

# Antimicrobial activity of *Agaricus brasiliensis* on *Plasmopara viticola* and its effect on the induction of resistance to the control of downy mildew on 'Isabel Precoce'

## Atividade antimicrobiana de *Agaricus brasiliensis* sobre *Plasmopara viticola* e seu efeito na indução de resistência para o controle de míldio em videiras Isabel Precoce

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**ABSTRACT:** *Agaricus brasiliensis* include bioactive compounds that can act as antibiotics, bacteriostatic, fungistatic and nematostatic substances. In this sense, this study aimed to evaluate the effect of a single application of aqueous mycelial suspension (AMS) of *A. brasiliensis* in control of downy mildew (*Plasmopara viticola*) and resistance induction in 'Isabel Precoce' grapevines under greenhouse conditions. Treatments consisted of three doses of 1%, 5%, 10%, 15% and 20% AMS *A. brasiliensis*, as well as treatment with acibenzolar-S-methyl (ASM). The variables analyzed were: sporangiospore germination, disease severity, represented by the area under the disease progress curve (AUDPC), catalase enzyme activity, peroxidase and polyphenol. The 10%, 15% and 20% doses of AMS caused approximately 80% reduction in germination of *P. viticola* sporangiospores. The treatments did not show significant effects in reducing both the AUDPC of mildew and polyphenol oxidase enzyme activity. The *A. brasiliensis* aqueous mycelial suspension showed a fungitoxic effect on the germination of sporangiospores; however, it was not enough to reduce the severity of mildew in the 'Isabel Precoce' grapevines, even when acting on the catalase and peroxidase enzymes. Thus, experiments should be performed to verify the viability of the reproductive structures of the pathogen externalized in the vines when treated with *A. brasiliensis* AMS.

**KEYWORDS:** *Plasmopara viticola*; *Vitis labrusca*; aqueous mycelial suspension; elicitor.

**RESUMO:** *Agaricus brasiliensis* possui compostos bioativos que apresentam atividade antimicrobiana e induz mecanismos de defesa em plantas contra patógenos. O objetivo deste trabalho foi avaliar a aplicação da suspensão miceliada aquosa de *A. brasiliensis* no controle do míldio (*Plasmopara viticola*) e indução de resistência em videiras Isabel Precoce. Os tratamentos foram: 0, 1, 5, 10, 15 e 20% da suspensão miceliada aquosa de *A. brasiliensis*, além do tratamento com acibenzolar-S-metil. As variáveis analisadas foram: germinação de esporangiósporos; severidade da doença, representada pela área abaixo da curva de progresso da doença; atividade da enzima catalase; peroxidase e polifenoloxidase. As doses 10, 15 e 20% de suspensão miceliada aquosa de *A. brasiliensis* proporcionaram redução de aproximadamente 80% na germinação dos esporangiósporos de *P. viticola*. Os tratamentos não apresentaram efeitos significativos na redução da área abaixo da curva de progresso da doença do míldio e na atividade da enzima polifenoloxidase. A dose de 10% da suspensão miceliada aquosa de *A. brasiliensis* reduziu a atividade de catalase e induziu a atividade da peroxidase. A suspensão miceliada aquosa de *A. brasiliensis* apresentou efeito fungitóxico na germinação de esporangiósporos, entretanto não foi suficiente para reduzir a severidade do míldio da videira Isabel Precoce, mesmo atuando na atividade das enzimas catalase e peroxidase. Assim, experimentos deverão ser realizados para verificar a viabilidade das estruturas reprodutivas do patógeno exteriorizadas nas videiras quando tratadas com suspensão miceliada aquosa de *A. brasiliensis*.

**PALAVRAS-CHAVE:** *Plasmopara viticola*; *Vitis labrusca*; suspensão miceliada aquosa; elicitor.

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## INTRODUCTION

The Brazilian wine sector is noteworthy for destining its final product for the production of table wines and juices. Among the cultivars used for this production, the rustic and fertile ink grape 'Isabel Precoce' stands out with its short cycle (CAMARGO et al., 2010).

However, this culture has some limitations that influences the yield and quality of the grapes produced, as is the case of diseases caused by phytopathogens. Among these diseases is mildew (*Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni), which mainly affects the leaves, reducing the active photosynthetic area, thus causing losses in the vine production (JERMINI et al., 2010).

