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Control of the fungi *Lasiodiplodia theobromae*, the causal agent of dieback, in cv. syrah grapevines

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ABSTRACT. Grapevine trunk diseases are among the most important limiting factors of worldwide viticulture. In this context, we aimed to verify the effect of chemical fungicides, biological agents and plant extracts on the control of *Lasiodiplodia theobromae* in pruning wounds and their physiological effects in cv. Syrah grapevines. Plant extracts (clove, cinnamon, garlic, rosemary and lemongrass), commercial fungicides (difeconazole, tebuconazole, mancozeb, sulfur, pyraclostrobin, fosetyl-Al, and azoxystrobin), chitosan, *Trichoderma harzianum* and *Bacillus subtilis* were used for the *in vitro* trials. The protection of pruning wounds in the potted vines was studied using fosetyl-Al, tebuconazole, *Trichoderma harzianum, Bacillus subtilis*, garlic extract and clove extract. The experiments were carried out through two vegetative cycles: 2015/2016 and 2016/2017. The length of wood discoloration, pathogen re-isolation percentage, fresh mass of the pruning material, peroxidase activity, fluorescence, chlorophyll index, phenological stages, shoot length and leaf area were evaluated. The treatments with clove, garlic extract, tebuconazole, pyraclostrobin, mancozeb, fosetyl-Al and *B. subtilis* reduced mycelial growth by more than 90%. In the grapevines, the use of *T. harzianum* decreased the re-isolation of *L. theobromae*, but no differences were verified for the other plant evaluations. We concluded that the use of *T. harzianum* would be a potential option for wound protection without altering the physiological aspects of cv. Syrah grapevines.

Keywords: *Trichoderma harzianum*; pruning; protection; biological control.

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Introduction

Grapevine trunk diseases (GTDs) are among the most important limiting factors of viticulture in the world. Several trunk fungi may be responsible for these diseases, affecting both *Vitis vinifera* L. and *Vitis labrusca* cultivars. Among these fungi, the genus *Lasiodiplodia* is the causal agent of grapevine dieback, which is also referred to as black dead arm or Botryosphaeria canker. In symptomatic plants, *Lasiodiplodia theobromae* was reported to be the most prevalent (Úrbez-Torres, 2011). In Brazil, *Lasiodiplodia theobromae (*Pat.) Griff. & Maubl (Correia et al., 2013) and *Lasiodiplodia brasiliense* (Correia et al., 2015) were isolated from grapevine decline symptomatic plants.

These pathogens usually infect plants via pruning wounds and grafting points (Úrbez-Torres & Gubler, 2009); furthermore, they can be passed from the grapevine mother plants to the young through grafting or other processes of propagation at nurseries (Gramaje & Armengol, 2011). The infection is also favoured by conditions that reduce plant vigour, such as frost, high temperatures in the summer months, poor nutrition and poorly conducted pruning, and is associated with several disease symptoms, including foliar chlorosis, stunted growth, the dieback of shoots, spurs and side-branches, canker of the trunk or side-branches, wedge-shape cankers in the vascular tissue and mortality (Larignon & Dubos, 2001).

Strategies to reduce the incidence of vine trunk pathogens are limited to the management of the crop, using healthy plants, disposing of contaminated parts, pre-pruning or double pruning (Weber, Trouillas, & Gubler, 2007). Several works have been carried out *in vitro* and *in vivo* to obtain products that promote the control of these pathogens, such as chemical or biological products. However, the efficacy ranges broadly

according to the species studied (Rolshausen et al., 2010; Amponsah, Jones, Ridgway, & Jaspers, 2012; Díaz & Latorre, 2013).

The use of some biological control agents for the control of GTD fungi has also been tested, demonstrating the *in vitro* and *in vivo* efficacy of these agents. Alfonzo, Conigliaro, Torta, Burruano, and Moschetti (2009) evaluated *in vitro Bacillus subtilis,* getting control of *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, *Verticillium dahliae* and *Botryosphaeria rhodina.* Using biological control agents triggers the colonization of the plants' wounds, preventing the entry of trunk pathogens. Thus, these agents become a plant-protection option over time (Mutawila, Fourie, Halleen, & Mostert, 2011). Mutawila, Halleen, and Mostert (2016) reported that the application of *Trichoderma* species on grapevine pruning wound surfaces reduces wound infection by trunk pathogens. The protection of wounds with chemical or biological products can also reduce the presence of disease in the area (Halleen, Fourie, & Lombard, 2010; Díaz & Latorre, 2013; Sosnowski, Loschiavo, Wicks, & Scott, 2013).

