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Influence of Fungicide Application and Vine Age on *Trichoderma* Diversity as Source of Biological Control Agents

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Abstract: Fungi from the genus *Trichoderma* have a worldwide distribution and are commonly found in agricultural lands. Further, it has been described as a non-virulent and symbiont microorganism that can contribute to minimize the pernicious effects of pathogens. In the present work we have isolated *Trichoderma* spp. from bark of grapevine in different orchards in order to determine the influence of fungicide application and vine age on *Trichoderma* diversity in plant. An opposite correlation between the number of fungicides sprayed per campaign and the diversity of *Trichoderma* spp. isolates was found. Moreover, the older are grapevine plants the higher is the diversity of *Trichoderma* spp. isolates. The different *Trichoderma* strains isolated were tested against *Phaeoacremonium minimum*, a grapevine trunk pathogen, to evaluate their biocontrol capacity. Three *Trichoderma* strains shown a significant capacity to control *P. minimum* and were selected as candidates to be used as biological control agents. In addition, a rapid and easy method for isolating *Trichoderma* spp. from grapevine plants has been developed, which allowed to determine that the reduction in the amount of pesticide use, together with the preservation of old vineyards, lead to healthier agroecosystems containing higher levels of beneficial microorganisms.

Keywords: biological control; grapevine trunk diseases; viticulture; sustainability; *Phaeoacremonium minimum*

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1. Introduction

Some of the most commonly isolated saprotrophic fungi growing in soil, wood, or bark are *Trichoderma* spp. Rifai, showing a great capability to adapt to different ecological conditions [1]. In addition, some *Trichoderma* spp. are well known as biological control agents, which exhibit different mechanisms of action against pathogens such as (i) mycoparasitism, (ii) antibiosis, and (iii) competition with pathogens and soil microbial community [2]. Direct confrontation in dual culture assays have been widely used to test in vitro the ability of a particular *Trichoderma* sp. isolate to control fungal pathogens, concluding that mechanisms that prevail are mycoparasitism and antibiosis [3,4]. As result of these studies the number of commercial products based on *Trichoderma* spp. has grown exponentially [5].

The availability of *Trichoderma* products is a great concern due to the reduction of conventional pesticides in European agriculture [6], and the great losses that some diseases such as Grapevine Trunk Diseases (GTDs) could develop due to a lack of efficient measurements of control [7]. So that, many *Trichoderma* species have been assayed against vine pathogens, such as *Botrytis cinerea* Pers. [8], *Plasmopara viticola* (Berk. and M.A. Curtis) Berl. and De Toni [9], and specially against GTDs [10].

Nowadays, one of the most important vine destructive diseases are these GTDs [11,12]. Thus, great efforts are needed to search for alternative control strategies to reduce costs and dependence of chemicals but no curative methods have been obtained yet [7], and *Trichoderma* can be revealed as an ecological, sustainable, and safe alternative against GTDs.

Some of them such as *T. atroviride* SC1 has shown an effective biocontrol activity in nursery and field conditions [13]. Further, Remedier® (a mix of *T. asperellum* and *T. gamsii*) has shown positive results in terms of reduction of incidence [14]. Another example are *T. atroviride* isolate UST1 and Eco-77 (*T. harzianum*) that were tested over grapevine pruning wound surfaces, resulting in a reduction of infection from trunk pathogens [15]. However, not all commercial strains have shown an effective reduction of the disease or ecological adaptation to specific conditions in some vineyards [16,17].

Some examples of successful indigenous strains sprayed can be found, e.g., the selection of indigenous *Trichoderma* spp. strains from bean plants has shown promising results; these *Trichoderma* strains were assayed in vitro against *Rhizoctonia solani* JG Kühn (dual confrontation assay and membrane assay) and some of them were able to biocontrol this pathogen, in addition they could induce plant defense resistance [18]. Further, other indigenous bacteria strains isolated from grapevine rootstocks against GTDs were able to control these diseases [19]. In addition, some other studies suggested that there are no differences between native strains and *Trichoderma* commercial products in terms of biocontrol [20].

Furthermore, the importance of crop management influencing microbial communities has been described since many years ago [21], including in vineyards and its soils [22–24]. There is an important influence of crop management over fungal endophytic communities in grapevines, *Vitis vinifera* L. cv. Merlot and cv. and cv. Chardonnay, obtaining that fungal endophytic from organically managed farms were different from farms that are cultivated under Integrated Pest Management [25].

However, none of them have related the role of the *Trichoderma* communities on grapevine plants and their management.

So that, the application of indigenous *Trichoderma* strains in vineyards with similar agroecological characteristics, could improve the adaptation of this biological control agent (BCA) to each specific agroecosystem [20]. Further, it has been proved that commercial products of fungi and bacteria in comparison to strains that are isolated from vines can be used potentially for controlling crown gall disease in both cases [26].

Our main objective is searching for indigenous strains adapted to the agroecosystem to be applied on the points of disease penetration.

One of the most common and widely distributed species of GTDs is *Phaeoacremonium minimum* W. Gams, Crous, M.J. Wingf., and L. Mugnai (formerly *Phaeoacremonium aleophilum*) [27]. It is considered a pioneer in esca disease and can colonize bark, pith, and xylem fibers [28]. It has been described also in Petri and esca diseases, where it is presented as the most prevalent and virulent pathogen [7]. *P. minimum* can remain not only in spurs and old vascular tissues [29,30] but also it can survive as a soilborne pathogen [31]. This pathogen is also the main cause of wood disease as a vascular pathogen in other crops such as *Actinidia deliciosa* [32]. Moreover, this specie has been described as a pathogen in human beings who could develop phaeohyphomycosis [27]. Therefore, this pathogen can be considered as a reference for evaluating the efficiency of biological control agents.

