A protocol for selection of *Trichoderma* spp. to protect grapevine pruning wounds against *Lasiodiplodia theobromae*

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Abstract

There is no standardized protocol to select *Trichoderma* against trunk disease pathogens in grapevine. This study describes a protocol to select *Trichoderma* isolates for protecting the pruning wounds of grapevine against *Lasiodiplodia theobromae*. Thus, four assays were performed: (1) *in vitro* antagonism of *Trichoderma* isolates, molecularly identified, to control *L. theobromae*; (2) *in vitro* effect of grapevine fungicides on the conidia of the antagonists; (3) capability of *Trichoderma* to survive and endophytically penetrate in grapevine canes; and (4) efficiency of *Trichoderma* and fungicides to protect the pruning wounds of grapevine shoots against *L. theobromae*. *T. asperelloides* (02/03), *T. koningiopsis* (09/02), and *T. asperellum* (01/13) were the best antagonists for *L. theobromae*. The conidia of the three isolates were affected only by tebuconazole, with the first two fungi being the most tolerant to the fungicides. The *Trichoderma* isolates survived on the cane tip for 15 days, but only *T. asperelloides* (02/03) penetrated endophytically 4 cm in the cane and showed preventive and curative capability to control *L. theobromae*, with similar efficiency as that of the fungicides in both the periods tested post inoculation. These four assays are suggested to select *Trichoderma* against *L. theobromae* or other trunk disease pathogens.

Keywords Biocontrol · Fungicides · Niagara Rosada · Decline

Introduction

The Brazilian viticulture is a crucial activity for the small farmers. It includes the production of juice, wine, and grapes for consumption *in natura* or as raisins, as well as for rural tourism [5,27]. However in Brazil, “Niagara Rosada” is one of the most cultivated grapes for consumption *in natura* [19,28]. It is the result of a somatic mutation that occurred in the “Niagara Branca” grapevine (*Vitis labrusca* L. × *Vitis vinifera* L.) in 1933, in Louveira city, São Paulo State, Brazil, predominating the original form [28].

In Brazil, grapevine plantations still face several problems, particularly regarding the incidence of diseases. Among the diseases, botryosphaeria dieback, caused mainly by fungus *Lasiodiplodia theobromae*, deserves special attention.

This fungus causes decline symptoms (dieback), fruit rot, retarded growth of plants, brown necrosis and black spots in the vascular tissue of the trunk, foliar chlorosis and necrosis and progressive defoliation, spur dieback, shoot dieback, cankers, and plant death. The symptoms are generally observed in plants under hydric and/or nutritional stress [1,26,31]. This disease has spread because of some neglected control measures such as ignoring the chemical protection of wounded sites during grafting and pruning.

L. theobromae colonizes the inner tissues of the plant, causing immeasurable losses to the production system and the post-harvest properties. It is highly difficult to control the pathogen because of its ecological features and a wide variety of hosts. The most suitable control method is to prevent the plant from being exposed to the pathogen or prevent its reproduction in the crop remains. The efficiency of the chemical control method is questionable. The recommended control procedures include

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using healthy seedlings, eliminating plants with severe symptoms of wood necrosis, dieback, and crop remains, protecting pruning wounds with fungicides, disinfecting pruning tools, discarding infected crop remnants, and irrigation management to prevent prolonged water stress and keep the plants well nourished [2,13,14].

The biological control of botryosphaeria dieback and other diseases in grapevine in Brazil have been inadequately studied. The fungi of the genus *Trichoderma* are the most known and used biocontrol agents worldwide to control several plant pathogens, either soil-borne or aerial. These fungi are used for the treatment of pruning wounds in plants, showing effective protection results mainly in grapevine and are compatible with fungicides, insecticides, botanicals, and biopesticides [13,22,30].

Despite the product used, whether chemical or biological, protection against *L. theobromae* or other trunk disease pathogens of grapevines should last for at least 20 days after pruning, because the plant susceptibility to the pathogen is greater in the first 2 weeks after pruning, whereas the total wound healing occurs 4 weeks later according to Sônego et al. [26] and Van Niekerk et al. [32].

