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Antifungal activity of proteolytic fraction (P1G10) from (*Vasconcellea cundinamarcensis*) latex inhibit cell growth and cell wall integrity in *Botrytis cinerea*

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ABSTRACT

The aim of this study was to determine the antifungal activity of the proteolytic fraction P1G10 from *Vasconcellea cundinamarcencis (ex-Carica candamarcensis)* against *Botrytis cinerea*, the causative agent of pre- and postharvest damaging disease in fruit and vegetables. The survival of *B. cinerea* at different concentrations of P1G10 showed that 1 mg/mL inhibited 50% of mycelium growth after 72 h incubation. The kinetic of growth inhibition fits the Weibull distribution function, and the data was confirmed by the IC_{50} survival assay. The study shows that P1G10 inhibits conidia germination and germ tube elongation of *B. cinerea* relative to untreated conidia. Hypersensitivity to cell wall-perturbing agents (Calcofluor white and Congo red) was observed in mycelium cells treated with P1G10. In addition, P1G10 exhibited inhibitory effect on the adhesion of conidia, provoked alterations in membrane integrity and induced production of reactive oxygen species accompanied by cellular damage. Our results highlight the effect of P1G10 on mycelium growth, cell wall alterations, membrane integrity and adhesion. P1G10 emerges as promising antifungal to control disease causing agents in the food agroindustry.

1. Introduction

Botrytis cinerea is an ubiquitous pathogenic fungus, responsible for severe damage in more than 200 plant species worldwide, including grapes, stone-fruit, berries and vegetables, causing important economic losses pre- and post-harvest (Olmedo et al., 2016; Wang et al., 2013). The fungus infects either by direct penetration, or through wounds caused by farming practices and is responsible for severe loss during long-distance transport and storage of vegetables, especially when environmental conditions are appropriate for fungal development (Soylu et al., 2010). Traditionally, *B. cinerea* is primarily controlled by synthetic fungicides. For example, the chemical control applied to table grapes is mainly based on different groups of fungicides (dicarboximides, anilinopyrimidines, phenylpyrroles, carboxamides and hydroxyanilides), with applications programmed four to six times during the season (Aqueveque et al., 2016). However, use of fungicides is linked to hazardous effects including carcinogenicity, teratogenicity, high acute residual toxicity, and delayed degradation period, impacting human health (Kast-Hutcheson et al., 2001). Hence, human health hazards and the adverse environmental impact occasioned by conventional pesticides prompted a search for new antifungal agents to meet the needs of food industry (Martínez-Romero et al., 2007).

Several less aggressive alternatives, including near-harvest spraying with biological controlling agents and postharvest application of bicarbonates, or antagonistic microorganisms, sanitization and addition of natural products with antimicrobial properties have been investigated to control fungal diseases. In the last decade these studies focused on plant extracts and essential oils with antimicrobial activity, and they emerge nowadays as good alternatives instead of conventional synthetic fungicides (Aqueveque et al., 2016; Burt, 2004; Olmedo et al.,

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2016; Qin et al., 2010; Soylu et al., 2010). On the other hand, a wide variety of latex producing plants species have been described, some of them known to display plant protective-like properties (Konno, 2011). Bioactive molecules have been identified in these fluids and are generally classified into secondary metabolites and proteins. For instance, the proteolytic enzyme bromelain from *Ananas comosus* inhibits the growth of filamentous fungi *Fusarium verticillioides, F. oxysporum* and *F. proliferatum* (López-García et al., 2012). Also, the proteolytic fractions from different lattices including *V. cundinamarcensis* inhibited spore germination and mycelium growth of six phytopathogens (Ramos et al., 2014). Out of five plant lattices analyzed *Calotropis procera* and *V. cundinamarcensis* exerted the best phytopathogenic effect. The phytopathogenic effect was significantly reduced following iodoacetylation of the proteolytic fraction thus confirming the relevance of the proteolytic activity. The bulk of the proteolytic fraction (P1G10) in *V. cundinamarcensis* is rich in cysteine proteinases; its proteolytic activity is about five times higher than papain from *Carica papaya* (Baeza et al., 1990). Later, Teixeira et al. (2008) identified 14 isoforms of cysteine proteinases in P1G10 from *V. cundinamarcensis*.

Additional pharmacological properties have been associated to P1G10, like the mitogenic activity in cultured fibroblasts, skin healing in different models of wounds, including gastric ulcers, and anti-inflammatory, antitumoral and immunomodulatory effects (Freitas et al., 2017; Salas et al., 2008; Silva et al., 2003). In a recent report we demonstrated the healing efficacy of P1G10 in a model of chronic diabetic foot ulcers (Tonaco et al., 2018). While we cannot confirm the antibacterial effect of P1G10 in these models, there are reports that papain from *C. papaya* inhibit formation of bacterial films in the buccal cavity and displays antimicrobial action against *A. actinomycetemcomitans*, a major pathogen involved in periodontal disease (Kush et al., 2015; Mugita et al., 2017). Currently, we do not know if P1G10 has any effect on human pathogenic fungi, but the subject deserves future attention.

In view of this evidence, we investigate now the antifungal effects of P1G10 against to *B. cinerea*. In the present study, the antifungal effects of P1G10 in mycelium growth, cell wall, membrane integrity and adhesion of *B. cinerea* are investigated.

2. Materials and method

2.1. *Vasconcellea cundinamarcencis latex proteins*

Latex from *V. cundinamarcensis* was collected by making several longitudinal incisions onto the surface of unripe fruits with the aid of a sharp scalpel. A voucher specimen of the plant is being deposited at the herbarium of the Universidad de La Serena, Chile, with #15063. The collected sap was freeze-dried and stored at −20 °C for further processing. Dried latex is composed primarily by 70% protein, 18% carbohydrates and 4% residual water, similar in composition to *C. papaya* latex (Baeza et al., 1990). Three grams of dried latex were dissolved in 20 mL of 1 M sodium acetate solution containing 25 mM L-cysteine, 5 mM DTT and 10 mM EDTA pH 5.0. After low speed centrifugation and filtration (Whatman #1, Wilmington, MA, USA), the clear solution was chromatographed through Sephadex G-10 previously equilibrated with 1 M sodium acetate pH 5.0 at room temperature (Mello et al., 2008).

The proteins containing fractions were screened by their absorbance at 280 nm followed by ultrafiltration on Amicon membranes (MW 10,000) and the final concentrate dehydrated by lyophilization (Silva et al., 2003). The first eluting peak containing the amidase activity represents the fraction P1G10. Each batch of P1G10 was analyzed for its amidase activity (13.5 ± 0.5 nM/mg/min), SDS-PAGE profile (Fig. S1A, lanes 1–5 different stocks of P1G10) and HPLC retention time Fig. S1B, as described by (Mello et al., 2008). A mass spectroscopic analysis of P1G10 shows a characteristic profile (Fig. S2) of peptides whose masses are in the range compatible with major proteolytic forms (Dittz et al., 2015). This well characterized fraction was further used to evaluate the antifungal activity.