The main goal of this article is to evaluate the influence of fungicide application and vine age in *Trichoderma* diversity in vine as source of biological control agents.

2. Materials and Methods

2.1. Location and Vine Age

We have chosen 4 Spanish winegrowing regions belonging to Castilla y León (Spain) (placed in municipal districts of: 42°35′59″ N 6°43′32″ W (Cacabelos); 41°35′51″ N 4°07′22″ W (Peñafiel); 41°19′59″ N 5°28′09″ W (El Pego); 42°08′03″ N 5°24′10″ W (Gordoncillo); 42°10′45″ N 5°41′22″ W (La Antigua); 42°17′51″ N 5°54′06″ W (La Bañeza). All of them are placed in the inner plateau of Spain (northern subregion) under the same weather conditions. It is a Mediterranean-continental climate that possess long cold winters and short and heat dried summers. Even though, there is a strong contrast of temperature between day and night. We selected 10 plots that have different crop management. All these plots and their characteristics can be identified in Table 1. These plots are involved into a PDO. PDO is a certification to distinguish quality schemes for agricultural products and foodstuff of a specific region (EC Reg. n. 1493/1999. 8 August 2009, OJC 187).

Table 1. Places and code of the soil sampling used for this study.

Site	Location	Vine Varieties	Dates Sampled	Year Vineyard Stablished
		PDO LEÓN		
1	La Antigua, Castilla y León	Prieto Picudo	January 2017	1934
2	La Bañeza, Castilla y León	Tempranillo	January 2017	2001
3	Gordoncillo, Castilla y León	Albarín blanco	February 2017	2006
4	Gordoncillo, Castilla y León	Prieto Picudo	February 2017	1997
		PDO TORO		
5	El Pego, Castilla y León	Tempranillo	February 2017	1926
		PDO BIERZO		
6	Cacabelos, Castilla y León	Mencía	January 2017	1995
7	Cacabelos, Castilla y León	Godello	January 2017	2011
8	Cacabelos, Castilla y León	Godello	January 2017	1937
		PDO RIBERA DEL DUERO		
9	Peñafiel, Castilla y León	Tempranillo	February 2017	2001
10	Peñafiel, Castilla y León	Cabernet Sauvignon	February 2017	2008

There were four plots (1, 2, 3, and 4) that belongs to PDO León; (5) to PDO Toro; (6, 7, and 8) to PDO Bierzo; and (9 and 10) to PDO Ribera del Duero. Woody tissues sampling was undertaken during winter season (between the end of January and the end of February). For bark sampling, fifteen samples of bark were taken from each grapevine plots of 8 different plants (trunk and arm) of *V. vinifera* (there are 15 samples per plot in 10 plots, obtaining 150 total samples). To collect the samples, a zip-lock bag was used that was kept at 4 °C until use. The bark (rhytidome) from trunk/branch vines was selected as a suitable place for searching for new *Trichoderma* isolates due to be the most commonly isolated saprotrophic fungi [1].

2.2. Fungicide Treatment and Vine Age

Wineries were surveyed during last five campaigns according to management practices. Number of fungicides spraying was recorded every year and media value of them during this period was annotated. Further, type of fungicide sprayed was recorded, Table 2. Plot (1) is an abandoned vineyard and plot (5) is a vineyard certified as organic production. The other plots (2, 3, 4, 6, 7, 8, 9, and 10) are being cultivated under an integrated pest management.

Table 2. Characteristics of fungicides treatments.

Site	Media Number of Fungicides Spraying per Campaign	Sulphur	Cooper	Synthetic Fungicides
	PDO LEÓN			
1	0	no	no	no
2	5.2	yes	yes	yes
3	4	yes	no	yes
4	4	yes	no	yes
	PDO TORO			
5	3	yes	yes	no
	PDO BIERZO			
6	5.9	yes	yes	yes
7	5.1	yes	yes	yes
8	5.9	yes	yes	yes
	PDO RIBERA DEL DUERO			
9	5	yes	yes	yes
10	5	yes	yes	yes

2.3. Sample Processing, Trichoderma Isolation and Quantification

Pruning shears were used for collecting bark samples. They were disinfected using 70% ethanol between different samples in the field. Eight plants were selected in each of the ten plots. Isolation of samples was performed as follows, a square segment of bark that had a dimension of (3 cm × 3 cm) was cut from vine plant, this process was done for extracting bark from trunk and from one of the branches of each vine plant. Plants were selected randomly through each plot in order to obtain a representative sample, in some plants no branches were found so that it was decided to define a total number of samples as 15. They were kept in plastic bags at 4 °C until processing.

These segments were treated in 1.5% sodium hypochlorite solution for one minute, and then washed with abundant sterile distilled water. After that, bark segments were finally dried for 15 min in a laminar flow chamber. These segments were cut using a sterile scalpel in a laminar flow cabinet and seven wood chips (1–2 mm approx. diameter; 0.5–1 cm approximately length) of each segment were placed on a Rose Bengal–Chloramphenicol Agar medium (Conda Laboratory, Torrejón de Ardoz, Madrid, Spain) plate. The wood chips were incubated at 25 °C in darkness until fungi grew to a size at which *Trichoderma* could be morphologically identified (between 3 and 4 days) [33]. From these data the percentage of presence of *Trichoderma* spp. was determined as the mean percentage of samples, giving a value =1 in case of finding *Trichoderma* species in a plate and a value = 0 in case of absence (*Trichoderma* abundance in grapevine plant = (presence of *Trichoderma* spp. in each plate/15 plates) × 100). For further analyses, isolates from the same plants that have the same morphological and cultural characteristics were discarded. In total 8 different samples were examined, and each of them had two technical replicates except for one that had just one technical replicate, obtaining 15 subsamples.