The application of plant extracts can be an alternative method for disease control with lower environmental impacts. In vines, the use of garlic extract had an effect on the *in vitro* control of *Botryosphaeria dothidea, Diplodia seriata*, *Eutypa lata, Ilyonectria macrodidyma*, and *Phaeoacremonium aleophilum* and inhibited the development of *D. seriata* and *P. chlamydospora* in grapevine branches (Cobos et al., 2015).

As described, various management methods, including chemicals, biological control, natural molecules and material sanitation, have been tested against pathogens causing diseases of the stems of vines. Despite the reported experiments and the wide range of products registered for grapevines in Brazil, until now, there are no registered products for the control of *L. theobromae*.

In this context, this work aimed to verify the effect of chemical fungicides, biological agents and plant extracts on the control of *Lasiodiplodia theobromae* in pruning wounds and its physiological effects on grapevine plants cv. Syrah.

Material and methods

In vitro trials

Trials were carried out at the Phytopathology Laboratory of UNICENTRO (Guarapuava, Paraná State, Brazil) using an isolate of *Lasiodiplodia theobromae* (CMM 307) from the fungal collection of the Federal University of Pernambuco obtained by the isolation of symptomatic vines (*Vitis vinifera*) in Petrolina, Pernambuco State, Brazil.

For the tests with plant extracts, flower buds of clove (*Caryophyllus aromaticus* L.), bark cinnamon (*Cinnamomum zeylanicum*), fresh bulbs of garlic (*Allium sativum* L.), dried leaves of rosemary (*Rosmarinus officinalis*), and lemongrass (*Cymbopogon citratus*) were applied. The plant extracts were prepared using 30 g of plant material in 100 mL of distilled water, shredding the mixture in a blender, and subsequently filtering the mixture through a cheesecloth. A commercial garlic extract was also used (Natualho®, ai: 70%). The plant extracts were added to a PDA culture medium (potato dextrose agar, K25-610102, KASVI, Lot: 100713203) to obtain final concentrations of 0, 5, 10, 15, and 20% (v/v).

To test the volatile substances, the same plant extracts were used at a concentration of 20% of each extract, adapted from the methodology of Celoto, Papa, Sacramento, and Celoto (2008), using bipartite Petri dishes.

For the experiment with commercial products, the following treatments were added to the PDA culture medium: difeconazole (Score®, 1 mL L^{-1}, ai: 250 g L^{-1}), tebuconazole (Folicur® 200 EC, 8.75 mL L^{-1}, ai: 200 g L^{-1}), mancozeb (Dithane®, 3 g L^{-1}, ai: 800 g kg^{-1}), sulfur (Kumulus®, 3 g L^{-1}, ai: 800 g kg^{-1}), pyraclostrobin (Comet®, 5.33 mL L^{-1}, ai: 250 g L^{-1}), fosetyl-Al (Aliette®, 2.5 g L^{-1}, ai: 450 g L^{-1}), azoxystrobin (Amistar®, 0.24 g L^{-1}, ai: 500 g L^{-1}), chitosan (Fish Fértil Quitosana®, 16 mL L^{-1}, ai: 2%), *Bacillus subtilis* (Serenade®, 80 mL L^{-1}, ai: 13.68 g L^{-1}), *Trichoderma harzianum* (Ecotrich®, 4 g L^{-1}, ai: 300 g kg^{-1}) and *Trichoderma harzianum* (Predatox®, 4 mL L^{-1}, ai: 20 g L^{-1}).

The biological control agents *Bacillus subtilis* and *Trichoderma harzianum* were submitted to direct pairing tests with the pathogen and further evaluation of the volatile metabolites; this was performed using bipartite Petri dishes filled with PDA medium in the two compartments. First, the biological control agents were inserted in a partition of the plate. After 48h of recording, the mycelial discs of the phytopathogen were inserted into the other partition of the dish.