2.2. *Inhibition of P1G10 with iodoacetamide*

Two-hundred milligrams of P1G10 dissolved in 50 mM sodium acetate buffer (pH 5.0) were incubated with 1 mM IAA at 25 °C for 1 h followed by extensive dialysis against distilled water to eliminate free IAA and then concentrated by freeze-drying. This fraction was named P1G10-IAA after confirming that remaining activity is $< 10\%$ (data not shown).

2.3. *B. cinerea strain and growth conditions*

A B05.10 isolate from *Vitis vinifera* (Germany) was used as wild type (WT) strain and recipient for genetic modifications (Büttner et al., 1994; Quidde et al., 1998). *B. cinerea* strain was grown on malt extract agar (MEA, 2% malt extract and 2% agar), malt extract broth (MEB), potato dextrose agar (PDA, AppliChem). Radial growth and conidiation rates were measured from cultures on MEA. Ten microliters of conidial suspension (2.5×10^5 conidia/mL) was incubated in MEA agar plates for 2 weeks at 20 °C under a 24 h photoperiod (12 h light/12 h darkness). A conidial suspension was prepared in sterile water and filtered through a sterile 5 mL pipette tip containing glass wool.

2.4. *Modeling of antifungal agent inactivation kinetics*

To evaluate the effect of P1G10 on mycelial growth of *B. cinerea* we carried out a survival assay. One hundred microliters of P1G10 suspension were added into 96 well microtiter plates (polystyrene, JetBiofil, China) and twelve serial dilutions from 8 mg/mL to 0.250 mg/mL were made. Later, each 96 well plate was inoculated with 100 μL of conidial suspension of *B. cinerea* (2.5×10^5 conidia/mL) and incubated with agitation at 22 ± 1 °C in the dark. After 72 h of incubation, mycelial growth was quantified by determination of OD_{595} nm (Victor X3 Perkin Elmer 2030 workstation).

2.5. *Weibull model*

Experimental inhibition data were fitted to the Weibull distribution function (1) (Scholze et al., 2001), the cumulative form of which yields:

$$F(c) = exp\left[-\left(\frac{c}{\alpha}\right)^{\beta}\right] \tag{1}$$

where “*c*” is P1G10 concentration (mg/mL), and α and β are scale and shape factors, respectively; β interprets the shape of the inhibition curve, so that when $\beta < 1$, the inhibition curve is concave, when $\beta > 1$, the inhibition curve is convex, and when $\beta = 1$, a straight line is present. The following statistical indices served to evaluate the quality of fit into Weibull model: the root mean square error (RMSE), which measures the average deviation between the observed and fitted values, given by Eq. (2). Small RMSE values in the model indicate a close fit between the data and the model (González et al., 2009)

$$RMSE = \sqrt{\frac{\sqrt{(fitted - observed)^2}}{n - p}} \tag{2}$$

where “n” is the number of observations and p is the number of parameters to be estimated.

2.6. *Effect of P1G10 on mycelial growth of B. cinerea in solid media*

Fungitoxicity of P1G10 was assessed using the radial growth test on malt yeast extract agar. P1G10 was dissolved in sterile water at different final concentrations (0.02, 0.04, 0.08, 0.16 mg/mL). An aliquot of each solution (200 μL) was added to 7 mL of malt-yeast extract agar. The medium in presence or absence of P1G10 was poured onto 6 cm diameter Petri dishes. Then, the dishes were inoculated with 0.5 cm agar discs containing thin mycelium of *B. cinerea*. Cultures were incubated in the dark at 22 ± 1 °C during several days. Mycelial growth diameters were measured daily, and the inhibition values were calculated. Significant differences were evaluated with a two-way analysis of variance (Tukey's test; $p < 0.05$). The determination of IC_{50} values for B05.10 mycelial growth at 48 h incubation was analyzed with PROBIT Test using the MINITAB V.16 (Robles-Kelly et al., 2016).

2.7. Effect of P1G10 on mycelial growth of B. cinerea malt extract broth

This assay was performed according to the methodology described by Ramos et al. (2014). Conidia were collected and washed with sterile water, thereafter, the conidia concentration was adjusted to 2.5×10^5 conidia/mL in 100 µL MEB medium, and plated in 96 wells plastic plates, complemented with 100 µL P1G10 (1 mg/mL). Treatment of B05.10 conidia with active P1G10 was designated B05.10 + P1G10. On the other hand, the treatment named B05.10 + P1G10 T° corresponds to heat-inactivated P1G10 by boiling 40 min to 100 °C and P1G10-IAA corresponds to P1G10 inhibited with iodoacetamide (IAA). The 96 well plate was then incubated (100 rpm) at 22 ± 1 °C in the dark. After 72 h incubation, mycelial growth was quantified by determination of OD_{595} nm (Victor X3 Perkin Elmer 2030 workstation).

2.8. Adhesion capacity of B. cinerea

The effect of P1G10 on adhesion capacity of *B. cinerea* was determined using a modified version of a protocol developed by Plaza et al. (2015). Briefly, after incubating conidia (untreated and treated with P1G10) for 72 h at 20 °C, the medium containing unbound conidia was aspirated, and remaining non-adherent conidia were removed by washing the wells three times with 200 µL sterile water. Then, 100 µL 0.1% methyl violet in sterile water was added, and further incubated with the adhered cell material for 5 min. Finally, the wells were washed three times with 200 µL of sterile water and monitored at 595 nm (Victor X3 Perkin Elmer 2030 workstation). The OD values were proportional to the quantity of biofilm formed under the mentioned conditions, which comprises mycelium and extracellular polymeric material.

2.9. Effect of P1G10 on conidial germination and germ tube elongation

The effect of P1G10 on conidia germination *B. cinerea* was performed using a modified version of Ji and Kuc (1996). Aqueous samples (500 µL) of conidia suspension (2.5×10^5 conidia/mL) were incubated with 500 µL of P1G10 (1 mg/mL) in MEB medium, for 0, 4 and 6 h at 22 ± 1 °C with constant agitation. In assays of conidia without inhibitor, P1G10 T° or P1G10-IAA, the final volume was completed with 1 mL MEB medium. Thereafter, conidia were washed twice in sterile water (500 µL) with centrifugation at 12,000 rpm for 5 min; the pellet was resuspended in 100 µL of sterile water. Then, 10 µL of sample were placed on microscope glass slides, covered with glass cover slip and examined using light microscope (Eclipse E-200). The percentage of germinating conidia and the lengths of germ tubes were estimated on microphotographs of each preparation. The conidia were considered germinated when the germ tube was twice the size of the conidia. The germ tube was measured using Image J software. Three replicates were conducted for each treatment and a minimum of 100 conidia were counted in each replicate.