2.4. DNA Extraction, PCR Amplification, and Sequencing

Genomic DNAs were isolated from 100 mg of mycelia of each fungal isolate. The manufacturer's protocol for fungi of the Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) was performed. A NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to estimate DNA concentration. PCR amplifications were performed using 50 ng of template DNA in a final volume of 50 µL, containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 M dNTP, 400 nM of each primer, and 1.5 U of DreamTaq DNA polymerase (Thermo Scientific, Wilmington, DE, USA). The primer pair ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify nuclear rDNA-ITS regions [34].

PCR products were first purified by the Nucleo Spin Extract II kit (Machery-Nagel, Düren, Germany) and were then sequenced using primer ITS5 and the kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the automatic capillary sequencer ABI 3130xl (Applied Biosystems), according to the manufacturer's instructions. DNA sequences were introduced in databases such as the NCBI Genbank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov, accessed on 20 December 2020) using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, accessed on 20 December 2020) to identify the fungi [35].

2.5. Biocontrol Assays

In vitro evaluation of *Trichoderma* isolates as potential biological control agents was performed by modification of previously described methods by Kotze et al. (2011) [30] and Hermosa et al. (2000) [3]. In this case, it will be tested against a harmful trunk disease pathogen such as *P. minimum* (formerly *P. aleophilum*) strain Y038-05-3a that is significantly aggressive to grapevine plants [36]. A mycelial plug of *P. minimum* from a 14-day-old culture grown on potato dextrose agar medium (PDA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), was placed on a fresh PDA plate and incubated in dark conditions at 25 °C for 14 days. After that, a *Trichoderma* 7-day-old culture from a PDA plate was placed on the opposite side of the plate, 4.5 cm far from the pathogen. Growth parameters were evaluated after 5 days. Percentage Growth Inhibition relative (%GI) was calculated as $\%GI = \left\{ \frac{(G2-G1)}{G1} \right\} \times 100$. In total 25 isolates were chosen for biocontrol assays, seven isolates from plot 1, two isolates from plot 2, one isolate from plot 3, two isolates from plot 4, six isolates from plot 5, two isolates from plot 6, three isolates from plot 8, one isolate from plot 9 and one isolate from plot 10. Each *Trichoderma* isolate had four replicates and the radial mycelial growth (G1), was assessed by calculating the mean diameter from two perpendicular measurements of *P. minimum* in presence of each *Trichoderma* strain. (G2) was calculated regarding to the control, mean diameter from two perpendicular measurements in control plates, and it was calculated for the day 5. This value was chosen as the principal criteria for selecting them.

In addition, morphological characteristics of the sporulation on the colony pathogen and production of a yellow pigment on the surface of the medium were recorded by using a 0 to 3 scale in which the values were coded as follows: 0, absence; 1, weak; 2, heavy; and 3, very heavy (Figure 1). However, in order to facilitate the application of the selection, these values were transformed into a scale in which 1 was the maximum rate [3]. *Trichoderma* strains were selected as potential biological control agents in case that a media value equal or over a 0.67 was achieved for each strain in sporulation.

Statistical Analysis

Values of Percentage Growth Inhibition relative (%GI) were confirmed that had a normal distribution using Kolmogorov–Smirnov test, homogeneity of variances was evaluated using Leven Test and ANOVA one-way analyses were done to determine if there were significant differences. A post hoc tests (Tukey's HSD, $p < 0.05$) was performed to establish differences between groups. SPSS software (Statistics for Windows Version 26.0, IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