In all the trials, mycelial discs of 5 mm diameter of an isolated *Lasiodiplodia* were transferred to the centre of the Petri dishes containing culture medium with the treatments and incubated in a culture chamber at 25°C under 12h day/night photoperiods. The experimental design was completely randomized with five replications.

The evaluation of mycelial growth was performed daily by diameter measurements (cm) of the colony with a digital pachymeter 8" (ZAAS Precision, São Paulo, Brazil). From the evaluations of mycelial growth, the area under the mycelial growth curve (AUCMG) was estimated, adapted from Campbell and Madden (1990), and for the antagonism test, the index of antagonism following the methodology of Campanile, Ruscelli, and Luisi (2007) was used. The data were analysed using analysis of variance (ANOVA), a mean comparison by using a Scott-Knott test at 5% and a regression analysis for the doses. The percentage inhibition of each treatment was calculated in comparison with the control, and the means comparison was performed by a t-test at 5%. The statistical analyses were performed using the statistical software ASSISTAT Version 7.7 (Silva & Azevedo, 2016).

In vivo trials

The *in vivo* trials were conducted in a greenhouse in Guarapuava, Paraná State, Brazil (25°33' S and 51°29' W, altitude of 1,095 m). Nursery grapevines of cv. Syrah clone 174 (*Vitis vinifera*) grafted on rootstock 'Paulsen 1103' were transplanted into 3 L pots containing 2 L of soil, 0.9 L of organic compost and 0.1 L of dolomitic limestone.

The experiments were performed in two seasons: summer pruning and winter pruning. The grapevines were pruned at the height of the seventh bud in the vegetative period of the plant during the second week of December (summer 2015) and at the third bud spurs during the first week of August (winter 2016). The pruning scissors were disinfected with 70% (v/v) alcohol. Then, at the pruning wound sites, the treatments were applied with a micropipette, applying 2 mL of solution per wound (Díaz & Latorre, 2013).

The treatments used were an uninoculated control (water), inoculated control (water), fosetyl-Al (Aliette®, 2.5 g L^{-1}, ai: 450 g L^{-1}), tebuconazole (Folicur® 200 EC, 8.75 mL L^{-1}, ai: 200 g L^{-1}), *Bacillus subtilis* (Serenade®, 80 mL L^{-1}, ai: 13.68 g L^{-1}), *Trichoderma harzianum* (Ecotrich®, 4 g L^{-1}, ai: 300 g kg^{-1}), plant extracts of clove (*Caryophyllus aromaticus* L., dose 20%) and plant extracts of garlic (*Allium sativum* L., dose 20%).

For each pruning season, 24h after the application of the treatments, the fresh pruning wounds were inoculated with a mycelial disc (5 mm in diameter), taken from the PDA cultures of *L. theobromae*, which was placed upside down over the fresh pruning cut and wrapped with Parafilm M (Pechiney Plastic Packaging) to avoid rapid dehydration. For the control, without inoculation, the PDA discs without fungi colonization were used.

The experimental design was in randomized blocks with eight treatments, six replicates and an experimental plot consisting of one pot.

In August (winter 2016), 230 days after the first season treatments, and in January (summer 2017), 156 days after the second season treatment, spurs were removed from the vines and returned to laboratory for assessment. Then, the branches were cut longitudinally, and the lesions, i.e., the length of wood discoloration (LWD) extending from the point of inoculation, were measured with a digital pachymeter 8" (ZAAS Precision, São Paulo, Brazil). To confirm that the lesions were caused by trunk pathogens, 12 small pieces of discoloured wood from the margin of each lesion were surface-sterilized following the methodology of Almança et al. (2013), in which the pruned branches were disinfected with 70% alcohol for 30 seconds, with 3.5% sodium hypochlorite for 2 minutes and again immersed in 70% alcohol for 30 seconds and placed in PDA. The percent re-isolation was calculated.