Kotze et al. [13] assessed *Trichoderma*-based bioproducts and some antagonist isolates under laboratory and field conditions to protect grapevine against trunk pathogens on pruning wounds. For the tests performed on the field, spore suspensions of the fungi *Neofusicoccum australe*, *N. parvum*, *Diplodia seriata*, *Eutypa lata*, *Diaporthe ampelina*, *Phaeomoniella chlamydospora*, and *L. theobromae* were applied 7 days after the application on pruning wounds. Furthermore, after 8 months of inoculation, most biocontrol agents showed efficacy similar or superior to the fungicide benomyl. *T. atroviride* USPP-T1 was highly effective in reducing the incidence of *D. ampelina*, *E. lata*, *P. chlamydospora*, *N. australe*, *N. parvum*, *D. seriata*, and *L. theobromae* by 69%, 76%, 77%, 78%, 80%, 85%, and 92%, respectively. This is the first study to show the biological protection of grapevine pruning wounds against a wide range of trunk disease pathogens [13].

Despite a broad study conducted by Kotze et al. [13], there is a need for a standardized protocol to select *Trichoderma* for the protection of grapevine pruning wounds against trunk fungi. Therefore, this study aimed to propose a protocol with several assays to select *Trichoderma* isolates capable of protecting the pruning wounds of “Niagara Rosada” grapevine against the fungus *L. theobromae* or any other trunk pathogen. Thus, four assays were performed: (1) *in vitro* antagonism of *Trichoderma* isolates, molecularly identified, to control *L. theobromae* using dual culture test; (2) *in vitro* effect of grapevine fungicides on the conidia of the best antagonist isolates; (3) the capability of *Trichoderma* to survive and endophytically penetrate in “Niagara Rosada” grapevine canes; and (4) the efficiency of *Trichoderma* and fungicides to protect the pruning wounds of “Niagara Rosada” grapevine shoots against *L. theobromae* [13].

Material and methods

Identification of *Trichoderma* isolates

The nine isolates of *Trichoderma* spp. (Table 1) were grown on a potato dextrose agar (PDA) medium for 14 days at 25 °C, and DNA was extracted according to the CTAB method, described by Doyle and Doyle [6]. Polymerase chain reaction (PCR) was performed to amplify the translation elongation factor 1-alpha gene using a pair of primers, tef71F (5'-CAAAATGGGTAAGGAGGASAAGAC-3') and tef997R (5'-CAGTACCGGCRGCRATRATSAG-3'), generating products of approximately 930 bp [24].

PCRs were performed in a PTC100 thermal cycler (MJ Research) as per the following program: initial denaturation at 94 °C for 2 min, 40 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, and the final extension at 72 °C for 4 min. PCR products were purified by precipitating with polyethylene glycol according to the protocol described by Schmitz and Riesner [23].

Sequencing was done by the chain termination method using BigDye 3.1 reagent (Life Technologies, Carlsbad, CA) and a 3500× L automated sequencer (Life Technologies, Carlsbad, CA). After sequencing, a phylogenetic tree was generated with the translation elongation factor 1-alpha gene sequences of isolates and different *Trichoderma* species obtained from the GENBANK/NCBI. The sequences were aligned using ClustalW, and a maximum likelihood tree with 1.000 bootstrap replicates was constructed using MEGA 6.0 software. Suitable nucleotide substitution model, based on the Bayesian information criterion, was found to be Kimura 2-parameter that allowed some sites (46.36%) to be evolutionarily invariable.

In vitro antagonism

L. theobromae Griffon & Maubl. (Pat.) was identified according to the cultural and morphological characteristics described by Punithalingon [18] and Rodrigues [21].

The inocula of *Trichoderma* spp. isolates and *L. theobromae* (Table 1) were obtained by growing the fungi on the PDA medium incubated for 7 days in a growth chamber (BOD type) at 25 °C in the dark.

The experimental design was completely randomized, including nine isolates of *Trichoderma* spp., one isolate of *L. theobromae* (Table 1), and ten replicates. A replicate consisted of one Petri dish containing a plug of the pathogen and antagonists (5-mm diameter) on the PDA medium plated on opposite sides (dual culture test). The dishes were kept in the growth chamber (BOD type) at 25 °C in the dark for 14 days. The experiment was performed two times.

