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Research Article

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Evaluation of *Bacillus subtilis* PTA-271 and *Trichoderma atroviride* SC1 to control Botryosphaeria dieback and black-foot pathogens in grapevine propagation material

Catarina Leal,[a,b] David Gramaje,[c] Florence Fontaine,[b] Nicolas Richet,[b] Patricia Trotel-Aziz[b] and Josep Armengol[a*]

Abstract

Background: Grapevine trunk diseases (GTDs) are a complex group of diseases that lead to major economic losses in all wine-producing countries. The investigation of biocontrol agents (BCAs) capable of forestalling or at least minimizing the development of GTDs has, recently, become a priority. Nursery experiments were set up to (i) assess the biocontrol effect of *Trichoderma atroviride* (*Ta*) SC1 and *Bacillus subtilis* (*Bs*) PTA-271, alone and in simultaneous application, against Botryosphaeria dieback (BOT)- and black-foot (BF)- associated pathogens during the grapevine propagation process and (ii) evaluate the success of the BCA inoculation during the grapevine propagation process, using quantitative reverse-transcription polymerase chain reaction techniques.

RESULTS: The results demonstrated a significant reduction in the percentage of potentially infected plants and the percentage of fungal isolation from wood fragments of BOT and BF pathogens in nursery material treated with *Ta* SC1 and *Bs* PTA-271, respectively. In one of the experiments, simultaneous treatments with *Bs* PTA-271 and *Ta* SC1 caused a reduction in percentages of potentially infected plants and fungal isolation, from wood fragments containing BOT and BF pathogens.

CONCLUSION: These biological treatments may be relevant components of an integrated approach, using complementary management strategies to limit infection by GTD pathogens, but further research is still needed to elucidate the effectiveness of *Bs* PTA-271 and the benefits of simultaneous application with *Ta* SC1 for the control of GTD pathogens in nurseries.

Supporting information may be found in the online version of this article.

Keywords: Botryosphaeria dieback; black foot; biocontrol agents; grapevine trunk diseases; grapevine propagation process; pathogen control

1 INTRODUCTION

Grapevine trunk diseases (GTDs), despite having been extensively studied since the early 20th Century, are still considered one of the most relevant challenges for viticulture, leading to important economic losses all around the world.[1–3] Botryosphaeria (BOT) and Eutypa diebacks, and Esca, Petri and black-foot (BF) diseases, are the predominant GTDs currently found in vineyards.[1,2,4]

The control of GTDs is a major challenge for grape growers, nurserymen and scientists, mostly because of their complexity compared to other grapevine diseases. It is well-known that more than one GTD can be expressed within the same plant, and one of the most problematic aspects of the fungal pathogens associated with GTDs is their indeterminate latency (endophytic status).[5] By the time the first external symptoms appear, the grapevine wood may already be extensively damaged,[3,6] leaving viticulturists with few options to reduce the impact of GTDs in vineyards.[2] Latent infections also are dangerous during nursery propagation processes, as asymptomatic planting material infected by GTD pathogens during the various steps of nursery plant production (e.g. hydration, cold storage, grafting, callus formation) can preserve and then transmit the infections to newly planted vineyards.[2] The nursery grapevine material is very susceptible to GTD

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infections as a consequence of the several cuts and wounds made during the nursery steps,[7] and there may be an unsuspected spread of infected plants if these infections are not controlled at an early stage, first in the nursery and then in the vineyard.[2,7–10] In this sense, the most important aspects to be covered for the sustainable management of GTDs are the improvement of the phytosanitary quality of the vines produced in nursery and the subsequent prevention of pruning wound infections in the vineyard from the time of planting.[11]

Investigation of biocontrol agents (BCAs) capable of forestalling or at least minimizing the impact of GTDs is viewed as a research priority. In the past ten years, many efforts have been made to develop new microbial antagonists, including fungi, bacteria and oomycetes.[11–20] *Trichoderma* spp. currently represent one of the most studied fungal-based BCAs used in agriculture. *Trichoderma* have a broad range of benefits for plants.[21,22] They can parasitize and suppress other fungi (mycoparasitism),[23,24] and produce several secondary metabolites, such as antibiotics[21,23] that help to control fungal pathogens. Besides pathogen control, *Trichoderma* can increase both plant and root growth by enhancing nutrient and nitrogen uptake and inducing plant systemic defenses.[21,24] In grapevine, *T. atroviride* strains USPP-T1, USPP-T2, I-1237 and SC1 have shown good performances in the protection against GTDs, as have other *Trichoderma* spp. such as *T. harzianum*, *T. asperellum* and *T. gamsii* strains.[11,19,20,25,26]