The plants inoculated with *L. theobromae* were also examined for the presence of physiological changes. In both trial periods, the fresh mass (g) of the pruning material (precision balance model M2202, BEL Equipamentos Analíticos) and the chlorophyll index, with results expressed in ICF values (Falker chlorophyll index) according to Falker, (2008) (chlorophyll meter ClorofiLOG® model CFL 1030, Falker Automação Agrícola, Porto Alegre, Brazil) and chlorophyll *a* fluorescence (Fv/Fm - maximum PSII quantum yield - measured with a modulated fluorometer (PAM-2500 model, Walz, Effeltrich, Germany)) were evaluated.

After summer pruning, after 24h of inoculation, the activity of the guaiacol peroxidase enzyme was analysed in a UV-visible spectrophotometer (UV-1800 model, Shimadzu, Kioto, Japan) at 480 nm (Urbanek,

Kuzniak-Gebarowska, & Herka, 1991). A unit of GPX activity was defined as the change of 1.0 unit of absorbance (480 nm) per milligram of soluble protein per minute (UA mg P^{-1} $min.^{-1}$).

After winter pruning, were also evaluated, the length of the branches (measured monthly with a tape measure), the phenological stages according to Eichhorn and Lorenz (1984) and the leaf area, determined with the model leaf area LI 3100C (Licor, Nebraska, United States of America) installed on bench, and the results were expressed in cm^2.

The data were analysed with an analysis of variance and a mean comparison by a Scott-Knott test at 5% significance using the statistical programme ASSISTAT Version 7.7 (Silva & Azevedo, 2016).

Results and discussion

In vitro trials

Antifungal activity is considered to be high when aqueous extracts provide inhibition equal to or greater than 50% (Venturoso et al., 2011). Thus, the results obtained in the experiment demonstrated the antifungal efficiency of cinnamon, clove and garlic. These treatments had a quadratic effect according to dose, with inhibitions between 90 and 100% of the mycelial growth of the pathogens at all the doses used (Figure 1a). The antifungal activities of clove and garlic extracts was proven, demonstrating the potential of these two extracts for the *in vitro* control of *L. theobromae*. The effect of garlic extract on the *in vitro* control of disease-causing fungi such as *D. seriata* and *P. chlamydospora* in grapevines was also reported by Cobos et al. (2015).

The cinnamon and rosemary extracts had quadratic effects according to dose. The highest inhibition using a 20% dose of cinnamon was verified with a reduction of 98.5%, and for the rosemary extract, the maximum inhibition was verified at a 10% dose (-24%). The lemongrass extract had a linear effect according to dose and a maximum inhibition at 20% (-31%) (Figure 1b).

The action of the extracts could be directly related to its composition. In addition to the efficiency of the extracts when added to the culture medium, the diffusion of traces of volatile compounds may induce or inhibit germination or growth or trigger changes in the development of plants and fungi (French, 1992). Volatile oils, essential oils, and ethereal oils or essences are complex mixtures of volatile substances, lipophilic, usually odoriferous and liquid, and soluble in apolar organic solvents such as ether but have limited solubility in water (Simões & Spitzer, 2001), indicating that the extraction of the aqueous extract carries a lesser amount of active principles. The lower drag of the active principles may explain the low efficiency of the volatile compounds in this experiment.

