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The Influence of Temperature on the Growth, Sporulation, Colonization, and Survival of *Trichoderma* spp. in Grapevine Pruning Wounds

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Abstract: *Trichoderma* is a genus of fungi used for the biological control of plant diseases and a large number of its bio-formulates are available in the market. However, its efficacy under field conditions remains unclear, especially for the protection of grapevine plants against Grapevine Trunk Diseases (GTDs). These diseases are caused by a complex of fungal pathogens whose main point of entrance into the affected plants is through pruning wounds. In this research, different *Trichoderma* native strains have been evaluated according to their ability to grow at different temperatures and their capacity to colonize pruning wounds in adverse climatic conditions. Strains from section *Trichoderma* have adapted to cooler conditions. On the other hand, strains from clade *Harzianum/Virens* grow at higher temperatures. However, differences can also be found between strains inside the same clade/section. Native strains were able to colonize more than 70% of vine pruning wounds in winter conditions. The *Trichoderma* strain T154 showed a significantly higher re-isolation degree from vine plants and its concentration was optimized for spraying onto vine plants. In conclusion, *Trichoderma* native strains are better adapted to survive in a changing environment, and they could give better protection to grapevine plants in co-evolution with each specific vineyard.

Keywords: grapevine trunk diseases; microbial biological control agents; native strains; climate change; crop protection; abiotic factors

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1. Introduction

Trichoderma spp. Pers. are ubiquitous filamentous fungi, some of which are able to produce beneficial outcomes in crop production and have been used as natural pathogen antagonists in agricultural fields for a long time [1]. Some of them have been isolated and selected as biological control agents to be used in integrated pest management strategies to reduce the use of chemical pesticides [2]. This selection is due to their capacity to use diverse mechanisms involving the microorganisms themselves, their genes, and their metabolites [3].

Therefore, introducing *Trichoderma* as a biological agent in viticulture could have a positive impact on grapevine plants [4]. *Trichoderma* has been proven to be an effective biological control agent (BCA) against *Botrytis cinerea* Pers. together with integrated pest management strategies [5]. This biological control agent also reduced Grapevine Leaf Stripe Disease (GLSD) symptoms in vine plants over a long period of time [6]; these symptoms are associated with fungal species of *Phaeoacremonium minimum*, and other species of *Phaeoacremonium* and *Phaeomoniella chlamydospora* [7]. Moreover, this fungus can induce

resistance against downy mildew caused by *Plasmopara viticola* (Berkeley and Curtis) and Berlese and de Toni in grapevine plants [8]. Moreover, its secondary metabolites are able to control some other pathogens such as *Neofusicoccum parvum* (Pennycook and Samuels), P.W. Crous, Slippers and A.J.L. Phillips and *Eutypa lata* (Pennycook and Samuels) Crous, Slippers, and A.J.L. Phillips [9].

In fact, in the last few years, species of *Trichoderma* have been widely studied for their action against Grapevine Trunk Diseases (GTDs), which has enabled important advances in terms of reducing the impact of GTDs [4]. This complex of diseases can be divided individually into six different types of disease: Petri disease, Black foot, Botryosphaeria dieback, Eutypa dieback, Phomopsis dieback, Esca, and Grapevine Leaf Stripe Diseases, and each of them are associated with a complex of fungal pathogens that cause these diseases [10]. Petri disease is caused by *Phaeomoniella chlamydospora*, 29 species of *Phaeoacremonium*, *Pleurostoma richardsiae*, and 6 species of *Cadophora* [11–13]. Black foot is caused by up to 24 species belonging to the genera *Campylocarpon*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria*, *Neonectria*, and *Thelonectria* [14–16]. Esca and Grapevine Leaf Stripe Disease are described as successive invasions of fungi, where the pioneer fungi are *P. chlamydospora* and/or species of *Phaeoacremonium* and after that, basidiomycetous species continue to colonize the grapevine wood [10]. Phomopsis is caused by the genera *Diaporthe* but seven species have been described as pathogenic on grapevine wood [17,18]. Botryosphaeria dieback is mainly associated with taxa from the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, and *Neofusicoccum* in grapevines [19–21]. Eutypa dieback is caused by species from the genera *Diatrypaceae* and the most virulent and common is *Eutypa lata* [22,23]. All spores of these GTD fungi can infect grapevine plants and annual pruning wounds are the primary point of entry [10].

However, *Trichoderma* species do not always act as effective biocontrols for grapevine diseases and fungal species of GTDs. For example, *Trichoderma* did not significantly reduce the incidence of black foot (the majority of fungal species associated to *D. torresenis*, *D. macrodidyma*, *Dactylonectria novozelandica*, and *Ilyonectria liriodendri*) in comparison to other treatments [24]. In addition, no positive effects were obtained in relation to the shoot and root weight in grapevine plants including a non-significant reduction in symptoms caused by black foot disease (caused by *Dactylonectria torresensis*, *Dactylonectria macrodidyma*, *Ilyonectria liriodendri*, and *Dactylonectria alcacerensis*) and Petri disease (caused by *Cadophora luteo-olivacea*, *Phaeoacremonium minimum*, and *Phaeomoniella chlamydospora*) and diseases in all BCAs assayed such as *Trichoderma*, *Streptomyces*, *Pythium*, *Pseudomonas*, and *Bacillus* spp. [25]. Different *Trichoderma* bio-formulated-based treatments applied on grapevine roots reduced their weight significantly during the first year, and these fungi were not able to prevent infections caused by the black foot disease pathogens identified as *Campylocarpon* spp., *Dactylionectria*, *Ilyonectria* spp., and *Ilyonectria liriodendri* [26]. Moreover, some experiments that compared *Trichoderma* bio-based commercial agents and chemical products have concluded that chemicals are more efficient in terms of pathogen control (*Eutypa lata*, *Diplodia seriata*, and *Phaeomoniella chlamydospora*) than the *Trichoderma* biological control agents assayed [27,28].

The different effects observed could be strongly influenced by the disease tetrahedron concept, where the pathogen, host, environment, and biotic factors play a key role [29]. Among them, environmental factors are thought to have a major impact on disease development [30]. The effects of temperature are considered to be an important parameter for proper biocontrol activity [31]. Recent research has found that temperature and moisture conditions affect the growth and survival of the theoretical BCA significantly. They could be considered key factors for efficient biocontrol [32]. Moreover, another experiment showed that temperature and moisture levels could be used to make good predictions for effective biocontrol using *Trichoderma* among other BCAs against *Botrytis cinerea* in grape berries [33]. Moreover, an increase of 5 °C can significantly influence the response of *Trichoderma* in relation to the induction of the plant response in tomato plants [34]. All these environmental factors and the alteration in temperatures due to climate change [35]

represent major challenges in wine production. Temperatures are increasing worldwide, and this change is leading to a lack of water, shifts in the ripening phase, and modifications in grape composition, along with other changes such as the increase in extreme climatic events [36]. Rising temperatures lead to an advanced phenology and sugar accumulation in *Vitis vinifera* L. [37]. On the other hand, it has been reported that the negative effects caused by increasing temperatures and higher CO_2 could be mitigated by a higher ultraviolet-B radiation that could modulate the accumulation of sugars and upregulate anthocyanin biosynthesis in *V. vinifera* cv. Tempranillo [38]. Furthermore, many other harmful effects have been described and have pointed to the need to use new suitable molecular techniques in order to mitigate these effects [39].