After 7 and 14 days of inoculation, the antagonism level of *Trichoderma* was assessed on the basis of the criteria proposed

Table 1 Information about the nine isolates of *Trichoderma* spp. obtained from the Collection “Mário Barreto Figueiredo” of the Biological Institute / São Paulo (SP), Brazil, and about the isolate of *Lasiodiplodia theobromae* obtained from the Collection of the Phytosanitary Research and Development Center of the Agronomical Institute, Campinas / SP (IAC), Brazil

Code of <i>Trichoderma</i> isolates	Cities SP - Brazil	Altitude (m)	Notes
01/13	Aguaí	–	Cultivated soil - tomato
02/03	Aguaí	–	Cultivated soil - pepper
08/07	São Paulo	749	“University São Paulo, Reserva CUASO”
09/02	São Paulo	746	Botanical Institute
26/10	Caraguatatuba	19	Highway SP 055
34/01	Itapetininga	632	Highway SP 270 - Km 154
46/08	Votuporanga	483	Highway SP 320
47/02	São José do Rio Preto	560	Highway SP 320 - Km 454
Quality®	–	–	“Grupo Farroupilha”
<i>L. theobromae</i> isolate	Cities SP - Brazil	Altitude (m)	Notes
N. 8387	Salto	–	“Niagara Rosada” grapevine

by Bell et al. [2], which are as follows: (1) *Trichoderma* covers the entire Petri dish; (2) *Trichoderma* covers two thirds of the dish; (3) *Trichoderma* and *L. theobromae* each cover one half of the dish; (4) *L. theobromae* covers two thirds of the dish; and (5) *L. theobromae* covers the entire Petri dish.

Effect of grapevine fungicides on conidia of *Trichoderma*

The conidia inocula of the isolates 02/03, 09/02, and 01/13 of *Trichoderma* spp., selected as the appropriate antagonists in the *in vitro* assay, were obtained by growing the fungi on the PDA medium incubated in the growth chamber (BOD type) at 25 °C in the dark for 7 days.

The fungicides used were registered in Brazil for grapevine: Forum Plus-Dimethomorph+Chlorothalonil (active ingredient); Bravonil 500-Chlorothalonil; Aliette-Fosetyl-Al; Dithane NT-Mancozeb; Pomme-Thiophanate methyl; Amistar WG-Azoxystrobin; Folicur 200 EC-Tebuconazole; Kumulus-Sulfur and Recop-Copper oxychloride. The fungicides were added to the medium according to the methodology described by Edgington et al. [7] and modified by Menten et al. [17]. The fungicides were tested at four concentrations: 1, 10, 100, and 1000 ppm. After autoclaving and still in the semi-solid state (approximately 40–45 °C), the amount of active ingredients of each fungicide was added on the PDA medium to prepare the desired concentrations [20,22,30].

To verify the effect of fungicides on the *Trichoderma* conidia, the experimental design was completely randomized as a factorial arrangement, containing nine fungicides plus control (no fungicide), four fungicide concentrations (1, 10, 100, and 1000 ppm), three *Trichoderma* isolates, and four replicates. Each replicate consisted of one Petri dish containing PDA + oxytetracycline (0.05 mg/mL) plus fungicides with 0.1 mL aliquot of a conidial suspension (1×10^7 spores/mL). The aliquot of each isolate was spread over the medium surface using a Drigalski handle.

The dishes were sealed with the synthetic plastic polymer polyvinyl chloride PVC film and kept in a growth chamber (BOD type) at 25 °C in the dark.

The dishes were evaluated after 14 days of inoculation to verify colony inhibition using the following scale ranging from 1 to 4: 1 = no colony growth (100% inhibition); 2 = sparse colony growth on the medium with white coloration; 3 = colony growth in parts of the medium with green coloration (sporulation); and 4 = colony growth over the medium with green coloration (sporulation), highly characteristic of *Trichoderma* (0% inhibition). This assay was performed two times.

Survival and endophytic penetration of *Trichoderma* in “Niagara Rosada” grapevine canes

The *Trichoderma* isolates used in this assay were the same as those used in the fungicide assay.

The inocula of *Trichoderma* isolates were produced on cooked rice [4]. For each isolate, an autoclavable polyethylene bag containing 300 g of rice previously immersed in distilled water for 50 min was used. The bags were sealed and autoclaved for 30 min at 121 °C. After bags were cooled, 10 mL of a conidial suspension from each *Trichoderma* isolate (1×10^6 conidia/mL) was added to each bag. The suspension was spread over the rice grains, and the bags were incubated in a growth chamber (BOD type) at 25 °C in the dark for 14 days. After each *Trichoderma* isolate was grown on the rice, the content was blended in a blender using 150 mL of autoclaved distilled water to prepare a pasty mixture.

