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Antifungal activity and mechanism of action of monoterpenes against *Botrytis cinerea*

Atividade antifúngica e mecanismo de ação de monoterpenos contra *Botrytis cinerea*

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ABSTRACT

Botrytis cinerea is considered one of the most important post-harvest pathogens being the causative agent of gray rot. To reduce the use of synthetic fungicides, it is important to explore alternative products with antifungal properties. Among these alternative products are essential oils, which present monoterpenes as major compounds. The objective of this study was to evaluate the effects of eight monoterpenes (1,8-cineole, carvacrol, citral, citronellal, citronellol, geraniol, linalool, and thymol) on the control of *B. cinerea*. The mycelial growth of *B. cinerea* was assessed after treating it with the monoterpenes at a concentration of 1,000 mg/L. Subsequently, the minimal inhibitory concentrations (IC_{90}) of the monoterpenes that showed the greatest antifungal potential were determined. Carvacrol and thymol were tested on *B. cinerea* cell membrane integrity, intracellular accumulation of reactive oxygen species (ROS), and mitochondrial membrane potential of the conidia. Among the tested monoterpenes carvacrol, citral, citronellal, citronellol, geraniol, and thymol demonstrated complete inhibition of mycelial growth at a concentration of 1,000 mg/L. Carvacrol and thymol exhibited the lowest IC_{90} values against *B. cinerea*, with an IC90 of 125 mg/L. Furthermore, carvacrol and thymol induced conidial death in a dose-dependent manner, resulting in the disruption of cell membrane integrity, increased intracellular ROS levels, and decreased mitochondrial membrane potential. These findings highlight the potential of carvacrol and thymol as alternative means of controlling *B. cinerea*.

Index terms: Gray rot; carvacrol; thymol; alternative control.

RESUMO

Botrytis cinerea é considerado um dos mais importantes patógenos pós-colheita, sendo o agente causador da podridão cinzenta. Para reduzir o uso de fungicidas sintéticos, é importante explorar produtos alternativos com propriedades antifúngicas. Entre esses produtos alternativos estão os óleos essenciais, que apresentam os monoterpenos como compostos majoritários. O objetivo deste estudo foi avaliar os efeitos de oito monoterpenos (1,8-cineol, carvacrol, citral, citronelal, citronelol, geraniol, linalol e timol) no controle de *B. cinerea*. O crescimento micelial de *B. cinerea* foi avaliado após tratamento com monoterpenos na concentração de 1.000 mg/L. Posteriormente, foram determinadas as concentrações inibitórias mínimas (CI90) dos monoterpenos que apresentaram maior potencial antifúngico. Carvacrol e timol foram testados em *B. cinerea* quanto à integridade da membrana celular, acúmulo intracelular de espécies reativas de oxigênio (ROS) e redução do potencial da membrana mitocondrial dos conídios. Entre os monoterpenos testados, carvacrol, citral, citronelal, citronelol, geraniol e timol demonstraram inibição completa do crescimento micelial na concentração de 1.000 mg/L. Carvacrol e timol exibiram os menores valores de IC90 contra *B. cinerea*, com IC90 de 125 mg/L. Além disso, o carvacrol e o timol induziram a morte dos conídios de maneira dose-dependente, resultando na ruptura da integridade da membrana celular, aumento dos níveis intracelulares de ROS e diminuição do potencial da membrana mitocondrial. Essas descobertas destacam o potencial do carvacrol e do timol como meios alternativos de controle de *B. cinerea*.

Termos para indexação: Podridão cinzenta; carvacrol; timol; controle alternativo.

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Introduction

Botrytis cinerea is a phytopathogenic fungus responsible for causing gray mold diseases in a wide range of hosts, resulting in losses in numerous plants, including fruits, vegetables, ornamental plants, and crops. It has been considered one of the most destructive and significant pathogens worldwide (Dean, 2012). This pathogen infects a broad specter of wild plants and crops, affecting approximately 170 plant families that hold agricultural importance. Moreover, the fungus ability to have multiples sources of inoculum, combined with its capacity to survive for extended periods as conidia and sclerotia in crop debris, poses significant challenges for control (Williamson et al., 2007; Elad et al., 2016).

