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Control of mildew in vines with cinnamon extract and catalase activity in organic production

Controle do míldio em videiras com extrato de canela e atividade da catalase em produção orgânica

Control del mio en vides con extracto de canela y actividad de la catalasa en producción orgânica

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Abstract
Management with synthetic fungicides in the control of phytopathogens in viticulture can cause environmental pollution and contamination with residues in grape cluster. The objective of this study was to verify the effect of aqueous cinnamon extract on the in vitro and in vivo control of *Plasmopara viticola* on catalase activity on 'Isabel Precoce' vines. The treatments used were: aqueous cinnamon extract (ACE) at concentrations of 0.12; 0.25 and 0.50% plus 0.25% vegetable oil (VO); being the standard treatments VO (0.25%), Bordeaux mixture 1:1:100 (lime: copper sulfate: water) and water only. The germination tests of P. viticola sporangia were carried out in incubation periods of 4 and 24 hours of the pathogen in contact with the treatments. In addition, the area under the disease progress curve (AUDPC) and the activity of the catalase enzyme were estimated in plants grown in the greenhouse. The results indicated that the treatments with 0.12%, 0.25% and 0.5% ACE with VO reduced the germination of *P. viticola*. In relation to the AUDPC, the 0.25% dose of VO associated ACE reduced 65% and 67% in leaf discs and vines in the greenhouse, respectively. This fact is related to the induction of CAT activity provided by this dose in the periods of 2HBA, 2HAI and 4HAI. Thus, it can be said that the ACE associated with VO can be used to control the downy mildew of the 'Isabel Precoce' vine.
Keywords: *Cinnamomum zeylanicum*; Enzymes; Plant extracts; *Plasmopara viticola*.

Resumo
O manejo com fungicidas sintéticos no controle de fitopatógenos na viticultura pode causar poluição ambiental e contaminação com resíduos nos cachos de uva. Para isso, objetivou-se com o trabalho verificar o efeito do extrato aquoso de canela no controle in vitro e in vivo de *Plasmopara viticola* e na atividade da catalase em videiras 'Isabel Precoce'. Os tratamentos utilizados foram: extrato aquoso de canela (EAC) nas concentrações de 0,12; 0,25; e 0,50% acrescidos de 0,25% de óleo vegetal (OV); sendo os tratamentos padrões OV (0,25%), calda bordalesa 1:1:100 (cal: sulfato de cobre: água) e somente água. Os testes de germinação dos esporângios de P. viticola foram realizados nos períodos de 4 e 24 horas de incubação do patógeno em contato com os tratamentos. Além disso, foi estimada a área abaixo da curva do progresso da doença (AACPD) e a atividade da enzima catalase em plantas cultivadas na casa de vegetação. Os resultados indicam que os tratamentos com 0,12%, 0,25% e 0,5% de EAC com OV reduziram acima de 56% a germinação de *P. viticola*. Em relação, a AACPD a dose 0,25% de EAC associada ao OV reduziu 65% e 67%

em discos de folha e em videiras na casa de vegetação, respectivamente. Esse fato está relacionado com a indução da atividade da CAT proporcionada por essa dose nos períodos de 2 HAA, 2HDI e 4HDI. Dessa forma, pode-se dizer que o EAC associado ao OV pode ser empregado no controle do míldio da videira 'Isabel Precoce'.
Palavras-chave: *Cinnamomum zeylanicum*; Enzimas; Extrato de plantas; *Plasmopara viticola*.

Resumen
El manejo con fungicidas sintéticos en el control de fitopatógenos en la viticultura puede causar contaminación ambiental y contaminación con residuos en los racimos de uva. Para ello, se objetivó con el trabajo verificar el efecto del extracto acuoso de canela en el control in vitro e in vivo de *Plasmopara viticola* y en la actividad de la catalasa en vides 'Isabel Precoce'. Los tratamientos utilizados fueron: extracto acuoso de canela (EAC) en las concentraciones de 0,12; 0,25; y 0,50% más el 0,25% de aceite vegetal (AV); y los tratamientos estándar AV (0,25%), calda bordalesa 1: 1: 100 (cal: sulfato de cobre: agua)(CB) y sólo agua. Las pruebas de germinación de los esporangios de P. viticola se realizaron en los períodos de 4 y 24 horas de incubación del patógeno en contacto con los tratamientos. Además, se estimó el área debajo de la curva del progreso de la enfermedad (ADCPE) y la actividad de la enzima catalasa en plantas cultivadas en la casa de vegetación. Los resultados indican que los tratamientos con 0,12%, 0,25% y 0,5% de EAC con OV redujo un 56% la germinación de *P. viticola*. En relación, la ADCPE la dosis del 0,25% de EAC asociada al AV redujo el 65% y el 67% en discos de hoja y en vides en la casa de vegetación, respectivamente. Este hecho está relacionado con la inducción de la actividad de la CAT proporcionada por esa dosis en los períodos de 2 HAA, 2HDI y 4HDI. De esta forma, se puede decir que el EAC asociado al AV puede ser empleado en el control del mio de la vid 'Isabel Precoce'.
Palabras clave: *Cinnamomum zeylanicum*; Enzimas; Extractos vegetales; *Plasmopara viticola*.