The paste (0.1 g) obtained for each *Trichoderma* isolate (1×10^6 conidia/g rice) was applied using a sterile spatula on the fresh wounds at the tip of the cane of a healthy “Niagara Rosada” grafted onto IAC 766 rootstock growing in a plastic sack of soil. After inoculation, the plants were kept in a greenhouse (26 ± 2 °C), under shaded conditions, and irrigated after every 2 days.

The experimental design was completely randomized as a factorial arrangement, with three *Trichoderma* isolates, three different periods of evaluation post inoculation (5, 10, and 15 days), and four replicates (plants). The control plants were not inoculated with *Trichoderma*. The experiment was performed two times.

To assess the *Trichoderma* species at the tip (0 cm) and in the inner tissues of the cane at a distance of 2 and 4 cm from the tip, cane fragments were removed from the cited sites of each plant. In a laminar flow cabinet, the fragments were superficially disinfected for 5 s in 70% ethanol solution and 1 min in 1.5% sodium hypochlorite solution. Then, the fragments were successively washed in sterile distilled water, dried on sterile filter paper, and finally sectioned in smaller fragments. Furthermore, three replicates were generated for each plant and each sampled site (tip and inner tissues at a distance of 2 and 4 cm from the tip). Each replicate consisted of a Petri dish containing PDA + oxytetracycline and four small fragments. The dishes were kept in the growth chamber (BOD type) at 25 °C in the dark for 14 days. The evaluation was based on the percentage of fragments with the growth of isolates per Petri dish (25 to 100%).

Biological and chemical protection of “Niagara Rosada” grapevine shoots against *L. theobromae*

Grapevine shoots were used in this study because canes removed from plants in the field have typically been infected with the fungi of *Botryosphaeriaceae* genus. Therefore, healthy Niagara Rosada grapevine shoots (sanity attested) were obtained and segmented into 7-cm-long stems. These stems were disinfected by a 1.5% NaOCl solution for 1 min, washed in sterile distilled water and dried using a sterile filter paper, and transferred to Petri dishes containing a glass slide over the filter paper (all previously sterilized).

One end of each stem was covered with sterile cotton moistened with sterile distilled water to keep the segment moisturized. The other edge was treated with the treatments.

The most suitable *Trichoderma* isolate selected in the previous assays was *T. asperelloides* 02/03. The methods used to obtain the inoculum and inoculate were similar to those used in the previous assays (survival and endophytic penetration of *Trichoderma* in “Niagara Rosada” grapevine canes).

The inoculum of the fungus *L. theobromae* was produced on PDA + oxytetracycline culture medium incubated in the growth chamber (BOD type) at 25 °C in the dark for 30 days.

The fungicide applied for the preventive control of *L. theobromae* was fluazinam (Frownicide 500 SC) and that applied for curative control was tebuconazole (Folicur 200 CE). [21] Furthermore, 100 mL of fungicides (commercial product) was applied in 100 L of latex paint instead of water. The edge of the long stems was immersed in the paint for 1 min. The latex paint helped to fix the fungicide on the

edges for preventing the removal of the fungicides by exudates from the grapevine. According to Van Niekerk et al. [32], water and paint have a similar effect in protecting grapevine pruning wounds against trunk pathogens. Therefore, water was used in the control treatment.

The fungus *L. theobromae* was inoculated on the wound with a plug of PDA medium (0.5-cm diameter) containing mycelium + pycnidia, followed by covering it with a sterile gauze embedded in sterile distilled water and sealed with para film.

For preventive control, *Trichoderma*, fungicide, and water were first applied. Then, the fungus *L. theobromae* was inoculated after 3 days. The assay was evaluated after 10 and 20 days of the fungus inoculation.

For curative control, *L. theobromae* was first inoculated. Then, 3 days later, *Trichoderma*, fungicide, and water were applied. The assay was evaluated similarly to the preventive control.

The experimental design was completely randomized as a factorial arrangement with three treatments (fungicide, *T. asperelloides* 02/03, and water), two methods of application (prevention and curative), two evaluation periods, and three replicates. Each replicate consisted of a glass Petri dish containing two moisturized filter papers, two glass slides, and one long stem of “Niagara Rosada” grapevine shoot. The experiment was performed three times.

