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Effect of the Combined Treatments with LC2017 and *Trichoderma atroviride* Strain I-1237 on Disease Development and Defense Responses in Vines Infected by *Lasiodiplodia theobromae*

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Abstract: Grapevine trunk diseases constitute one of the major problems for viticulture worldwide, with Botryosphaeria dieback considered as one of the most important of these diseases. In this work, we aimed to (i) evaluate the effect of the combination of two products, Esquive® (a *Trichoderma*-based product) and LC2017 (a low-copper-based product), in the control of *Lasiodiplodia theobromae*, by evaluating the internal lesion length caused by inoculation of this pathogen on greenhouse kept grapevines of cvs Cabernet Sauvignon and Touriga Nacional and, (ii) investigating their elicitor effect on plant defense responses, through the analysis of the expression of a set of genes. The pathogen was always re-isolated from the infected tissues and was able to cause wood discoloration. Touriga Nacional exhibited longer lesions than Cabernet Sauvignon, and the application of both products did not appear to reduce lesion length when compared to LC2017 applied alone. The elicitor effect of LC2017 on plant defense was confirmed by gene expression analysis, and no significant differences were found between plants treated with LC2017 and with both products. Moreover, a specific response related to the cultivar was verified, but this apparently unique interaction between product, cultivar and pathogen remains to be further investigated.

Keywords: Botryosphaeria dieback; internal necrosis length; gene expression; cuttings; Touriga Nacional; Cabernet Sauvignon; sustainable control

1. Introduction

Grapevine trunk diseases (GTDs) are a group of fungal diseases currently affecting grape-growing regions throughout the entire world. These diseases attack the wood of the perennial organs of the grapevine, resulting in yield reduction, progressive decline and, finally, increasing costs for winegrowers [1–3]. GTD control in vineyards is extremely complex, especially after the prohibition of several active ingredients, namely sodium arsenite and benzimidazoles, which are efficient but dangerous for humans and the environment (for review, see [2,4]). The increasing consumer demands for healthier and sustainable agriculture have also led to the need for new strategies and Plant Protection Products (PPPs) to control diseases in viticulture.

Among disease control strategies, Integrated Pest Management (IPM) is currently one of the most promising. With the harmonic and coordinated use of natural products as well as biocontrol agents (BCA), cultural practices and PPPs, the IPM could yield rapid results in terms of increasing and protecting biodiversity and health in the agroecosystem [5,6]. For these reasons, and to reduce the high number of treatments in vineyards required to control the main fungal diseases, such as downy and powdery mildew, gray mold and GTDs, the research on BCAs in viticulture has strongly developed. Among the BCAs tested in viticulture, the fungal genus *Trichoderma* has shown promising results towards several GTD pathogens, such as black-foot and Petri diseases, Botryosphaeria-dieback and Esca [7–17]. Currently, several *Trichoderma*-based products are available for winegrowers as wound protectants since the main entry for GTD pathogens are the wounds produced by pruning practices in vineyards. *Trichoderma*-based products could act against GTD pathogens both directly, by competition for space and nutrients, mycoparasitism, antibiosis and the production of cell-wall degrading enzymes [18–22], and indirectly, as a plant growth promoter and defense elicitors [8,18–22]. One of the main problems for treatment with *Trichoderma* spp. and, more generally, with BCAs, is their optimal integration in IPM strategies. The use of PPP with biocide action can strongly interfere with the BCA efficacy, even leading up to their elimination. For these reasons, Mutawila et al. [11] attempted to facilitate the introduction of *Trichoderma* spp.-based treatments into IPM strategies using Benzimidazole-resistant strains of *Trichoderma*.

In viticulture, copper has been used for over 130 years as an active ingredient to control the most important grapevine fungal disease, the downy mildew (*Plasmopara viticola*) [23]. The continuous use of Cu-based fungicides in vineyards has led to an increase in its concentrations in soils due to the tendency to accumulate in the upper layers [24]. This has caused severe negative impacts on soil microbial biodiversity and on the surrounding ecosystems [23]. The environmental problems have led the European Union to limit the use of copper-based products in viticulture to a maximum of 28 kg·ha^{-1} of metallic copper in 7 years, i.e., 4 kg·ha^{-1} per year (1981/2018 EC regulation, 1107/2009 EC regulation) [23,25]. This limitation is a major concern, especially for organic viticulture [26], where there is no alternative to cupric fungicides for the control of downy mildew. Since copper-based products are not easily replaceable in organic viticulture, the agrochemical producing industry changed its focus, synthesizing new copper formulations with lower ecotoxicological profiles. Among these innovative formulations, the HA + Cu (II), namely LC2017, containing a low copper amount (3.5%) with hydroxyapatite (HA), has recently revealed promising results in the control of GTD pathogens [16,27–29]. Moreover, LC2017 showed interesting elicitation properties towards several genes linked with plant defense systems when tested in controlled conditions [28–30]. Recently, Reis et al. [16] successfully demonstrated the compatibility between *Trichoderma atroviride* strain I-1237 and LC2017 treatments to control GTDs in vineyards. Due to the ascertained abilities of both LC2017 [29] and *Trichoderma* spp. [22] in eliciting the host plant defense system, it seems interesting to further characterize this combination of treatments in grapevines.

Therefore, the main objective of this study was to evaluate the efficacy of the combination of LC2017 and *T. atroviride* strain I-1237 applications for the control of *Lasiodiplodia theobromae*, an agent of the Botryosphaeria-dieback [31], under controlled conditions. A greenhouse bioassay was thus conducted on Cabernet Sauvignon and Touriga Nacional cuttings to evaluate the effects of both products on the control of *L. theobromae* infection by assessing (i) the internal lesion length analyses and the pathogen re-isolation, and (ii) plant defense responses by studying the induction of the expression of targeted genes.

2. Materials and Methods

2.1. Fungal Isolates and Inoculum Preparation

The *Lasiodiplodia theobromae* isolate Bt105 used in this study was isolated in 2009 from internal wood necrosis of grapevine cv Castelão, in Portugal, and is currently stored at the culture collection of Instituto Superior de Agronomia, Lisbon, Portugal. This particular

isolate was selected due its previously shown aggressiveness towards the two cultivars under study [16,32]. The isolate maintained on Potato Dextrose Agar (PDA, Difco, Sparks, MD, USA) slants was first transferred to Petri dishes containing PDA to promote colony growth and then incubated in the darkness, for 8 days at 25 °C. To promote the production of pycnidia, the fungus was thus transferred into Petri dishes containing 2% water agar (Difco, Sparks, MD, USA) with sterilized pine needles (*Pinus pinea*) and incubated at 25 °C with a 12 h photoperiod (fluorescent light) [33–35]. Prior to inoculation, conidia were harvested by removing the pycnidia from the pine needles' surface to a 1.5 mL Eppendorf tube containing sterile distilled water (SDW). Pycnidia were crushed with the use of a pestle, and the tubes were shaken in a vortex to release the conidia. The spore suspension obtained was filtered through cheesecloth, and the final concentration was adjusted to 1×10^5 spore mL^{-1} with the help of a hemocytometer (Brand, Wartheim, Germany).