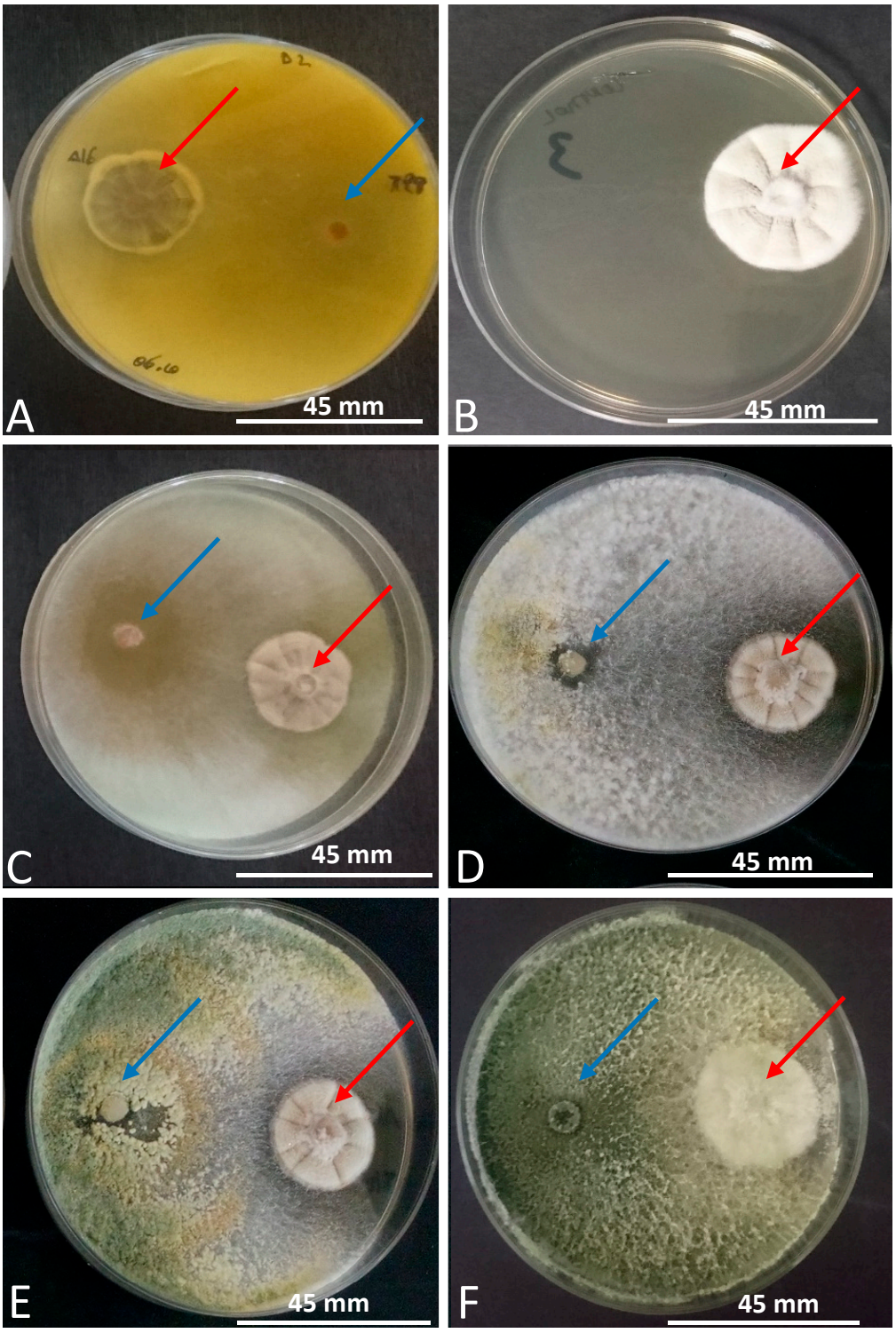

Figure 1. Dual culture evaluations of *Trichoderma* spp. (blue arrow) and *P. minimum* (red arrow) in Petri dishes with potato-dextrose-agar medium (PDA) after 5 days of co-culture. (**A**) *Trichoderma* isolate (T79) that produces yellow pigmentation. (**B**) *P. minimum* control plate. (**C**) Degree 0 of sporulation (T106) in dual confrontation (absence). (**D**) Degree 1 of sporulation in dual confrontation (weak) (T85). (**E**) Degree 2 of sporulation (T138) in dual confrontation (heavy). (**F**) Degree 3 of sporulation (T154) in dual confrontation (very heavy).

2.6. Identification of Parameters Involved in Trichoderma spp. Diversity

Data used in this section are media number of fungicide treatments per campaign, age of vines and percentage of *Trichoderma* abundance in plants. The R software was used for performing an statistical analysis in order to establish a correlation between abundance of *Trichoderma* in plants and the other variables [37]. First, it was checked that variables had a normal distribution using Kolmogorov–Smirnov test. After that, a Pearson's correlation analysis between data was done.

3. Results

3.1. Trichoderma Isolation, Quantification and Identification

Plates obtained from sample processing were checked and they were examined morphologically for identifying *Trichoderma* isolates. They were transferred onto a PDA plates and after seven days, *Trichoderma* spp. isolates were examined morphologically. They were selected preliminary and counted as *Trichoderma* spp. as [38] suggests. The 10 different plots have a percentage of *Trichoderma* spp. presence as follows: 73.33% (11/15) in plot 1, 13.33% (2/15) in plot 2, 6.67% (1/15) in plot 3, 26.67% (4/15) in plot 4 from PDO León; 26.67% (4/15) in plot 5 from PDO Toro; 6.67% (1/15) in plot 6, 0.00% (0/15) in plot 7, 20.00% (3/15) in plot 8 from PDO Bierzo; and 13.33% (2/15) in plot 9 and 13.33% (2/15) in plot 10 from PDO Ribera del Duero in Figure 2.

