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Technical Note

EVALUATING THE EFFECT OF *TRICHODERMA ATROVIRIDE* (I-1237) ON GRAPEVINE PHOMOPSIS CANE AND LEAF SPOT: A PROMISING AND REPRODUCIBLE TRIAL

AVALIAÇÃO DO EFEITO DE *TRICHODERMA ATROVIRIDE* (I-1237) NA ESCORIOSE DA VIDEIRA: UM ENSAIO PROMISSOR E REPRODUTÍVEL

Jorge Sofia[1,2,*], Raul Sofia[3], João Vila-Maior[4]

[1]Instituto Nacional de Investigação Agrária e Veterinária, I.P., Polo de Dois Portos/Estação Vitivinícola Nacional, Quinta da Almoínha, 2565-191 Dois Portos, Portugal

[2]Center for Functional Ecology, Associate Laboratory TERRA, Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Portugal.

[3]University of Coimbra, Centre for Informatics and Systems of the University of Coimbra, Department of Informatics Engineering, Coimbra, Portugal.

[4]Independent Researcher.

* Corresponding author: Tel.: + 351261710500; e-mail: jorge.sofia@iniav.pt

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SUMMARY

A field trial with artificial infection was designed and performed to verify the potential effect of the application, as a pruning wound protectant for grapevine trunk diseases immediately after pruning, of a commercial product containing the biological control agent *Trichoderma atroviride* (I-1237), on later symptom expression of Phomopsis cane and leaf spot (PCLS) caused by *Diaporthe* spp.. The trial comprised four modalities: “Witness”, that remained in natural conditions without artificial infection and was sprayed with distilled water during the treatments; “Infected Witness”, with artificial infection and was sprayed with distilled water during the treatments.; “Reference Product”, with artificial infection and reference fungicide application at bud burst (BBCH 09), and “Test Product” in which the *T. atroviride* commercial formulation was applied as per the label, during dormancy (BBCH 00) immediately after pruning, and subject to artificial infection. The trial consisted of six replicates per modality with three plants per replicate. Incidence and severity of PCLS were assessed on the first four internodes in all the canes of all trial plants during development stages “fruit set” to “berries groat sized” (BBCH 71 to 73). The effectiveness of the artificial inoculation stands out; all the modalities showed lower incidence and severity than those of the "infected witness", and the reduction in incidence, compared to the "infected witness", was statistically significant for all modalities. Due to its short duration, the test did not allow the initial premise to be validated, but it validated the methodology used and its reproducibility. The suggestion that the pruning application of the *T. atroviride* formulation influences the subsequent manifestation of PCLS will need further testing in the following years to gauge these preliminary results.

RESUMO

Foi delineado e executado um ensaio com infeção artificial para verificar um potencial efeito sinérgico da aplicação, no momento da poda, de um produto comercial contendo *Trichoderma atroviride* (I-1237), homologado para doenças do lenho da videira à poda, no controlo posterior de Escoriose causada por *Diaporthe* spp.. O ensaio compreendeu quatro modalidades: “Testemunha”, modalidade que permaneceu em condições naturais sem infeção artificial e que, aquando dos tratamentos, foi pulverizada com água destilada; “Testemunha Infetada”, modalidade que sofreu infeção artificial e que na altura dos tratamentos foi pulverizada com água destilada; “Produto de Referência”, que sofreu infeção artificial e aplicação de fungicida de referência ao estado de ponta verde (BBCH 09); “Produto Teste” tratamento com um produto fitofarmacêutico contendo *T. atroviride* (I-1237) efetuado durante a dormência (BBCH 00), à poda, e em que todos as réplicas foram sujeitas a infeção artificial. O ensaio compreendeu seis repetições por modalidade com três plantas por repetição, sendo avaliadas a incidência e a severidade de Escoriose nos quatro primeiros entrenós em todas as plantas desde o vingamento ao bago de chumbo (BBCH 71 a 73). Destaca-se a efetividade da inoculação artificial; todas as modalidades ensaiadas apresentaram valores de incidência e severidade inferiores aos da “testemunha infetada”, sendo a diminuição da incidência face à modalidade “testemunha infetada” estatisticamente significativa. O ensaio, pela sua curta duração não permitiu validar a premissa inicial, contudo validou a metodologia utilizada, e a sua reprodutibilidade. A sugestão de que a aplicação à poda de *T. atroviride* (I-1237) influencia a manifestação posterior de sintomas de Escoriose só poderá ser verificada mediante a repetição do ensaio para validação destes resultados preliminares.