Currently, many studies are being conducted to characterize the effects of abiotic factors due to climate change which involve changes in the physiology, phenology, and harvesting of grapevine plants [40–42]. Some studies concluded by emphasizing the importance of evaluating the effects of these changing environmental conditions on the biological control microorganisms in grapevine plants, in order to predict their possible adaptations to the changing temperatures and relative humidity conditions [43–45]. Most *Trichoderma* species are mesophilic, and low winter temperatures might be a problem for their biological activity and their emergence, which is also due to dry conditions [31]. To summarize, it is important to evaluate the influence of different temperature conditions of *Trichoderma* on the different species, and isolate selected ones as putative biocontrol agents. These assays must be carried out previously under semi-field conditions before being sprayed in the field, because an effective biocontrol activity in in vitro conditions could lead to failure in the field due to a deficient adaptation to the environment, and as a result prevent the growth and the performance of any biological activity. The importance of using native strains to obtain a better protection for grapevine pruning wounds could be a key factor due to the possibility that they possess better adaptative responses to these conditions.

Based on this, we described and assessed different studies that would be required in order to carry out different assays to characterize a biological control agent under the current changing environmental conditions before being sprayed in the field. These experiments have been divided into two stages: (i) testing different *Trichoderma* strains at different temperatures and their effects on fungal mycelia and spore development to select the best strains, and (ii) evaluating the colonization of pruning wounds under winter conditions in the event of climate change.

2. Materials and Methods

2.1. Fungal Isolates

Four *Trichoderma* strains (T065, T071, T154, and T214) were obtained from the culture collection of the Plant and Pest Diagnostic Laboratory under the accession numbers ULET065, ULET071, ULET154, and ULET214, respectively (University of León, León, Spain). All isolates were selected due to their biocontrol activity and were isolated from vineyards from Castilla y León (Spain) (Table 1). *Trichoderma* spp. T065 and T214 were selected based on their high potential to produce secondary metabolites; T214 showed a remarkable mycoparasitic activity against the pathogenic fungus *Phaeoacremonium minimum* (unpublished data); *Trichoderma* T071 is effective against the *Xylotrechus arvicola* insect pest [46]; and *Trichoderma* sp. T154 also possesses significant mycoparasitic activity against *P. minimum* [47].

Table 1. *Trichoderma* isolates used in this study.

Number Isolate	Reference [1]	Origin	Main Mechanism of Biocontrol
T065	This study	Vineyard soil	-
T071	*T. gamsii* (Section *Trichoderma*) [46].	Vineyard soil	Mycoparasistim in insects
T154	*Trichoderma* spp. (Clade *Harzianum/Virens*) [47].	Grapevine bark	Mycoparasistim of fungi and niche exclusion
T214	This study	Grapevine bark	-

[1] Section/clade identification performed according to Kubicek et al., 2019 [48].

2.2. Fungal Identification

The identification of *Trichoderma* was performed using sequences of five housekeeping genes (*act1* (encoding for the actin); *cal1* (calmodulin); *fas1* (fatty acid synthase alpha subunit); *rpb2* (RNA polymerase 2nd largest subunit), *lcb2* (sphinganine palmitoyl transferase subunit 2); and *tef1* (translation elongation factor 1-alpha)) retrieved from the genome sequence of T065 and T214 isolates. BLASTn software was used to compare these six genes of the genome of *T. harzianum* CBS 226.59 against the genome sequences of T065 and T214, using the sequences of fungal homologous genes as queries. (Supplementary Material Table S1). Finally, intron regions from the six housekeeping genes obtained were manually removed, the resulting coding sequences were compared to NCBI Genbank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov, accessed on 21 July 2021) databases using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, accessed on 21 July 2021), and a phylogenetic tree was performed as indicated in the legend of Supplementary Material Figure S1. In conclusion, *Trichoderma* T065 belongs to Section *Trichoderma* (ST) and *Trichoderma* T214 belongs to Clade *Harzianum/Virens* (HV) (Supplementary Material Figure S1).

Trichoderma T154 was classified into Clade *Harzianum/Virens* (HV) [47] and *Trichoderma* T071 was assigned to Section *Trichoderma* (ST) [46]. The criteria followed for this assessment was applied according to Kubicek et al., 2019 [48].

2.3. Growth Evaluation

An in vitro evaluation of the four potential BCAs (T065, T071, T154, and T214) was carried out to compare the effects of temperature in cultures grown on 90 mm Petri dishes containing a potato dextrose agar (PDA) medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Mycelial development and sporulation were evaluated in order to determine their capacity to face constantly changing environmental conditions. This protocol was designed to evaluate the colonization capacities of the *Trichoderma* native strains with regard to biocontrol in relation to GTDs, protecting vine pruning wounds in our local region (Castilla y León, Spain), and trying to avoid the entrance of GTD pathogens, the most harmful diseases in viticulture and the ones without any available effective control [10]. First, the most common fungi associated with the species of GTDs in Castilla y León region (Spain) were selected and identified according to Cobos et al., 2007 [49]. The range of growth of these pathogens was then retrieved from the bibliography (Table 2) and a range of temperatures from 4 °C to 35 °C was chosen to test the ability of *Trichoderma* native strains to grow at these temperatures.

Mycelial growth was assessed as follows: each plate was inoculated with one agar plug (7 mm diameter) of a *Trichoderma* strain collected from a 7-day-old culture grown on a PDA. Plates were incubated in the dark at 4, 15, 25, and 35 °C. Mycelial growth was assessed daily except for the plate at 4 °C that was evaluated after six weeks. The experiment was performed with a completely randomized design where each plate was considered a biological replicate, and a total of three replicates were evaluated; moreover, this experiment was conducted twice in order to ensure reproducibility. Data from both

experiments were combined ($p < 0.05$), and a total of six biological replicates were used for the statistical analysis.

Table 2. Temperature growth range of the main fungi associated with GTDs in Castilla y León region.