In order to evaluate the treatments and control, fragments of 1 cm were removed from 0.5 cm below the inoculation site of each replicate. The fragments were superficially disinfected similar to that in the previous assay (survival and endophytic penetration of *Trichoderma* in “Niagara Rosada” grapevine cane), sectioned in small fragments, and distributed four fragments in Petri dish containing PDA + oxytetracycline. Furthermore, eight Petri dishes were prepared for each replicate, forming 24 replicates or 96 fragments per treatment. The percentage of *L. theobromae* per fragment (25 to 100%) was evaluated per replicate.

Data analysis

All the data were analyzed according to the technique of the nonparametric analysis of variance for the model with two or three factors complemented with Dunn’s multiple comparison test [33], considering 5% significance level, except for assay data on the effect of grapevine fungicides on *Trichoderma* conidia. For this assay, the data were subjected to Probit Analysis, followed by the determination of LC50 values [9]. The LC50 of each *Trichoderma* isolate was classified into four efficiency categories according to an adapted scale of Edington et al. [7]: (1) LC50 < 1 ppm = *Trichoderma* highly sensitive to the fungicides (S), (2) LC50 between 1 to 10 ppm = *Trichoderma* moderately sensitive to the fungicides (MS), (3) LC50 between 10 to 50 ppm = *Trichoderma*

moderately resistant to the fungicides (MR), and (4) $LC_{50} > 50$ ppm = *Trichoderma* resistant to the fungicides (R).

Results

Identification of *Trichoderma* isolates

According to the phylogenetic tree (Fig. 1), the isolates of *Trichoderma* 46/08, 26/10, 47/02, and 08/07 were molecularly identified as *T. harzianum*, whereas the isolate 09/02 was identified as *T. koningiopsis*. The isolate 02/03 was identified as *T. asperelloides*, and the isolate of the Quality® was determined as of *T. asperellum*. Silva et al. [25] identified the isolates 01/13 and 34/01 (Table 1) as *T. asperellum* and *T. harzianum*, respectively.

In vitro antagonism

After 7 days of inoculation, *T. asperelloides* 02/03, *T. harzianum* 47/02, and *T. asperellum* 01/13 were highly antagonists to *L. theobromae*, differing from other isolates ($p < 0.05$; Table 2). Fourteen days, the isolates of *T. asperelloides* 02/03, *T. asperellum* 01/13, and *T. koningiopsis* 09/02 showed the best antagonisms against *L. theobromae* ($p < 0.05$; Table 2). Therefore, it is the most appropriate time to assess the antagonism of *Trichoderma* against *L. theobromae* (Table 2).

Effect of grapevine fungicides on conidia of *Trichoderma*

Tebuconazole was the only active ingredient that affected the conidia of the three *Trichoderma* isolates; *T. asperelloides* 02/03 and *T. koningiopsis* 09/02 were moderately resistant, whereas *T. asperellum* IB 01/13 was moderately susceptible

Table 2 *In vitro* antagonism notes for *Trichoderma* isolates to *Lasiodiplodia theobromae*, grapevine pathogen

Isolates	Days after inoculation	
	7	14
<i>T. harzianum</i> 46/08	2.0 ± 0.5 bc ¹ B ²	2.0 ± 0.5 b A
<i>T. harzianum</i> 26/10	2.0 ± 0.5 bc B	1.0 ± 0.5 ab A
<i>T. koningiopsis</i> 09/02	2.0 ± 0.5 bc B	1.0 ± 0.0 a A
<i>T. harzianum</i> 34/01	2.0 ± 0.5 bc A	2.0 ± 0.5 ab A
<i>T. harzianum</i> 08/07	2.0 ± 0.5 bc A	1.5 ± 0.5 ab A
<i>T. asperelloides</i> 02/03	1.0 ± 0.0 ab A	1.0 ± 0.0 a A
<i>T. harzianum</i> 47/02	1.0 ± 0.5 ab A	1.0 ± 0.5 ab A
<i>T. asperellum</i> 01/13	1.0 ± 0.5 ab A	1.0 ± 0.0 a A
<i>T. asperellum</i> – Quality®	2.0 ± 0.5 c B	2.0 ± 0.5 b A