Pesticides are used to control this disease, which leads to several environmental impacts and selection of resistant strains. As a way to eliminate or at least reduce these problems, production technologies that are less aggressive to man and the environment are sought, such as active compounds present in mushrooms, which are able to induce resistance against phytopathogens in plants (SCHWAN-ESTRADA et al., 2012).

Among the mushrooms used as resistance inducers is *Agaricus brasiliensis* (Synonymy: *A. blazei* or *A. subrufescens*), which has bioactive compounds that can act as antibiotics, bacteriostatic, fungistatic and nematostatic substances (SCHWAN-ESTRADA et al., 2012). FIORI-TUTIDA et al. (2007) found that the *A. blazei* and *Lentinula edodes* mushrooms have a fungitoxic effect on the germination of *Puccinia recondita* f.sp. *tritici* spores. ARRUDA et al. (2012) also point out that aqueous extracts of *A. blazei* at a 10% dose induced the accumulation of phytoalexin gliceolin in 'CB202' soybean cotyledons.

In this context, this study aims to verify the effect of a single application of the aqueous mycelial suspension (AMS) from the *A. brasiliensis* mushroom in the control of mildew and in the induction of resistance on 'Isabel Precoce' grapevines.

## MATERIALS AND METHODS

### Obtainment, maintenance and preparation of the *A. brasiliensis* inoculum

The *A. brasiliensis* mushroom from the fungi collection of the Bioprocesses Laboratory, Food Engineering Department, Universidade Estadual do Centro Oeste (UNICENTRO), was isolated from the fruiting body of a commercial culture. The inoculum was collected in an Erlenmeyers flask, and the methodology proposed by DALLA SANTA et al. (2009) was followed for its mycelial production.

To obtain the aqueous mycelial suspension (AMS) of *A. brasiliensis*, the mycelium (94% moisture) was thawed and weighed according to the dose determined for the experiments, and then macerated into a mortar. Subsequently, distilled water was added and the substance was triturated in a blender for two minutes.

### Evaluation of in vitro germination of *P. viticola* sporangiospores

A volume of 100 mL of sterile distilled water containing 20 µL of Tween 80 were added on vine leaves with typical symptoms of mildew, which were collected in the UNICENTRO orchard and, with a Drigalski handle, a smear was made on the pathogen's mycelium, which consequently caused the sporangiospores to be released. This suspension was standardized at  $1 \times 10^6$  sporangiospores mL<sup>-1</sup> with a Neubauer chamber count (hemocytometer).

The treatments tested in the germination control of these sporangiospores were 1%, 5%, 10%, 15% and 20% doses of *A. brasiliensis* AMS; acibenzolar-S-methyl (ASM) (0.005% diluted in water) as standard treatment; and an absolute control (water only). Aliquots of 40 µL of the pathogen suspension and another of 40 µL of the double-dose treatments (due to dose dilution occurring in the presence of the pathogen suspension) were placed in individual wells of ELISA test plates.

The plates were then kept in a growth chamber at 25°C in the dark, each corresponding to a period (4, 6, 12 and 24 hours). In order to stop sporangiospore germination, 20 µL of lactophenol cotton blue dye was added to each well at the time scheduled for evaluation. Subsequently, the percentage of germinated sporangiospores was evaluated at random in an inverted microscope, totaling 100 sporangia, in four replicates. Those that showed release of zoospores were germinated. The experimental design was completely randomized, with seven treatments and five replications.

### Effect of aqueous mycelial suspension on downy mildew control used on 'Isabel Precoce' grapevines under greenhouse conditions

'Isabel Precoce' vine seedlings grafted on 'Paulsen 1103' rootstocks were planted on 01/23/2013 in 1 L pots containing Plantmax® commercial substrate, and kept in a greenhouse for two cycles in the year 2013.

The experimental design was in randomized blocks, with seven treatments and six replicates, and experimental plot constituted by one plant. When the seedlings presented eight leaves, the treatments were applied (02/08/2013 for the first cycle, and 11/04/2013 for the second cycle) with a hand sprayer, to the point of drainage, with 1%, 5%,

10%, 15%, and 20% doses of *A. brasiliensis* AMS (doses corresponding to a dry weight content of 0.06%, 0.3%, 0.6%, 0.9%, and 1.2 % *A. brasiliensis*), plus a treatment with the commercial resistance inducer 0.005% acibenzolar-S-methyl (ASM), and control (no treatment). After 24h of the applications, the pathogen was inoculated with suspension of  $1 \times 10^4$  sporangiospores  $\text{mL}^{-1}$  in all leaves of the vine. In order to favor the pathogen's penetration and infection, the plants were placed in a humid chamber for 24 hours. After 14 days for the first cycle and 7 days for the second cycle, the first symptoms of the disease were observed, when severity assessments were initiated. Five evaluations were carried out every three days. Afterwards, the area under the disease progress curve (AUDPC) was calculated, based on the following Equation 1:

$$\text{AUDPC} = \sum (y_i + y_{i+1})/2 * (t_{i+1} - t_i), \quad (1)$$

where:

n = number of evaluations;

y = disease severity (%);

t = time (days).