1. Introduction

Viticulture is faced with problems caused by phytopathogens that can cause qualitative and quantitative losses, due to the costs incurred in the control measures. (Pons et al. 2018). Among these pathogens the oomycete *Plasmopara viticola* [Berk. & Curt] Berl. & de Toni, causal agent of vine mildew, which causes losses in productivity due to the compromise of the plant aerial part, which subsequently worsens and adversely affects the vegetative development (Gessler et al. 2011).

For the control of grape mildew, synthetic fungicides are used, which despite efficiency, select pathogens resistant to the active principles, cause environmental pollution and also remain as residues in grape clusters (Peruch et al. 2007).

As a way to eliminate or at least reduce these problems, alternative products that are less polluting to the environment are sought, as well as effective in disease control, being able to act directly on the pathogen or trigger the elicitor effect of resistance inducers on the vine (Pinto et al. 2013).

Among the alternative products that may provide fungitoxic action are cinnamon (*Cinnamomum zeylanicum*), which presents volatile compounds, such as eugenol and cinnamaldehyde, that may adversely affect the development of microorganisms (Jham et al. 2005). Flávio et al. (2014) verified that the extract of cinnamon at a concentration of 30% reduced the infection of sorghum seeds by the pathogens of *Penicillium* sp., *Aspergillus* sp., *Trichoderma* sp., *Curvularia* sp., *Rhizopus* sp., *Colletotrichum* sp., *Fusarium* sp., *Dresheslera* sp., *Alternaria* sp., *Chaetomium* sp. Additionally, cinnamaldehyde may enhance the antioxidant activity of the enzymes superoxide dismutase, glutathione S-transferase and catalase, which are important in inducing plant resistance to pathogen attack (Brugalli 2003).

As a way to prevent the volatilization of these active ingredients present in cinnamon, allowing the expression of the fungitoxic effect as well as the activation of defense enzymes, such as catalase increasing the adhesion of the product on the leaf, the vegetable oil can be used as an adjuvant (Bogorni & Venturoso 2003).

In this context, the present work aimed to verify the effect of cinnamon (*C. zeylanicum*) and vegetable oil (adjuvant) in the control of grapevine mildew in vitro and in vivo and its effect on the activity of the enzyme catalase on 'Isabel Precoce' vines in organic production system.

2. Methodology

The experiments were conducted in the Phytopathology laboratory and in a greenhouse, both belonging to the Department of Agronomy of the State University of the West Center (UNICENTRO), Guarapuava-PR.

To prepare the treatments, the cinnamon bark (*C. zeylanicum*) obtained in the local trade was immersed in distilled water at 70ºC to obtain the aqueous solutions at the concentrations of 0.12%, 0.25% and 0.50% (w/v). Subsequently, the infusions were rested for 24 hours in a container. After this time, the preparation was filtered and then 0.25% vegetable oil (Natur'l oil ® Stoller, 930 ml/L (93% v/v), (Cosmópolis, São Paulo).

For all experiments the treatments were as follows: ACE at concentrations of 0.12%; 0.25% and 0.50% with 0.25% VO as adjuvant; 0.25% VO, 1:1:100 Bordeaux mixture (BM) (lime: copper sulphate: water) and absolute control (water only).

In the germination of *P. viticola* sporangia, 100 mL of sterilized distilled water containing Tween 80 on leaves with typical symptoms of vine mildew was added and, with a Drigalski loop, the mycelium was removed for the sporangia release. This suspension was calibrated to 5x104 mL^{-1} sporangia in Neubauer chamber (hemocytometer).