2.10. DAPI-stained nuclei in mycelium

To determinate whether P1G10 interferes during nucleus formation in mycelium of *B. cinerea*, a nuclear staining assay of mycelial body with Fluoromount G with DAPI (4′,6-diamidino-2-phenylindole) (Electron Microscopy Sciences, USA) was performed. Germinated conidia incubated in presence of P1G10 (1 mg/mL) for 6 h were washed twice in sterile water and resuspended in 100 µL. Five microliters of conidia and 5 µL DAPI were placed onto a microscope glass slide covered with cover slip. After 10 min incubation, conidia were observed under a light microscope with epifluorescence system (Eclipse E-200). A microphotograph of each preparation was obtained (0 and 6 h) and the nuclear staining was visually assessed.

2.11. Membrane integrity assay

The membrane integrity of *B. cinerea* was determined according to the method of Qin et al. (2010) with some modifications. A *B. cinerea* isolate was initially grown on MEA and conidia were collected and washed with sterile water. Then, a suspension of 5×10^5 conidia/mL of *B. cinerea* was treated with 1 mg/mL of P1G10 or P1G10 inhibited (P1G10 T° or P1G10-IAA) in MEB medium and incubated during 4 or 6 h in a shaker at 22 ± 1 °C. Conidia in MEB medium were collected by centrifugation at 5000 rpm for 10 min at 25 ± 1 °C, washed twice with 50 mM sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 2 min. After suspension, conidia were stained with 10 µg/mL propidium iodide (PI) for 5 min at 30 °C. Finally, conidia were collected by centrifugation, washed twice with the buffer to remove residual dye and observed under a light microscope with epifluorescence system (Eclipse E-200).

2.12. Sensitivity to cell wall perturbing agents

The sensitivity of *B. cinerea* to cell wall perturbing agents Congo red (CR, 500 µg/mL) and Calcofluor white (CFW, 500 µg/mL) was performed according to Plaza et al. (2015). Conidia were collected and washed with sterile water. Then, concentration was adjusted to 2.5×10^5 conidia/mL. Later, 500 µL of conidia suspension were preincubated with 500 µL of P1G10 (1 mg/mL) or P1G10 inhibited (P1G10 T° or P1G10-IAA) in MEB medium, for 4 h with agitation at 22 ± 1 °C. Subsequently, 20 µL of each preparation was inoculated into growing medium, previously prepared with of Congo red (CR, 500 µg/mL), Calcofluor white (CFW, 500 µg/mL) and 2%, MEA respectively. Cultures were incubated in the dark at 22 ± 1 °C for 6 days. Mycelial diameters were measured daily. All experiments were performed in triplicate.

2.13. Production of reactive oxygen species

The production of reactive oxygen species (ROS) was evaluated using ROS-GLOtm H_2O_2 assay kit (Promega, Madison, USA) according to a method described by Kelts et al. (2015). Conidia (79 µL) were plated in a 96-well plate at a concentration of 1×10^5 conidia/mL/well. Then, each well was incubated in presence of 1 µL P1G10 (1 mg/mL), 20 µL of H_2O_2 substrate and cultured for 2 h at 21 ± 1 °C. After this interval, 100 µL of ROS-GLOtm reagent was added to each well and incubated for 20 min at room temperature. ROS production was measured using a luminometer (Tecan infinite m200pro). Menadione (0.01 mg/mL) was used as positive control, following the manufacturer's instructions. Mean values displaying significant differences ($p < 0.05$) were considered.

2.14. Real-time PCR and gene expression

For gene expression analysis, conidia were inoculated in sterile Erlenmeyer flasks with malt-yeast medium and cultured without stirring for 3 days at 21 ± 1 °C in presence of 1 mg/mL of P1G10. Then, the mycelium was extracted and grinded with liquid nitrogen. Total RNA extraction and cDNA synthesis was performed according to the procedure described by (Silva-Moreno et al., 2016). At least three independent cDNA preparations obtained from independent biological replicates were used for analysis. Each target gene was measured in triplicate and the mean Ct value was calculated. Expression of each gene was determined by the method of Livak and Schmittgen (2001).

Expression levels were normalized against the reference gene ubiquitin-conjugating enzyme E2 (ubce) according to (Silva-Moreno et al., 2016). Primers for Real-time PCR assays and amplification efficiencies (E) are shown in Table S1 (Supplementary material).

2.15. *Statistical analysis*

All the experiments were repeated at least three times, and each experiment was conducted with three or more duplicates. One-way analysis of variance (ANOVA) (Statgraphics Plus® 5.1 software, Statistical Graphics Corp., Herndon, USA) was used to demonstrate significant differences among samples. Significance testing was performed using Fisher's least significant difference (LSD) test and Student's *t*-test; differences were accepted with significance 95%. The Multiple Range Test (MRT) included in the statistical program was used to assess for the existence of homogeneous groups within each of the parameters analyzed.

3. Results

3.1. *Fit of the Weibull model*

To determine whether P1G10 induced microbial inhibition, a growth assay of *B. cinerea* was carried out in the presence of different concentrations of P1G10. The experimental OD_{595} data associated to mycelial growth at different concentration of P1G10 were fitted to the Weibull distribution function. The Weibull model is being applied because of its potential to describe microbial, enzymatic and the kinetic of chemical degradation. The main advantage of this model is that it is simple and sufficiently robust to describe inactivation (downward concave function) (Criado et al., 2015). The concave survival curve and its β parameter (0.0001) shows a typical example of microbial inactivation; the adjusted mean r^2 value and the statistical parameter RMSE were 0.961 and 0.009, respectively. These values indicated that the Weibull function adjusted closely to the empirical data. The growth of *B. cinerea* was reduced by increasing P1G10 concentration and attained a minimum at about 1 mg/mL. Meanwhile, survival between 1 and 8 mg/mL P1G10 remained invariable without further decrease ($p < 0.05$) (Fig. 1). This model provided an adequate fit, enabling the study of *B. cinerea* survival after P1G10 treatment. From the data, it was determined that P1G10 at 1 mg/mL reduced by 50% fungal growth.

3.2. *Determination of P1G10 IC_{50} affecting mycelial growth of B. cinerea*

The antifungal activity and the IC_{50} for P1G10 were evaluated by measuring *B. cinerea* mycelial growth in solid medium at different concentrations of the inhibitor. Fig. 2A shows the kinetic of inhibition of *B. cinerea* mycelial growth in presence of P1G10. The results indicate that P1G10 reduce *B. cinerea* mycelium growth between 0.08 and 0.16 mg/mL after 48 h incubation. To determine the IC_{50} value of P1G10 on mycelial growth, Probit analysis was carried out. The antifungal IC_{50} of P1G10 capable of reducing 50% growth in solid medium was 1 mg/mL (Fig. 2B). Based on the Weibull model and the determined IC_{50} value, 1 mg/mL P1G10 was further applied in remaining assays.