Botrytis cinerea may exhibit evident disease symptoms during the pre-harvest period or remain quiescent until the post-

harvest period (Fillinger & Elad, 2016). It has been regarded as one of the most challenging post-harvest pathogens in vegetables and fruits (Zhang et al., 2014). Therefore, the control of *B. cinerea* diseases has received a lot of attention, with a constant search for new strategies to control the fungus.

In modern agriculture, the primary method for controlling gray rot is the application of synthetic fungicides (Hou et al., 2020). These substances are applied from before the first blooms until the pre-harvest period of the fruits. It is essential to intersperse products with different mechanisms of action when spraying fungicides. The main pesticides used to control gray rot include benzimidazoles, dicarboximides and carbamates (Maia et al., 2021; Shao, Zhao, Y., & Ma, 2021). However, the indiscriminate use of these agrochemicals can lead to the selection of resistant fungal strains, resulting in a loss of their effectiveness (Bardas et al., 2010; Liu et al., 2019; Leroux et al., 2002). In addition, the use of these synthetic fungicides contributes to biodiversity loss, has high toxicity risks, and often harms human health and the environment (Romanazzi & Feliziani, 2014; Mesnage & Séralini, 2018; Toral et al., 2018). Therefore, alternative treatments for controlling *B. cinerea* should prioritize eco-friendly approaches with minimal potential toxic effects on humans. One such alternative method is the use of essential oils, which have a direct action in controlling phytopathogenic diseases and also demonstrate a reduced selection of resistance. Furthermore, essential oils are capable of activating defense routes in plants, delaying the infection of various phytopathogens during the pre-harvest stage (Toral et al., 2018).

Essential oils are compounds that are extracted by hydrodistillation or other systems from various parts of higher plants, including leaves, roots, stems, flowers, and fruits (Akdağ & Öztürk, 2019). These extracts are complex mixtures of substances derived from the secondary metabolism of plants, which include terpenes, phenolic compounds, nitrogenous compounds, and other volatile compounds. Among these, the most abundant group of compounds in essential oils is monoterpenes, which are ten-carbon molecules biosynthesized by the condensation of two isoprene units (Hanif et al., 2019). Monoterpenes have been reported to exhibit inhibitory activities against bacteria (Lang & Buchbauer, 2012), fungi (Scariot et al., 2020), and nematodes (Echeverrigaray, Zacaria, J., & Beltrão, 2010). Moreover, monoterpenes posses various bioactive properties such as preservative, antioxidant, allelochemical, anticancer, antiobesity, and other therapeutic properties (Vermaas et al., 2018; Mancianti & Ebani, 2020).

Studies investigating the mechanism of action of monoterpenes on *B. cinerea* are still limited. Their antifungal activities are attributed to their hydrophobic nature, which allows them to interact with cell membrane components, disturbing cell membrane integrity and increasing cell membrane permeability (Yu et al., 2015; Zhang et al., 2019). Some monoterpenes, such as limonene, terpinene-4-ol, and γ-terpinene, have also been observed to potentially destroy the fungal cell wall (Pellegrini et al., 2017; Yu et al., 2015). Another possible mode of action of monoterpenes could involve the intracellular accumulation of ROS and inhibition of key enzymes. In previous studies with *Colletotrichum,* another phytopathogenic fungus, treatment with monoterpenes such as citral, carvacrol, citronellol, geraniol, and thymol resulted in intracellular ROS accumulation (Scariot et al., 2020). Moreover, Ma et al. (2015) demonstrated that exposition to an essential oil rich in carvone and limonene leads to the inhibition of enzymes associated with the tricarboxylic acid cycle in *Sclerotinia sclerotiorum*.

In this study, we aimed to evaluate the potential antifungal effect of a group of monoterpenes against a strain of *B. cinerea*. The *in vitro* antifungal activity of eight monoterpenes was evaluated. Moreover, we evaluated the mechanism of action of the monoterpenes with higher antifungal potential using fluorescent dyes to evaluate cell membrane integrity, intracellular ROS accumulation, and mitochondrial membrane potential.

Material and Methods

Fungal isolate and monoterpenoids

The assays were carried out with the *B. cinerea* isolate CX1-8-001/11, which was obtained from grapefruits with symptoms of gray rot and provided by the Laboratory of Phytopathology of the University of Caxias do Sul. The isolate CX1-8 001/11 was maintained on potato-dextrose-agar (PDA) culture medium and incubated at 25 ºC for 10 days before being used for the tests.