Keywords: *Trichoderma atroviride*, *Diaporthe spp.*, field trial, Phomopsis cane and leaf spot, Dão.
Palavras-chave: *Trichoderma atroviride*, *Diaporthe spp.*, ensaio de campo, Escoriose, Dão.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are a group of diseases affecting grapevines, caused by various fungal pathogens that inhabit and colonize the wood of perennial organs. These diseases lead to wood necrosis, discoloration, vascular infections, and white decay (Mugnai *et al.*, 1999; Bertsch *et al.*, 2013). Externally, affected vines exhibit a general and progressive decline, including delayed bud burst, dead buds, dieback, stunted development, chlorosis, and apoplexy. These symptoms are often associated with specific foliar manifestations depending on the disease and can initially result in loss of productivity, and ultimately lead to the death of the vine. Under the designation GTDs are included fungal diseases like Eutypa dieback, Botryosphaeria dieback, esca and grapevine leaf stripe disease, and Phomopsis dieback, primarily spread through the dispersion of airborne spores, and also through the use of infected cuttings. Baumgartner *et al*. (2013), Urbez-Torres *et al.* (2013) and Gramaje *et al.* (2018) verified that Phomopsis dieback presence was particularly high in vineyards severely affected by Phomopsis cane and leaf spot (PCLS), associated to *Diaporthe* spp., namely *Diaporthe ampelina* (= *Phomopsis viticola*) (Gomes *et al.*, 2013; Guarnaccia *et al.*, 2018). PCLS is a common disease throughout the winegrowing world, the occurrence of which depends on spring weather conditions and the health history of the vineyards (Pereira *et al.*, 2012). The first reports of its presence in Portugal date back to 1951, but it was not until the 1970s that it became a recurring concern in Portuguese viticulture (Tomaz and Rego, 1990). Symptoms of the disease appear from bud burst, at the base of the new shoots, and are characterized by small dark brown cortical necrotic spots, initially elongated and later confluent. In autumn and winter, it is possible to observe bleaching of the first internodes of the affected canes with small black dots, which are nothing more than the pycnidia of the fungus. After pruning, the affected buds left to produce new shoots, tend to die or may produce stunted shoots.

In the Dão Wine Region, the problem of PCLS has been taking on increasing proportions and is currently a major concern for producers (Sofia *et al.*, 2013). Notifications are usually issued by local warning stations, considering the phenological development of the grapevines, advising of the need to control this disease, and always endeavouring to alert growers to the opportunity, need, and products available (SNAA, 2023). Although there are no systematic surveys of the situation regarding this disease, it is recognized that cv. 'Touriga Nacional', an emblematic cultivar of that region, is one of the most susceptible (Bohm *et al.*, 2011; Eiras-Dias *et al.*, 2011; Pereira *et al.*, 2012; Magalhães, 2015).

Traditionally, PCLS control in Portugal has been based on the preventive application, at "bud burst" stage (BBCH 09) of fungicides containing sulphur, phthalimides, phosphonates, QoI-fungicides, quinones, and dithiocarbamates, single or in a mixture (SIFITO, 2023).

Due to global restrictions on the use of many chemicals, there is growing interest in non-chemical and natural barriers (Soares *et al.*, 2023), including biological control agents (BCAs), for managing GTDs (Gramaje *et al.*, 2018). Furthermore, developing more diverse long-term control solutions for integrated disease management programs has become a priority. In particular, *Trichoderma* spp. have been extensively studied (Köhl *et al.*, 2019).

Several *Trichoderma* species have shown success as pruning wound protectants against GTDs (Mutawila *et al.*, 2016a,b; Pertot *et al.*, 2016) with effect on *Phomopsis viticola* (Król, 2004; Kotze *et al.*, 2011; Mutawila *et al.*, 2016a). *Trichoderma atroviride* I-1237 was demonstrated to be effective in wound protection against esca, Botryosphaeria dieback, and Eutypa dieback, reducing leaf symptom expression after at least a 2-year application (Mounier *et al.*, 2016), and reducing the infections on pruned canes artificially inoculated with *Phaeomoniella chlamydospora*, associated to the esca complex and *Neofusicoccum parvum*, associated to Botryosphaeria dieback (Reis *et al.*, 2017). *Trichoderma atroviride* (I-1237) is registered in Portugal as a pruning wound protectant against GTDs to be applied immediately after pruning although not for PCLS caused by *Diaporthe* spp.