Aliquots of 40 µL of the suspension and another in the same amount of the solution from each of the treatments were placed into individual wells of ELISA test plates.

The plates were then maintained in a growth chamber at 25 °C in the dark, each corresponding to the period of 4 and 24 hours. In order to stop the sporangium germination, 20 µL of the blue cotton dye of lactophenol was added to each well, at the time scheduled for evaluation. Subsequently, the percentage of sporangia germinated (100 spores per repetition) was evaluated, observed in an inverted objective optical microscope. Germinated sporangia were those that showed release of zoospores. The experimental design was completely randomized with six treatments and five replications (Garcia et al., 2018).

In the evaluation of the severity of mildew (*P. viticola*) on 'Isabel Precoce' vine leaves, healthy leaves with 5 cm diameter were used, surface disinfested with 2% sodium hypochlorite for 5 seconds and dried at room temperature. Subsequently, the leaf discs were immersed for 1 minute in containers containing the different treatments and then distributed on foam moistened in Gerbox boxes at room temperature.

After 24 hours, 5x104 sporangia mL-1 water suspension of *P. viticola* was inoculated onto the leaf discs, and after 48 hours the mildew severity was evaluated for seven days according to the diagrammatic scale proposed by Azevedo (1997). Subsequently severity data were transformed into an area under the disease progression curve (AUDPC) based on the formula: $AUDPC = \Sigma (yi + yi+1) / 2 * (ti+1 – ti)$, where: n = number of assessments; y = disease severity (%); t = time (days). The experimental design was completely randomized with six treatments and four replicates with six disks each.

In order to evaluate the severity of mildew (*P. viticola*) on 'Isabel Precoce' vines in greenhouse, the grafts grafted on the 'Paulsen 1103' rootstock were planted in 1 L pots and filled with commercial substrate (Plantmax®, composed of 60% Pinus bark, 15% vermiculite, "fine" granulometry and 15% "superfine" and 10% humus) and stored in a greenhouse under sprinkler irrigation. After the first leaves of the vines were sprouted, treatments were sprayed every 7 days with a hand sprayer. These sprays were carried out in the period from 02/05/2014 to 03/19/2014, totaling 7 applications.

On 02/26/2014 the inoculation of the suspension of 5x104 sporangia of *P. viticola* was carried out and then the vines were kept in a humid camera for 2 days and after 7 days, severity assessments were initiated (% of injured area), according to the scale of Azevedo (1997), in 4 leaves previously identified in each plant. Severity data were transformed into an area below the disease progression curve (AUDPC) based on the formula: $AUDPC = \Sigma (yi + yi+1) / 2 * (ti+1 – ti)$, where: n = number of evaluations; y = disease severity (%); t = time (days). The experimental design was in randomized blocks with six treatments and four replicates, each vessel being an experimental plot.

For catalase enzyme activity in vines that were under greenhouse conditions, leaf discs of approximately 5 cm in diameter were collected after four treatments. These collections were carried out 2 hours before (2HBA) of the fourth

application of treatments and inoculation of *P. viticola*, as well as 2 (2HAI), 4 (4 HAI) and 6 hours later (6 HAI).

The leaf samples were protected with foil, cooled on ice and stored in a freezer at -80 °C until preparation of extracts for analyzes of total protein and catalase activity.

Leaf discs were weighed and then macerated in liquid nitrogen mortar and mechanically homogenized with 1% (w/w) PVP (polyvinylpyrrolidone) and 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. Afterwards the solution was centrifuged at 13,000 g for 30 min at 4 °C, the supernatant obtained being considered as an enzymatic extract, which was stored at -80 °C until the analyzes were performed. From these extracts the content of total proteins and catalase activity were determined.

For the determination of the protein content according to Bradford (1976) for each 50 µL of the supernatant was added, under stirring, 2.5 ml of the Bradford reagent. After 5 min the absorbance was read at 595 nm in a spectrophotometer (Shimadzu - Model UV-1800). The concentration of proteins, expressed in mg per mL of sample (mg protein mL^{-1}), was determined using a standard curve of bovine serum albumin (BSA) concentrations of 0 to 0.5 mg mL^{-1}, obtained by the Bradford method y = -0,0456+0,733x.