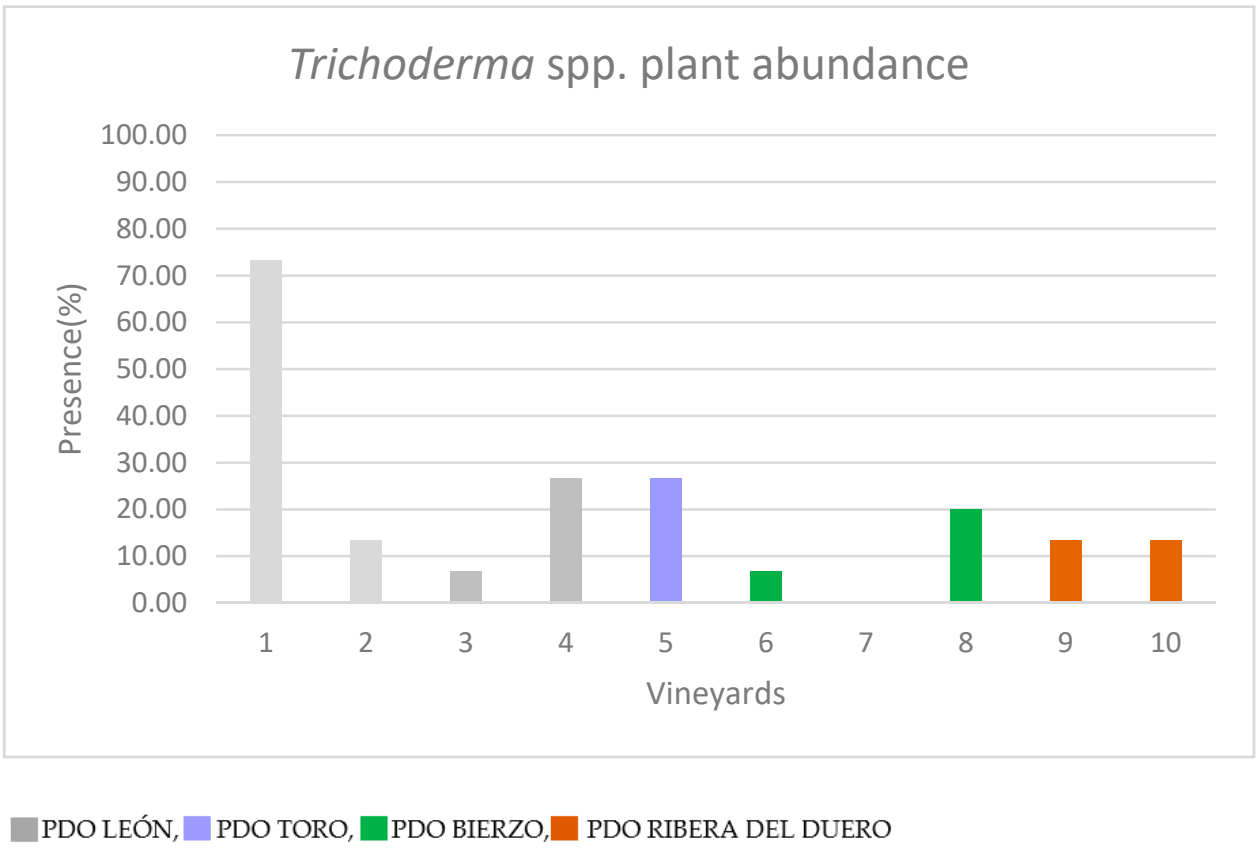

Figure 2. *Trichoderma* plant abundance. The code of each soil is detailed in Table 1.

A selection was performed to avoid duplicity of same isolates in same plots due to their morphological and cultural characteristics in case that they belonged to the same plant, and finally they were confirmed using genetic identification by PCR amplification and sequencing. As result, 25 *Trichoderma* isolates from bark vine (rhytidome) were finally selected in Table 3. *T. gamsii* was identified at the highest frequency (48%; 12/25). In

second place *T. koningiopsis* was obtained in a (20%; 5/25). After that, they were chosen for biocontrol analysis.

Table 3. Isolates of *Trichoderma* spp. in each vineyard.

Isolate	Species	Accesion Number	Site
	PDO LEÓN		
T72	*Trichoderma gamsii*		1
T73	*Trichoderma gamsii*		1
T74	*Trichoderma koningiopsis*		1
T75	*Trichoderma koningiopsis*		1
T76	*Trichoderma* spp.		1
T77	*Trichoderma koningiopsis*		1
T78	*Trichoderma gamsii*		1
T138	*Trichoderma* spp.		2
T139	*Trichoderma gamsii*		2
T100	*Trichoderma gamsii*		3
T105	*Trichoderma gamsii*		4
T106	*Trichoderma* spp.		4
	PDO TORO		
T79	*Trichoderma* spp.		5
T80	*Trichoderma koningiopsis*		5
T81	*Trichoderma harzianum*		5
T82	*Trichoderma gamsii*		5
T84	*Trichoderma koningiopsis*		5
T85	*Trichoderma gamsii*		5
	PDO BIERZO		
T136	*Trichoderma gamsii*		6
T137	*Trichoderma citrinoviride*		6
T135	*Trichoderma gamsii*		8
T170	*Trichoderma gamsii*		8
T171	*Trichoderma gamsii*		8
	PDO RIBERA DEL DUERO		
T154	*Trichoderma* spp.		9
T151	*Trichoderma atroviride*		10

3.2. Biocontrol Assays

Dual confrontation assays were performed with all *Trichoderma* spp. isolated as indicated above, against *P. minimum*. %GI values were obtained and were combined with data regarding two additional parameters, i.e., sporulation on the plate, and sporulation on the pathogen colony in PDA plates. In this case, *Trichoderma* strains that had the maximal values for these three parameters, significant higher values in percentage of GI, a value of sporulation on plate higher than 67%, and a value of over-sporulation on pathogen higher than 67% were selected as effective biological control agents. According to a significant percentage of GI, *Trichoderma* strains selected are as follows, from DPO León (T72, T74, T75, T77,T78, and T105); from DPO Toro (T79,T80, T82, T84, and T85); none of them from DPO Bierzo and from DPO Ribera del Duero (T154). Among these selected *Trichoderma* strains, sporulation on plate reached a maximum value (= 1.00) in T79 and T154; and 0.92 in T75. Moreover, T84 exhibited a 0.67 coefficient in sporulation. Sporulation on the pathogen had a value of 0.67 as higher in T75, T79, T84, and T154. In summary, a total of four strains (T75 identified as *T. koningiopsis*, T79 identified as *Trichoderma* spp., T84 identified as

T. koningiopsis and T154 *Trichoderma* spp.) were selected as potential biological control agents due to its capacity of antibiosis and/or mycoparasitism. Table 4.