Winegrowers, who applied the *T. atroviride* phytopharmaceutical, reported a side-effect of the application of the wound protectant on reducing PCLS symptoms, when applied *as per* label, meaning after pruning during dormancy (BBCH 00).

Field trials play a pivotal role in evaluating the efficacy of phytopharmaceutical products in addressing PCLS, a significant concern in grapevine cultivation. The aim of this study was to present preliminary results obtained on an in-field trial designed to evaluate the potential effect, reported by winegrowers, of a commercial formulation containing *Trichoderma atroviride* (I-1237), authorized for protecting pruning wounds against GTDs, on the control of PCLS symptoms in new shoots, and to validate the methodology for reproducibility in future trials on the same subject.

MATERIALS AND METHODS

Plant material

The experiment was conducted in 2020 in a commercial twenty-year-old cv. 'Touriga Nacional' grafted on 1103 Paulsen vineyard, located at the Dão Wine Region in the municipality of Nelas, Viseu, Portugal (40°33'41.12"N, 7°50'28.16"W). Grapevines were trained in single Guyot, usual in that region. The plot showed mild symptoms of PCLS, that were observed during the prospection carried out in an earlier season. All the replicates were pruned on the same day, 6th March 2020.

PCLS assessment

To secure plant material for artificial inoculation, in 2019, a survey was carried out in vineyards throughout the Dão wine region to detect grapevines with canes presenting severe symptoms of PCLS, that would be collected later during winter. To ensure that most of the PCLS lesions on collected canes were associated with *Diaporthe* spp., small tissue pieces (<5 mm) were excised from the margins of the first internode of 30 canes of grapevines showing symptoms of PCLS. Each piece was surface disinfected in a 1,5% sodium hypochlorite solution, for one minute, rinsed with sterile distilled water to remove the excess of sodium hypochlorite and then dried with sterile filter paper. The excised piece was then sectioned into four fragments and plated in PDA (PDA; Difco, Beckton, Dickinson and Co, Sparks, MD, USA) amended with 250 µg of chloramphenicol (BioChemica, AppliChem, Germany), four fragments per PDA plate, sealed and placed to incubate at 24°±1°C, under complete darkness. Plates were observed every two days until fungal growth and until pycnidia were visible and well-formed allowing the identification of *Diaporthe* spp.. Confirmation of the presence of *Diaporthe* spp. was based on the morphological and cultural characters of the colonies and microscopic observation of spores (Van Niekerk *et al.* 2005; Gomes *et al.*, 2013).

Inoculum preparation

The success of natural contamination is dependent on favorable spring weather conditions, that is rainfall and mild temperatures, during the sensitive bud stage. The risk of a dry spring could therefore cause the experiment to fail, hence the decision to proceed to artificial infection by two different methods, natural inoculum, and spore spraying. Canes showing evident symptoms of PCLS were collected from diseased grapevines in the Dão wine region, during the previous winter, and kept at 4°C. Two weeks before the expected date for artificial infection, canes were taken out of the cold and left outside out of the rain, to allow pycnidia reactivation, according to Bugaret (1997). For preparation of the spore suspension for spraying, fragments of the infected canes containing the first four internodes were cut off and placed in a moist environment three days before inoculation. After testifying the presence of abundant *cirri*, the fragments were left to wash on a tray containing approximately 2 L of sterile distilled water and placed on a reciprocal shaker (125 strokes/min) for 24 hours at room conditions, to produce a stock solution. Six hours before the spraying, the conidial suspension was adjusted to 2×10^5 conidia/mL (α-conidia) with sterile distilled water using a hemocytometer (Burker, HBG, Giessen, Germany) as indicated in Bugaret (1997) and kept at room temperature until use.

Inoculation procedures

To ensure the success of the contamination and allow strong parasite pressure on the phenological stages considered most receptive to the disease, natural infection was also promoted by suspending fragments containing the first four internodes of PCLS-affected canes hung on the trellising wire, positioned well above the grapevines in each plot, at approximately 20 cm from each other. The contaminating fragments were placed about 15 days before "bud burst" stage (BBCH 07/09) and were kept in place throughout the susceptibility of the young shoots, with at least 30 to 40% of buds reaching the beginning of "bud burst" stage (BBCH 07) to 30 to 40% of buds reaching the "three leaves unfolded" stage (BBCH 13). Spore spraying was carried out at the end of the day, with high relative humidity, to keep the plant moist for the time needed for the spores to germinate. The spore suspension spraying was carried out on the same day of its preparation by copious spraying of the entire canes using a small pre-pressure sprayer.