## Determination of catalase, peroxidase and polyphenoloxidase activity

Leaf discs with approximately 5 cm in diameter were randomly collected from 'Isabel Precoce' grapevine plants only in the first cycle, at periods of 6, 48, 72 and 96 hours after treatment application, which were then protected with aluminum foil, cooled on ice and stored in a freezer at  $-80^\circ\text{C}$ , until the preparation of the extracts for biochemical analyzes. For the analyzes, they were weighed and macerated into a mortar with liquid nitrogen, and mechanically homogenized with 1% (w/w) PVP (polyvinylpyrrolidone) and with 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The solution was centrifuged at 15,000 g for 40 min at  $4^\circ\text{C}$ , and the obtained supernatant was considered an enzymatic extract.

From the enzymatic extract, it was possible to determine:

- Protein content, according to BRADFORD (1976);
- Catalase activity (CAC) (EC 1.11.1.60), according to TOMÁNKOVÁ et al. (2006);
- The activity of the enzyme guaiacol peroxidase (POD) (EC 1.11.1.7), according to LUSSO; PASCHOLATI (1999) and;
- The enzymatic activity of polyphenyl oxidase (PPO) (EC 1.10.3.1), according to DUANGMAL; APENTEN (1999).

## Statistical analysis

All results were submitted to the analysis of variance and, when significant, polynomial regression was carried out, as

well as a comparison of the means by the Tukey test at a 5% of error probability level, using statistical program SISVAR (FERREIRA, 2011).

## RESULTS AND DISCUSSION

### In vitro germination of sporangiospores of *P. viticola*

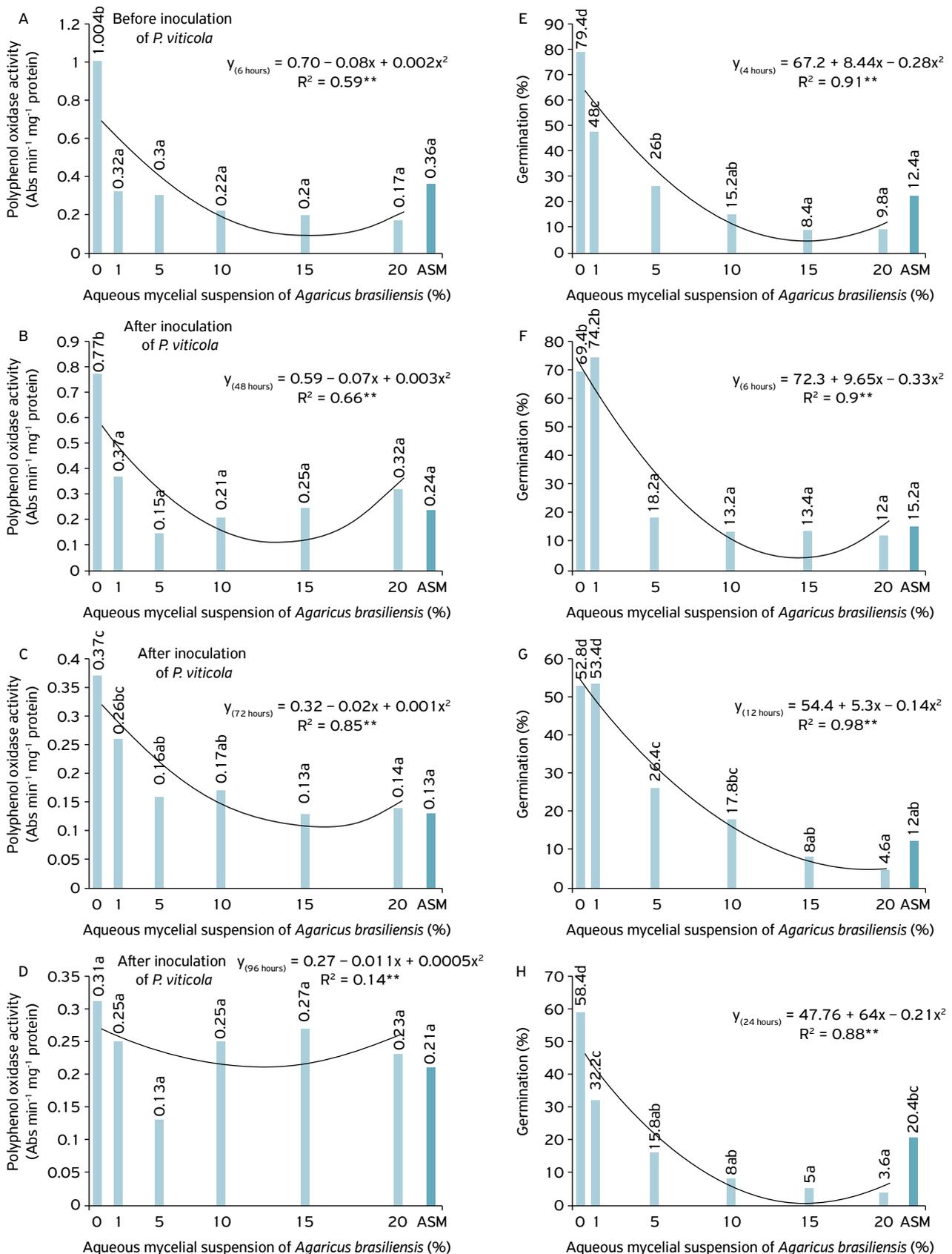
For the germination of *P. viticola* sporangiospores, there was a quadratic effect as a function of the doses of the *A. brasiliensis* AMS in all evaluation periods. The 10%, 15% and 20% doses of the *A. brasiliensis* AMS caused an 80% reduction in the germination of *P. viticola* sporangiospores in the periods of 4, 6 and 24 hours, and in 12 h, the reduction was 66%, 85%, and 91%, respectively. The treatment with 1% of the suspension, in the 6 and 12 hour periods, induced the germination of the pathogen in 107% and 101%, respectively, when compared to the control treatment (Figs. 1E to 1H).