Scale of notes: (1) *Trichoderma* covers the entire Petri dish; (2) *Trichoderma* covers 2/3 of the dish; (3) *Trichoderma* and *L. theobromae* each cover one-half of the dish; (4) *L. theobromae* covers 2/3 of the dish and (5) *L. theobromae* covers the entire Petri dish

*Median and semi-amplitude interquartile of two assays. Each assay with 10 replicates per isolate

^{1,2} Lowercase letters: comparison of isolates, fixed evaluation day and uppercase letters: comparison of the evaluated day for each isolate, according to Dunn's multiple comparison test [33] with 5% significance level

to the fungicides (Table 3). Therefore, the isolates that showed the maximum resistance to the grapevine fungicides were of *T. asperelloides* 02/03 and *T. koningiopsis* 09/02.

Survival and endophytic penetration of *Trichoderma* in “Niagara Rosada” grapevine cane

For the three periods evaluated, all *Trichoderma* isolates had a higher survival rate at the tip than in the inner tissues of the cane (2 and 4 cm from the tip). At the cane tip, no differences

Fig. 1 Phylogenetic tree based on the maximum likelihood method, showing the relationship between the nine isolates of *Trichoderma* sp. obtained from the Collection “Mário Barreto Figueiredo”, of the Biological Institute, São Paulo - Brazil, and the species of *Trichoderma* deposited in the GenBank - NCBI. The sequences used are of the alpha-elongase gene. The access numbers of GenBank sequences are within parentheses beside the name of isolates. The “bootstrap” values for 1000 replicates are indicated at the tree branch

Table 3 Lethal concentration (LC 50) of fungicides for conidia of *Trichoderma asperelloides* IB 02/03, *T. asperellum* IB 01/13, and *T. koningiopsis* IB 09/02, onto Potato Dextrose Agar culture medium

Fungicides	<i>Trichoderma</i> isolates		
	02/03	01/13	09/02
Dimethomorph + Chlorothalonil	244 (R) ¹	233 (R)	282 (R)
Chlorothalonil	215 (R)	244 (R)	215 (R)
Fosetyl-Al	95 (R)	67 (R)	126 (R)
Mancozeb	93 (R)	100 (R)	100 (R)
Thiophanate methyl	90 (R)	144 (R)	90 (R)
Azoxystrobin	1.0 × 10 ³ (R)	* (R)	485 (R)
Tebuconazole	43 (MR)	7 (MS)	42 (MR)
Sulfur	* (R)	7.0 × 10 ³ (R)	94 (R)
Copper oxychloride	94 (R)	144 (R)	94 (R)

*The antagonist was inhibited at all fungicide concentrations; ¹ Adapted scale of Edington et al. [7]: (1) LC 50 < 1 ppm = *Trichoderma* highly sensitive to the fungicides (S); (2) LC 50 between 1 to 10 ppm = *Trichoderma* moderately sensitive to the fungicides (MS); (3) LC 50 between 10 to 50 ppm = *Trichoderma* moderately resistant to the fungicides (MR); and (4) LC 50 > 50 ppm = *Trichoderma* resistant to fungicides (R)

were found in the survival rate among isolates at different periods ($p < 0.05$; Table 4).

For the control, *Trichoderma* was not detected at the tip and in the inner tissues of the cane in the three evaluation periods ($p < 0.05$; Table 4).

T. asperellum 01/13 penetrated up to 4 cm at 5 days post inoculation. However, after 5 days post inoculation, the percentage of “Niagara Rosada” grapevine fragments with this antagonist in the inner tissues (2 and 4 cm from the tip) were low, not differing significantly from the control ($p < 0.05$; Table 4).

Regarding the endophytic penetration, *T. koningiopsis* 09/02 and the control showed equal penetration ($p < 0.05$; Table 4).

T. asperelloides 02/03 showed endophytic penetration up to 4 cm in the cane, differing considerably from the other isolates. Moreover, after 5 days post inoculation, the percentage of “Niagara Rosada” grapevine fragments with this antagonist in the 4 cm inner tissue increased, differing significantly from the control ($p < 0.05$; Table 4).