Experimental design

The reference product, chosen as recommended in the OEPP PP 1/55(2) standard (EPPO, 1996), is a commercial product containing a mixture of 25% de folpet and 50% fosetyl-Al, authorized for PCLS control with a single application at phenological stages BBCH 07/09, at a dose of 900 g (commercial product) per hectare. A commercial formulation containing 10^8 UFC/g of *Trichoderma atroviride* (I-1237) was used as the test product. The formulation is registered in Portugal as a pruning wound protectant against fungi associated with GTDs, although not for PCLS caused by *Diaporthe* spp., at a dose of 4 kg/ha on one application per year as soon as possible after pruning.

Both products were prepared *in situ* at the time of spraying. The trial comprised four modalities: "Witness", which remained in natural conditions without artificial infection and no fungicide spray; "Infected witness", with artificial infection and no fungicide spray; "Reference product", with artificial infection and reference product (RP) application, and

"Test Product" in which the test product (TP) was applied as per the label, shortly after pruning, during dormancy (BBCH 00), and where all replicates were subject to artificial infection. At BBCH 09 the reference product was applied just to "Reference Product", while all the other modalities were sprayed just with water.

The trial was laid out as a randomized complete block design, consisting of six replicates per modality and three plants per replicate.

Incidence of PCLS, as the percentage of infected canes and severity of PCLS, as the percentage of necrotic surface on the infected canes, were assessed on the first four internodes in all the canes of all trial plants during growth stages "fruit set" to "berries groat sized" stage (BBCH 71 to 73).

Weather data

Weather data were acquired and recorded using a "Davis Vantage Pro 2 Plus" Automatic Weather Station positioned approximately 3 km from the experimental plot. Air temperature, relative humidity, wind speed and direction, and rainfall, were monitored. The proximity of the weather station to the plot minimized the potential for spatial variability in weather parameters and ensured that the recorded data accurately reflected the microclimate conditions experienced by the experimental plot.

Data analysis

The collected data set was analyzed using the statistical software PAST (version 4.05) through a one-way analysis of variance (ANOVA), with the null hypothesis stating that there were no significant differences between treatments. Data expressed as proportions were transformed using the arcsine square root transformation, and the homogeneity of variances was verified with Levene's test. Differences were considered significant when $p < 0.05$. If the null hypothesis was rejected, Tukey's post-hoc test was applied to distinguish between the treatments at that level of significance. Data visualization was performed using R software (version 4.3.1) and the ggplot2 package (version 3.3.2). The data pipeline, from raw to visual representation, consisted of preprocessing, using custom R scripts to address outlier management and normalization steps needed for some of the plots, and finally feeding them to the visualization engine.

RESULTS AND DISCUSSION

Figure 1 shows the weather data recorded in March 2020. Pruning of all the plants in all the replicates was delayed avoiding the risk and consequences of late frosts, taking place during BBCH 00 on the 6th of March.

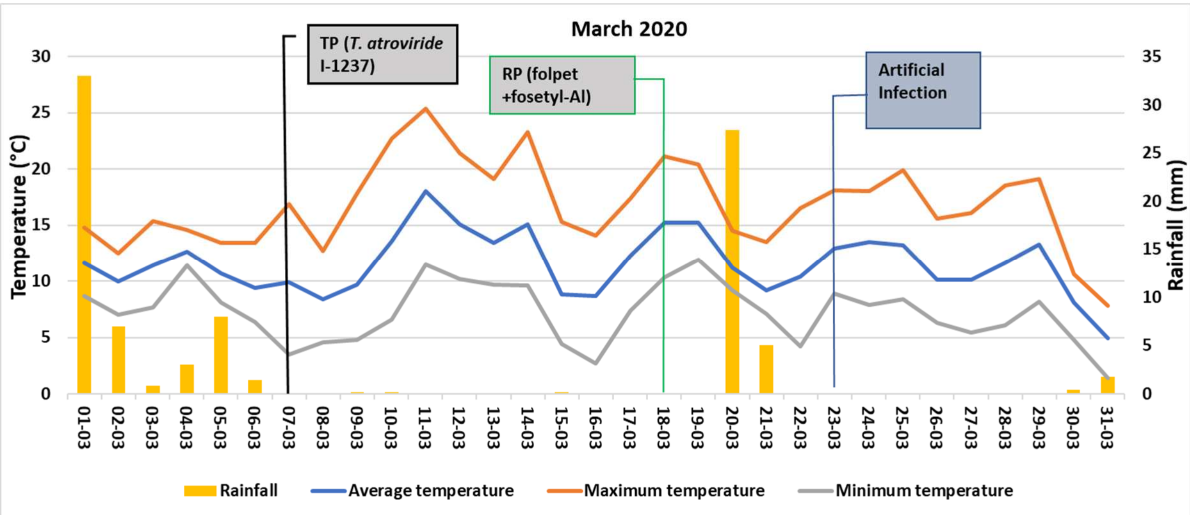

Figure 1. Weather data recorded in March 2020. The dates of artificial contamination, reference product and test product applications are indicated.