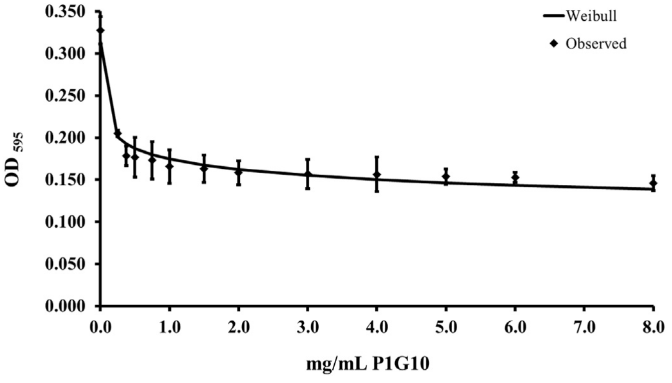

Fig. 1. Effect of P1G10 on survival of *B. cinerea*. (◆) represents the observed data. The solid line corresponds to estimated values from the Weibull distribution function. Error bars represent the standard deviation of 5 experimental values.

3.3. *Antifungal effects of P1G10 on mycelial growth and adhesion in B. cinerea*

The kinetic inhibition and IC_{50} data showed that 1 mg/mL of P1G10 had a critical effect on *B. cinerea* mycelial growth. To investigate if the antifungal effect is caused by the proteolytic activity in P1G10; *B. cinerea* was grown for 72 h at 22 ± 1 °C in MEB medium supplemented with 1 mg/mL of P1G10 or P1G10 inhibited (P1G10 T° or P1G10-IAA). The results showed that 1 mg/mL P1G10 reduced mycelial growth ≥ 50% (Fig. 3A), while fungal biomass in medium without inhibitor or containing inhibited P1G10 was unaffected. This result shows that thermal denaturation or iodoacetylation at cysteinyl residues in proteolytic enzymes from P1G10 obliterated the inhibition of *B. cinerea* growth.

As known, adhesion is an important attribute of virulence in phytopathogenic fungi securing the colony from being removed in a suitable environment. Therefore, the adhesion capacity of *B. cinerea* growing mycelia was analyzed in polystyrene microtiter plates supplemented with 1 mg/mL of P1G10. Mycelia were stained with crystal violet and exposed to a detachment force to measure the effect of P1G10 on adhesion of *B. cinerea* onto the polystyrene surface. The results were visualized by fluorescence microscopy and showed that P1G10 inhibited about 50% of the adhesion capacity of *B. cinerea* after 72 h incubation at 22 ± 1 °C (Fig. 3B and C). This analysis revealed that the proteolytic action of P1G10 interferes with the adhesion capacity of *B. cinerea* to polystyrene surface, while fungi grown with inactivated P1G10 exhibited adhesion similar to *B. cinerea* without P1G10.

3.4. *Effect of P1G10 on conidial germination and germ tube elongation*

The antifungal effect of 1 mg/mL P1G10 in germination and germ tube elongation of *B. cinerea* were next performed. The inhibitory effect after incubation of *B. cinerea* with P1G10 assessed germ tube elongation at 0, 4 and 6 h post incubation at 22 ± 1 °C (Fig. 4A). The conidia were considered germinated when the germ tube was twice the size of the conidia and then the percent of germination was calculated. As observed before, during mycelial growth inhibition experiments, fungal isolates without inhibitor or treated with P1G10 T°, or P1G10-IAA exhibited normal germination after 6 h incubation. Meanwhile, a significant reduction in germination was observed in conidia treated with P1G10 at 4 h interval (Fig. 4B). A stronger germination decline approaching 70% was observed after 6 h in contact with P1G10; while germination in conidia without inhibitor or the inactivated inhibitor remained close to the 100% (Fig. 4B).

Fig. 4C shows the germ tubes size after 6 h incubation; the average conidial length was 26.93 ± 3.28 μm for B05.10, 23.59 ± 2.02 μm for B05.10 + P1G10 T°, 25.25 ± 1.69 for B05.10 + P1G10-IAA and 13.86 ± 2.80 μm for and B05.10 + P1G10. A difference in length higher than 10 μm was observed between conidia without inhibitor and added P1G10 (Fig. 4C).

On the other hand, a possible nuclear alteration in mycelia arising from P1G10 treatment was analyzed by staining conidia with DAPI. DAPI is normally excited at UV wavelengths incompatible with live-cell imaging assays (Besserer et al., 2008). In Fig. 5 it is shown a microphotograph of *B. cinerea* mycelium grown for 6 h in presence of 1 mg/mL P1G10. The microphotograph was obtained by epifluorescence microscopy and showed that P1G10 does not affect the nuclei numbers

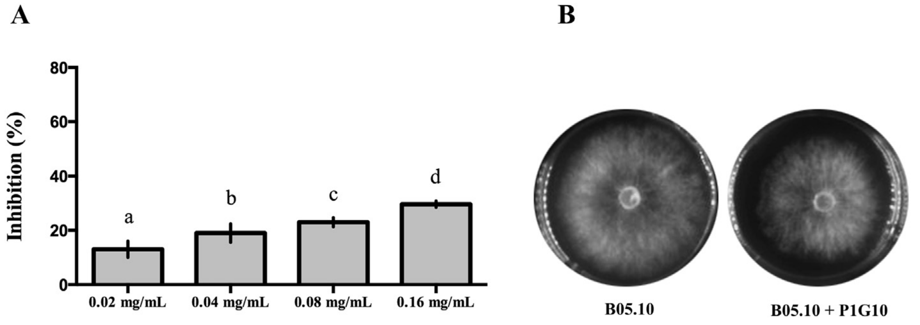

Fig. 2. Effect of P1G10 on growth of *B. cinerea* in solid media. (A) Kinetic of growth inhibition determined after several h of incubation at different concentrations of P1G10 i.e. 0.02, 0.04, 0.08 and 0.16 mg/mL. Each point represents the average of at least three independent experiments ± standard deviation. (B) Effect of P1G10 on mycelial growth of *B. cinerea* in solid media at 48 h of incubation. P1G10 was dissolved in sterile water at a final concentration of 0.02, 0.04, 0.08 and 0.16 mg/mL. The percent growth inhibition was determined after 48 h of incubation. Every bar represents the average of at least three independent experiments ± standard deviation. (letters) Represents significant differences evaluated with a two-way analysis of variance ($p < 0.05$).