Table 4. Dual-culture assay of *Trichoderma* spp. isolates after 5 days at 25 °C with the plant pathogenic fungi *P. minimum* on PDA. The letters indicate means within which there are no statistically significant differences ($p = 0.05$), according to Tukey's honestly significant difference (HSD) procedure applied to normalized data. An asterisk (*) indicates *Trichoderma* strain selected as potential biological control agent.

Isolate	Dual Culture (%GI)	Sporulation on Plate	Sporulation on Pathogen	Production of Yellow Pigment	Origin
		DPO LEÓN			
T72	20.42 ^{abc}	0.33	0.00	0.00	1
T73	14.62 ^{bcde}	0.50	0.00	0.00	1
T74	20.42 ^{abc}	0.83	0.17	0.00	1
T75 *	19.51 ^{abcd}	0.92 *	0.67 *	0.00	1
T76	11.66 ^{cdefg}	0.33	0.00	0.00	1
T77	22.02 ^{ab}	0.75	0.17	0.00	1
T78	22.92 ^{ab}	0.75	0.00	0.00	1
T138	4.04 ^{g}	0.33	0.00	0.00	2
T139	9.25 ^{efg}	0.33	0.00	0.00	2
T100	−5.19 ^{h}	0.08	0.00	0.00	3
T105	23.77 ^{a}	0.42	0.00	0.00	4
T106	17.30 ^{abcde}	0.25	0.00	0.67 *	4
		DPO TORO			
T79 *	20.30 ^{abc}	1.00 *	0.67 *	0.33	5
T80	22.40 ^{ab}	0.42	0.67	0.00	5
T81	12.36 ^{cdefg}	0.75	0.50	0.08	5
T82	16.14 ^{abcde}	0.58	0.00	0.00	5
T84 *	17.84 ^{abcde}	0.67 *	0.67 *	0.00	5
T85	15.33 ^{abcde}	0.75	0.00	0.00	5
		DPO BIERZO			
T136	13.15 ^{cdef}	1.00	0.17	0.00	6
T137	12.69 ^{cdefg}	1.00	0.00	0.00	6
T135	11.65 ^{cdefg}	0.33	0.00	0.00	8
T170	10.93 ^{defg}	0.33	0.00	0.00	8
T171	12.86 ^{cdefg}	0.08	0.00	0.00	8
		DPO RIBERA DEL DUERO			
T154 *	20.38 ^{abc}	1.00 *	0.67 *	0.00	9
T151	4.57 ^{fg}	0.58	0.00	0.00	10

3.3. Identification of Parameters Involved in Trichoderma spp. Diversity

Pearson's correlation analysis were performed for evaluating the correlation between data of management: age of vines (Age), number of fungicides sprayed per campaign (Fungicides) and *Trichoderma* abundance in grapevine plants (*Trichoderma*_plant_abundance) was performed.

Pearson's correlation coefficient analysis showed significant differences ($p < 0.05$) between *Trichoderma* plant abundance and Fungicides and also in the combination *Trichoderma* plant abundance and Age. Regarding Fungicides and age combination, there was no significant differences (Table 5) and they were represented in Figure 3.

Table 5. Pearson's product-moment correlation. 95 percent confidence interval (data in bold means significant differences).

Combination of Management	Correlation	*p* Value
Trichoderma plant abundance and Fungicides	−0.88382	0.00069
Trichoderma plant abundance and Age	0.67559	0.03202
Fungicides and Age	−0.52270	0.1211