TP was immediately applied afterwards, to ensure better product protection and efficacy (Mutawila *et al.*, 2016a). The reference product was applied at the beginning of BBCH 09, on the 18th of March with fair weather. Artificial inoculation by spraying a spore suspension was performed on the 23rd of March, after the rainfall of the 21st and 22nd of March, ensuring good conditions for spore germination and contamination, with mild temperatures above 12 °C and relative humidity above 60%. The weather conditions in the following month (April) were favourable to the onset of the disease and the consequent manifestation of PCLS symptoms due to high rainfall and favourable temperatures (Figure 2). Analysing the incidence of PCLS in this trial, Table I and Figure 3, show undoubtedly that the incidence ranged in "Infected Witness" from 74,2% to 100%, with 50% of all the plants showing values of incidence up to 89%, thus demonstrating the effectiveness of artificial inoculation.

The "Test Product", "Reference Product" and "Witness" modalities showed significantly lower incidence values than the "Infected Witness".

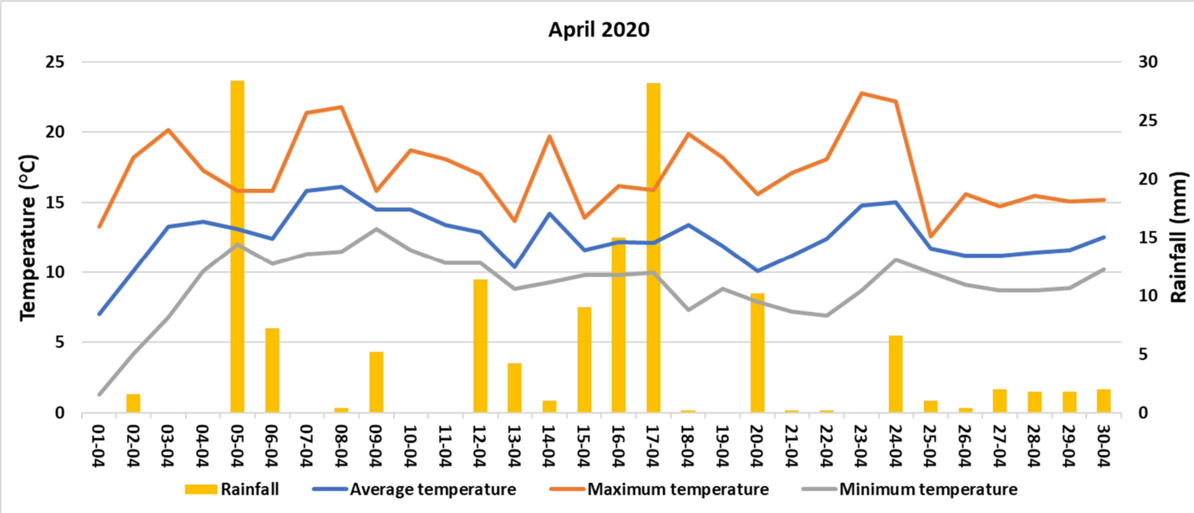

Figure 2. Weather data recorded in April 2020.

As expected, "Reference Product", by its nature, performed better in controlling the incidence of PCLS with 50% of the plants showing up to 46,7 % of the incidence of PCLS with a maximum value of 51,4% and a minimum of 33,3%. Modality "Witness" showed 50% of the plants with a value up to 39,7%, a minimum of 29,2%, and a maximum of 47,8%, indicating probable natural contamination. Regarding "Test Product", 50% of the plants showed incidence up to 50%, with a maximum of 74% and a minimum of 38,5%

Table I

Values of incidence (%)[1]

Modality	Min	Max	Q1	Median	Q3	Mean
Test Product	**38.5**	**72.4**	**40.3**	**50.0**	**64.8**	**52.4 b**
Reference Product	**33,3**	**51.4**	**40.5**	**46.7**	**46.7**	**43.7 b**
Witness	**29,2**	**47.8**	**38.8**	**39.7**	**47.8**	**40.7 b**
Infected Witness	**74.2**	**100**	**77.7**	**89.0**	**97.2**	**87.9 a**

[1]Mean values followed by different letters were significantly different according to Tuckey's test for p<0.05.