Biological and chemical protection of “Niagara Rosada” grapevine shoots against *L. theobromae*

T. asperelloides 02/03 was similar to the fungicides and both treatments differed from the control for preventive and curative control in the two assessed periods, aiming to protect

Table 4 Percentage of “Niagara Rosada” grapevine fragments with *Trichoderma asperelloides* 02/03, *Trichoderma asperellum* 01/13, and *T. koningiopsis* 09/02: The pathogens were detected at the tip of the

Treatments	Periods								
	5 days			10 days			15 days		
	<i>Trichoderma</i> Isolates								
	01/13	02/03	09/02	01/13	02/03	09/02	01/13	02/03	09/02
Tip	100.0*± 50.0 c ¹ A ² α ³	100.0± 50.0 c A α	100.0± 50.0 b A α	100.0± 37.5 b AB α	100.0± 25.0 c A α	75.0± 37.5 b B α	100.0± 25.0 b A α	100.0± 25.0 c A α	87.5± 50.0 b A α
2 cm	0.0± 37.5 ab A α	25.0± 37.5 b B β	0.0± 37.5 a A α	0.0± 50.0 a A α	25.0± 37.5 b B β	0.0± 50.0 a A α	0.0± 25.0 a A α	0.0± 50.0 ab A α	0.0± 37.5 a A α
4 cm	25.0± 37.5 b B β	12.5± 37.5 ab AB α	0.0± 37.5 a A α	0.0± 25.0 a A α	25.0± 37.5 b B α	0.0± 25.0 a A α	0.0± 25.0 a A α	25.0± 50.0 b B α	0.0± 50.0 a A α
Control	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α	0.0± 0.0 a A α

*Median and semi-amplitude interquartile of two assays. Each assay with 12 replicates per isolate, per treatment and per evaluation day

^{1,2,3} Lowercase letters: comparison of treatments, fixed periods and *Trichoderma* isolates; uppercase letters: comparison of *Trichoderma* isolates, fixed treatments and periods and Greek letters: comparison of periods, fixed treatments and *Trichoderma* isolates, according to Dunn’s multiple comparison test [33] with 5% significance level

grapevine pruning wounds against *L. theobromae* ($p < 0.05$; Table 5).

Discussion

T. koningiopsis, *T. asperellum*, and *T. asperelloides* were among the *Trichoderma* isolates that had not been assessed previously to control *L. theobromae*. The *Trichoderma* species that have already been assessed to control *L. theobromae* are *T. atroviride*, *T. viride*, *T. harzianum*, and *T. virens* [5,10,17,23].

All *Trichoderma* isolates were antagonists to *L. theobromae* *in vitro*. However, the most suitable three isolates were *T. asperelloides* 02/03, *T. asperellum* 01/13, and *T. koningiopsis* 09/02. A previous study showed that *Trichoderma* was an antagonist to *L. theobromae* [3].

Tebuconazole was the only fungicide that affected the conidia of *T. asperelloides* 02/03, *T. koningiopsis* 09/02, and *T. asperellum* 01/13, with the first two fungi showing moderate resistance to the fungicide. In addition, McLean et al. [15] observed the influence of tebuconazole on the germination of *T. harzianum* conidia. However, according to Melo [16], *Trichoderma* may be an opportune alternative to the fungicides, developing resistance to the products when exposed to it, which suggests new studies to assess the adaptability of the isolates of *Trichoderma* under frequent exposure to tebuconazole.

T. asperelloides 02/03, *T. asperellum* 01/13, and *T. koningiopsis* 09/02 survived up to 15 days post inoculation at the cane tip of “Niagara Rosada” grapevine with fresh wounds. *T. asperelloides* 02/03 survived and grew endophytically inside the cane. In addition, the capability of

Trichoderma to penetrate inner tissues of grapevine and other plants was observed by Spradling [29], John et al. [12], and Kotze [13].

Spradling [29] reported that after 2 days of inoculation, *T. lignorus* penetrated 4 cm into *Pinus taeda* sapwood previously steamed for 30 min at 100 °C compared with 1.1 cm into *P. taeda* that was unsteamed. According to a field study conducted by John et al. [12], *T. harzianum* penetrated 3 cm deep from the inoculation point of a mature vine after 4 months.

Therefore, the penetration of *T. asperelloides* 02/03 up to 4 cm in the inner tissues of “Niagara Rosada” grapevine at 15 days post inoculation shows its excellent endophytic capability in comparison with *T. harzianum* in the study of John et al. [12].