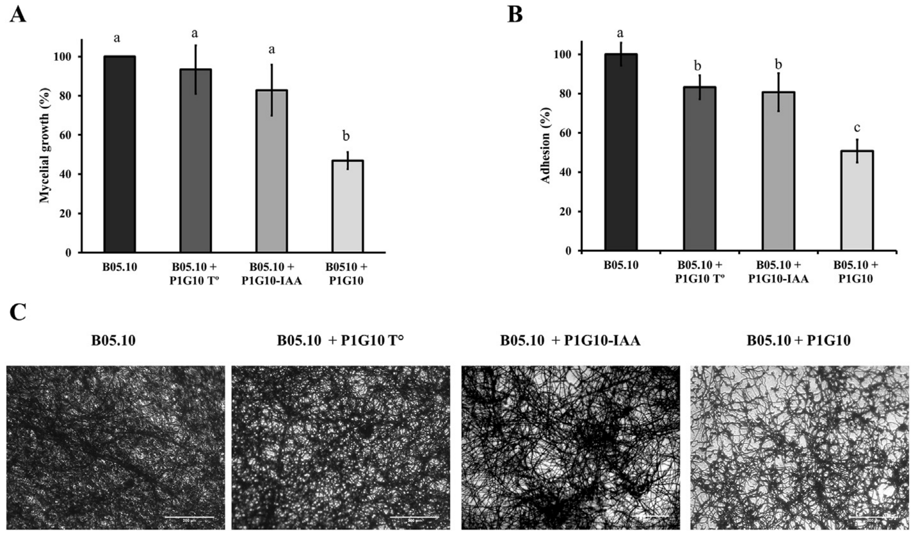

Fig. 3. Antifungal effect of P1G10 on mycelial growth and adhesion in *B. cinerea*. (A) Percentage inhibition of P1G10 on mycelial growth. (B) and (C). Percent inhibition of P1G10 on the adhesion and biofilm formation of *B. cinerea* (B05.10, treatment with P1G10 or B05.10 + P1G10 T° or P1G10-IAA) on polystyrene after incubation for 72 h at 20 °C. Bound mycelia were stained with crystal violet and visualized by fluorescence microscopy (10×). All experiments were performed 3 times, with n = 6. Different letters indicate significant differences (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in conidia. No significant differences were found in nucleus number between treated or untreated conidia or mycelia (data not shown).

3.5. Effect of P1G10 on membrane and cell wall integrity

We used propidium iodide (PI) to determine whether P1G10 led to a loss of membrane integrity in *B. cinerea*. Conidia treated or untreated with P1G10 were collected, stained with PI and observed under fluorescence microscopy. According to the data in Fig. 6A, the plasmatic membrane of conidia of *B. cinerea* was markedly damaged in presence of P1G10. The integrity of membrane declined in presence of P1G10 after 4 h and 6 h of incubation. However, integrity of membrane remained unaltered in conidia incubated without P1G10 or inhibited P1G10.

Therefore, we also analyzed the effect of P1G10 on the cell wall of *B. cinerea*. To evaluate the effect of P1G10 in cell wall integrity, conidia treated with P1G10 was analyzed for its sensitivity to antifungal drugs, including Calcofluor white (CFW) (alters the assembly of chitin fibrils in the cell wall), and Congo red (CR) (alters the assembly of microfibrils of β1,3-glucan). Fig. 6B and C show that P1G10 alters the cell wall in *B. cinerea*. An increase in sensitivity to CFW was observed in cells treated with P1G10 presenting a significant reduction in growth rate on day 6th (76.6%), this pattern was also observed on days 3, 4, and 5. Similar result was found in conidia treated with P1G10 and grown in presence of CR, confirming that the cell wall in these cells had been affected.

3.6. Assessment of P1G10 effect on production of reactive oxygen species

The induction of reactive oxygen species (ROS) was examined. Fig. 7 shows the luminescence measured in presence of P1G10 (1 mg/mL) after 2 h incubation. The ROS-Glo™ H_2O_2 assay is a, sensitive luminescent method that measures directly in cell culture the level of hydrogen peroxide (H_2O_2), a reactive oxygen species (Robles-Kelly et al., 2016). Menadione was used as positive control in this assay. The results show that P1G10 induces approximately a 50% increase in luminescence relative to the control, and therefore enhances ROS production.

3.7. Determination of expression of specific genes related with cellular damage

To elucidate a possible mechanism involving the action of P1G10 against *B. cinerea*, several genes associated to cellular damage were studied. The first gene analyzed was *hex* that encodes for the principal protein of the Woronin body. It has been described that *hex* increases its expression along with mycelium damage, because Woronin bodies appear to plug the septal pores within a few minutes after damage (Aguayo et al., 2011). The second gene was *aox*, a gene associated to oxidative stress in fungi (Honda et al., 2012; Magnani et al., 2007). Finally, we analyzed *nma* and *cas*-1 that are related to programmed death cell (PDC). These genes have been bioinformatically identified in *B. cinerea*. The cas-1 gene, orthologue to cas-A, corresponds to a metacaspase and nma is a homologue of the yeast NMA11 gene, which belongs to the high temperature requirement (HtrA) family of serine proteases, and is an homologue of human HtrA2/Omi, a mitochondrial protein with pro-apoptotic activity (Shlezinger et al., 2011). Fig. 8 shows the results of the expression of these genes in presence of 1 mg/mL of P1G10. All the genes examined exhibit increase transcription in presence of P1G10 after 72 h incubation, suggesting substantial cellular damage.