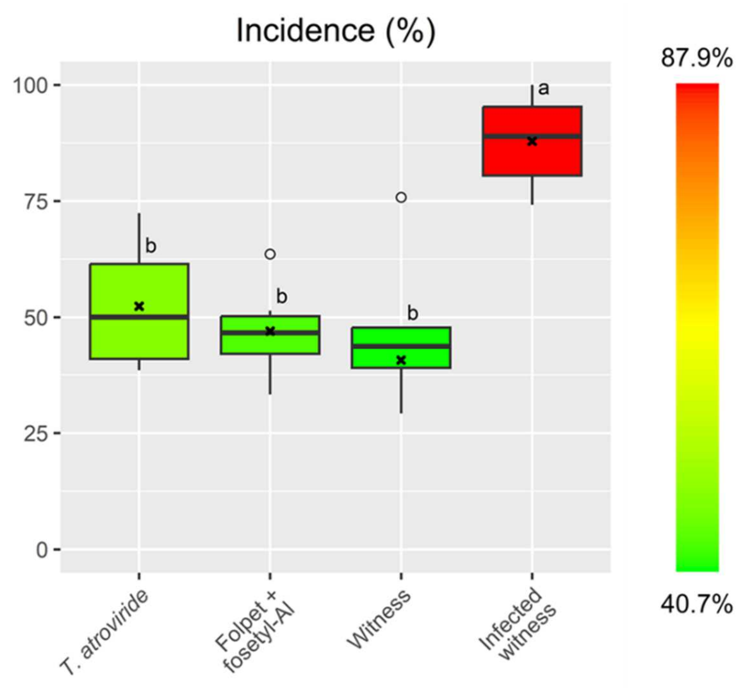

Figure 3. PCLS incidence (%). X represents the average. Color gradient scale from lowest incidence (green) to highest incidence (red). Different letters indicate significant differences according to Tuckey's test for p<0.05.

Analysis of the severity of PCLS (Table II and Figure 4) in this trial did not reveal statistically significant differences between the different modalities. Nevertheless, "Reference Product", "Test Product" and "Witness" presented lower values of severity than "Infected Witness". "Infected Witness" presented a severity of up to 35,3% for 50% of the plants with a maximum of 42,4% and a minimum of 24,7%. The modality "Reference Product", with 50% of the observations with a severity up to 21,8%, presented a maximum of 23,6% and a minimum of 12,4%, values expectable as this is a well-known mixture for preventive control of PCLS. "Test Product" with 50% of the samples showing a severity up to 27,2%, showed an intermediate performance. As to the modality "Witness", 50% of the observed showed a severity up to 18,7% with a maximum of 32,9% and a minimum of 12,6%, once again indicating a probable natural inoculation, as PCLS symptoms had been recorded in the plot.

Table II

Values of severity (%)[2]

Modality	Min	Max	Q1	Median	Q3	Mean
Test Product	**17.4**	**38.3**	**20.6**	**27.2**	**36.5**	**28.0**
Reference Product	**12.4**	**23.6**	**15.7**	**21.8**	**22.6**	**19.8**
Witness	**12.6**	**32.9**	**13.1**	**18.7**	**29.1**	**20,7**
Infected Witness	**24.7**	**42.4**	**29.0**	**35.3**	**41.2**	**34.8**

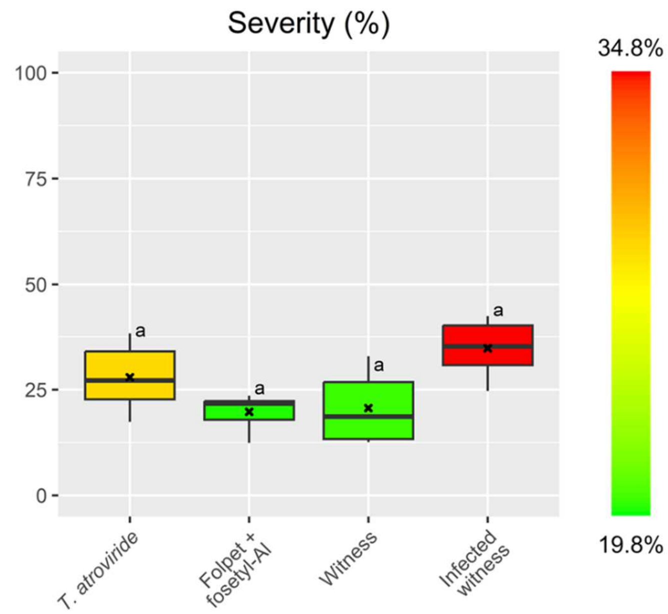

Figure 4. PCLS severity (%). X represents the average. Color gradient scale from lowest severity (green) to highest severity (red).