Catalase activity (CAT) (EC 1.11.1.6) was quantified by the stable complex formed by ammonium molybdate with hydrogen peroxide (Abs 405 nm). The enzyme extract (0.2 mL) was incubated in 1 mL reaction mixture containing 60 mM hydrogen peroxide in 60 mM potassium phosphate buffer pH 7.4 at 38 ° C for 4 min. The addition of 1mL of 32.4 mM of ammonium molybdate after 4 min of incubation was done to stop the consumption of hydrogen peroxide by the enzyme present in the extract. A blank was prepared for each sample by addition of ammonium molybdate to the reaction mixture, omitting the incubation period. The yellow complex of molybdate and hydrogen peroxide were measured at 405nm. The difference between the blank absorbance and the incubated sample indicated the amount of hydrogen peroxide used by the enzyme. The H_2O_2 concentration was determined using the extinction coefficient $\epsilon = 0.0655\ mM^{-1}\ cm^{-1}$.

The results were submitted to analysis of variance and comparison of means by the Tukey test at the 5% probability level, with the statistical program SISVAR (Ferreira 2011).

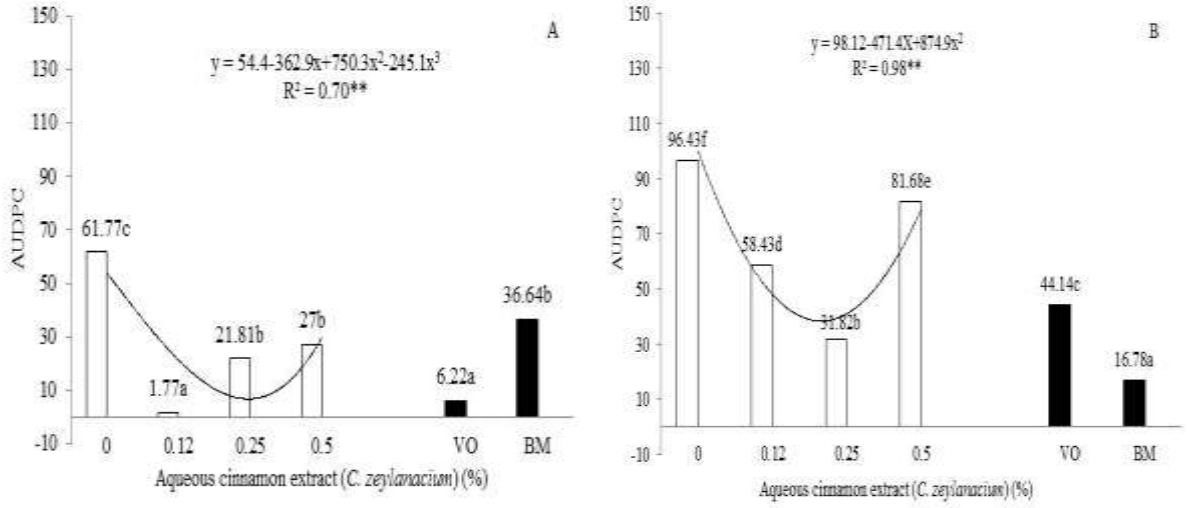
3. Results

For the germination of sporangia of *P. viticola*, at the times evaluated, there was a quadratic effect as a function of the doses of aqueous cinnamon extract with vegetable oil. It was observed that at 4 and 24 hour treatments of 0.25% and 0.5% ACE with VO reduced in 60% and 56%, 70% and 73.8%, respectively,

the germination of *P. viticola* when compared to the control. It was observed that the direct contact of the VO with the pathogen in these two evaluation periods had a fungitoxic effect reducing germination by 62.3% and 67.6%. Regarding BM, these values are 34.6% and 52.7%, respectively (Figure 1A and 1B).

For the AUDPC of mildew on leaves of vines, a quadratic effect was observed as a function of the doses. It should be noted that the 0.12% and 0.25% ACE doses reduced the AUDPC of grapevine mildew by 97.2% and 65%, and did not present statistical difference with the treatment composed only of VO, which decreased by 90 %, both with respect to the control treatment (Figure 2A).