Most Frequent Fungi Associated with GTDs Isolated in Castilla y León Region [1]	Minimal Temperature for Mycelial Growth (°C)	Maximum Temperature for Mycelial Growth (°C)	Reference of Temperature Conditions
Botryosphaeria dothidea (Moug.) Ces. and De Not	4	35	[50]
Botryosphaeria parva Pennycook and Samuels	4	35	[50]
Botryosphaeria stevensii Shoemaker	15	33	[51]
Diplodia seriata De Not	4	35	[50]
Phaeoacremonium minimum (Tul. and C. Tul.) Gramaje, L. Mostert and Crous	10	30	[52]
Phaeomoniella chlamydospora (W. Gams, Crous, M.J. Wingf. and Mugnai) Crous and W. Gams	10	35	[52]

[1] species described according to Cobos et al., 2007 [52].

Spore production was evaluated for each *Trichoderma* strain from a 7-day-old culture grown on a PDA medium grown at 25 °C. Spore suspensions were prepared from these cultures by dislodging the spores with a sterile loop and filtering them through a sterile cheese cloth to remove mycelium fragments. The spores were counted using a haemocytometer. Two technical replicates per plate and three replicates were evaluated. This experiment was performed twice.

In addition, the evaluation of the mycelial growth from the spores was also assayed at low temperatures. They were incubated at 4 °C and 15 °C, and two concentrations of spores were evaluated, 2×10^{-6} and 2×10^{-7} colony forming units (CFU)/mL. Mycelial growth was assessed daily at for the plate at 15 °C and for the plate at 4 °C it was evaluated after six weeks. In all cases, radial growth was evaluated usually after seven days, which is the time that the fastest fungus needed to completely colonize a Petri dish.

2.4. Evaluation of Trichoderma Survival over Pruning Wounds in Planta

The survival of the *Trichoderma* strains was assessed over pruning wounds in planta under semi-field conditions. This condition involved potted vine plants in a protected environment. *Trichoderma* strains T071 and T154 were selected for this experiment; in the case of T071, this strain was selected due to its capacity to grow at low temperatures, and T154 was selected for its ability to produce a high number of spores and also for its ability to grow significantly at higher temperatures. They were grown on a PDA medium at 25 °C in the dark for 7 days. It was decided to inoculate a total amount of 50 µL in a concentration of 2×10^{-6} CFU/mL and 2×10^{-7} CFU/mL suspensions of T071 and T154 which were sprayed over a pruning wound to prevent the entrance of fungal pathogens within a period of 24 h after being pruned [53].

One-year-old dormant grapevine grafted plants of the Tempranillo/110 Richter combination from Vivai Cooperativi Rauscedo (Rauscedo, Italy) were potted in June 2017. The pots contained a sterile mixture of vermiculite and peat (1:1 *v/v*). Each plant was considered a biological replicate and eight biological replicates per treatment were performed. These plants grew during the vegetative season and, after that, during dormancy, when the plants were pruned to one spur and *Trichoderma* was later sprayed in November 2017. The experiment was carried out in controlled conditions where warmer and dryer conditions were used in order to simulate climatic change parameters. A thermohygrometer (Electroson, Valladolid, Spain) was used (defined as a local weather station) to measure these conditions and was compared to the standard automatic weather station located close to the trial sites. Data were retrieved from the website (http://www.inforiego.org/opencms/opencms, accessed on 21 July 2021).

After 12 weeks of inoculation (February 2018), the effectiveness of the colonization was evaluated following this protocol: eight wood chips were cut (approx. diam. 1–2 mm; approx. length 0.5–1 cm) over the inoculation point, and placed 3, 6, 9, 12, and 15 cm

from the inoculation point, over Rose Bengal agar (RBGA, Conda Laboratory, Torrejón de Ardoz, Madrid, Spain) plates. They were incubated at 25 °C in darkness for seven days and the growing isolates were morphologically identified [54]. The colonization index was determined as the mean percentage of wood chips (8 chips × 8 plants = 64) placed by the inoculated fungus in order to avoid fungal pathogens that could develop GTDs.

2.5. Statistical Data Analysis

To compare the degree of colonization between the isolates, an evaluation of growth, spore production, and pruning wound colonization were performed using a simple analysis of variance (ANOVA). The Duncan post hoc test at $p = 0.05$ was evaluated. Analyses were conducted using Statistics for Windows Version 26.0, IBM Corp., Armonk, NY, USA.

3. Results

3.1. Evaluation of Fungal Growth

Mycelial growth was evaluated at temperatures ranging from 4 °C to 35 °C and different behaviours were identified depending on the *Trichoderma* strain. At 4 °C, the total radial growth over the PDA plate after 6 weeks of *Trichoderma* T071 was significantly greater ($p < 0.05$) than the other *Trichoderma* strains (Figure 1). T154 did not grow at all at this temperature, and no significant differences were seen compared to the *Trichoderma* T065 strain. However, the rest of the *Trichoderma* strains exhibited very small growth (Figure 1).

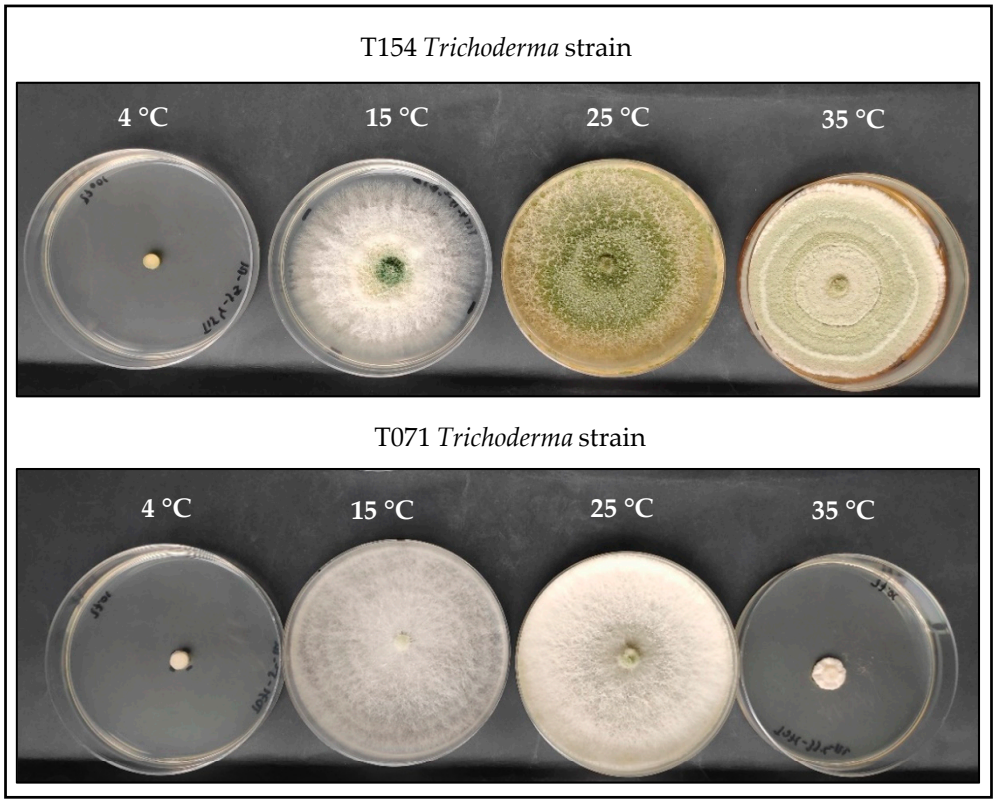

Figure 1. Growth of the *Trichoderma* strains T154 and T071 after 7 days at 4 °C, 15 °C, 25 °C, and 35 °C.