2.2. Plant Material, Experimental Design and Treatments

2.2.1. Plant Material

Inoculations were performed on cuttings of two different cultivars, Touriga Nacional and Cabernet Sauvignon, coming from a commercial nursery. The cuttings were rooted at 24 °C under natural light on a rooting table containing perlite, and a month later, the rooted cuttings were individually potted in a sandy soil mixture (1/3 sand, 1/3 soil, 1/3 organic matter). In the end, the rooted cuttings were placed in a ventilated greenhouse at 24 °C under natural light and watered as needed.

2.2.2. Tested Products

The product and treatment plans used in this study are reported in Table 1. We tested two products: (i) Esquive® (product developed by Agrauxine S.A., Quimper, France, and commercialized by Idai Nature S. L., Valencia, Spain), a commercially available *Trichoderma*-based wound protectant containing *Trichoderma atroviride* strain I-1237 and (ii) LC2017, a copper sulphate pentahydrate salt (Cu (II) 35 g· L^{-1}), transported throughout the plant by hydroxyapatite molecules. Both products were applied according to the label and/or manufacturer recommendation or previous works [29]. A total of 6 conditions were set up for both cultivars in a completely randomized design with 8 replicates for each treatment, for a total of 48 plants per cultivar.

Table 1. Treatments tested on cultivars Cabernet Sauvignon and Touriga Nacional.

Treatment	Product	Inoculation
1	LC2017	Water (control)
2	LC2017	*L. theobromae* (Bt105)
3	Esquive®	Water (control)
4	Esquive®	*L. theobromae* (Bt105)
5	LC2017 + Esquive®	Water (control)
6	LC2017 + Esquive®	*L. theobromae* (Bt105)

2.2.3. Product Application and Pathogen Inoculation

The scheme for product application and inoculation is reported in Figure 1. After three months of pot individualization, a pruning wound was made with scissors in the upper part of the cuttings, it was immediately treated with Esquive®/water suspension (27 g·L^{-1}). For the condition with only LC2017 treatment, no pruning wound of the upper part of the cuttings was made, and the product was applied to the whole plant (cutting + annual shoot) using a backpack sprayer. For the condition with both treatments, cuttings were pruned in its upper part, the wounds were treated with Esquive®, and after letting the product dry, LC2017 application to the whole plant (cutting + annual shoot) was carried out. Twenty-four hours after product applications, two different kinds of inoculations with *L. theobromae* were performed. The first one was with 20 μL of *L. theobromae* spore suspension (≈2000 spores) directly put on the pruning wounds of cuttings treated with

Esquive® only. The second one used mycelial plugs put in wounds created between the 2nd and 3rd internodes using a cork-borer [36] in the annual shoot of cuttings treated with LC2017 only. For cuttings treated with both Esquive® and LC2017, the two kinds of inoculation were performed on the same plant. For the controls, STW and sterile plugs of PDA were inoculated. Being not in the aim of the study, no untreated plants were inoculated with the pathogen. After inoculation, to prevent dehydration and promote spore germination, the pruning wounds were protected for one week using Parafilm M® (Bemis, Sheboygan Falls, WI, USA). The site of inoculations on the annual shoots was covered with moist wool cotton and sealed with Parafilm M® (Bemis, Sheboygan Falls, WI, USA) for one week to prevent dehydration.
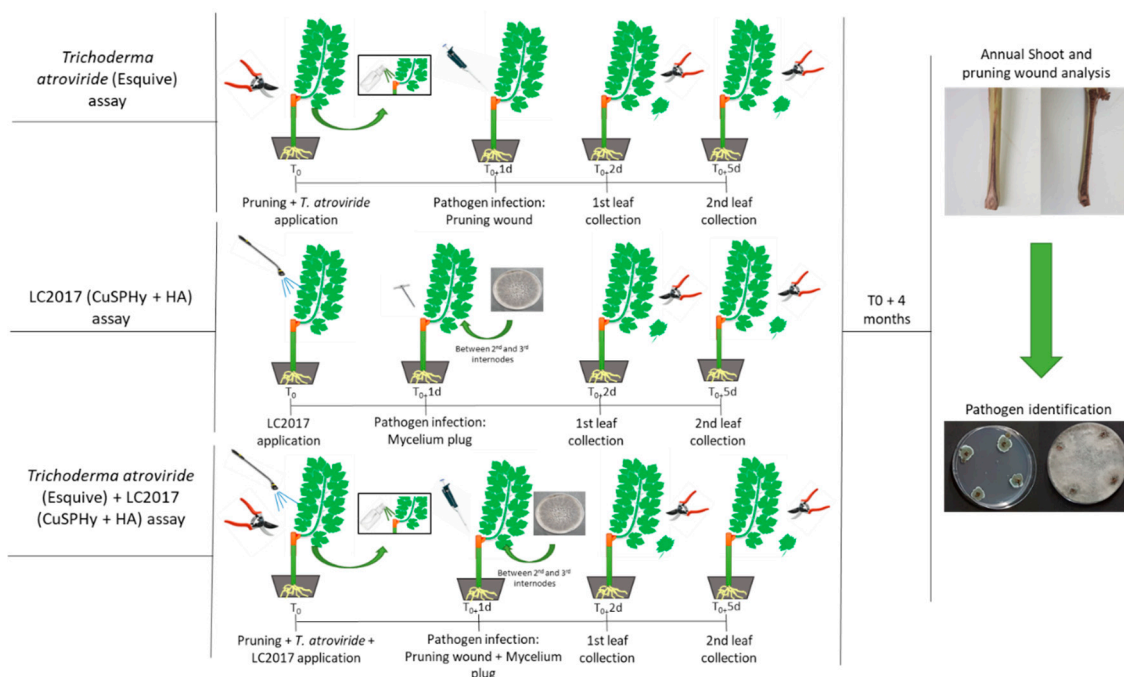
Figure 1. Diagram showing the several steps of treatment and inoculation of greenhouse-kept grapevines of cultivars Cabernet Sauvignon and Touriga Nacional. Information on the treatments and products used can be found in Table 1.

2.3. Gene Expression Analysis

To study the effects of the treatments (Esquive®, LC2017, Esquive® + LC2017) and of the GTD pathogen *L. theobromae* on plant defense response in both cultivars, a targeted gene expression analysis was set up. The leaf sampling for this transcript analysis was performed at T0 + 2d and at T0 + 5d (T0 = Esquive® or LC2017 application; d for days). Each sample consisted of the two leaves immediately above and below the inoculation point, with three cuttings per condition being used as replicates for each time point. The leaves collected were immediately frozen in liquid nitrogen and stored at −80 °C. Following the RNA extractions, quantitative real-time RT-PCR analysis was carried out according to [37,38], respectively. The results were expressed as the values of relative expression levels (ΔΔCt), corresponding to the mean of three independent biological replicates. *39SRP*, *60SRP* and *EF-1α* genes were used as housekeeping genes. Since our objective was to evaluate the interaction between the two treatments (Esquive® and LC2017), both analyses were compared to the Esquive® T0 + 2d condition, which represented our control herein.