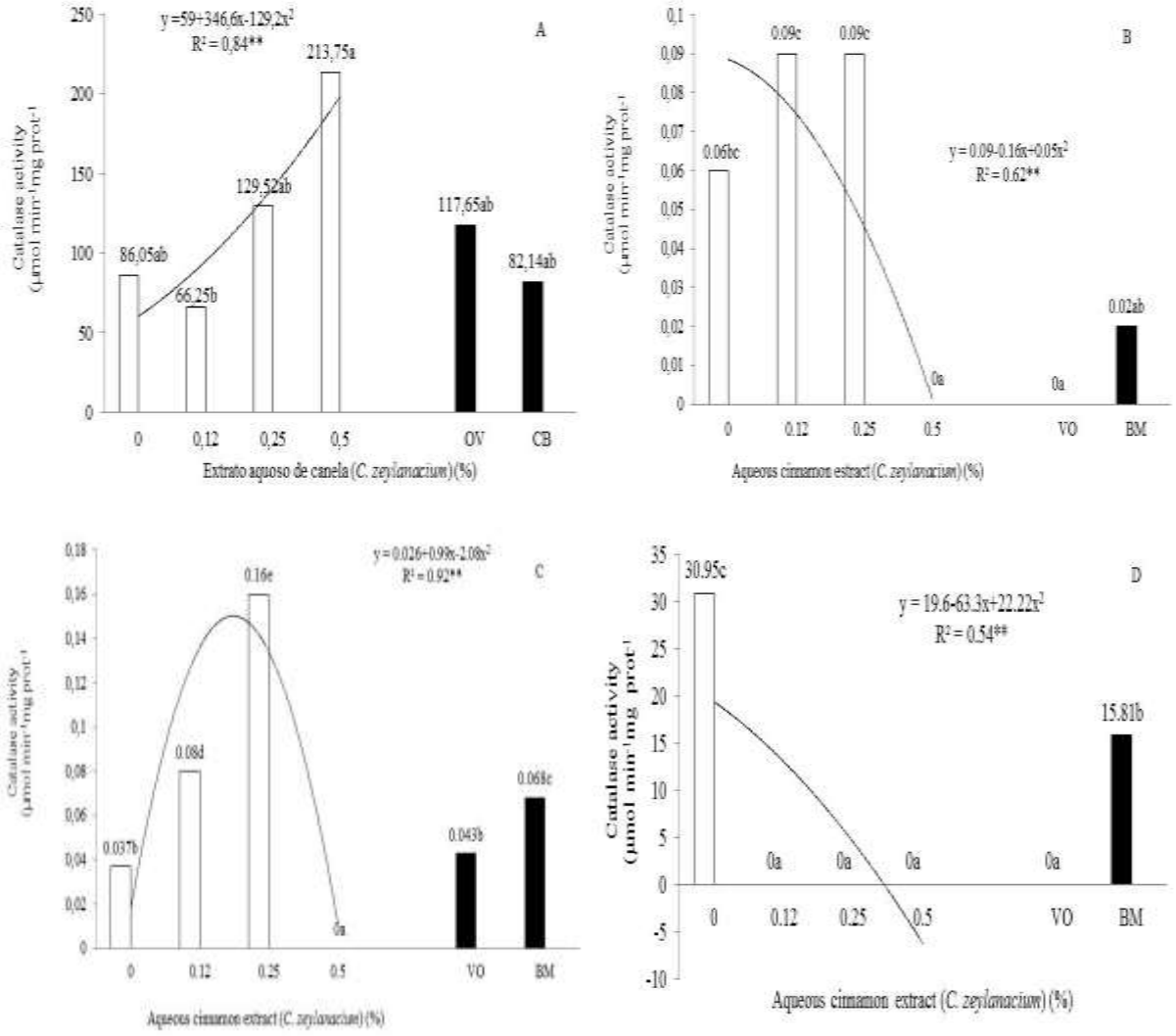
Figure 1. Sporangia germination of *Plasmopara viticola* on the effect of 0.12%, 0.25% and 0.5% aqueous cinnamon extract (*C. zeylanicum*) (ACE) plus 0.25% vegetable oil, treatment with 0.25% vegetable oil only, standard with Bordeaux mixture (BM) and absolute control (water only) in the periods of: (A) 4 hours and (B) 24 hours after incubation at 25 °C.



*Statistically significant at 5% probability. Source: Authors.

Regarding the catalase enzyme, the ACE dose of 0.25% induced its activity in 50.5%, 50% and 332.4% in 2HBA, 2HAI and 4HAI, respectively, in relation to the control treatment. As in 6HAI the activity of this enzyme was inhibited by this treatment. VO also inhibited CAT in 2HAI and 6HAI, and BM reduced its activity by 4.54% (2HBA), 66.6% (2HAI) and 49% (6HAI) (Figure 3).