Afterwards, they were grown at 25 °C and were all able to colonize the plate after 7 days (data not shown).

At 15 °C, strains T065, T071, and T214 showed a significantly higher growth ($p < 0.05$) in comparison to T154 on day six (Figure 1). The lag phase (little or no observable growth) was considered as 1 day. At 25 °C, no significant differences were found between them (Figure 1). On day three, all the *Trichoderma* strains were able to entirely colonize the

plate. At 35 °C, the growth of T154 was significantly greater ($p < 0.05$) than the rest of the *Trichoderma* strains (Figure 1). T214 showed a moderate growth rate and a significantly greater rate of growth than T065 and T071. These last two strains were not able to grow at 35 °C, thus showing a lag phase for the whole experiment (7 days). (Figure 2).

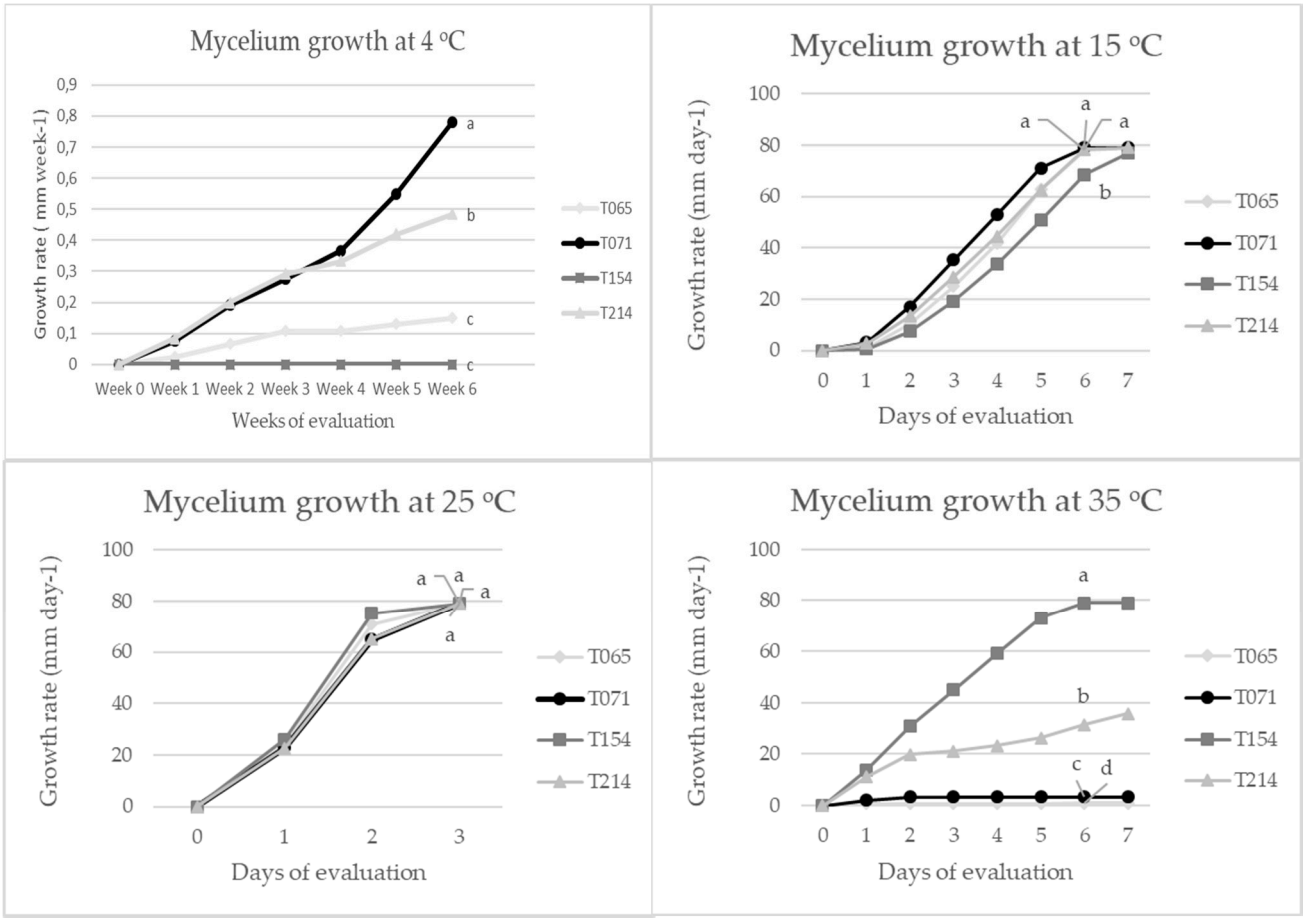

Figure 2. Evaluation of the growth of the *Trichoderma* strains at a different range of temperature (4 °C, 15 °C, 25 °C, and 35 °C). Values in each graph followed by the same letter do not differ significantly ($p = 0.05$).

Spore production was assessed at 25 °C in darkness in order to determine their suitability for scale production. The *Trichoderma* strain T154 produced a significantly higher ($p < 0.05$) number of spores in comparison to the rest of *Trichoderma* strains (Figure 3). T214 also produced a significantly higher production of spores than T065 and T071. The lowest value of spore production was found for T065 and T071 and no significant differences were found between them.

The mycelial growth of the *Trichoderma* spp. in the PDA plates was evaluated from 5 microliters from two different spore concentrations (2×10^{-7} CFU/mL and 2×10^{-6} CFU/mL) and in combination with two low values of temperatures (15 °C and 4 °C). At 15°C and 2×10^{-7} CFU/mL, the *Trichoderma* strains T065 and T071 showed a growth rate significantly higher ($p \leq 0.05$) than the others. When the concentration was 2×10^{-6} CFU/mL and the temperature was 15 °C, the *Trichoderma* strain T071 had a significantly greater growth rate ($p < 0.05$) in comparison to the other *Trichoderma* strains. The lag phase was 2 days for all of the strains. (Figure 4). At 4 °C and using both concentrations, none of the strains were able to develop a mycelium or had observable growth after 6 weeks.