The specific primers used in the gene expression study are listed in Supplementary Table S1. These genes were selected based on similar previous studies [29,30,37–42]. Specifically, 11 genes were chosen to evaluate the grapevine cultivars' response to the above-mentioned treatments and are related to: the phenylpropanoid pathway (*PAL* and *STS*) and other defense protein markers (*CHIT4C*, *GLUC*, *PR1* and *PR10*), detoxification

processes (*GTS1*) and the "recovered" health-status markers highlighted in leaves of GTD-infected vines treated with sodium arsenite (*PME25*, *MSR*, *WRKY* and *HYD2*; *unpublished data*, Fontaine, F.). The genes analyzed were considered to be up or down-regulated when changes in their expression were either >2-fold or <0.5-fold.

2.4. Determination of Internal Lesions and Pathogen Recovery

Four months after pathogen inoculation, both inoculated shoots and grafted cuttings showed internal lesions after being cut longitudinally. Internal lesions were measured longitudinally both on the woody part of the rooted cuttings and the annual shoots. The lesions were measured using an electronic caliper, and four fragments of necrotic tissue from the margin of the lesion were collected. These fragments were surface disinfected with a 7% sodium hypochlorite solution for one minute, rinsed three times by immersion in sterile distilled water (SDW) for one minute each and platted onto 9 mm Petri dishes containing PDA amended with 250 mg·L^{-1} of chloramphenicol (PanReac, AppliChem, Darmstadt, Germany). After incubation at 25 °C for one week, the Petri dishes were assessed for the presence of Botryosphaeriaceae colonies, which were sub-cultured onto fresh PDA dishes and again incubated at 25 °C for one week in the darkness. A representative set of *L. theobromae* isolates was selected from each of the treatments to undergo molecular confirmation of species identity. Two weeks after inoculation, the shoots showed some external discoloration, not easily observable after their lignification. Thus, re-isolation of the pathogen was only performed from internal wood lesions. The re-isolation percentage was calculated as the mean percentage of wood fragments showing pathogen growth for each treatment.

2.5. Statistical Analysis

Statistical analysis was performed using the R-program v. 4.0.5 (www.r-project.org (accessed on 20 March 2022)). Assumptions for variance analysis were assessed. For data normality and homogeneity of variance Shapiro-Wilk and Levene's tests were performed, respectively. According to the results, the influence of distinct levels was assessed using the non-parametrical Kruskal-Wallis test. For significant differences ($p < 0.05$), a comparison between the distinct levels was performed using the ranks. The re-isolation percentage data (RP) were transformed into the arcsine of the square root of the proportion (RP/100) 1/2. Statistical significance ($p \leq 0.05$) for the data collected for gene expression was determined with Mann–Whitney non-parametric tests using GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Relationship between the Lesion Length Induced by L. theobromae and Treatments

The mean lesion lengths of wood discoloration caused by L. theobromae on both cultivars are presented in Figure 2. *Lasiodiplodia theobromae* Bt105 was able to cause wood discoloration in both cultivars independently to the treatments. For Cabernet Sauvignon (CS) cuttings, the mean lesion length values were significantly lower than for Touriga Nacional (TN). For TN, the pathogen determined larger lesions in the inoculated cuttings than in annual shoots. The mean lesion length for the pruning wounds ranged from 0.63 cm for the control cuttings inoculated with PDA and treated with Esquive® or LC2017 to 2.24 cm for those inoculated with the pathogen and treated with LC2017 + Esquive®. In this case, no significant differences are observed between the inoculated cuttings treated with Esquive® and the respective controls, while the cuttings treated with both Esquive® and LC2017 showed differences compared to the control cuttings (Figure 2).
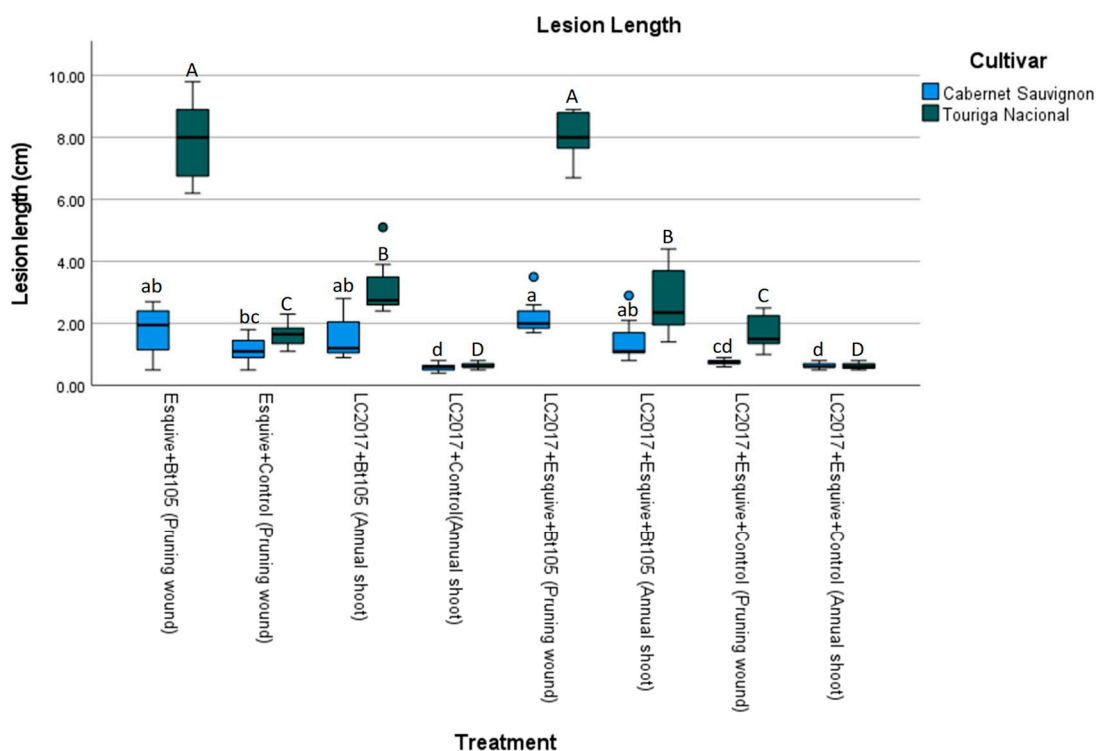
Figure 2. Boxplot representing the average lesion lengths recorded for the inoculation of cuttings from two different grapevine cultivars, Cabernet Sauvignon and Touriga Nacional, with *L. theobromae* isolate Bt105 under greenhouse conditions. The median is represented by the solid line. Different letters in the column correspond to significant differences ($p < 0.05$) based on ranks assessed by Kruskal–Wallis analysis. Upper-case letters refer to Cabernet Sauvignon, and lower-case letters to Touriga Nacional.