The heatmaps presented in Figures 5 and 6, show the layout of the different modalities and their respective repetitions in the test plot and the gradation of incidence and severity. It is noticeable that both the incidence and severity values were evenly distributed across the plot, with no apparent bias. In the period following inoculation, a north wind with speeds of 20 to 43 km/h was recorded, but the distribution of the intensity of incidence and severity in the different modalities seems to favour the thesis that infection in the "Witness" modality was due more to the natural contamination by inoculum already present than to air-borne spores at the time of inoculation, performed in the late afternoon when winds were less intense.

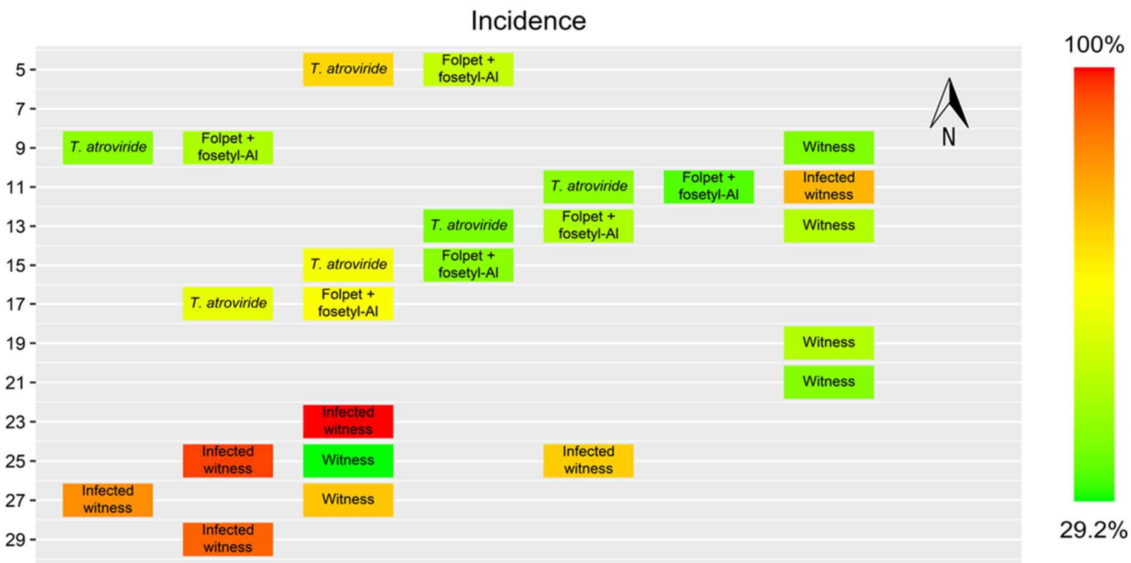

Figure 5. Heatmap of the distribution of the incidence of PCLS in the trial plot with the indication of the modalities. The numbers on the left indicate the trial lines. Color gradient scale from lowest incidence (green) to highest incidence (red).