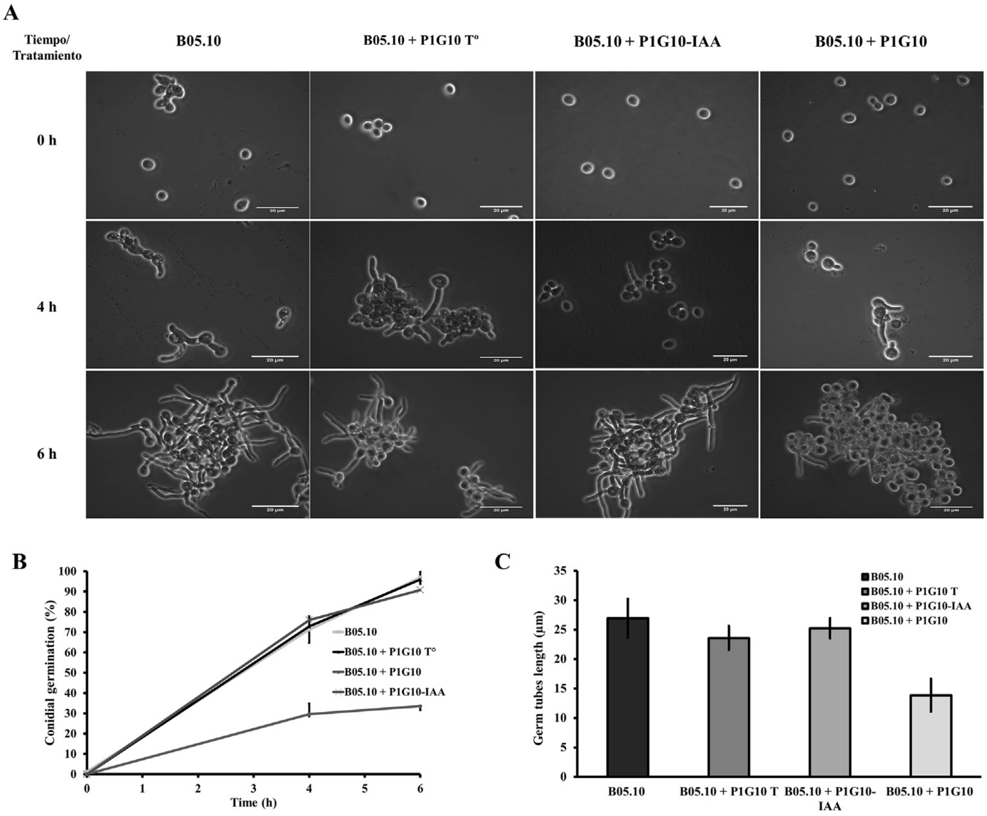

Fig. 4. Inhibition of germination of *B. cinerea* by P1G10. (A) Image (40 ×) of germinating conidia at different intervals. (B) Percent germination of *B. cinerea* after 4 and 6 h incubation with P1G10 at 22 °C, respectively. (C) Germinal tube length after 6 h incubation.

4. Discussion

Food safety is safeguarded by addition of antimicrobials that prevent or considerably retard spoilage caused by microorganisms (Soylu et al., 2010). In recent years, extracts from edible plants and their purified sub-products have shown antimicrobial activity. A similar situation applies to some plant proteins for which an antifungal activity was reported. In a review of antifungal metabolites are described several plant extracts including peptides (Abad et al., 2006). Latex from *V. cundinamarcencis,* the source of P1G10 was initially studied for its high protein content and strong proteolytic activity (Bravo et al., 1994). According to Ramos et al. (2014), cysteine proteases from *Cryptostegia grandiflora* latex inhibit spore germination of phytopathogenic fungi *Fusarium solani*. Also, the same fraction studied here (P1G10) exerted antifungal activity against *Fusarium solani, Colletotrichum glorosporioides, Neurospora sp, Rhizoctonia solani* and *Aspergilus niger* (Souza et al., 2011). Nonetheless, no account exists about the effect of plant proteases against *B. cinerea,* the agent causing grey mold disease. The present work is the first report describing the antifungal activity of *V. cundinamarcensis* P1G10 fraction against *B. cinerea*.

The results demonstrate that P1G10 exhibits an important inhibitory activity against *B. cinerea* in solid and liquid media. As shown in Fig. 1 different concentrations of P1G10 fitted into the Weibull distribution function demonstrating an inhibitory effect. Fig. 1 shows that P1G10 at 1 mg/mL, inhibits mycelium growth after 72 h incubation at 22 °C. Thus, the mathematical model was applied to experimentally find the dose effect of P1G10 and to assist for selection of the appropriate concentration in subsequent assays (Scholze et al., 2001). The Weibull function provides a fair estimation of experimental data and a low root mean square error (RMSE). A good fit of the survival curve is important to obtain valid estimates of the modeled parameter. The result of Weibull model was supported by the IC_{50} determined for P1G10 ≈ 1 mg/mL in solid medium. A similar IC_{50} value (1.368 mg/mL) has been described using latex proteases from *Calotropis procera* (CpLP) (Souza et al., 2011) against *Aspergillus niger*. Based on the available data it seems that the efficacy of the inhibitory effect by proteases varies with the fungal species (Souza et al., 2011). The authors also showed that CpLP was more efficacious than P1G10 against several fungi except *A. niger* where P1G10 was more active than CpLP.

To determine whether the proteolytic activity was relevant for the antifungal effect, P1G10 samples were heat-denatured or inhibited with IAA (irreversible inhibitor of proteolytic enzymes containing cysteinyl groups at the active site) before assaying. It was observed that the inhibition of fungal growth was suppressed when P1G10 was heat-denatured or treated with IAA, confirming that the protease activity was important for the inhibitory action. A similar suppression of the antifungal effect was also observed when P1G10 or the proteolytic fraction from *C. procera* was exposed to thermal denaturation, treated with IAA or E64 (inhibitor of cysteine proteases), thus confirming the role of proteolytic activity in the antifungal effect (Souza et al. (2011)). Proteolytic action is not the only response elicited by plants to combat pathogens; it may involve secondary metabolites (Abad et al., 2006) and macromolecules like chitinases, ß-l,3-glucanases, and thionins or a group of peptides participating in the resistance phenomenon known as the hypersensitive response (HR) (Salas et al., 2015). In *C. papaya* latex, a species related to *V. cundinamarcensis* minor amounts of lysozyme, beta-1,3-endoglucanase, chitinase, Kunitz-type serine protease inhibitor, and glutaminyl cyclase were reported (Azarkan et al., 2006). The three first of these enzymes display antifungal activity, but we have not yet identified these enzymes in *V. cundinamarcensis*. While we cannot rule that a minor portion of the antifungal effect seen is caused by one of the enzymes identified in *C. papaya*, independent experiments using CMS2MS2, a purified proteinase isoform found in P1G10, confirmed its antifungal activity (Souza et al., 2011). Also, the SDS-PAGE and mass spectroscopic analyses of P1G10 fraction do not show a profile compatible with the presence of chitinase or beta-1,3-