Regarding annual shoot inoculation, the mean lesion length varied between 0.59 cm for the LC2017 control cuttings and 1.53 cm for cuttings treated with LC2017. Significant differences are observed for all the treatments with inoculated cuttings compared with their respective controls. Regarding the TN cuttings, the mean lesion length on the pruning wounds varied between 1.6 cm for the control cuttings inoculated with PDA and treated with Esquive® and 8.1 cm on the cuttings inoculated with the pathogen and treated with LC2017 + Esquive®. Although the mean lesion length value recorded for the control cuttings is apparently high, as in the pruning wounds of cuttings treated with LC2017 + Esquive® (1.7 cm), this may be attributed to the healing and the accumulation of phenolic compounds due to the process of wounding through pruning. Moreover, significant differences were found among all of the inoculated and the control plants that underwent pruning wound inoculation (Figure 2). The mean lesion length for the annual shoot inoculations was also shorter on average than the ones recorded for the pruning wounds with values ranging from 0.62 cm for controls treated with both LC2017 and Esquive® to 3.15 cm on cuttings treated only with LC2017. Significant differences were observed for most of the isolates inoculated compared to the controls (Figure 2).

On average, the mean lesion lengths recorded were significantly higher in TN than in CS. Moreover, the mean lesion lengths were longer on the inoculated pruning wounds than on the inoculated annual shoots for both cultivars.

3.2. Pathogen Recovery

The re-isolation of *L. theobromae* was higher from the inoculated annual shoots than from the pruning wounds for both cultivars. Moreover, no pathogen was recovered from the control plants inoculated with PDA plugs or water for both cultivars, and no pycnidia

formation was observed during the period of the experiment, both on inoculated annual shoots and cuttings. Cabernet Sauvignon inoculations recorded a maximum re-isolation percentage ranging from 18.7% for the inoculated pruning wounds of LC2017 + Esquive® treated cuttings to 81.2% for annual shoots treated with LC2017 + Esquive®. For TN, the re-isolation percentages from inoculated annual shoots showed the highest minimum value of 43.7% on cuttings treated with both products but also the lowest maximum value of re-isolation percentage of 65.6% on cuttings treated with LC2017 (Figure 3). The re-isolation percentages at pruning wounds were the same (21.8%) for the cuttings treated with only Esquive® and for cuttings treated with both products. For both cultivars, significant differences were found between the inoculated annual shoots and the inoculated pruning wounds. Nevertheless, for TN, significant differences could also be observed on the re-isolation percentage obtained from the inoculated annual shoots treated with LC2017 alone and in combination with Esquive®. On average, slightly higher percentages of re-isolation are reported for CS than for TN, in contrast to what was observed for the lesion length, where TN showed the highest values. Further, no product applied conjointly or alone appears to be more efficient in reducing the presence of *L. theobromae*.
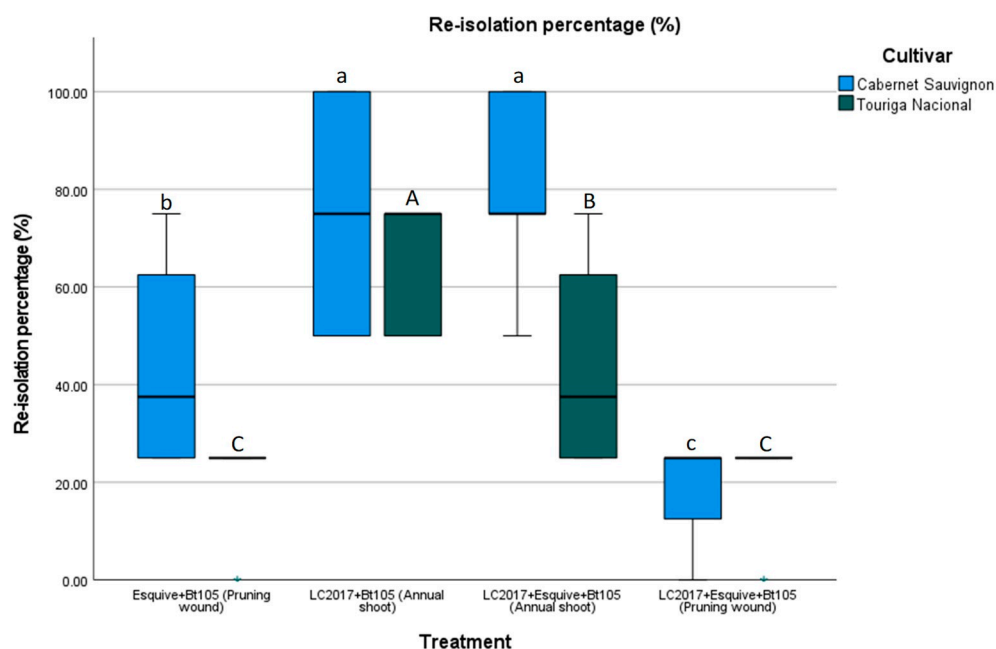
Figure 3. Boxplot representing the re-isolation percentage of *L. theobromae* (isolate Bt105) were recorded from the cuttings of two cultivars, Cabernet Sauvignon and Touriga Nacional. The re-isolation percentage was calculated as the mean percentage of wood fragments showing pathogen growth for each treatment. The median is represented by the solid line. Different letters in the same column correspond to significant differences ($p < 0.05$) based on ranks assessed by Kruskal–Wallis analysis. Upper-case letters refer to Cabernet Sauvignon, and lower-case letters to Touriga Nacional.

T. atroviride was only recovered from cuttings treated with Esquive®. Lower *T. atroviride* recovery rates were obtained from treated cuttings of CS than TN. Moreover, although no application of Esquive® was performed on the annual shoots of cuttings, still small percentages of recovery were reported from this part of the cuttings treated with both Esquive® and LC2017. Although extreme caution was used when applying Esquive® to pruning wounds, this presence may be due to some spray drift to adjacent plant organs. For CS, recovery rates ranged from 3.1% to 59.4% for annual shoots of cuttings treated with LC2017 + Esquive® and pruning wounds of cuttings treated with Esquive® alone, respectively. Concerning *T. atroviride* recovery rates from TN cuttings, as referred to before, higher values were obtained with 12.5% for the annual shoots of cuttings treated with both products and a maximum of 81.2% for pruning wounds treated with Esquive® alone.

