces the expression of the ATP-binding cassette (ABC) transporters in *B. cinerea* to eliminate this toxic compound. It has been reported that some compounds as broad spectrum antibiotics produced by *Pseudomonas* spp., resveratrol and the fungicide fenpiclonil, induced expression of several ABC transporter genes in *B. cinerea* (*32*, *33*).

It has been described that some natural products produced by plants can cause inhibition of cutinases and laccase production by phytopathogenic fungi (*34*, *35*). In *Monilinia fructicola*, cutinase production was suppressed by phenolic acids as chlorogenic and caffeic acid. In *B. cinerea*, caffeic acid also decreased cutinase production in liquid cultures (*35*). On the other hand, cucurbitacin repressed the laccase production by *B. cinerea* (*34*).

In this work, it was shown that production of *p*-nitrophenyl butyrate esterase and laccases by *B. cinerea* was stimulated by the diterpenoids. In *B. cinerea*, it has been reported that phenolic compounds or pectin induce laccase production and *p*-nitrophenyl butyrate esterase production is induced by cutin or cutin monomers (*26*, *36*). Therefore, the results obtained in this work suggest that other compounds than those already described can induce the production of *p*-nitrophenyl butyrate esterases and laccase by *B. cinerea*.

In conclusion, the inhibition of conidia germination and mycelium growth of *B. cinerea* produced by 3β-hydroxy-kaurenoic acid protected the tomato leaves against the infection by this fungus. The increased production of *p*-nitrophenyl butyrate esterase and laccase would not affect the colonization of the tomato leaves by *B. cinerea*.

The action mechanism of the antifungal diterpenoids is not known so far. It has been reported that the antifungal diterpene (*E*)-8β,17-epoxylabd-ene-15,16-dial isolated from *Alpinia galanga* would interact with the fatty acid from the cytoplasmatic membrane of *C. albicans* and would produce a change of membrane permeability (*28*).

In the present work, it was shown that the 3β-hydroxy-kaurenoic acid induced efflux of phosphorus from the *B. cinerea* mycelium. Therefore, these data suggest that 3β-hydroxy-kaurenoic acid affects growth of *B. cinerea* mainly by affecting membrane permeabilization. This permeabilization could be by interaction of the diterpene with some membrane constituents.

It has been reported that the kaurenoic acid has a selective antibacterial activity against Gram-positive bacteria and synthetic and natural derivates that protected the carboxyl group at C-19 have not antibacterial activity (*37*). The action mechanism of this diterpenoid on bacteria is unknown, but the alteration of the electron transport and subsequently the oxidative phosphorylation has been postulated as a possible mechanism of action (*37*). Therefore, the action mechanism of the diterpenoids on fungi and bacteria would be different.

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