The eight monoterpenes used in the assays were purchased from Acros-Organics or Sigma-Aldrich. Stock solutions of each monoterpene were prepared in Tween-20® (1:1 v/v) at a concentration of 100 mg/mL. The chosen monoterpenes were 1,8-cineole (99%), carvacrol (98%), citral (95%), citronellol (95%), citronellal (97%), geraniol (96%), linalool (97%), and thymol (99%). These specifics monoterpenes were selected based on previous studies that showed their efficiency in controlling various phytopathogenic fungi (Tsao & Zhou, 2000; Kordali, Kotan, R., & Cakir, 2007; Hou et al., 2020; Scariot et al., 2020).

In vitro evaluation of the antifungal activity of monoterpenes

The antifungal effect of each monoterpene on *B. cinerea* was evaluated by measuring the mycelial growth on Petri dishes containing PDA culture medium supplemented with each monoterpene at a final concentration of 1,000 mg/L. The plates were inoculated with a 5.0 mm diameter fragment of fungal mycelia from 10-days-old cultures. The control group consisted of PDA plates containing only Tween-20 at the

same final concentration as the treatments. The cultures were incubated at 25 °C with a photoperiod of 16 h for 8 days, and the colony diameters were measured using a millimeter ruler. These assays were performed in triplicate. The percentage of mycelial inhibition (*Mi%*) was calculated based on the average diameter of the colonies using the following formula (1), where "*Dc*" represents the control diameter and "*Dt*" represents the treatment diameter (Maia et al., 2021):

$$Mi\% = (100-(Dc-Dt)/Dc*100) \quad (1)$$

Inhibitory concentration of selected monoterpenoids

Monoterpenes that exhibited antifungal potential with over 80% inhibition of mycelial growth at concentrations of 1,000 mg/L were selected for determining their respective IC_{90} values, which represent the concentration needed to inhibit 90% of fungal growth. The monoterpenes used in these tests were: carvacrol, citral, citronellal, citronellol, geraniol, and thymol. The evaluation was carried out in Petri dishes containing the PDA culture medium supplemented with the six selected monoterpenes at concentrations of 0, 31.2, 62.5, 125, 250, and 500 mg/L. The plates were inoculated with a 5.0 mm diameter disc of the fungus mycelia from 10-days-old cultures. The cultures were then incubated at 25 °C with a photoperiod of 16 h, and the colony diameters were measured daily using a millimeter ruler. The assay was performed in triplicate, and the percentage of mycelial inhibition was calculated as previously described. IC_{90} values were calculated by PROBIT regression (Finney, 1947) using SPSS software, version 28.0.1 (IBM, USA).

Evaluation of the effect of monoterpenes on *B. cinerea* conidia

Conidia of *B. cinerea* were obtained from 14-day-old cultures grown on a PDA medium (25 °C with 16 h photoperiod). To collect the conidia, 5.0 mL of sterilized water was added to the cultures, and the surface was gently scraped using a sterilized scrapper. The resulting suspension was filtered through a layer of cotton fiber, and the concentration was adjusted to $1x10^6$ conidia/mL. The two monoterpenes with the highest activity in previous tests, carvacrol and thymol, were used in these experiments at different concentrations (0, 62.5, 125, 250, and 500 mg/L), in a volume of 1,0 mL and inoculated with $1x10^5$ conidia/mL. The control and monoterpenes samples were incubated for 4 h at 25 ºC on an orbital shaker (150 rpm). Subsequently, the following parameters were evaluated using flow cytometry: cell membrane integrity, intracellular accumulation of ROS, and mitochondrial membrane potential.

To assess cell membrane integrity, propidium iodide dye (PI, Sigma) was used. PI binds to DNA but can only penetrate cells with compromised cell membranes. For staining, 500 µL of the samples were mixed with 1.0 µL of PI (5.0 mM) and incubated for 30 min in the dark. The samples were then analyzed by flow cytometry using the FL3 channel (488/670). The intracellular accumulation of ROS was evaluated using the fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH, Sigma). Conidia were incubated with 5.0 µg/mL of the dye for 30 min in the dark and analyzed by flow cytometry using the FL1 channel (488/533). The mitochondrial membrane potential of the conidia was determined by staining with 175 nM of 3,3'-dihexylxacarbocyanine iodide (DiOC6, Sigma) for 30 min in the dark. After staining, the conidia were analyzed by flow cytometry using the FL1 channel.