3.3. Plant Responses at Gene Expression Levels According to the Treatments

The results of the gene expression studies on cv Cabernet Sauvignon and Touriga Nacional submitted to the different treatment conditions are reported in Figures 4 and 5, respectively.
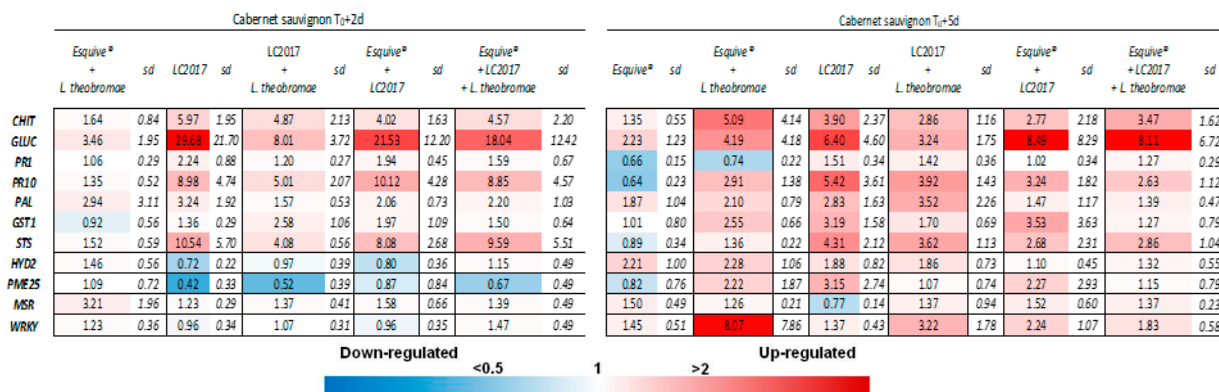
Cabernet sauvignon T0+2d

	Esquive® + L. theobromae	sd	LC2017	sd	LC2017 + L. theobromae	sd	Esquive® + LC2017	sd	Esquive® + LC2017 + L. theobromae	sd
CHIT	1.64	0.84	5.97	1.95	4.87	2.13	4.02	1.63	4.57	2.20
GLUC	3.46	1.95	29.68	21.70	8.01	3.72	21.53	12.20	18.04	12.42
PR1	1.06	0.29	2.24	0.88	1.20	0.27	1.94	0.45	1.59	0.67
PR10	1.35	0.52	8.98	4.74	5.01	2.07	10.12	4.28	8.85	4.57
PAL	2.94	3.11	3.24	1.92	1.57	0.53	2.06	0.73	2.20	1.03
GST1	0.92	0.56	1.36	0.29	2.58	1.06	1.97	1.09	1.50	0.64
STS	1.52	0.59	10.54	5.70	4.08	0.56	8.08	2.68	9.59	5.51
HYD2	1.46	0.56	0.72	0.22	0.97	0.39	0.80	0.36	1.15	0.49
PME25	1.09	0.72	0.42	0.33	0.52	0.39	0.87	0.84	0.67	0.49
MSR	3.21	1.96	1.23	0.29	1.37	0.41	1.58	0.66	1.39	0.49
WRKY	1.23	0.36	0.96	0.34	1.07	0.31	0.96	0.35	1.47	0.49

Cabernet sauvignon T0+5d

	Esquive®	sd	Esquive® + L. theobromae	sd	LC2017	sd	LC2017 + L. theobromae	sd	Esquive® + LC2017	sd	Esquive® + LC2017 + L. theobromae	sd
CHIT	1.35	0.55	5.09	4.14	3.90	2.37	2.86	1.16	2.77	2.18	3.47	1.62
GLUC	2.23	1.23	4.19	4.18	6.40	4.60	3.24	1.75	8.49	8.29	8.11	6.72
PR1	0.66	0.15	0.74	0.22	1.51	0.34	1.42	0.36	1.02	0.34	1.27	0.29
PR10	0.64	0.23	2.91	1.38	5.42	3.61	3.92	1.43	3.24	1.82	2.63	1.12
PAL	1.87	1.04	2.10	0.79	2.83	1.63	3.52	2.26	1.47	1.17	1.39	0.47
GST1	1.01	0.80	2.55	0.66	3.19	1.58	1.70	0.69	3.53	3.63	1.27	0.79
STS	0.89	0.34	1.36	0.22	4.31	2.12	3.62	1.13	2.68	2.31	2.86	1.04
HYD2	2.21	1.00	2.28	1.06	1.88	0.82	1.86	0.73	1.10	0.45	1.32	0.55
PME25	0.82	0.76	2.22	1.87	3.15	2.14	1.07	0.74	2.27	2.93	1.15	0.79
MSR	1.50	0.49	1.26	0.21	0.77	0.14	1.37	0.94	1.52	0.60	1.37	0.23
WRKY	1.45	0.51	8.07	7.86	1.37	0.43	3.22	1.78	2.24	1.07	1.83	0.58

Down-regulated <0.5   1   >2 Up-regulated

Figure 4. Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Cabernet Sauvignon at T0 + 2d and T0 + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta Ct$) of treatments relative to the control (condition Esquive® T0 + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls.
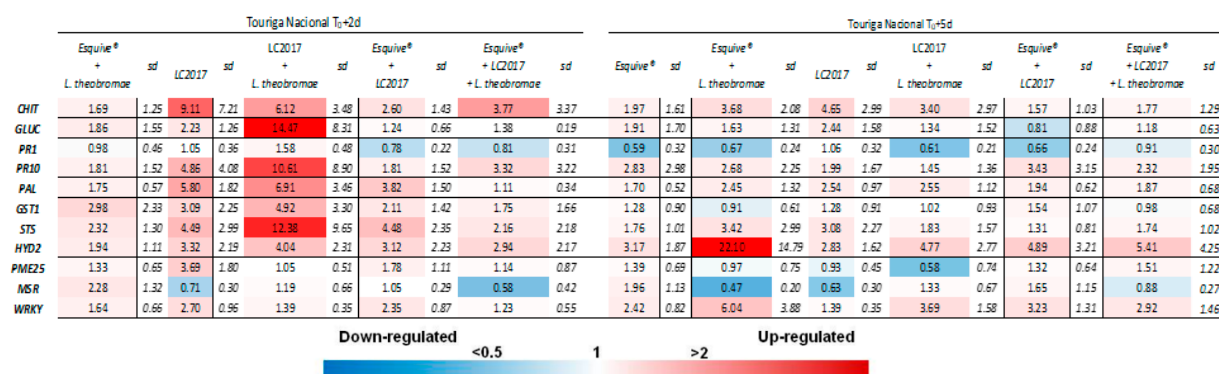
Touriga Nacional T0+2d