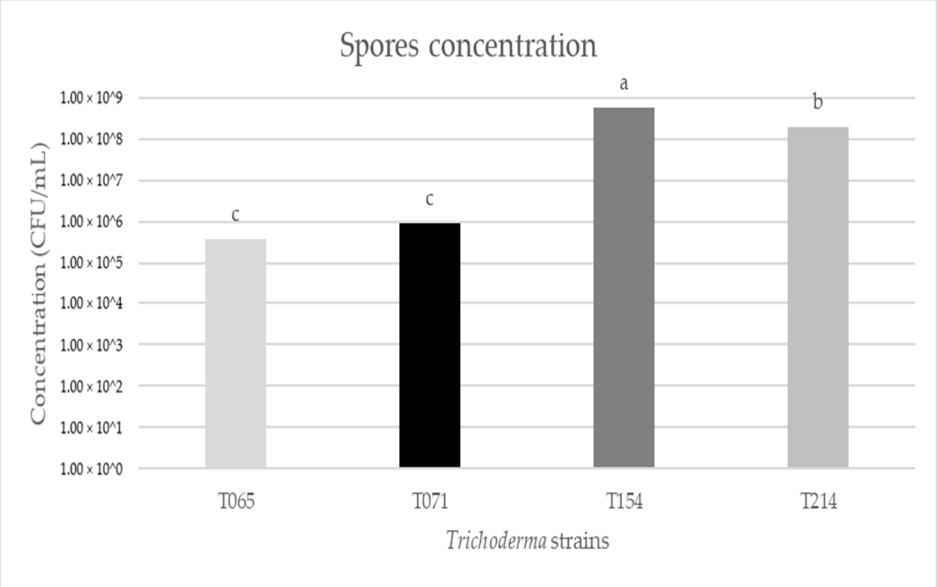

Figure 3. Spore production at 25°C after 7 days in a PDA plate. Values followed by the same letter do not differ significantly ($p = 0.05$).

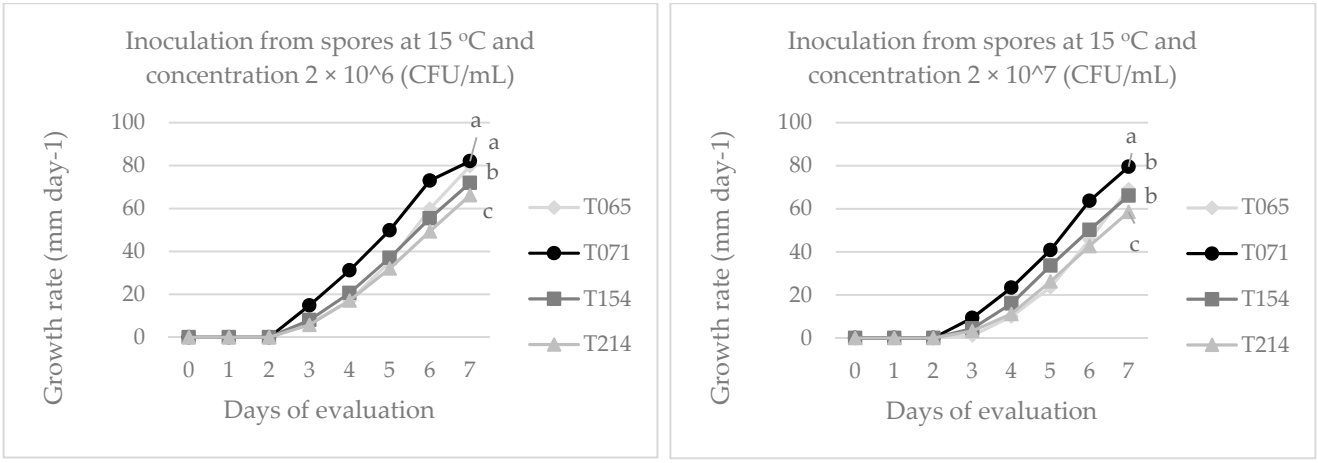

Figure 4. Growth rate of the different *Trichoderma* strains analyzed in this work from two spore concentrations at 15 °C. Values followed by the same letter do not differ significantly ($p = 0.05$).

3.2. Evaluation of Survival over Pruning Wounds in Planta

The *Trichoderma* strains T071 and T154 were inoculated over pruning wounds to colonize and prevent the entrance of other fungal pathogens (Figure 5A,B) at 6 °C and a relative humidity of 56.00%. Both strains were able to survive after 12 weeks in in planta conditions during winter, from November 2017 to February 2018. Data from the minimum mean temperatures were selected as references to evaluate possible climate change events. Weather conditions recorded in a local weather station had a higher temperature media of 4.06 °C compared to a standard weather station and a lower than standard relative humidity media of 27.30%. The average temperature and relative humidity per week are recorded in (Figure 6A,B).

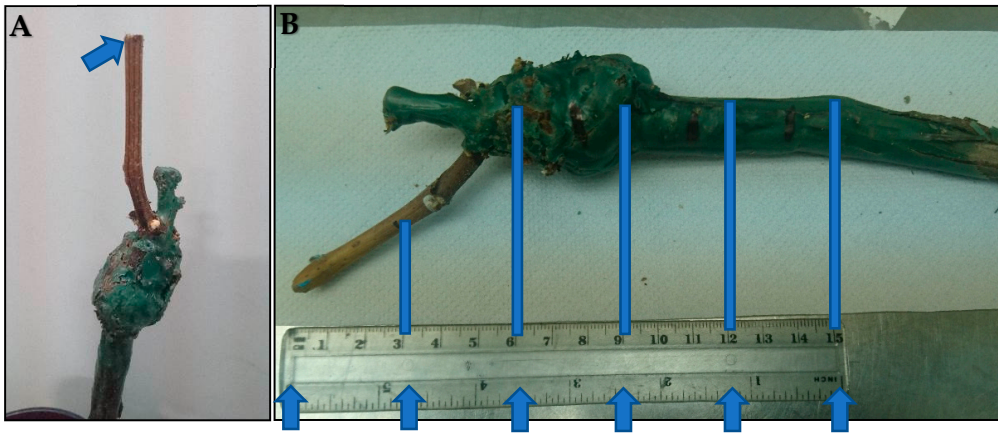

Figure 5. (**A**) Inoculation point. (**B**) Points of evaluation after inoculation.