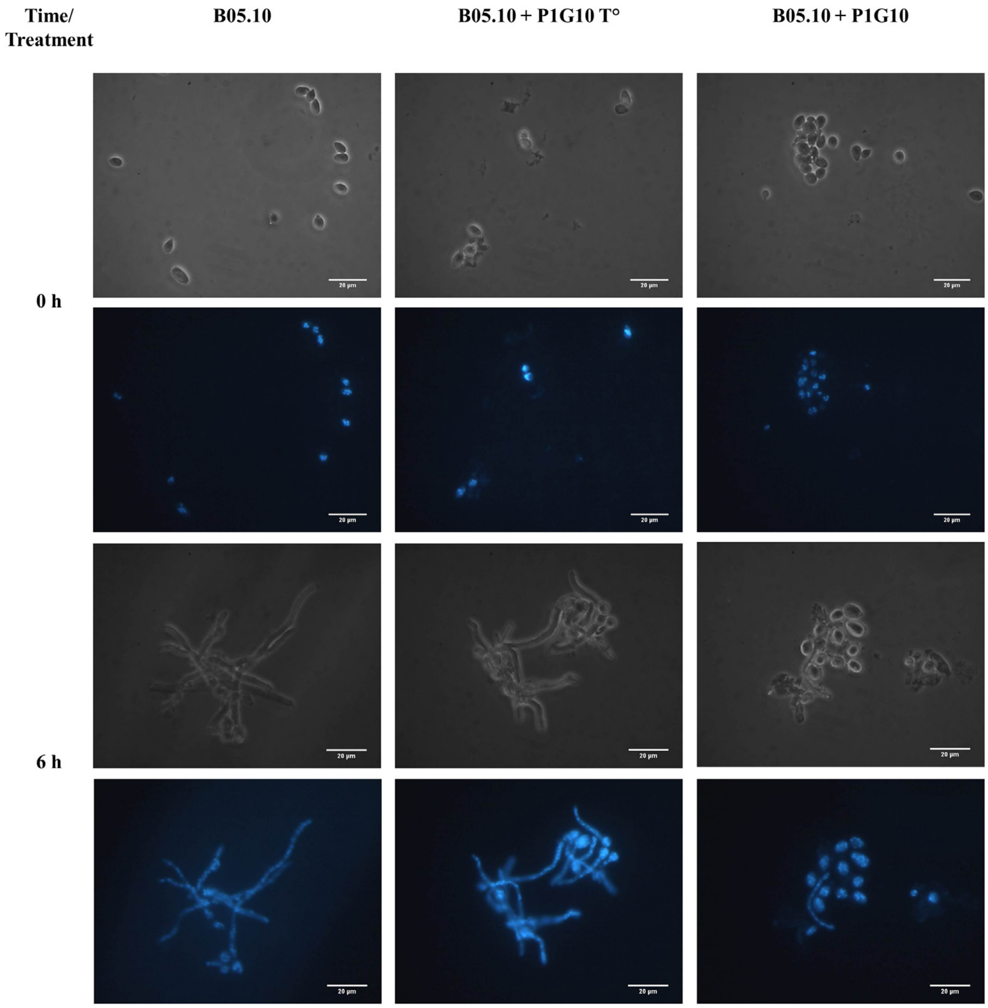

Fig. 5. Effect of P1G10 on *B. cinerea* cells treated with DAPI. *Botrytis* conidia treated and untreated with P1G10 were exposed to the indicated concentrations of Fluoromount-G Dapi for 10 min. Images are representative of three independent experiments.

endoglucanase (> 40 kDa) (Fig. S1).

Adhesion proteins in *B. cinerea* are involved during recognition and fungal binding to host tissues (Cormack et al., 1999; Staab et al., 1999). These proteins play vital roles in cell wall integrity, adhesion and virulence (González et al., 2013; Plaza et al., 2015; Wagener et al., 2008). The data show that P1G10 impairs the adhesion, conidial germination and germ tube elongation of *B. cinerea*. In this assay, P1G10 impaired the interaction between *B. cinerea* cell wall proteins and the polystyrene surface. A similar effect is observed when mammalian cultured cells are incubated with P1G10. In the latter case, cell agglomerates accumulate in the culture but, reverse to the original state upon removal of P1G10 without noticeable effects in cell viability (unpublished observations). Other authors have reported a correlation between adhesion and germination in *B. cinerea* conidia (Doss et al., 1995; Slawecki et al., 2002). In our study, germination of *B. cinerea* was reduced in presence of P1G10. This result is similar to that observed with the flavonoid 5.7-dihydroxy-3.8-dimethoxyflavone from *Psaudognaphalium robustum* and chitosan from crustaceans on conidial germination of *B. cinerea* or *Penicillium expansum* (Cotoras et al., 2011; Liu et al., 2007). It is uncertain if proteases from P1G10 directly affect conidial germination of *B. cinerea*.

Our data suggest that membrane integrity was also affected in conidia exposed to P1G10 at various intervals. Fig. 6A shows that *B. cinerea* treated with P1G10 exhibited higher number of stained conidia in experiments with propidium iodide. Accordingly, the plasmatic membranes of *B. cinerea* were markedly damaged by P1G10 (Fig. 6A). Similarly, chitosan induced membrane lesions, decreased membrane integrity and increased membrane permeability in *B. cinerea* and *Penicillium expansum* (Liu et al., 2007). It is unclear how P1G10 interferes with the membrane but, there is a certain possibility that P1G10 proteases degrade the proteins in plasmatic membrane, but further studies are necessary to confirm this possibility.

It is known that fungi cell wall is responsible for the cell form and offers protection against harmful environmental conditions (Costa-de-Oliveira et al., 2013). Fungi cell walls are mainly composed of mannoproteins, β-glucan and chitin (Delgado-Silva et al., 2014). In line with this notion, P1G10 enhanced the sensitivity to cell wall-perturbing agents CFW and CR, as shown in Fig. 6B and C. As known, CFW preferentially binds to chitin in fungi interfering with cell wall assembly, while CR binds to β1,3-glucan and alters the assemblage of microfibrils at the cell wall (Ram and Klis, 2006). Increases in cell wall chitin are often observed in mutants which are more sensitive to CFW and CR drugs and it is viewed as part of a mechanism induced by fungi to compensate for loss of cell wall integrity (Kapteyn et al., 1997; Popolo et al., 1997). The increased susceptibility to CFW or CR observed here was indicative of cell wall defects in conidia treated with P1G10 + CFW or P1G10 + RC, suggesting that P1G10 is also a cell wall perturbing agent. This cell wall alteration may also explain the reduced adhesion on polystyrene surface by *B. cinerea* treated with P1G10, mentioned above. Cell wall damage can be induced by a pore formation mechanism (Salas et al., 2015). Antifungal peptides display a particular structural activity arrangement involving polar and neutral amino acids