The flow cytometry data acquisition was performed using a FACSCalibur flow cytometer equipped with an argon ion laser emitting at 488 nm. Data from 10,000 cells were obtained using Cell Quest Pro software (Becton-Dickison) and the data analysis was conducted using FlowJo v.10 software (TreeStar, Inc).

Statistical analysis

The test data were submitted to analysis of variance (ANOVA) and the means were compared using Tukey's test with a significance level of $p \leq 0.05$. These statistical analyzes were performed using SPSS software, version 28.0.1 (IBM, USA).

Results and Discussion

The antifungal effect of the monoterpenes on the mycelial growth of *B. cinerea* was evaluated at a fixed concentration of 1,000 mg/L (Fig. 1A). Most of the tested monoterpenes exhibited inhibitory effects on fungal growth at this concentration. Carvacrol, citral, citronellal, citronellol, geraniol, and thymol completely inhibited mycelial growth at 1,000 mg/L. However, linalool and 1,8-cineol showed lower fungal growth inhibition, less than 80%, and were not included in further assays.

The IC_{90} of the six selected monoterpenes against *B. cinerea* was determined (Figure 1B). Citral, citronellol, and geraniol exhibited higher IC_{90} values, without significant differences among them, with IC_{90} values close to 400 mg/L. Citronellal, with an IC_{90} of about 300 mg/L, was considered with some potential to control the fungus. The monoterpenes carvacrol and thymol were the two compounds with significantly lower IC_{90} (125 mg/L) against *B. cinerea* and with more potential to control the fungus.

Monoterpenes are the main components of many essential oils. They are originated from secondary metabolites of plants and other organisms and have several biological activities, including antifungal properties (Wang et al., 2018; Oliveira et al., 2019; Xing et al., 2019; Da Silva et al., 2020). Monoterpenes are nonpolar with hydrophobic and lipophilic characteristics, allowing them to interact with fungal cell membranes. This interaction is considered essential in disrupting cellular and energy homeostasis, leading to cell membrane damage and metabolic alterations (Lemos & Santos, 2006; Viriato, 2014; Da Silva et al., 2020).