Inhibition of *P. viticola* germination is probably due to the fungitoxic phenolic compounds present in the *A. brasiliensis* mycelial suspension (STANGARLIN et al., 2011; OKE; ASLIM, 2011). Similar results were observed by VIECELLI et al. (2010) using 20% of the mycelium extract of the mushroom *Pycnoporus sanguineus*, in which they verified a reduction of up to 70% in the spores germination of *Pseudocercospora griseola*, in relation to ASM.

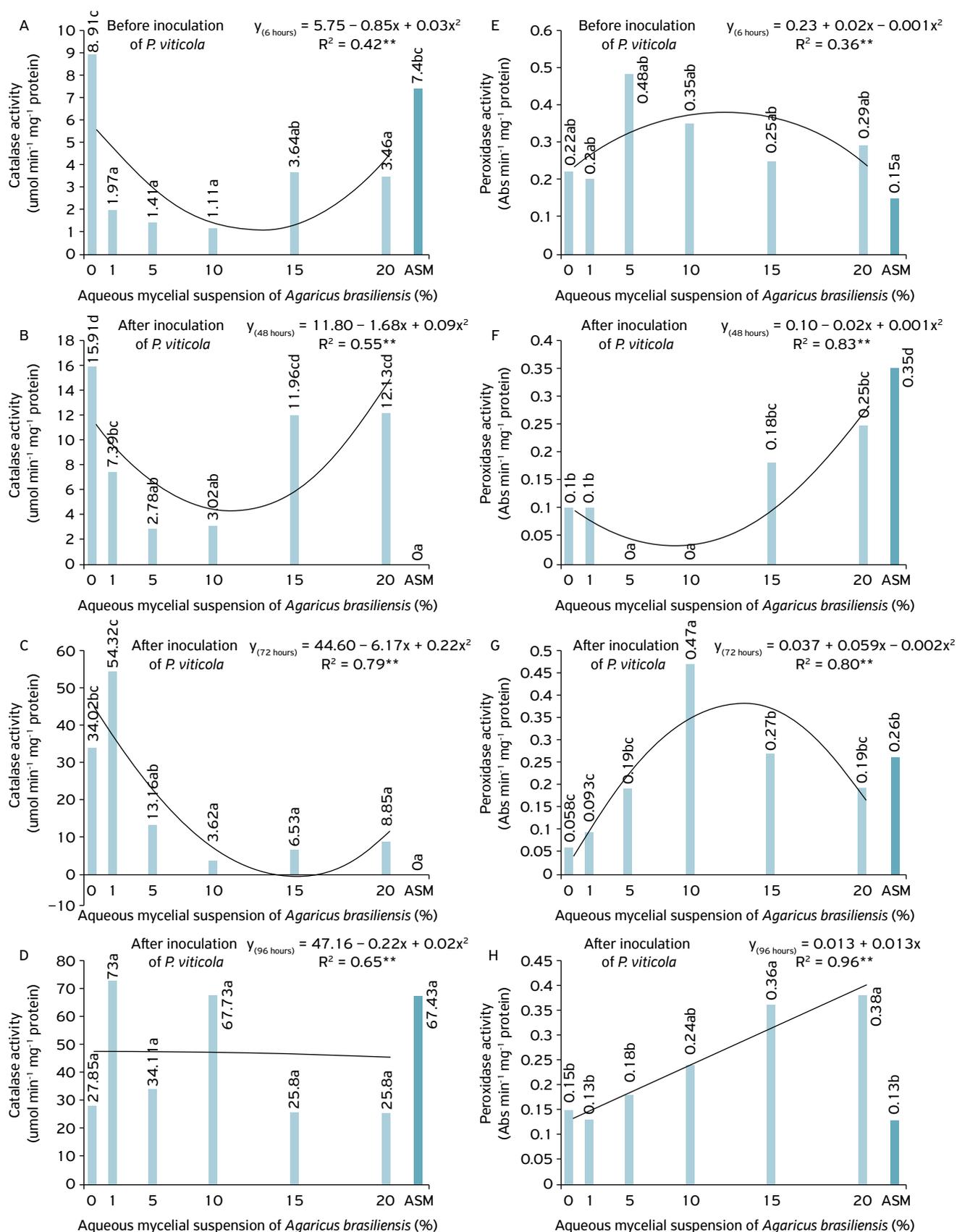
In the present study, treatment with ASM in the 4 h, 6 h, and 12 h periods did not present different results from the 20% dose of AMS. Similar results were obtained by MUÑOZ; MORET (2010), who verified the inhibition of the mycelial growth *Botrytis cinerea* when using acibenzolar-S-methyl in a potato-agar-dextrose (PAD) medium. This suggests that, in addition to the inductive effect, ASM can act directly on the pathogen, since it contains sulfur and methyl substance groups that are present in fungicides recommended for mildew control in grape crops (AGROFIT, 2016).

### Effect of aqueous mycelial suspension on downy mildew control used on 'Isabel Precoce' grapevines under greenhouse conditions

The AMS doses of *A. brasiliensis* and acibenzolar-S-methyl, in both cycles of the crop, did not present significant effect on the AUDPC of mildew on 'Isabel Precoce' vines under greenhouse conditions. The mean values observed in the two crop cycles were 344.5; 401.3; 273.7; 339.6; 301; 360.4 and