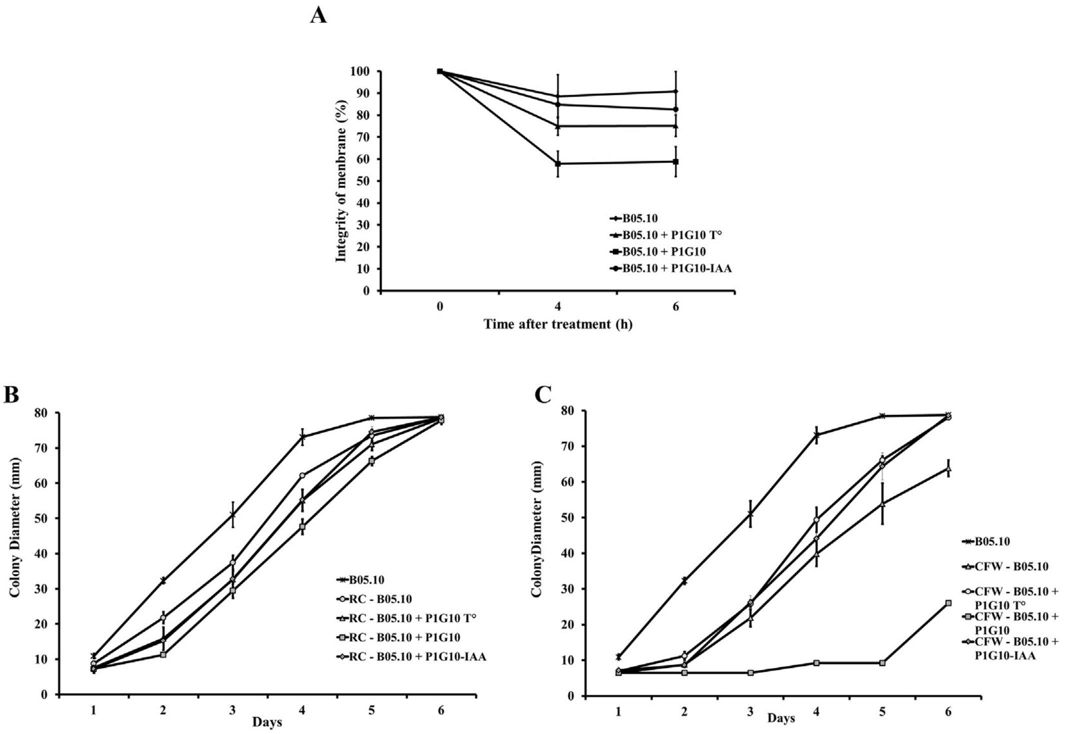

Fig. 6. The effect of P1G10 on plasma membrane integrity and the cell wall in *B. cinerea*. (A) Effects of P1G10 on plasma membrane integrity of conidia of *B. cinerea*. (B) and (C) Sensitivity of conidia with or without P1G10 and growth in agar media with Congo red (CR) or Calcofluor white (CFW). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

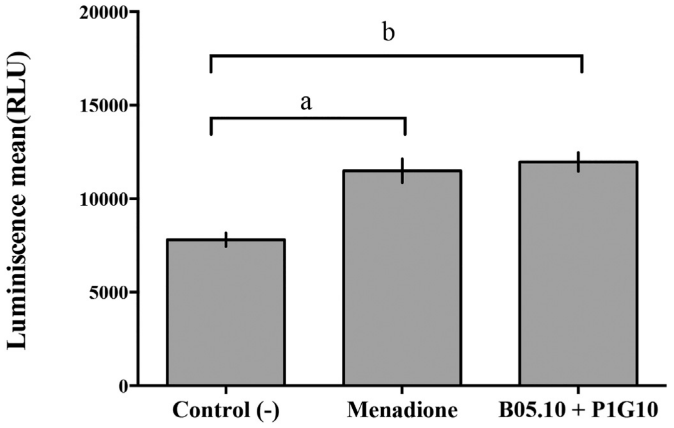

Fig. 7. P1G10 induced production of reactive oxygen species in *B. cinerea*. Measured luminescence was converted to concentration of H_2O_2 after background correction using a standard curve. Menadione (0.01 mg/mL final concentration) was used as positive control. Different letters indicate significant differences ($p < 0.05$) (Student *t*-test).

that target chitin residues which are abundant components of fungal cell wall (Yokoyama et al., 2009). It is unknown if structural determinants from P1G10 contain potential antifungal peptides, but, a structural analysis of proteases in *V. cundinamarcensis* whose structure have been elucidated reveals the presence of peptides with pore forming potential (unpublished observations).

The results of gene expression experiments show that there is an increase in *hex* and *aox* transcripts in presence of P1G10 compared to the control condition, indicating a possible hyphal damage. This result is supported by membrane integrity experiments with Calcofluor white and Congo red mentioned here. Similar results have been reported using other natural compounds displaying antifungal activity against *B. cinerea* (Robles-Kelly et al., 2016). In addition, genes associated to programmed death cell, *nma* y *cas*, exhibit increased transcription in presence of P1G10 (Fig. 8), indicating a positive transcriptional modulation induced by this compound, the evidence suggest that P1G10 induce significant damage to *B. cinerea*. Hence, next experiments will be aimed to elucidate this issue. Our results highlight the effect of P1G10 on *B. cinerea* and based on these results it might be an interesting candidate to design a new and effective natural control of this pathogen responsible for significant losses to food agroindustry.

5. Conclusions

We report for the first time the antifungal effect of P1G10 against *B. cinerea*, being 1 mg/mL, the most effective concentration. Our data shows that this compound inhibited mycelial growth, adhesion capacity, conidia germination and germ tube elongation of *B. cinerea*. Moreover, P1G10 causes massive cellular damage on plasma membrane and cell wall in *B. cinerea*. From these studies, it is possible to suggest that P1G10 affects propagation of *B. cinerea* and that this bioactive fraction could be an interesting candidate to design a new and effective natural control of this pathogen.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.08.027.

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Authors and contributors

AV, LC and MT participated in the design of the antifungal assays. CS purified and identified P1G10. MT conceived and developed the research ideas, performed experiments, collected data. JR and ES did

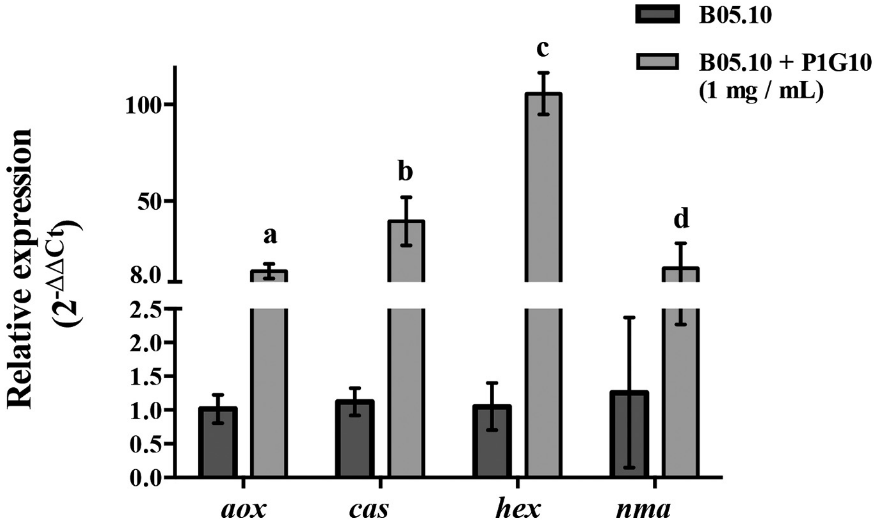

Fig. 8. The effect of P1G10 on gene expression of *B. cinerea*. Effect of P1G10 on mRNA levels of *nma*, cas-1, *hex* and *aox* genes, at 72 h post incubation. All values represent the mean ± standard deviation of three independent experiments with at least three replicates each. Different letters indicate significant differences ($p < 0.05$) (Student t-test).

genetic assay and data analyses. LC and MT drafted the manuscript. CS, AV, ES, LC and MJ analyzed the results, revised the manuscript and approved the final version.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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