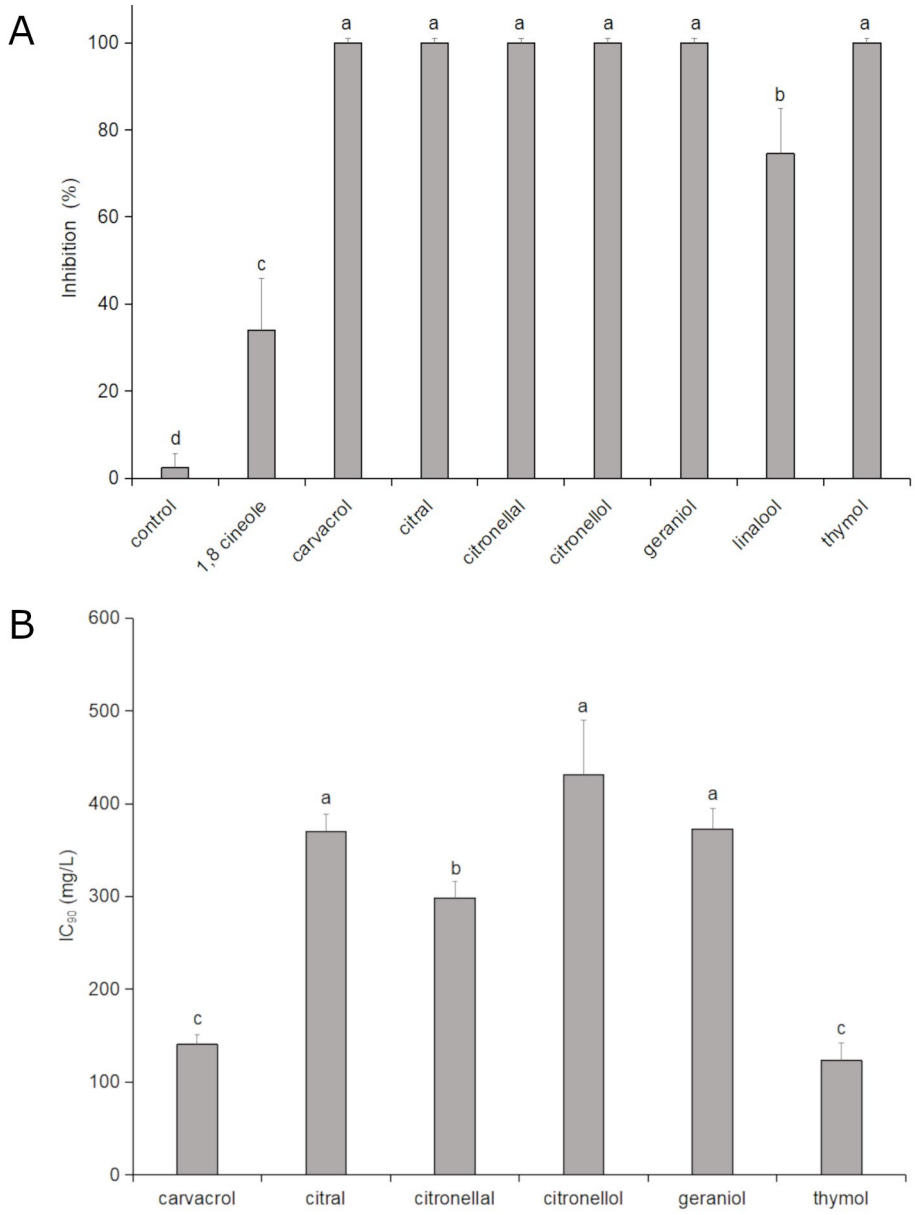

Figure 1: (A) *Botrytis cinerea* mycelial growth inhibition using eight monoterpenes at a concentration of 1,000 mg/L (B) Evaluation of the 90 % inhibitory concentration (IC_{90}) of six selected monoterpenes on the growth of *B. cinerea* mycelia. The different letters indicate statistically significant differences between the mean values ($p \leq 0.05$) by Tukey's test.

Based on these results, carvacrol and thymol were chosen for evaluating their effect on *B. cinerea* conidia, aiming to better understand the mechanism of action of these monoterpenes on the fungus. Conidia of *B. cinerea* were treated with different concentrations of carvacrol and thymol, and analyzed using flow cytometry. The results showed a dose-dependent reduction in cell membrane integrity of the conidia when treated with carvacrol or thymol (Figure 2A and Figure 2B). At the highest concentration (500 mg/L) of carvacrol and thymol, 78.29 % and 72.16 % of conidia exhibited cell membrane disturbance, respectively. Carvacrol and thymol interaction with the conidia cell membrane integrity indicated an interaction of monoterpenes with the cell membrane that affects homeostasis and permeability.

Carvacrol is considered a powerful antimicrobial agent against a broad range of fungi and bacteria, even at low concentrations (Khan et al., 2015). Likewise, several studies report the important antifungal activity of thymol (Scariot et al., 2020; Zhang et al., 2022). Moreover, several authors considered a synergistic effect of carvacrol and thymol (Campos-Requena et al. 2015). Zhang et al. (2019) studied the effect of carvacrol and

thymol on an isolate of *B. cinerea* and showed that the minimal inhibitory concentration for carvacrol and thymol were 120 and 65 mg/L respectively.

The antifungal mechanism of action of monoterpenes is typically associated with their interaction with cell membranes, resulting in increased cell membrane permeability. Zhang et al. (2019) studied the effect of carvacrol and thymol against *B. cinerea* mycelia and concluded that the treatment with the monoterpenes alters the fungal morphology of hyphae by disrupting the mycelia and causing an increment of extracellular conductivity and release of cellular components. The antifungal effect of carvacrol and thymol against other fungal pathogens also are involved in the increasing of cell membrane permeability (Zhou et al., 2018; Scariot et al., 2020).