	Esquive® + L. theobromae	sd	LC2017	sd	LC2017 + L. theobromae	sd	Esquive® + LC2017	sd	Esquive® + LC2017 + L. theobromae	sd
CHIT	1.69	1.25	9.11	7.21	6.12	3.48	2.60	1.43	3.77	3.37
GLUC	1.86	1.55	2.23	1.26	14.47	8.31	1.24	0.66	1.38	0.19
PR1	0.98	0.46	1.05	0.36	1.58	0.48	0.78	0.22	0.81	0.31
PR10	1.81	1.52	4.86	4.08	10.61	8.90	1.81	1.52	3.32	3.22
PAL	1.75	0.57	5.80	1.82	6.91	3.46	3.82	1.50	1.11	0.34
GST1	2.98	2.33	3.09	2.25	4.92	3.30	2.11	1.42	1.75	1.66
STS	2.32	1.30	4.49	2.99	12.38	9.65	4.48	2.35	2.16	2.18
HYD2	1.94	1.11	3.32	2.19	4.04	2.31	3.12	2.23	2.94	2.17
PME25	1.33	0.65	3.69	1.80	1.05	0.51	1.78	1.11	1.14	0.87
MSR	2.28	1.32	0.71	0.30	1.19	0.66	1.05	0.29	0.58	0.42
WRKY	1.64	0.66	2.70	0.96	1.39	0.35	2.35	0.87	1.23	0.55

Touriga Nacional T0+5d

	Esquive®	sd	Esquive® + L. theobromae	sd	LC2017	sd	LC2017 + L. theobromae	sd	Esquive® + LC2017	sd	Esquive® + LC2017 + L. theobromae	sd
CHIT	1.97	1.61	3.68	2.08	4.65	2.99	3.40	2.97	1.57	1.03	1.77	1.29
GLUC	1.91	1.70	1.63	1.31	2.44	1.58	1.34	1.52	0.81	0.88	1.18	0.63
PR1	0.59	0.32	0.67	0.24	1.06	0.32	0.61	0.21	0.66	0.24	0.91	0.30
PR10	2.83	2.98	2.68	2.25	1.99	1.67	1.45	1.36	3.43	3.15	2.32	1.95
PAL	1.70	0.52	2.45	1.32	2.54	0.97	2.55	1.12	1.94	0.62	1.87	0.68
GST1	1.28	0.90	0.91	0.61	1.28	0.91	1.02	0.93	1.54	1.07	0.98	0.68
STS	1.76	1.01	3.42	2.99	3.08	2.27	1.83	1.57	1.31	0.81	1.74	1.02
HYD2	3.17	1.87	22.10	14.79	2.83	1.62	4.77	2.77	4.89	3.21	5.41	4.25
PME25	1.39	0.69	0.97	0.75	0.93	0.45	0.58	0.74	1.32	0.64	1.51	1.22
MSR	1.96	1.13	0.47	0.20	0.63	0.30	1.33	0.67	1.65	1.15	0.88	0.27
WRKY	2.42	0.82	6.04	3.88	1.39	0.35	3.69	1.58	3.23	1.31	2.92	1.46

Down-regulated <0.5   1   >2 Up-regulated

Figure 5. Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions) and relative standard deviation values (sd) recorded on leaves cv Touriga Nacional at T0 + 2d and T0 + 5d. The values (each the mean of three technical replicates) represent expression levels ($\Delta\Delta Ct$) of treatments relative to the control (condition Esquive® T0 + 2d). The expression of each gene was considered up-regulated when the value was >2-fold compared to the controls or down-regulated when the value was <0.5-fold compared to the controls.

3.3.1. Effect of LC2017 Treatment Alone

In both cultivars, treatments with LC2017 alone and in the absence of the pathogen determined the induction of several genes, already at T0 + 2d, such as *CHIT*, *GLUC*, *PR-proteins*, *PAL*, *STS* and *GST1*. Most of these genes were found also induced at T0 + 5d for Cabernet Sauvignon and, to a lesser extent, for Touriga Nacional. A similar behavior was recorded in both cultivars upon *L. theobromae* infection. According to the considered cv, some genes are either more (*GST1* in Cabernet Sauvignon, Figure 4; *GLUC*, *PR10*, *STS* in Touriga Nacional, Figure 5) or not induced (*PME25* and *WRKY* in Touriga Nacional, Figure 5) upon *L. theobromae* challenge.