**Figure 1.** Polyphenol oxidase activity (PPO) on vine discs ('Isabel Precoc'), treated with the doses of 1, 5, 10, 15 and 20% of the aqueous mycelial suspension (AMS) of *Agaricus brasiliensis*, Acibenzolar-S-methyl (0.005 %) and absolute control collected in the periods of 6h (A), 48h (B), 72h (C) and 96h (D), after the treatments. Germination of *Plasmopara viticola* sporangia submitted to doses of 1; 5; 10; 15; 20% AMS *Agaricus brasiliensis*, absolute control and acibenzolar-S-methyl (ASM) (0.005%). In the periods of: (E) 4h; (F) 6h; (G) 12h and (H) 24h, after application of the treatments.



**Figure 2.** Catalase activity (CAT) and peroxidase (POD) in vine discs ('Isabel Precoce'), treated with 1, 5, 10, 15 and 20% doses of the *Agaricus brasiliensis* aqueous mycelial suspension, Acibenzolar-S-methyl (0.005%), and absolute control collected 6h (A), 48h (B), 72h (C) and 96h (D) after application of the treatments for CAT, and E, F, G and H for POD activity, in the same periods, after application of the same treatments. Regression analysis performed only for the doses of *A. brasiliensis* AMS. For ASM treatment, averages followed by the same letter do not differ by the Tukey test at 5% confidence probability.