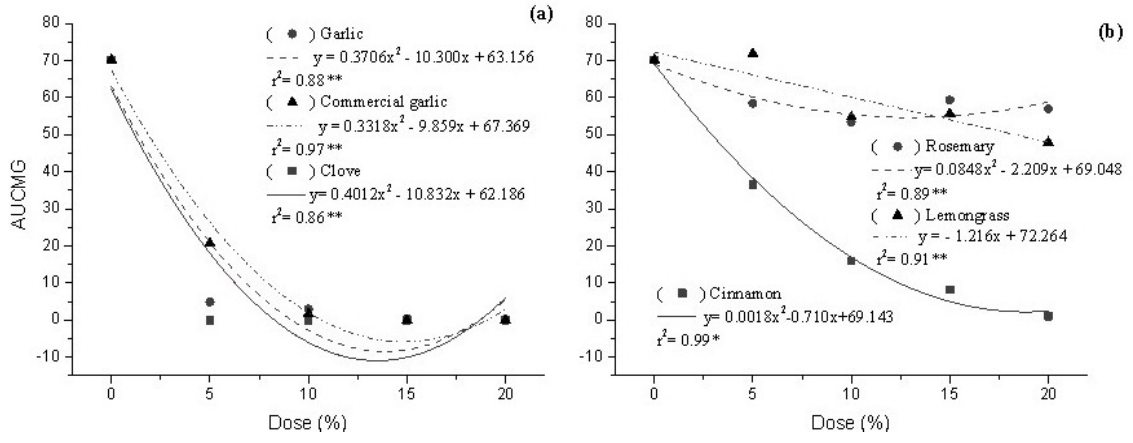

Figure 1. Area under the curve of the mycelial growth (AUCMG) of *Lasiodiplodia theobromae* subjected to different plant extracts. ** significant ($p \leq 0.01$). Guarapuava, Paraná State, Brazil, 2016.

The majority of the chemical treatments and biological control agents decreased the mycelial growth of *L. theobromae* (Figure 2a). The treatments tebuconazole, mancozeb, pyraclostrobin, fosetyl-Al, *B. Subtilis*, and *T. harzianum* (ai: 300 g kg-1) were the most effective, followed by difeconazole and *T. harzianum* (ai: 20 g L-1). The treatments with sulfur, azoxystrobin, and chitosan did not differ from the control, which showed the highest mycelial growth.

The volatiles of the plant extracts from cinnamon and garlic reduced the AUCMG, and the greatest reduction was obtained from the extracts of cinnamon (-35%) (Figure 2b). Although the effectiveness of control by the clove volatiles was not verified, they were efficient when diluted in culture medium, indicating that direct contact with the pathogen is necessary.

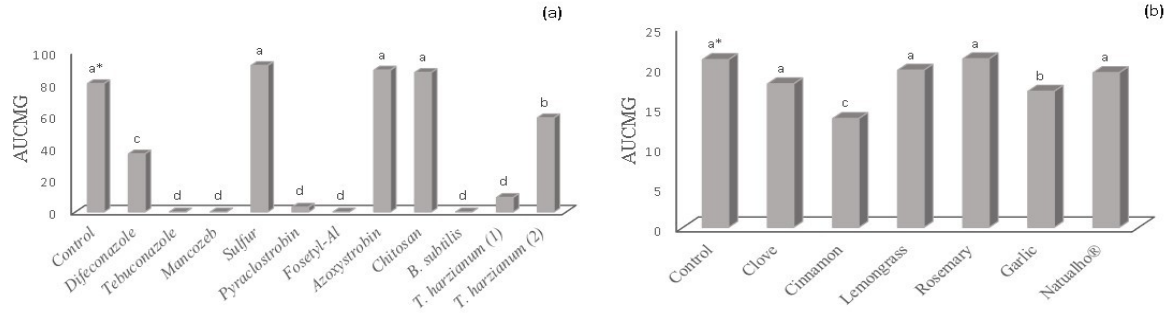

Figure 2. Area under the curve of the mycelial growth (AUCMG) of *Lasiodiplodia theobromae* submitted to treatments with chemical products and biological control agents (a) and to different volatile plant extracts (b). *Means followed by the same letter do not differ significantly by the Scott-Knott test ($p \leq 0.05$). (1) Commercial product with 300 g kg^{-1} of active ingredient, (2) Commercial product with 20 g L^{-1} of active ingredient. Guarapuava, Paraná State, Brazil, 2016.

These results corroborate those obtained by Amponsah et al. (2012), who reported the efficacy of carbendazim, flusilazole, methyl thiophanate, tebuconazole, iprodione, fenarimol, procymidone, mancozeb, and chlorothalonil in inhibiting mycelial growth of *Neofusicoccum australe, N. Luteum*, and *Diplodia mutila*, *the* causal agents of GTD. The efficacy of tebuconazole on the *in vitro* control of pathogens has also been reported for the control of Botryosphaeriaceae by Sosnowski et al. (2013).

Another chemical active ingredient with potential control is fosetyl-Al. In addition to the efficiency demonstrated *in vitro*, fosetyl-Al has the capacity to translocate into the plant via both the xylem and phloem (Kimati, 2011), which allows the use of this product in the protection of wounds. The *in vitro* efficiency of fosetyl-Al was verified in the control of *Phomopsis* spp. by Mostert and Crous (2000).