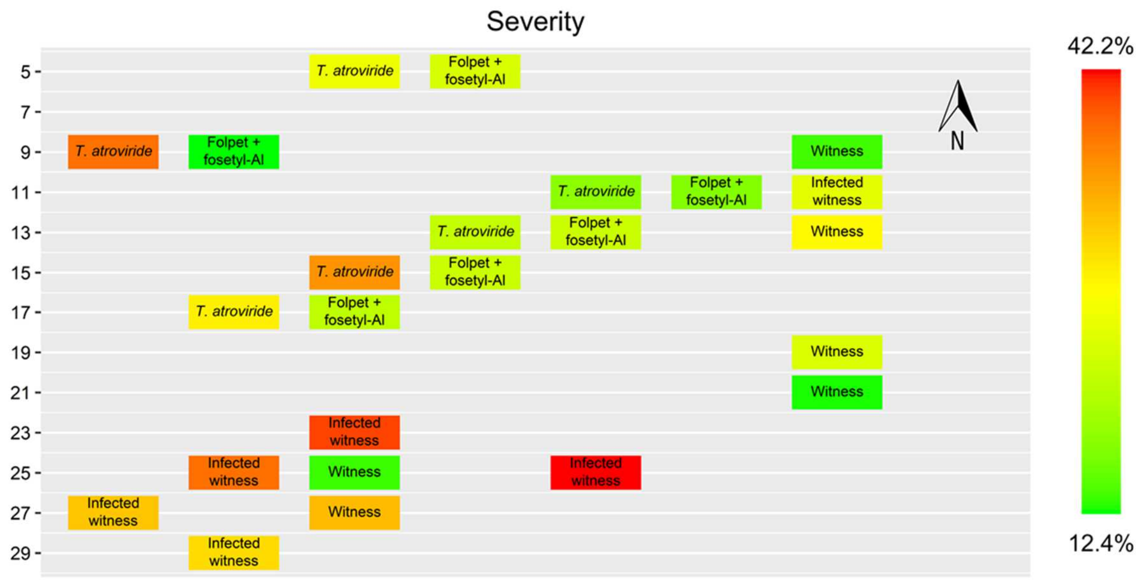

Figure 6. Heatmap of the distribution of PCLS severity in the trial plot with the indication of modalities. The numbers on the left indicate the trial lines. Color gradient scale from lowest severity (green) to highest severity (red).

The significant reduction in incidence observed in this preliminary trial aligns with the findings of Leal *et al.* (2024), who reported similar effects in a study conducted over three successive seasons in vineyards in France and Spain using the same formulation of *T. atroviride* (I-1237). These authors also noted high recovery percentages of *T. atroviride* from canes one year after infection, indicating its persistence in the field. This persistence likely contributed to the observed reduction in PCLS symptoms associated with *Diaporthe* spp. infections in the present study, particularly given the weather-favourable conditions following the application of *T. atroviride* (I-1237) (Mutawila *et al.*, 2015; Martínez-Diz *et al.*, 2020; Carro-Huerga *et al.*, 2021).

Future Prospects

These results are preliminary, and further trials are necessary in future grape-growing seasons and different locations, as environmental factors like climatic conditions significantly impact the manifestation of GTDs (Sofia *et al.*, 2018; Songy *et al.*, 2019). This study relied on artificial infections because natural infections do not guarantee robust results (Ayres *et al.*, 2022). It is generally accepted that a phytopharmaceutical proven effective under high inoculum pressure will also be effective under natural infection conditions (Ayres *et al.*, 2022). Discarding a formulation that is ineffective under high pathogen inoculum pressure could be misleading, as it might perform well under natural conditions. For instance, *T. atroviride* I-1237 was ineffective against artificial infections by *Diplodia seriata*, associated with Botryosphaeria dieback (Martínez-Diz *et al.*, 2021), but Leal *et al.* (2024) demonstrated its effectiveness against natural infections by Botryosphaeriaceae spp. Therefore, the continuation of this study should also consider the effect of *T. atroviride* I-1237 on PCLS incidence in naturally infected plants.

CONCLUSIONS

The integration of BCAs like *Trichoderma atroviride* into disease management strategies aligns with the ethos of reducing chemical inputs, fostering eco-friendly approaches, and enhancing sustainability in agriculture. Thus, investigating the efficacy of *T. atroviride* (I-1237) in mitigating PCLS not only addresses the immediate concerns of grapevine health but also explores the potential of natural compounds in meeting the standards set by the "Farm-to-Fork" policy.

The trial aimed to provide empirical evidence regarding the possibility of a side-effect of a phytopharmaceutical product containing *T. atroviride* (I-1237) in managing PCLS symptomatology, contributing insights into the role of natural substances in modern phytopharmaceuticals.

Ultimately, such endeavors are intended to benefit winegrowers by offering sustainable disease control options and aligned with broader agricultural policies promoting environmentally conscious practices in the Farm-to-Fork continuum. This study suggests a potential effect of the *Trichoderma atroviride* (I-1237) formulation in the subsequent control of PCLS. However, it also enabled validation of the methodology, confirming both the success of the artificial infection process and the suitability of the reference product, which proved effective in controlling PCLS, as its incidence and severity values did not differ greatly from the "Witness" modality that only showed slight natural infection. The reductions in severity and incidence, statistically significant in the latter, for all modalities when compared to "Infected Witness", are suggestive of the expected effect, but only the continuity and repetition of this work will be able to confirm the stated hypothesis.

Data availability statement

All relevant R scripts and data are available on request addressed to the authors.

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