3.3.2. Effect of the Double Treatment Esquive® and LC2017

The expression values recorded at T0 + 2d in rooted cuttings treated with both *T. atroviride* and LC2017 did not statistically differ from those recorded in the LC2017 condition, indicating a non-synergic effect of the double treatment on the targeted genes for both cultivars (Figures 6 and 7). The non-synergic effect between the two treatments could be supported by the expression level recorded at T0 + 5d in vines treated with *T. atroviride* only. These values were very similar to the control, except for a few genes (*GLUC* and *HYD2* in Cabernet Sauvignon and *PR10*, *HYD2* and *WRKY* for Touriga Nacional) that were slightly overexpressed with values ranging from 2.42 to 3.17 (Figure 6). The presence of *L. theobromae* showed globally no effect in vines treated with both *T. atroviride* and LC2017, with induction values similar to the related no-infected condition, except for *PAL* being significantly repressed in Touriga Nacional (Figure 7).
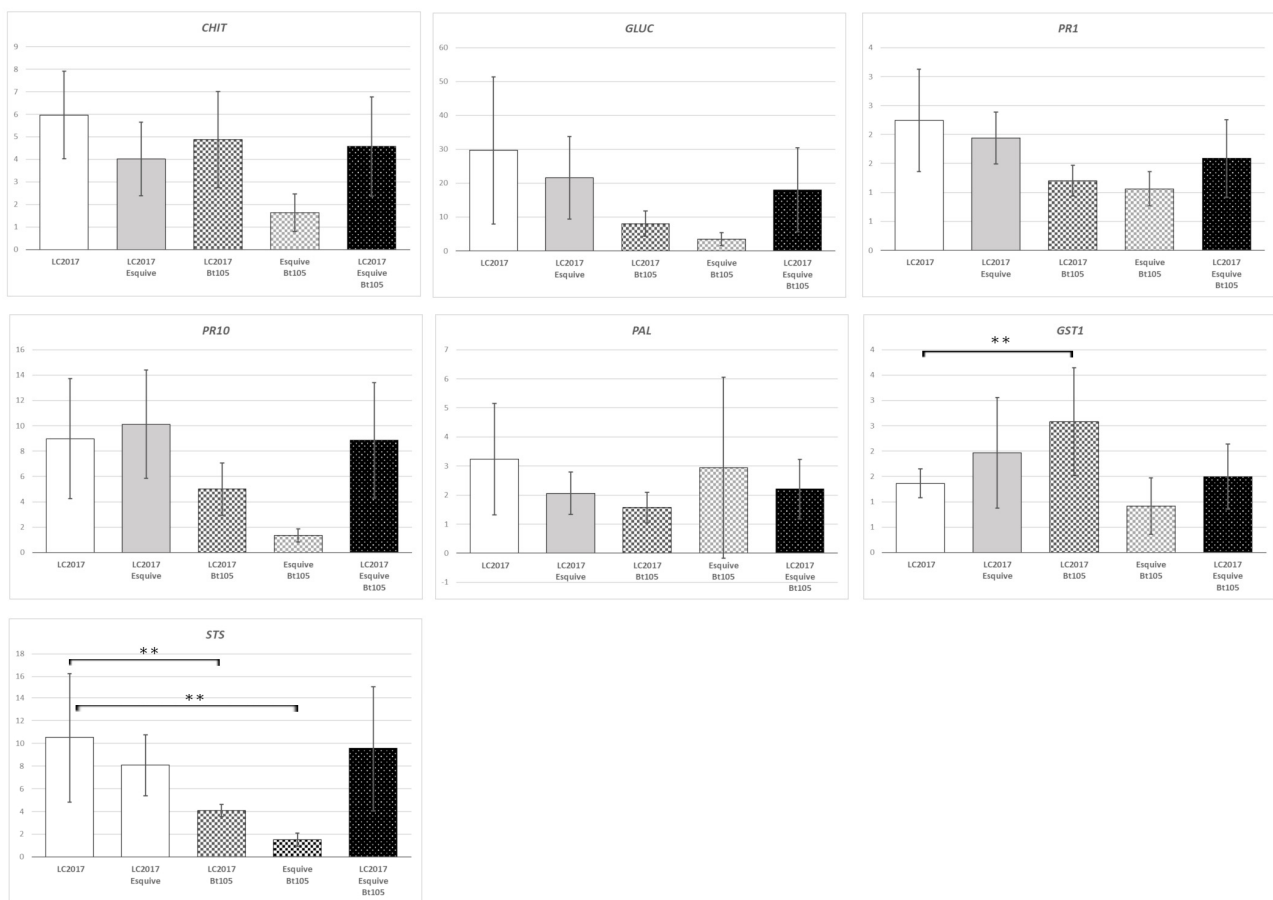
Figure 6. Overexpressed genes (values > 2) at T0 + 2d in Cabernet Sauvignon cuttings with single (LC2017) or double (LC2017 + Esquive®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney U test showed no significant differences between medians of LC2017 and LC2017 + Esquive® and between LC2017 + Bt105 and LC2017 + Esquive® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. ** = $p < 0.01$.
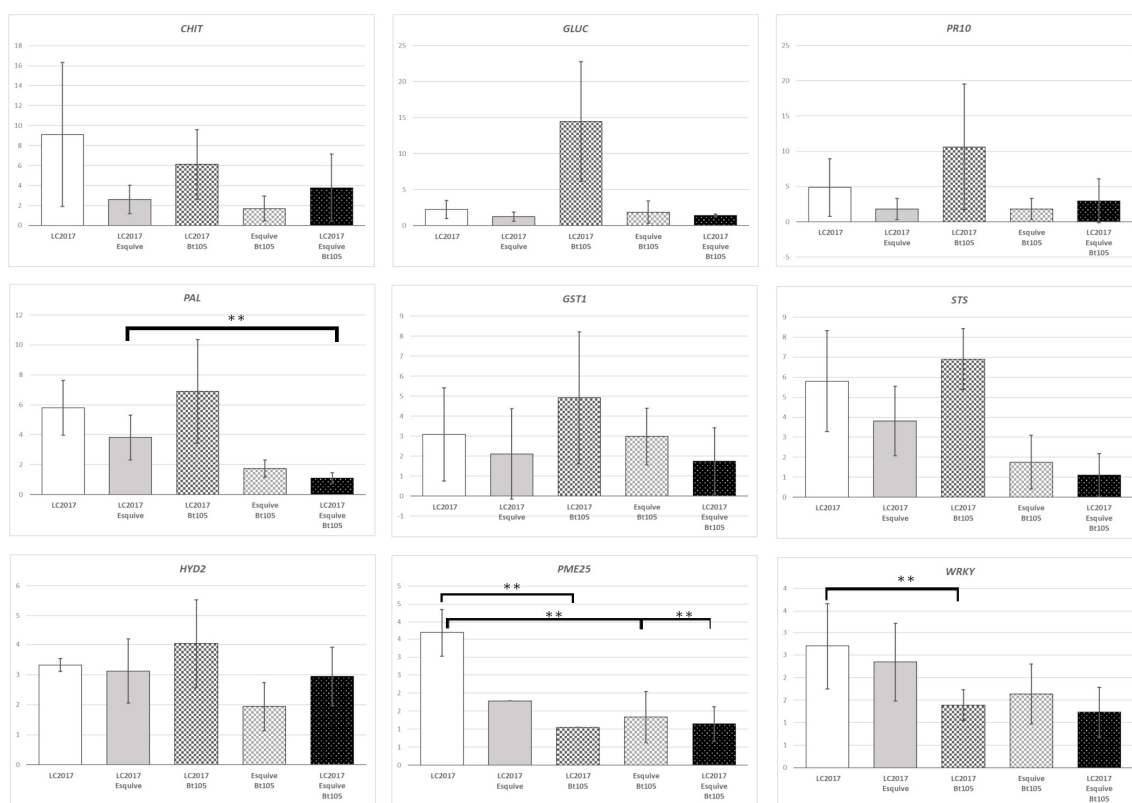
Figure 7. Overexpressed genes (values > 2) at T0 + 2d in Touriga Nacional cuttings with single (LC2017) or double (LC2017 + Esquive®) treatment, not inoculated or inoculated with *L. theobromae* strain Bt105. Statistical analysis with the Mann-Whitney U test showed no significant differences between the medians of LC2017 and LC2017 + Esquive® and between LC2017 + Bt105 and LC2017 + Esquive® + Bt105. Only a significant effect of pathogen presence was observed in some genes in LC2017-treated vines and in double-treated vines. The asterisk indicates a statistical significance (Mann-Whitney U test) between the medians of the two considered conditions. ** = $p < 0.01$.

3.3.3. Influence of Cultivar on Defense Responses

The gene expression study also highlighted differences in the expression levels according to the cultivars of the tested treatments. For Cabernet Sauvignon, *CHIT*, *GLUC*, *PR10*, *PAL* and *STS* were induced at both T0 + 2d and T0 + 5d when treated with LC2017 as well as the double treatment (Figure 4). At T0 + 5d, induction of several other genes, such as *GST1* and *PME25*, was also verified for both LC2017 alone treatment and double treatment with Esquive®. On the contrary, several genes were only overexpressed at T0 + 2d in Touriga Nacional, such as *PR10*, *GST1*, *PME* and *WRKY* in the LC2017 treatment and *CHIT*, *PAL*, *GST1* and *STS* in the double treatment (Figure 4). For this latter treatment, other genes, namely *PR10*, *HYD2* and *WRKY*, showed induction only at T0 + 5d in Touriga Nacional. Significant differences in the gene expression between both cvs were observed (Supplementary Figure S1), with values higher for Cabernet Sauvignon than for Touriga Nacional, except for *HYD2* and *WRKY*.