No significant differences were verified in the reduction of area under the curve of mycelial growth (AUCMG) of *L. theobromae* subjected to treatments with volatile metabolites of biological control agents (data not shown). Regarding the antagonist test, the biological control agents inhibited the growth of *L. theobromae,* obtaining an index of antagonism of 66.4 and 62.2% for *B. subtilis* and *T. harzianum*, respectively, with no significant difference between them.

The use of *B. subtilis* has also been reported to be effective in controlling *L. theobromae* on rubber trees (Sajitha, Florence, & Dev, 2014). Santos et al. (2016) observed that the antagonistic indices of *B. subtilis* were higher than those of *T. harzianum* in the control of *Dactylonectria macrodidyma*, a causal agent of black foot disease. The same authors reported the efficiency of the volatiles of biological antagonists for the tested pathogen. In this experiment, the volatiles of the biological control agents were not efficient in controlling *L. theobromae*, confirming that the effectiveness of the treatments can vary among fungal species.

Considering the results of the tests *in vitro*, the raw aqueous extracts of garlic and clove, the chemicals tebuconazole, mancozeb, fosetyl-Al, pyraclostrobin, and difeconazole, and the biological control agents *T. harzianum* and *B. subtilis* have potential for use in the control of *Lasiodiplodia theobromae* in grapevines.

In vivo trials

In the present experiment, the means of re-isolation of the inoculated plants without wound protection treatments were similar in both seasons, indicating that the cultivar is susceptible to the entry of pathogens in pruning wounds at different periods of the year, making it necessary to protect these wounds in any pruning condition.

Nevertheless, in general, infection with fungi that cause trunk diseases is favoured by conditions that reduce plant vigour, such as frost, high temperatures in the summer months, malnutrition and poorly conducted pruning (Larignon & Dubos, 2001). Another important factor is the spread and increase of infection rates when pruning is performed. Úrbez-Torres and Gubler (2011) observed that

the susceptibility in California vineyards of cvs. Chardonnay and Cabernet Sauvignon to infection by *Lasiodiplodia theobromae* and *Neofusicoccum parvum* was significantly higher when inoculation was performed in early winter rather than at the end of winter.

According to our findings, the plants treated with garlic extract after summer pruning differed from all the treatments, showing an increase in the area of internal vascular discoloration (Figure 3a). Nevertheless, for vines inoculated in winter pruning, the inoculated control and the treatments with tebuconazole and *T. harzianum* differed from the other treatments, obtaining the largest length of wood discoloration (Figure 3b).

For the inoculation performed in the summer pruning, there was a reduction in the percent re-isolation for the treatment with *T. harzianum* (Figure 3c). Similarly, in the winter pruning, the use of *T. harzianum* also decreased the percent of re-isolation of the pathogen, not differing from the control without inoculation and the treatment with the clove extract (Figure 3d).