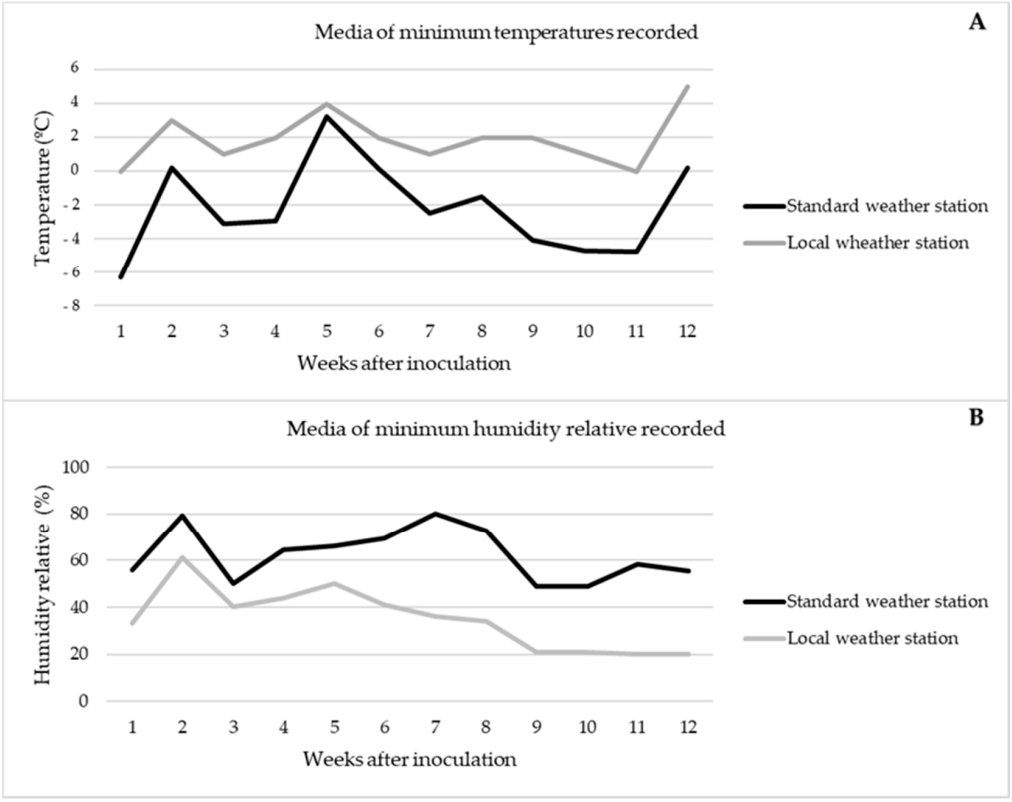

Figure 6. (**A**) Average temperature of each week. (**B**) Average relative humidity of each week.

The evaluation of the pruning wounds to study the capacity of *Trichoderma* to colonize the pruning wounds was performed by re-isolating the fungal strains from the inoculation points 12 weeks after the inoculation at a concentration of 2×10^{-6} CFU/mL from both the T154 and T071 strains.

The percentage of the re-isolation of T154 was 89.58%, a value significantly higher ($p < 0.05$) than that observed for T071, which reached 70.31% (Figure 7). Thus, T154 was chosen as more suitable strain for pruning wound colonization in order to avoid the entrance of possible pathogens that could lead to GTDs. Furthermore, a colonization of 91.67% was found for T154 when a concentration of 2×10^{-7} spores/mL was used. However, no significant differences were found when compared with the concentration of 2×10^{-6} spores/mL in terms of re-isolation (Figure 7).

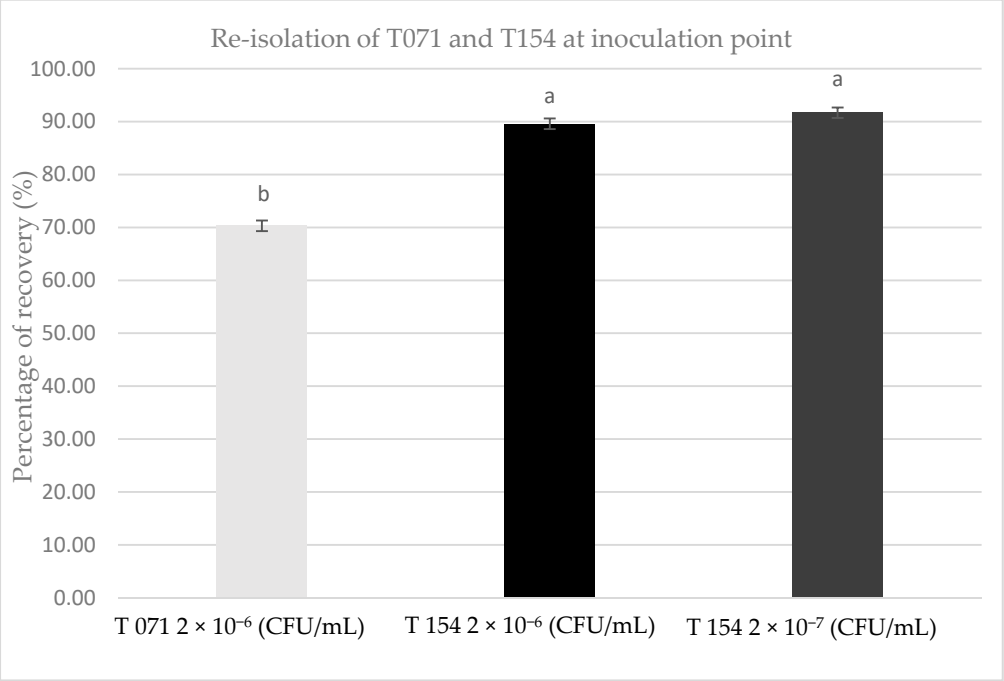

Figure 7. Percentage of re-isolation of pruning wounds after 12 weeks applying *Trichoderma* strain from T071 and T154. Values followed by the same letter do not differ significantly ($p = 0.05$).

Trichoderma strains were recovered 3 cm above the inoculation point in one of the eight vines for both of the strains. However, below the inoculation points at 6, 9, 12, and 15 cm, it was not possible to re-isolate *Trichoderma*.

4. Discussion

This work was conceived to try to efficiently evaluate the persistent performance of biological agents in semi-field conditions in winter to colonize pruning wounds as an assessment of a niche exclusion mechanism in order to block the entrance to other hypothetical fungal pathogens trough the xylem and phloem vessels. This is caused due to most biological control agents after obtaining positive laboratory tests. A poor efficacy rate was found for many biological control agents during the field tests; this has been attributed to host and abiotic factors that favour pathogen development [53,55]. In this article we evaluated abiotic factors and *V. vinifera* cultivar adequation before moving on to field conditions.

Under in vitro conditions and over grapevine plants, all *Trichoderma* strains showed different adaptations to different temperature conditions, demonstrating similar patterns according to its clade or section that it belonged to genetically. From the four *Trichoderma* strains evaluated under in vitro conditions, two of them (T154 and T071) were chosen to perform semi-field condition assays. They showed a good rate of survival over the inoculation points during the winter season over canes of grapevines, but T154 was re-isolated at a significantly higher rate than T071.