271.5 for the treatments of 0, 1%, 5%, 10%, 15% and 20% of *A. brasiliensis* AMS and ASM, respectively. Activation of the protective effect, according to VIECELLI et al. (2010), will depend on the concentration and the time interval between treatment applications. However, CABRAL et al. (2010), when evaluating the effect of acibenzolar-S-methyl, mananoligosaccharides and citrus bioflavonoids on the control of the watery spot caused by *Acidovorax citrulli* in melon plants (hybrid AF4945) and Frog Skin type (Nile hybrid), at different times (10 and 15 days after seedling emergence), found that, 10 days after seedling emergence, acibenzolar-S-methyl reduced disease incidence by 88%, and AUDPC by 94%.

## Determination of catalase, peroxidase and polyphenol oxidase enzyme activity

There was a quadratic effect as a function of the *A. brasiliensis* AMS doses for the activity of the catalase enzyme at all evaluated times. The 10% dose of mushroom AMS inhibited catalase activity in over 80% at all periods when compared to the control treatment, presenting greater efficiency than the synthetic inductor (ASM), which, in 6 and 48 hours, reduced the activity of this enzyme in 11% and 24%, respectively (Figs. 1A and 1B).

Catalase has the main function of degrading hydrogen peroxide ( $H_2O_2$ ). The low activity of this enzyme possibly results in high concentration of  $H_2O_2$ , which is a reactive oxygen species that can act as secondary messenger in the induction of genes related to pathogenicity, as well as strengthening the plant's cell wall through cross-links with structural proteins or the lipid peroxidation of the plasma membrane, thus increasing its integrity and inducing a certain resistance of these plants. However, it was not efficient to control vine mildew (TORRES et al., 2006; SOARES; MACHADO, 2007; VELLOSILO et al., 2010).

In 96 h, it was observed that doses of both AMS and ASM did not interfere with the catalase's enzymatic activity, because oxidative explosion-related enzymes often present a very rapid response, occurring within seconds, minutes and hours after the application of the elicitor treatment or addition of the pathogen (HEISER; OBWALD, 2008) (Fig. 2D). This may be related to the high activity of POD, which presented a stimulus with the increase of the dose of *A. brasiliensis* AMS (Figs. 1E to 1H) in the same period. POD probably consumes the  $H_2O_2$  present in the cell, which, in the previous period for oxygenation, and eventual polymerization of hydroxycinnamic alcohol, gives rise to lignin, which acts as a physical barrier to pathogen penetration (STANGARLIN; LEITE, 2008).

In addition, POD and PPO can oxidize the phenolic compounds, mainly chlorogenic acid, that are found in this classification, which originates quinones that are highly toxic to pathogens (BARROS et al., 2010). Both can compete for the same substrate. According to the results obtained, there was inhibition of PPO activity by POD (Figs. 2A to 2D).

Similar results were observed by SILVA et al. (2007), who reported that tomato plants treated with extract of basidiocarp of *A. blazei* and ASM inoculated with the bacterium *Ralstonia solanacearum* showed increased POD activity and PPO reduction. SILVA et al. (2008), when treating eggplant plants with basidiocarp extract of *A. blazei* and ASM, obtained an increase in POD activity. DI PIERO et al. (2006) found that cucumber plants treated with the aqueous extract of the *L. edodes* mushroom basidiocarp caused a reduction in anthracnose severity and increased peroxidase activity in leaves.

Most of the work with the *A. brasiliensis* mushroom is carried out with the extract made from the basidiocarp. In this study, mycelium extract was used, with a moisture content of 94%, in which the concentration of active principles can be smaller, which probably contributed to the absence of mildew's AUDPC reduction. Another factor to be considered is that *P. viticola* is part of the group of biotrophic pathogens, which have a specialized structure such as haustory, which, in addition to removing nutrients from host cells, can also produce and transport effectors for these cells. These effectors, in incompatible interactions, manipulate the metabolism of host cells and hinder the performance of the host plant's defense system (AMORIM; PASCHOLATI, 2011).

## CONCLUSIONS

The *Agaricus brasiliensis* aqueous mycelial suspension and acibenzolar-S-methyl inhibited sporangiospore germination of *P. viticola*.

The severity of the mildew was not reduced with application of the mycelial suspension of *A. brasiliensis* and acibenzolar-S-methyl.

The *A. brasiliensis* aqueous mycelial suspension and acibenzolar-S-methyl induced the activity of the catalase, peroxidase and polyphenol oxidase enzymes.

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