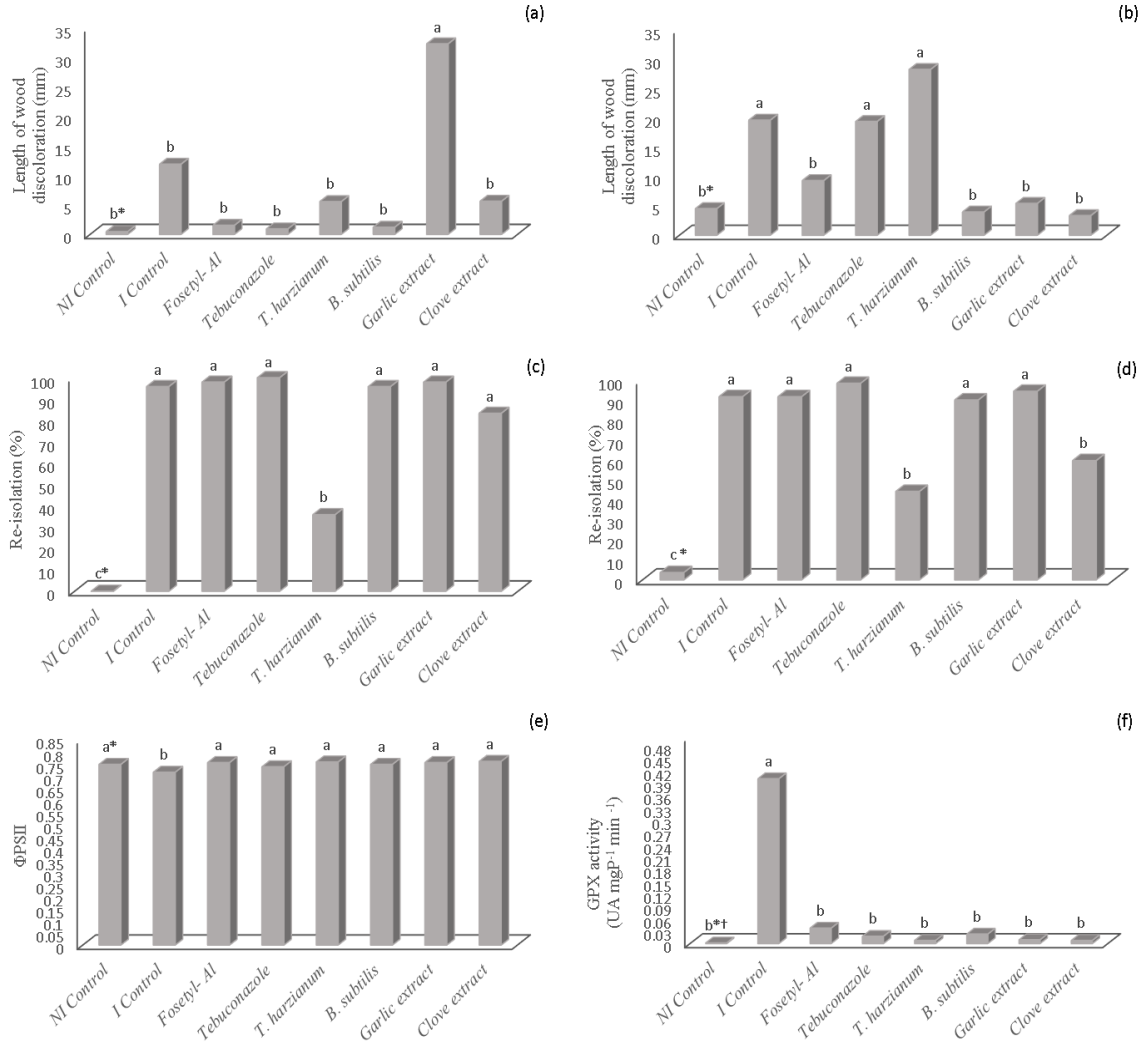

Figure 3. Length of wood discoloration (mm) 230 days after summer pruning inoculation (a), 156 days after winter pruning inoculation (b), percent re-isolation after summer pruning (c), percent re-isolation after winter pruning (d), PSII quantum yield (Fv/ Fm) after summer pruning inoculation (e), and peroxidase activity (UA mg P^{-1} min^{-1}) 24 h after summer pruning inoculation (f) of vines inoculated with *Lasiodiplodia theobromae* in cv. Syrah. †Data transformed by $\sqrt{x}$. *Means followed by the same letter do not differ significantly by the Scott-Knott test ($p \leq 0.05$). ns: not significant. NI: not inoculated. I: inoculated. Guarapuava, Paraná State, Brazil, 2017.

Some researchers have reported the effectiveness of chemical products in pruning wound protection to pathogenic fungi of the vine trunk and decreasing vascular symptoms (Díaz & Latorre, 2013; Sosnowski et al., 2013). Wound protection can also be performed with plant extracts and biological control agents. The use of wound protection decreases the severity of the disease. The use of fosetyl-Al, *B. subtilis*, garlic extracts and clove extracts reduced the severity of the pathogen after winter pruning,

but the garlic extract did not have the same effect in the different periods of inoculation, different from the reports of Cobos et al. (2015) that verified positive effects of wound treatment with garlic extract.