Firstly, in vitro evaluations of T065, T071, T154, and T214 led to the observation that they behave as mesophilic fungi because they are able to grow at 25 °C, which is an optimal temperature, as most of *Trichoderma* spp. do, as previously described [56,57]. T154 and T214 that belong to clade HV showed a good response at 35 °C in comparison to the T065 and T071 strains that are classified in the section SL, which have been described as able to grow under low temperatures [58,59]. *Trichoderma harzianum* have been described as a complex species that are adapted to warm climates [60]. At 15 °C, T154 showed a significant lower growth in comparison to the rest of fungi, confirming this trend. At 4 °C, T071 showed a significant growth but at a much lower level than other cold tolerant

Trichoderma strains [61]. This *Trichoderma* strain (T071) was the only one to have a significant growth rate in comparison to the others, which were able to grow slowly at a very low temperatures among all the strains analyzed in this work.

Moreover, the rate of spore production is an important characteristic since most commercial bio-formulated *Trichoderma* are presented in spore suspensions [2]. T154 and T214 that belong to the HV clade had a significantly higher production of spores than those belonging to section SL. In order to scale and optimize mass production, different substrates should be tested, such as agro-wastes, in order to reduce production costs, and also to obtain a higher and easier obtention of CFUs [62].

The performance of the spores was also evaluated at low temperatures in order to simulate in vitro the capacity to colonize a pruning wound at a low temperature during the winter season, i.e., at 15 °C. The same trend was observed with *Trichoderma* strains that belonged to section SL that had a significantly higher growth rate in PDA plates in comparison to species of clade HV. Thus, the use of biological control strains that belong to section SL could reduce the problems associated with low temperatures during winter, as result of the lower activity of the BCA [31]. Previous reports have also described a higher proportion of cold-tolerant strains among those belonging to the *T. viride* strains in comparison to the *T. harzianum* strains, and a higher optimal growth temperature for the species that belongs to clade HV [63,64]. All *Trichoderma* strains were able to sporulate and grow at 15 °C. Thus, all of them could eventually colonize and grow in winter conditions.

In conclusion, the in vitro assays, the range of temperatures assayed with these two different section/clades, and our results suggest that using the *Trichoderma* species belonging to section SL for the winter season and clade HV for the summer could be an option in order to optimize their capacities. Moreover, a combination of both kinds of *Trichoderma* strains could diminish the effects of climate change in viticulture [65].

The taxonomy of these strains is also an important issue. Thus, T065 and T071 have been identified as *T. gamsii*. This species was reassessed in 2006 [66] as a new species that belongs to section SL. Other *T. gamsii* strains have been described as effective BCAs in tomatoes [59,67]. Moreover, they have been described as effective BCAs against grapevine trunk diseases such as Esca—specifically for reducing the external symptoms of grapevine leaf stripe diseases, combined with *T. asperellum* and *T. gamsii* [6]. *T. harzianum* is a complex species distributed worldwide and is a very common BCA. However, not all *T. harzianum* strains can be described as efficient BCAs. Previously, T154 has been tested against *P. minimum* as an effective BCA that uses niche exclusion mechanisms for biocontrol [47], and T214 was shown to have a significant antifungal activity in our case (data not shown). These two strains belong to the clade HV that possesses a complex taxonomy and has not been fully identified due to its coexistence and interaction with all these organisms that together create this clade and have a lack of strict genetic borders between them [68].

The colonisation of grapevine pruning wounds is a key factor to ensure the effectiveness of these strains as BCAs against pathogens that could develop GTDs or a fungal infection after evaluating temperature performance. Annual pruning wounds are the main point of entry for pathogens that cause GTD pathogens [10]. Thus, a good colonization of *Trichoderma* strains would prevent the penetration of the main pathogens such as *Diplodia seriata*, *Phaeoacremonium minimum*, or *Phaeomoniella chlamydospore*, among others. An assay that evaluates the persistence of *Trichoderma* strains over a pruning wound will help to decide if these strains are suitable to be sprayed in field conditions. In order to reduce natural infection, early pruning (November) is recommended in combination with a biological or chemical wound protectant to reduce the risk of infection of *Eutypa lata*, *Neofusicoccum parvum*, *Botryosphaeria dothidea*, *Phomopsis* spp., *Cryptovalsa ampelina*, *Phaeomoniella chlamydospore*, *Diplodia seriata* [69], and the genera *Botryosphaeria*, *Cadophora*, *Cryptovalsa*, *Cytospora*, *Diaporthe*, *Diplodia*, *Eutypa*, *Neofusicoccum*, *Phaeoacremonium*, and *Phaeomoniella* [70] in comparison to late pruning (February) [57,58]. Moreover, delayed pruning may result in the sap-flow washing away any pruning wound protectant [53]. Thus, early pruning and immediate *Trichoderma* application is the most advisable practice.

In this semi-field experiment, the incidence of *Trichoderma* was evaluated after 12 weeks of inoculation. Its persistence over grapevine wounds was significantly higher at 89.58% when sprayed with T154 at 2×10^{-6} CFU/mL, in comparison to 70.31% for T071 at the same concentration of spores. Niche exclusion was described as one of the mechanisms used by T154 [47] in order to biocontrol *P. minimum* and it could be a reason for its successful colonization. Moreover, its capacity to produce a greater number of spores and its mycoparasitic activity would point to this strain as a suitable BCA [71]. In the present work, *Trichoderma* spores were sprayed during the dormant vine period and 24 h after pruning as is recommended [53]. No sap bleeding was detected after pruning and no rain fell after application. Similar results were obtained for the *T. atroviride* isolate UST1 (highest rate of incidence corresponded to 90.00%) and Eco-77 based on *T. harzianum* (highest rate of incidence corresponded to 87.00%), and no differences were found between the strains of clade HV and section SL [53].

Previous studies have determined that the percentage of prevalence recovery after eight months can vary according to the *Trichoderma* strain and the grapevine cultivar from 82.5% to 0.00% of mean prevalence [72]. Moreover, the highest incidence recovery of *Trichoderma* in a pruning wound was 59.03% of the colonization of the pruning wound after seven months [73]. Another experiment obtained a 33.41% incidence recovery in pruning wounds as the highest value after 8 months [74]. *Trichoderma* bio-based commercial products authorized currently in Spain such as Vintec®(*T. atroviride* SC1) and Esquive were assayed and the percentage of recovery after 12 months was as high as 13.5% and 9.8%, respectively, from pruning wounds [28]. In our case, the incidence of the recovery of *Trichoderma* was obtained after 3 months in semi-field conditions. Thus, further investigations would be needed to analyze a wider range of months (7–8) in a *Vitis vinifera* cv. Tempranillo field plant, in order to confirm the data from the in vitro and semi-field assays. This experiment revealed that *Trichoderma* strains were not able to penetrate but were able to colonize the pruning wound at a high percentage rate during the period of maximum risk of infection [75]. The period of evaluation in planta needs to be longer in order to validate the movement through the plant, as was found in the *Trichoderma* strain AG1 after 20 months of inoculation, where it was found 6 cm far from the inoculation point [76].