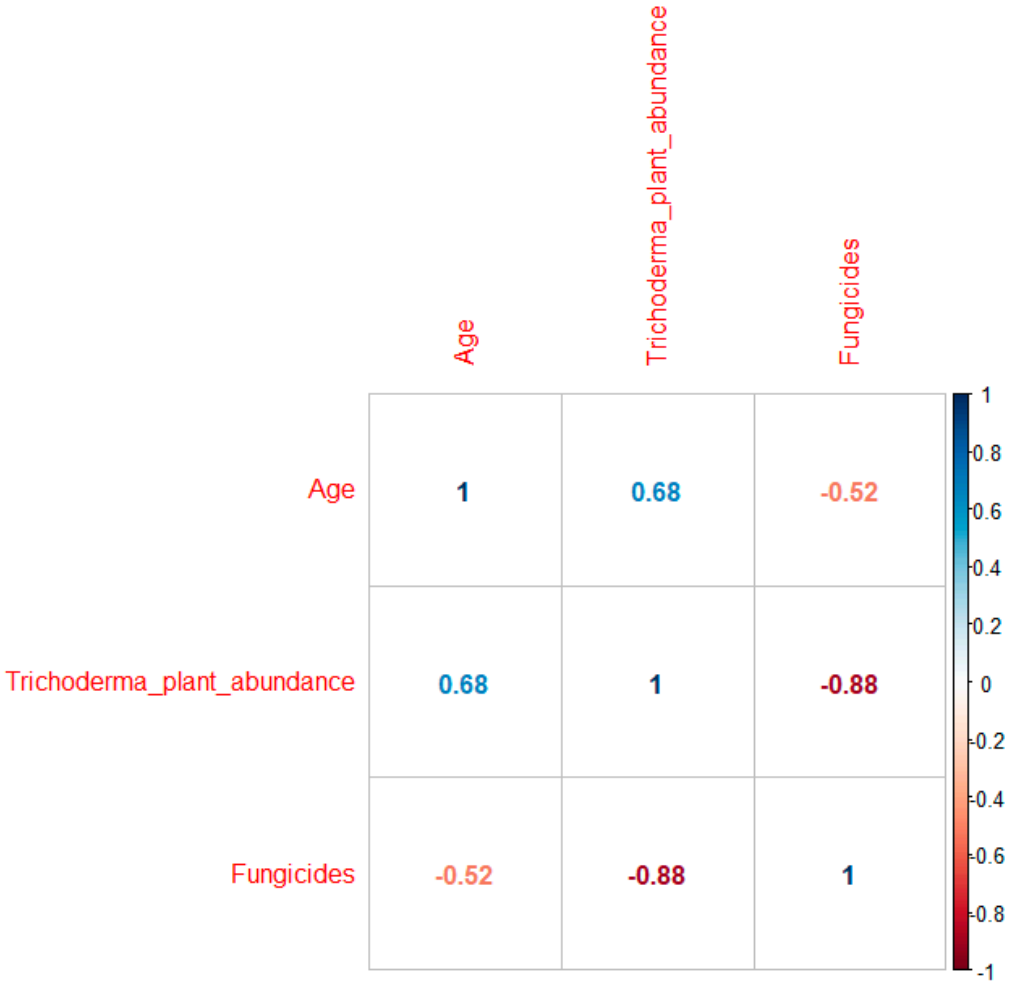

Figure 3. Correlation plot between variables Age, Fungicides, and *Trichoderma* plant abundance. Values closer to 1 refers a strong positive correlation. Values closer to −1 refers a strong negative correlation.

4. Discussion

Nowadays, it is well known that GTDs are a major threat for viticulture worldwide and losses associated to these diseases are increasing and having a great impact between grape growers. Furthermore, their etiology, specially of esca disease, is still uncertain due to not all pathogens described as possible causal agents of this disease have been proved following the Koch´s postulates [7]. Some recent studies are unravelling the importance of fungal communities inside grapevine trunks [39,40]. However, none of them have focused on *Trichoderma* communities over grapevine bark and their relationship to management practices in vineyards.

Using biological control agents reduce the possibilities of develop resistances to chemicals [41], and *Trichoderma* spp. are well-known fungi [1].

In the present work, for isolating these *Trichoderma* native strains from vineyards, the period from end January to first February was chosen as ideal of sampling for several reasons: in January the number of different species of isolates was greatest from woody tissues [39], during Winter season rapid growing taxa adapted to extreme weather conditions were more likely to be found [22] and most of farmer´s activities are carried out during growing season (May to October) so that no interference in agricultural activities are possible.

In our case, we have found *Trichoderma* species in 9 over 10 vineyards. Thus, this procedure, based on the use of Rose-Bengal agar medium [40], was possible for isolating *Trichoderma* species from grapevine plants bark. In addition, this traditional approach can be also recommended for accurately identify taxa [42].

Trichoderma species are one of the most frequently isolated from endophytic mycota associated with *V. vinifera* cv. Tempranillo, Moscatel, Grano Menudo, Cabernet-Sauvignon, Malvar, Syrah, Merlot, Garnacha, Albillo, and Airen in Spain [43], and according to [39], *Trichoderma* was the second most frequent genera identified from this niche. Furthermore, *Trichoderma* is one of most frequently saprotrophic and ubiquitous fungi [44,45]. Our results have shown a high percentage of *Trichoderma* isolates on bark.

Inside *Trichoderma* genus, *T. gamsii* was previously described as the prevalent species in *Trichoderma* grapevine plants according to [39], so that, these results agree with our data where *T. gamsii* represents 48% and it is the predominant specie. *T. atroviride* was also identified and others strains of this specie have been described as an effective biological control agent against GTDs [13,46].

The method for evaluating the biocontrol potential was performed by modification of previously described methods by Kotze et al. (2011) [30] and Hermosa et al. (2000) [3]. In this case we adapted it to our pathogen, giving to it an advantage of 14 days previous inoculation of *Trichoderma* according to [47]. Not a high value was obtained in terms of inhibition growth because the low rate of growth of *P. minimum*. Similar results were obtained using another method of %RGI (Radial Growth Inhibition) by [48], they obtained low rates but it is still useful for selecting isolates as potential biocontrol agents. Some strains were overgrowing the pathogen and sporulating over it and these previous methods did not evaluate these capacities. Further, other strains were able to stop quickly the pathogen, but they did not show a high production of spores or were not able to overgrow it. So that, we added another method for evaluating these abilities that it was described by [3] in order to determine their potential as future commercial product due to its capacity of antibiosis or mycoparasitism.

In dual cultures, all *Trichoderma* species were able to reduce the growth of the pathogen as shown by [47]. Thus, different behaviors were found according to each *Trichoderma* strain in terms of sporulation.

According to [3], *Trichoderma* species showed homogeneous behaviour against different pathogens, as indicates above, in our case a great number of *T. gamsii* were found, which exhibit remarkable differences in their behaviour, indicating that each isolate requires to be analysed to determine its application as a BCA in a particular environment, i.e., climate conditions, pathogens, crops.