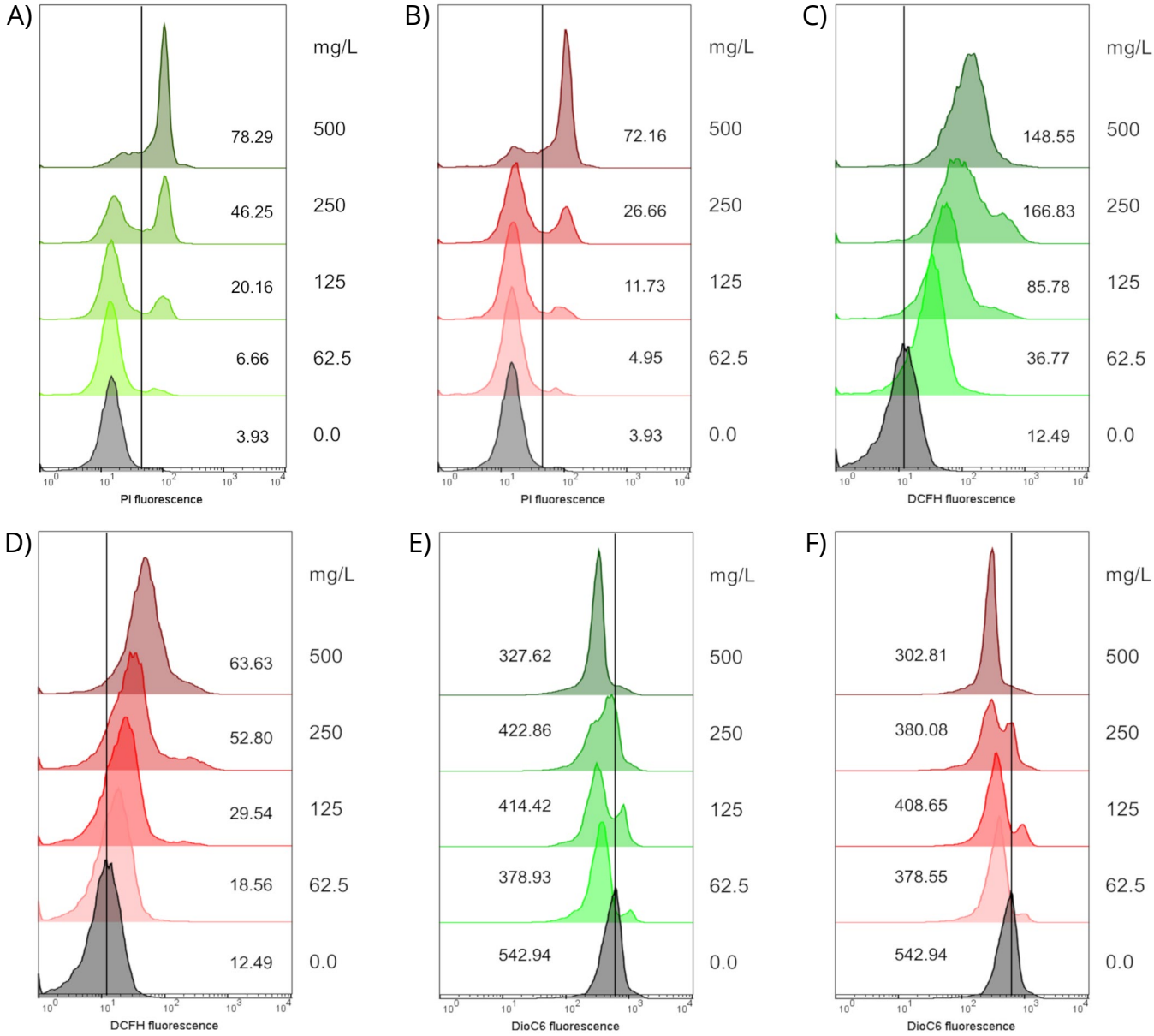

Figure 2: Evaluation of cell membrane integrity of *Botrytis cinerea* conidia using different concentrations of carvacrol (A) and thymol (B). The inner numbers in figures A and B indicate the percentage of PI-positive conidia (right side of the black line). Intracellular accumulation of reactive oxygen species in *B. cinerea* conidia using different concentrations of carvacrol (C) and thymol (D). Evaluation of mitochondrial membrane potential in *B. cinerea* conidia using different concentrations of carvacrol (E) and thymol (F). The numbers within the figures C, D, E, and F indicate the mean fluorescence value of each treatment and the black line indicates the mean fluorescence of the control.