Several factors have influenced these results. One of them is nutrient availability. Grapevine canes possess cellulose, hemicellulose, and lignin [77] in comparison to alpha polysaccharides that contain PDAs such as dextrose and potato extract (PDA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). This suboptimal nutrient availability and the effect of temperature can lead to a less effective substrate colonization [78] and it could be the reason for the lack of penetration through the grapevine cane. Temperature and moisture also play an important role during the colonisation along the cane, and higher temperatures and a humidity relative lower than 90% could generate lower rates of colonization for the *Trichoderma* species [33]. During the experiment in semi-field conditions, the media of the minimum temperatures barely reached 4 °C and the relative humidity minimum from week 2 to week 12 was lower than 50%, which led to very adverse conditions that did not allow it to grow far from the inoculation point. This follows the studies of John et al., 2008 [76], which obtained a colonization of 3 cm after 4 months. Moreover, T071 showed a significantly lower colonization over the grapevine cane despite having a better growth in in vitro assays under low temperatures. A different degree of colonization of the *Trichoderma* species was achieved in different grapevine varieties [72]. Thus, it is possible that T071, isolated from soil, would have a lower capacity of colonization than T154, isolated from the grapevine bark of *V. vinifera* cv. Tempranillo. These results are in agreement with data from Mayo-Prieto et al., 2020 [79], where *Trichoderma* native strains isolated from soil produced a higher percentage of germination and better agronomic parameters in comparison to those that were isolated from seeds. Both results demonstrate the importance of the source used for the fungal strain isolation. However, further research under field conditions is needed in order to confirm an early pruning time as a good option in order to combine it with the shorter period of infection of GTDs [70].

In addition, other important issues to take into account are the environmental conditions at the time of application. The temperature recorded was 6 °C with 56% relative humidity. Most experiments have not published these data [72,73,76,80] except Martínez-Díaz et al., 2020 [28], where the daily mean temperature was 6.5 °C and 8.2 °C during the week of the trials and *Trichoderma* did not colonize well. This is in contrast to the study by Mutawila et al., 2015 [53], where an average temperature of 15.6 °C and 12.9 °C was achieved during the first year at inoculation time and 12.0 °C and 13.4 °C during the second year. According to the relative humidity, the values of 59% and 65% during the first year of the experiment were obtained and 69% and 74% were recorded during the second season. In this case, *Trichoderma* obtained a high rate of re-isolation from the pruning wounds. Probably, higher temperature values and the relative humidity could influence the superior performance of *Trichoderma* colonization. Rainfall events were also recorded, and this factor is important for field experiments after pruning as the probability of infection increases due to GTDs [69,70]. Furthermore, it strengthens the idea that it is necessary to: (I) study the capacity of growing in these environmental and in vitro conditions and the same available nutrients; (II) measure environmental variables with the aim of characterizing the optimal conditions [81] for a good colonization, combining the knowledge of microbiology and engineering.

Regarding the concentration of spores, a greater range of spores at the inoculation point can vary in the vine plants from different *Trichoderma* species [27,72,74,76,80,82,83]. *T. harzianum* AG1 had the highest value, with a concentration of 10^{-9} spores/mL [80] and *T. harzianum* Biotricho had the lowest value, where 4.8×10^{-4} spores/mL were inoculated [74]. According to this range, it was decided to test 2×10^{-6} CFU/mL and 2×10^{-7} CFU/mL as two possible concentrations. Excessive concentrations of *Trichoderma* could have negative consequences for yield production [84]. Thus, the optimization of these parameters could help obtain a more sustainable biological product. In this case, no significant differences were found under in vitro or semi-field conditions in *Trichoderma* sp. T154 when these two concentrations were evaluated. The volume of the spore suspension could be closely linked to this issue. In our case we inoculated 50 µL in each pruning wound in 0.5–1 cm of the cane diameter in order to optimize the inoculation as much as possible. The inoculation of *Trichoderma* involves spraying a suspension of 2 mL [74] and other liquid formulations were applied using a 500 mL hand sprayer [28,53], and the results were totally different.

Another issue of discussion is the effectiveness of native strains versus commercial ones. In this regard, some previous reports have not found significant differences between them in terms of biological control efficacy [85]. Despite this, the studies performed with *Trichoderma* native strains isolated from bean soils showed an excellent in vitro biocontrol against *Rhizoctonia solani* J.G. Kühn [86]. Furthermore, native *Trichoderma* were able to colonize pruning wounds against *P. minimum* [47]. However, on the contrary, studies carried out with some commercial *Trichoderma*-based bioproducts concluded that they did not show a good plant colonization ability under the selected conditions [28]. Similar controversial results have been reported for other non-fungal biological control agents, as in the case of *Streptomyces* [25,87]. These data show how important it is to continue studying the biotic and abiotic factors that affect the success of a good colonization as a key step to a good biocontrol activity.

The studies carried out in the present work, including the determination of the range of temperatures under which the different *Trichoderma* strains can grow, and also the confirmation of the establishment of these strains over pruning canes, will allow a reduction in the negative impacts of pathogens in different currently changing abiotic conditions and would ensure the one health concept by which humans, animals, and plants interact based on the use of effective biological control products. Thus, the main result of this article is the description of a method for evaluating the *Trichoderma* response to different environmental conditions, which would allow us to determine the suitability of the analyzed strains to be used in a background of climate change. As a concluding remark, our results indicated that the *Trichoderma* native strains show a good ability of colonization and a good response

at different combinations of temperature. They could give better protection to grapevine plants in co-evolution with each specific environment.

5. Conclusions

Although *Trichoderma* strains from section *Trichoderma* were better adapted to cooler conditions and the others from clade *Harzianum/Virens* showed a greater capacity for growth under warmer temperatures during in vitro tests, *Trichoderma* T071 selected from the section *Trichoderma* had a significantly lower capacity to colonize the pruning wounds than *Trichoderma* T154 that belongs to Clade *Harzianum/Virens* during winter conditions. None of them were able to penetrate the grapevine cane but they were able to colonize successfully pruning wounds under adverse climate situations. *Trichoderma* T154 had a significantly higher colonization rate, probably due to its origin from grapevine wood and its effective mechanism of niche exclusion. Further studies are needed to evaluate these strains for longer periods of time in order to contrast temperature adaptation and test them against the main pathogens that cause grapevine trunk diseases infections.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11091771/s1, Figure S1: Trichoderma species phylogenetic tree. Table S1: *act1* resulting coding sequences retrieved from the genomic sequences.

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