Figure 3. Activity of catalase (CAT) in leaf discs extracted from cv. Isabel Precoce vines treated with doses of 0; 0.12%; 0.25% and 0.5% aqueous cinnamon extract (*Cinnamomum zeylanicum*) as well as the control treatment, water only, and standard with Bordeaux mixture, in the 2 hour before application (2HBA) (A), 2 hours (2HAI) (B), 4 hours (4HAI) (C) and 6 hours (6HAI) (D) after inoculation of *Plasmopara viticola*. Pathogen inoculation.



* Statistically significant at 5% probability. Source: Authors.

4. Discussion

Cinnamon extract (*C. zeylanicum*) presents in its composition eugenol and cinnamaldehyde that have a negative effect on the development of microorganisms, being able to act on the structures of hyphae growth and virulence of pathogens (Khan & Ahmad 2011). These compounds are potent antifungal agents that can control the development of *Colletrotrichum gloeosporioides*, *Alternaria* sp. and *Penicillium chysogenum* (Kumar et al. 2009).

This toxic effect was confirmed by cinnamon extract in results obtained by Venturoso et al. (2011) when verifying that the dose of 20% reduced by 43% the mycelial growth of *Phomopsis* sp.

Regarding VO, Garcia et al. (2015) also emphasize direct control over *P. viticola*. The authors note that the 0.80 mL L^{-1} dose of this product reduces the germination of *P. viticola* by 77% after 24 hours of incubation when compared to the control. Evidence that the longer the contact time of the product with the sporangia, the lower its germination.

BM showed to be effective for the control of grape mildew. However, it should be taken into account that the application of this compound in the vineyards increases the copper contents in the layers of 20-40 cm of the soil (Casali et al. 2008).

Dagostin et al. (2011) emphasize that VO has the same effect of copper (presents in BM) for the control of mildew. With the present study we show that this process was potentiated with the association with ACE, mainly with the dose of 0.25%. Possibly, this fact, is related to high adherence of ACE to the leaf surface allowed by VO (Zyl et al. 2010).

The application of plant extracts, such as cinnamon, has the advantage of producing organic plants free of toxic products and still present more than one antifungal compound that helps in the management of the crop (Shuping & Eloff 2017). This fungitoxic effect of cinnamon was confirmed by Flávio et al. (2014) who observed that the aqueous extract of this vegetable in the concentration of 30% reduced in 61% the fungus microflora of seeds of sorghum.

The effect of reducing AUDPC from mildew on vines treated with the 0.25% ACE dose is related to its direct effect on *P. viticola* (Figure 1) and also CAT activity. This enzyme highlights its performance as the main route of H_2O_2 degradation. Thus, its activation eliminates the excess of this molecule, which in high concentration can cause cell damage (Mittler 2017). So probably this treatment provided H_2O_2 synthesis prior to inoculation of *P. viticola*, activating plant defense mechanisms.

This activation occurs through the action of this molecule as a secondary messenger that activates genes related to the pathogenesis, induction of phytoalexins synthesis, in the reinforcement of the cell wall increasing the interconnections between hydroxyproline and glycoproteins to the matrix of polysaccharides, and/or acts directly toxic on fungal phytopathogen (Quan et al. 2008).

It should be considered that the pathogen also releases proteolytic enzymes that cause damage to the plasma membrane of the cell and consequently can also activate H_2O_2 (Quan et al., 2008). A fact that is observed in the treatment with 0.25% of ACE with 4HAI, potentializing the activation of defense mechanisms of these grapevine plants and the consequent reduction of AUDPC of mildew (Figures 2 and 3, C).

In general, it was observed that the 0.25% ACE dose had a direct effect on *P. viticola*, reducing the germination of the pathogen and also activated 'Isabel Precoce' vine defense mechanisms, reducing the AUDPC of mildew. It is also worth noting that ACE is easy to acquire and low cost, so it is recommended to carry out new experiments under field conditions so that the potential of this extract can be evidenced for the application in organic commercial vineyards.

5. Conclusion

The dose of 0.25% aqueous cinnamon extract (Cinnamomum zeylanicum) associated with vegetable oil had a fungitoxic effect directly on Plasmopara viticola, reduced AUDPC of mildew on leaf discs in greenhouse.

The 0.25% dose of the aqueous cinnamon extract also activated the catalase enzyme activity, inducing the resistance of these plants to vine mildew.

The treatments with cinnamon extract were efficient in controlling the vine mildew and with the advantage of use in the organic production in the cv. Isabel Precoce.

It is suggested that future work be carried out with these treatments under field conditions.

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