According to Mutawila et al. (2011), the effectiveness of protection based on *Trichoderma* spp. depends on the ability of vine pruning wound colonization by these fungi, and it depends on the interaction with the vine and is more or less effective according to cultivar. After the infection by pathogens in the wounds, the expression of external symptoms can occur; however, the appearance of symptoms is not often observed in the first year of infection, or the symptoms can be observed in one year and are more tenuous or even nonexistent in the following year (Mundy & Manning, 2011). Generally, grapevine decline symptoms are expressed in adult vineyards that are 7 years or older (Larignon & Dubos, 2001). In addition to the difficulty of symptom appearance in the early years, the external symptoms of the presence of the pathogen *Lasiodiplodia* tend to be easily confused with the presence of other pathogens or may result from causes that are not yet known (Úrbez-Torres, 2011).

There was no significant difference in the variables fresh mass of the pruning material, phenological stages and leaf area (data not shown). The presence of *L. theobromae* led to a significant reduction in chlorophyll *a* fluorescence in plants of the inoculated control relative to the other treatments (Figure 3e) and showed higher guaiacol peroxidase activity than all the other treatments (Figure 3f).

The inoculated plants, those without the use of a wound protection treatment, had shorter canes at the evaluations at 77, 119 and 156 days after winter pruning (DAWP) relative to the other treatments (Figure 4).

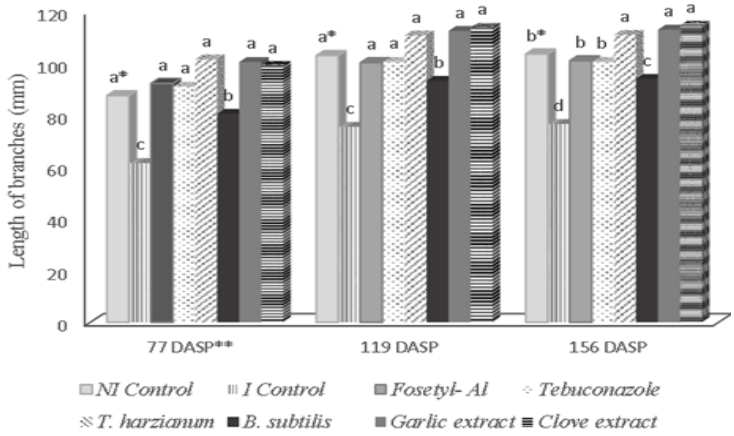

Figure 4. Length of the canes (cm) after inoculation in winter pruning of vines inoculated with *Lasiodiplodia theobromae* in cv. Syrah. *Means followed by the same letter do not differ significantly by the Scott-Knott test ($p \leq 0.05$). ns: not significant. **DAWP: days after the winter pruning. NI: not inoculated. I: inoculated. Guarapuava, Paraná State, Brazil, 2017.

Physiological stress in association with GTD fungi may contribute to the expression of symptoms in vines (Mundy & Manning, 2011). Among these parameters, peroxidase activity is indicative of intercellular stress due to the activation of the plant's antioxidant system to relieve oxidative stress. The increase in guaiacol peroxidase (GPX) activity in the inoculated control indicates that the entry of this fungus into the plants promotes stress. Another parameter, which confirms the stress caused by this pathogen in vines, was the lower chlorophyll *a* fluorescence, which was represented by the Fv/Fm ratio. It is also possible that the peroxidase and fluorescence evaluations of photosynthesis indicated a level of stress in the plant before they resulted in external symptoms. Letousey et al. (2010) observed that Chardonnay grapevine plants decreased their photosynthesis levels one week before the onset of apoplexy symptoms caused by Esca's disease, suggesting that the vine perceives some signs of the presence of the pathogen (probably toxins) and reacts by decreasing photosynthesis and triggering defence mechanisms. This level of stress caused a decrease in vine growth, reducing the cane length in some evaluations.

Conclusion

Plants infected with fungi that cause diseases of the vine wood alter their metabolism, regardless of the presence of symptoms, such as changes in chlorophyll fluorescence indices, peroxidase activity, and cane growth.

Regarding the management of *L. theobromae* in the grapevine cv. Syrah, the use of *T. harzianum* proved to be an effective option in the protection of wounds, reducing the mycelium growth *in vitro*, re-isolation of inoculated vines not altering the physiological conditions of the plant.

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