The survival of *Trichoderma* isolates in the cane tip and in the inner tissues during the time post inoculation is attributed by the different characteristics of the isolates itself, whereas the unexpected variations of the percentages of “Niagara Rosada” grapevine fragments obtained with each isolate in the different inner tissues were also observed in the study conducted by John et al. [12].

T. asperelloides 02/03 protected the pruning wounds of Niagara Rosada grapevine against *L. theobromae* similar to the fungicides fluazinam and tebuconazole for 20 days post inoculation. This protection for 20 days is extremely crucial because the grapevine is susceptible to the fungus during 2 weeks after pruning, particularly to *L. theobromae* as reported by Sônego et al. [26].

Kotze et al. [13] noted that, in most cases, the protection provided by *Trichoderma* to grapevine pruning wounds was similar or superior to the fungicide benomyl against trunk pathogens including *L. theobromae*. The study of Kotze et al. [13] supports the results obtained with *T. asperelloides* 02/03 in protecting grapevine pruning wounds in comparison with the fungicides fluazinam and tebuconazole against *L. theobromae*.

In the literature, *Trichoderma* was selected in a few isolated studies, such as *in vitro* antagonism of *Trichoderma* isolates against a target phytopathogen [8], screening of fungicides on *Trichoderma* [22,30], and application of isolates or *Trichoderma*-based products for the protection of grapevine wounds against trunk phytopathogen [10,13]. *Trichoderma*-based products were recorded in New Zealand for protection against infections caused by *Eutypa lata* in the grapevine [11], for example, the product Vinevax and others that contain isolates of *T. harzianum* and *T. atroviride*; however, the methodology used to select the *Trichoderma* isolates was not shown.

The sequence of assays used in this study may be considered a protocol for the selection of *Trichoderma* isolates to protect pruning wounds of grapevine against *L. theobromae*, with suggestions to be used for seeking the control of trunk phytopathogens.

Table 5 Incidence of *Lasioidiplodia theobromae* (percentage) in the long stems of “Niagara Rosada” grapevine shoots treated preventively and curatively with *Trichoderma asperelloides* 02/03 and with fungicides in two different periods post application

Treatments	Preventive		Curative	
	10	20 days	10	20 days
Fungicides	0.0* ± 0.0 a ¹ A ² α ³	0.0 ± 50.0 a A α	0.0 ± 50.0 a A α	0.0 ± 50.0 a A α
<i>Trichoderma</i>	0.0 ± 0.0 a A α	0.0 ± 0.0 a A α	0.0 ± 0.0 a A α	0.0 ± 0.0 a A α
Control	100.0 ± 50.0 b A α	100.0 ± 50.0 b A α	100.0 ± 50.0 b A α	100.0 ± 50.0 b A α

*Median and semi-amplitude interquartile of three assays

^{1,2,3} Lowercase letters: comparison of treatments, fixed periods, and application; uppercase letters: comparison of application, fixed treatments, and periods and Greek letters: comparison of periods, fixed treatments, and application forms, according to Dunn’s multiple comparison test [33] with 5% significance level

For the future, an appropriate formulation should be developed for protecting the inoculum of *Trichoderma* isolate against the harmful effects of environmental factors, such as sunrays, high or low temperatures, and climatic conditions such as rainfall or dry weather, assuring the survival of the antagonist at the grapevine cane tip and allowing *Trichoderma* to germinate and endophytically penetrate to protect grapevine pruning wounds against trunk phytopathogens, particularly *L. theobromae*. After the formulation, protection tests should be performed, particularly under field conditions, to show the biological efficacy of *Trichoderma*.

Conclusions

T. asperelloides (02/03), *T. asperellum* (01/13), and *T. koningiopsis* (09/02) were the best antagonists for *L. theobromae*. The conidia of the isolates were affected only by tebuconazole. *T. asperelloides* (02/03) and *T. koningiopsis* (09/02) were most tolerant to the fungicides. The *Trichoderma* isolates survived on the cane tip for 15 days; however, only *T. asperelloides* (02/03) showed endophytic penetration of 4 cm in the cane. *T. asperelloides* (02/03) showed preventive and curative capability to control *L. theobromae*, with similar efficiency as that of the fungicides fluazinam and tebuconazole in the periods of 10 and 20 days post inoculation. These four steps are suggested as a protocol to select *Trichoderma* for controlling *L. theobromae* or other phytopathogens causing trunk diseases in grapevine.

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