To evaluate intracellular ROS concentration in monoterpenes-treated conidia we used DCFH, a fluorescent dye commonly used to detect oxidative stress in cells due to the high sensitivity of fluorescence-based assays (Bonini et al., 2006). DCFH staining of conidia treated with carvacrol and thymol showed a dose-dependent increase in intracellular ROS content (Figure 2C and 2D). Carvacrol exhibited a greater induction of intracellular ROS accumulation compared to thymol. Treatment with 250 mg/L of carvacrol resulted in a fluorescence value 3.1 times higher than the same concentration of thymol.

Considering that the increase in intracellular ROS concentration can be associated with changes in mitochondrial activity, we evaluated the mitochondrial membrane potential of conidia treated with different concentrations of carvacrol and thymol. The evaluation of the potential of the mitochondrial membrane of the conidia showed a reduction in fluorescence when they were treated with carvacrol or thymol (Figure 2E and 2F), indicating loss of the mitochondrial membrane potential. Although all concentrations tested caused a reduction in mitochondrial membrane potential, the highest concentrations (500 mg/L) showed greater reductions, with fluorescence reductions of 40.0 % with carvacrol and 44.2 % with thymol.

Flow cytometry data revealed the accumulation of intracellular ROS in *B. cinerea* conidia treated with carvacrol and thymol, with the effect being dose-dependent. The relation between thymol and carvacrol with intracellular ROS accumulation was previously observed in spores of *Aspergillus flavus* (Shen et al., 2016) and *Colletotrichum acutatum* (Scariot et al., 2020) and in mycelia of *B. cinerea* (Hou et al., 2020). In this sense, RNA-seq transcriptome analysis of *Fusarium oxysporum* treated with thymol showed that genes involved in antioxidant activity were up-regulated in response to ROS accumulation caused by thymol (Zhang, Ge, & Yu, 2018). The accumulation of intracellular ROS can lead to cell death by necrosis or induce the apoptotic cascade, inhibiting the germination of fungal conidia, shrinkage of hyphae, collapse, and disorganization of conidia and hyphae (Hou et al., 2020; Scariot et al., 2020).

Responsible for producing energy, mitochondria are important organelles in fungal cells. Mitochondrial damage results in disruption of the respiratory chain and the tricarboxylic acid cycle pathway (Fernie, Carrari, & Sweetlove, 2004; Hou et al., 2020), inducing ROS accumulation and decreasing intracellular energy (Zorov, Juhaszova, M., & Sollott, 2014). Damage to mitochondria can lead to greater intracellular accumulation of ROS and accelerate the rate of apoptosis of fungal cells (Zorova et al., 2018). In our study, treatment with carvacrol and thymol caused a decrease in mitochondrial membrane potential in *B. cinerea* conidia. Previous studies have shown that carvacrol and thymol caused an increase in the fluorescence of rhodamine 123 (fluorescent dye with functionality similar to DioC6) in *B. cinerea* mycelia (Hou et al., 2020), while monoterpenes lead to a reduction in the fluorescence of DioC6 in *C. acutatum* (Scariot et al., 2020).

Conclusions

Results reveal the potent antifungal effect of carvacrol and thymol against *B. cinerea.* These compounds not only inhibit the growth of the fungus but also trigger conidia death through multiple mechanisms. Specifically, they cause mortality in fungal conidia by disturbing the cell membrane, increasing intracellular ROS levels, and reducing the mitochondrial membrane potential. These outcomes imply that carvacrol and thymol might be promising candidates for developing innovative antifungal agents or serving as natural fungicides in agricultural strategies targeting the control of *B. cinerea* infections.

Author Contribution

Conceptual idea: Delamare, A.P.L.; Echeverrigaray, S.; Scariot, F.J.; Methodology design: Delamare, A.P.L.; Echeverrigaray, S.; Scariot, F.J.; Data collection: Pedroso, M. B.; Rocha, R. K. M.; Scariot, F.J.; Data analysis and interpretation: Delamare, A.P.L.; Echeverrigaray, S.; Pedroso, M. B.; Rocha, R. K. M.; Scariot, F.J.; and Writing and editing: Delamare, A.P.L.; Echeverrigaray, S.; Pedroso, M. B.; Rocha, R. K. M.; Scariot, F.J.

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