In our case, four strains (T75 *T. koningiopsis*, T79 *Trichoderma* spp., T84 *T koningiopsis*, and T154 *Trichoderma* spp.) have the property to control *P. minimum* according to our criteria for being potential biological control agents. We identified two species *T. koningiopsis* (T75 and T84) as potential biological control agent, also another strain from this specie identified as *T. koningiopsis* (09/02) was selected as potential biocontrol agent against *Lasiodiplodia Theobromae* [49]. Strain T154 *Trichoderma* spp. belongs to green spored clade and it is close related to *T. harzianum*, being capable to remain as an endophyte in vine plants and mycoparasite *P. minimum* [50]. Other strains from the same species such as *T. harzianum* strain AG1 has been proved successfully against *Eutypa lata* and has performed a good colonization in grapevine canes [51]. Additionally, another strain like *T. harzianum* AG2 has shown effective results against GTDs [47,52] and it is commercial-

ized as Vinevax®. Another *T. harzianum* T39 strain favors a defense-related genes response against *Plasmopara viticola* [9]. Other positive effects have been demonstrated using *T. harzianum* M10 which improves crop yield, increases total amount of polyphenols and antioxidant activity in the grapes and suppresses the development of *Unicnula necator* [53]. Thus, *T. harzianum* strains show a great potential for reducing incidence of diseases in viticulture and improving sanitary status of vine plants.

An interesting possibility could be mixing different strains as cocktail of *Trichoderma* strains in one commercial product such as Remedier® or TUSAL® that have been tested against GTDs [14,17]. But, these strains need to be evaluated in order to test their intra- and inter-specific compatibility for determining their possible combinations [54].

Another method of evaluation was staining of PDA plates. Some of the strains obtained during this study were able to produce a yellow pigment, a typical characteristic of this genus, that over an agar media are able to produce 6-pentyl α-pyrone (6PP), a secondary metabolite that inhibits fungal growth [55]. So that, T106 strain from this work, could be preselected as a *Trichoderma* specie that potentially could produce high amount of secondary metabolites that are related to fungal inhibition as other strains of *T. atroviride* UST1 and UST2 isolated from vineyard [56]. This strain could be evaluated using a membrane assay as it was done in [18]. In order to perform an identification and characterization of its metabolic profile in case that *P. minimum* was inhibited using that test [56]. In case that positive results were found and identification of chemical compounds were achieved, it could be able to be mixed using those secondary metabolites and the previous selected *Trichoderma* strains [57].

Furthermore, these strains isolated from grapevine bark in this current work could be useful against insect pests such as *Xylotrechus arvicola* (Olivier) (Coleoptera: Cerambycidae) because both of them share the same ecological niche. Being an important insect pest in the Iberian Peninsula. This insect lays their eggs under the rhytidome of the grapevine plants and the action of the larvae, associated to the spread of wood fungi, causes a direct and indirect damage in this crop [58]. Moreover, biocontrol activity of other *Trichoderma* strains has been proved in in vitro tests with *Trichoderma* strains isolated from soil vineyards, a strain from work [59] that is described as T71 *T. gamsii* reached up to 100% of inhibition in eggs and an 87.5% against adults. *Trichoderma* is able to produce a range of compounds, such as quitinases, which could destroy the cell wall of the insect [59].

Related to the influence of management and the incidence on *Trichoderma* communities, this study is in concordance to [60], where they showed that fungal communities were significantly different depending on the age of the grapevines. In addition, numerous potentially plant-beneficial mycoparasites as *Trichoderma* were isolated from woody tissues of old grapevines, confirming our previous hypothesis indicating that *Trichoderma* are more likely to be found in old vineyards. In this way, we found that *Trichoderma* is one of the most common species in 58-year-old plants [60]. Thus, the older are the plants that we sampled the greater number of different *Trichoderma* strains were isolated. Furthermore, it has been suggested that other factors could affect to the presence of *Trichoderma* in the fungal communities. In agreement with that, we point out that management and specially a high amount of fungicides have a direct effect in the reduction in the fungal communities, and by the way in the BCAs present on them.

So that, using this method, as a fast method to isolate effective *Trichoderma*. Sampling a small portion of bark from vineyards, it can be obtained potential biological control agents. This classical microbiological method using our dual culture evaluation has an important advantage, that the species chosen can be obtained in the laboratory in large scale production due to its capacity to sporulate in a high proportion. Being until date, all the products commercialized as spores from *Trichoderma* isolates [5]. In comparison to other studies where only data of inhibition can be identified. In this case, sporulation on plate and sporulation over pathogen can give us an idea as a real potential in field. Thus, applying this entire protocol can be a useful tool for obtaining *Trichoderma* native strains as potential biological control agents. *Trichoderma* strains with biological properties

can be also isolated from young vineyards and under a high-pressure environment of fungicides spraying. So, they can have an important impact on biological control. However, in this case the older is the vineyard and a smaller number of fungicides per campaign is sprayed the more probabilities of finding a *Trichoderma* strain, but it does not mean that a strain has to be a potential biological control agent. This aspect remains unclear. Further studies on taxonomy and microbiome communities are necessary to unravel this hypothesis. Being their effectivity as biocontrol agent more related to biological aspects than to the management [45].

Chemical treatments with herbicides produced great changes in fungal communities according to [22]. The in vitro toxicity of some pesticides such as prochloraz, guazatine, and triticonazole over *Trichoderma* species such as *T. viride*, *T. harzianum*, *T. longibrachiatum*, and *T. atroviride* have a negative effects over these fungi, causing hyphal disruptions and extrusion of cytoplasmic content [61]. The abuse of fungicides has led not only to a lower number of *Trichoderma* strains as we have proved in our study but also to a contamination in soils as shown in [62]. So that, searching for new biological control agents, we will be able to reduce pesticide use and implement efficiently an integrated pest management [63].

5. Conclusions

A rapid and easy method for isolating *Trichoderma* from grapevine plants has been developed. The reduction in the use of fungicide treatments along with the preservation of old vineyards will lead to a healthier agroecosystem with a higher concentration of beneficial microorganisms. The best sources of *Trichoderma* BCAs are old vineyards with a reduced use of fungicide treatments.

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