4. Discussion

The aim of this study was to further test the strategy of combining *T. atroviride* (Esquive®) and HA + Cu (II) (LC2017), previously assessed as pruning wound protectants for the control of the Botryosphaeria-dieback-associated pathogen *L. theobromae* [16]. These products have been individually tested against several Botryosphaeria-dieback pathogens ([9,14–16] for Esquive®; [16,29] for LC2017). Nevertheless, no studies on the impact of the application strategy of the two formulations on plant defense responses at the gene level have been previously investigated. To achieve this goal, we studied the

interaction of these two formulations on two artificially inoculated cultivars with an isolate of *L. theobromae* (Bt105) under controlled conditions. The efficiency of this combination was assessed on both the pathogen and the disease progression by studying the pathogen's presence and internal lesions development, respectively. Furthermore, we studied the possible synergic elicitor effects of the two combined PPPs treatments on genes related to the grapevine defense system.

4.1. Effect of Treatments on Lesion Length Induced by L. theobromae

Despite the treatment and the type of inoculation used, the *L. theobromae* isolate Bt105 was always re-isolated and able to cause wood discoloration on both cultivars, though cuttings from CS always showed lower mean lesion length values than TN. These results are similar to those obtained in our previous study, carried out in the field with the same cultivars and *L. theobromae* isolate [16]. Further, for both cultivars, lesion lengths recorded on inoculated pruning wounds were longer than those on inoculated shoots, being the shortest lesion length found on the inoculated shoots treated with both formulations. Our results agree with Úrbez-Torres and Gubler [43], which reported that the variability of the aggressiveness of Botryosphaeriaceae observed in inoculation tests could depend on the isolate, type of inoculum used, inoculation tissue (e.g., mature canes vs. green shoots), as well as on the cultivar, age of the host and length of the inoculation period. Moreover, our previous study [32] showed that TN had a higher susceptibility towards *L. theobromae* than CS, both in controlled conditions and in the field. Thus, the higher susceptibility towards *L. theobromae* could explain the higher lesions on TN. The significant differences between cultivars on the lesion length in pruning wounds treated with Esquive® (alone and in combination with LC2017) cannot be related to the different colonization ability of *T. atroviride* of the two cultivars. In fact, higher re-isolation percentage values of *T. atroviride* were observed on TN rather than CS. This seems to be in contrast with what was reported by Mutawila et al. [12] about the correlation between the protection to grapevine conferred by *Trichoderma* sp. and its colonization abilities, influenced by intrinsic differences among cultivars. Thus, even if higher *T. atroviride* re-isolation percentages were found in TN, the susceptibility of this cultivar previously mentioned to *L. theobromae* may have masked the influence of the BCA on pathogen colonization. Nevertheless, lower RP were obtained from the inoculated pruning wounds of TN treated with Esquive®. *Trichoderma*-based products have been reported to need time for the BCA establishment in the treated plants [10]. Therefore, we hypothesize that in TN vines, the different rate of colonization between *L. theobromae* and *T. atroviride* has determined the higher susceptibility of this cultivar towards the pathogen, even if the BCA was able to establish itself, conferring some extent of control over the pathogen. However, this hypothesis still needs further research to clarify the interactions between the pathogen and BCA in grapevine wood. Similarly, the application of LC2017 did not show a significant effect on internal lesion length, as well as the double combined treatment. This concurs with previous works in which vines treated with LC2017 in controlled conditions and inoculated with Botryosphaeriaceae did not show evidence of a significant reduction of the internal lesion [29] nor increased the efficacy of pruning wound protection when combined with Esquive® [16].

4.2. The Combination of Esquive® with LC2017 Does Not Appear to Improve Plant Defense Response

Our study has confirmed the elicitation abilities of LC2017 on the grapevine defense systems, especially on the genes linked to molecules directly addressed to contrast fungal pathogen attack (*CHIT*, *GLUC* and *PR*-proteins) as well as the phenylpropanoid metabolism (*PAL*, *STS*) and detoxification process (*GST1*). Our results agree with previous studies, which reported that LC2017 is able to induce the host plant defense system in a similar manner to the commercial product (Bion) in healthy and GTD pathogens-infected vines [28–30]. Our transcript analysis results confirm those of Mondello et al. [29], in which *CHIT*, *GLUC*,

PAL and *STS* exert a role in grapevine tolerance to biotic and abiotic stresses [42,44] underwent elicitation.

Even if *Trichoderma*-based formulations have been reported to activate defense genes in planta [8,45], no significant differences in gene expression levels were found between cuttings treated with LC2017 alone and with Esquive® and LC2017 for both time points. Our results may be related to a host-plant effect on *Trichoderma* spp. induction abilities, as observed by Stempien et al. [45] on rootstock R110 and by Leal et al. [8] on Chardonnay. The low elicitation by *T. atroviride* could also be explained by both the short period of colonization (5 days) and the inoculation strategy (spores) adopted in this study, rather than a negative impact of LC2017 treatment on the BCA. In fact, despite the LC2017 ascertained fungistatic effect [29], *T. atroviride* was not affected by the LC2017 treatment [16]. It is to highlight the induction of genes linked to the "arsenite recovery effect" [29,30] in both cultivars, despite the considered treatment. These genes seem to play a role in the suppression of GTD foliar symptoms expression, observed in Esca-affected vineyards treated with LC2017 [30]. It will, therefore, be extremely interesting to carry out further research if this symptom suppression is to also be verified for field-grown vines infected with Botryosphaeria dieback-causing fungi.

Several other authors have shown that LC2017 has not only an eliciting effect on plant defense genes but also stimulates plant growth and photosynthesis [28–30]. These features, in addition to the capability of delivering a fungicide to a specific targeted area in the plant, may have the potential to block and/or limit infection by Botryosphaeria-dieback pathogens. Therefore, extended trials, especially on field-grown vines, are also recommended to assess the long-term impact of LC2017 on Botryosphaeria-dieback pathogens and to validate combination/s with other PPPs that may be of great interest in establishing an integrated management strategy against GTDs.

In conclusion, the use of the commercial product Esquive®, based *on Trichoderma atroviride*, is known to limit the annual GTD pathogens infection at pruning wounds in vineyards and contaminations in nurseries [7–9,13,15–17]. For LC2017, which is still under development, recent studies reported its fungistatic effect against GTD pathogens and an elicitor plant effect [28–30]. Firstly, the results confirmed the compatibility of Esquive® and LC2017, as reported by Reis et al. [16]. Then, the use of this combination to improve the control of *L. theobromae* associated with Botryosphaeria-dieback in our experimental conditions and according to the parameters targeted (lesion length, pathogen re-isolation, plant responses) does not appear to be relevant. Finally, the elicitor effect of LC2017 is confirmed, as well as a specific response related to the cultivar, as previously reported by Mondello et al. [29].

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