Bacillus subtilis strains also have been tested widely for use as BCAs against fungal pathogens.[18,20,27–29] They are well-known for their ability to enhance the systemic defenses of the plant against subsequent biotic stresses and promote plant growth by mobilizing nutrients, increasing their availability to the plant.[20,30,31] *Bacillus subtilis* strains also can directly suppress pathogens as a result of the production of several secondary metabolites such as antimicrobial molecules, siderophores, lytic enzymes and lipopeptides.[20,30–32] *Bacillus subtilis* PTA-271,[20] *B. subtilis* F62[33] and *B. subtilis* BBG127 and BBG131,[34] have been studied and shown potential to protect grapevines against GTDs.

When performing field experiments with BCAs, the most common methods to study the quality of BCA application are re-isolations in petri dishes. Although these methods are relatively easy, show visually fast results and can be cheaper than molecular techniques, they cannot ensure that the re-isolated strain corresponds to the inoculated one, and rely on molecular techniques to do so. Moreover, with these methodologies, it is not possible to accurately quantify BCAs. By using molecular techniques, especially quantitative reverse-transcription polymerase chain reaction (qRT-PCR), it is possible to not only detect BCA presence, but also quantify the microorganism. The qRT-PCR technique also allows us to distinguish between bioaugmented inoculated strains and the low-density native ones, even in complex samples such as root or soil samples.[35,36]

Thus, the objectives of this work were (i) to assess the biocontrol effect of *T. atroviride* SC1 and *B. subtilis PTA-271*, alone and in simultaneous application, against BOT- and BF-associated pathogens during the grapevine propagation process and (ii) to evaluate the success of the BCA inoculation during the grapevine propagation process, using qRT-PCR methods.

2 MATERIAL AND METHODS

2.1 Grapevine scion and rootstock material

Two independent nursery trials (experiments A and B) were performed using scions of cv. Tempranillo and cuttings of 110 Richter rootstock from a commercial nursery located in Valencia province, Spain in 2021. In each trial, before the grafting process, 25 scion fragments and 25 rootstock fragments were selected randomly from the plant material and analyzed for the presence of GTD pathogens. For that purpose, isolations were performed from 3-cm-long sections of each material type. The sections were washed with tap water, surface-disinfested using 70% ethanol and flamed. Then, ten internal wood fragments per section were placed on malt extract agar (MEA) complemented with 0.5 g L^{-1} streptomycin sulfate (Sigma-Aldrich, St Louis, MO, USA) (MEAS) (five fragments per two Petri dishes). Plates were incubated for 10–15 days at 25 °C in the dark, and all developing colonies were then transferred to potato dextrose agar (PDA). For confirmation of species identity, fungal mycelium and conidia from pure cultures grown on PDA for 15–20 days at 25 °C in the dark were scraped and mechanically disrupted using FastPrep-24™5G (MP Biomedicals, Santa Ana, CA, USA). Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, GA, USA) following the manufacturer's instructions. The quality and integrity of the DNA was visualized on 1% agarose gels stained with REALSAFE (Durviz S.L., Valencia, Spain). DNA samples were stored at −20 °C. The identification of all isolates was performed by analysis of the internal transcribed spacer (ITS) region amplified using the fungal universal primers ITS1F and ITS4.[37,38] Additional molecular identification was then conducted for specific groups of pathogens. *Cadophora* and *Phaeoacremonium* spp. were identified by sequence analysis of the β-tubulin gene, with the primers BTCadF and BTCadR, T1 and Bt2b, respectively.[39–41] Identification of Botryosphaeriaceae spp. was performed by analysis of the translation elongation factor *1-α* gen amplified using EF1F and EF2R primers.[42] Identification of *Cylindrocarpon*-like asexual morphs was performed by sequencing part of the *histone H3* gene with primers CYLH3F and CYLH3R.48.[43]

2.2 Biological control agent preparation

2.2.1 Bacillus subtilis *PTA-271*

Bacillus subtilis PTA-271 (GenBank Nucleotide EMBL accession no. AM293677 for 16 S rRNA and DDBJ/ENA/GenBank accession no. JACERQ000000000 for the whole genome), therefore *Bs* PTA-271, was isolated in 2001 from the rhizosphere of healthy Chardonnay grapevines on a vineyard located in Champagne (Marne, France).[27,31] Bacterial growth started with addition of 1 mL glycerol stock suspension to sterile Luria Bertani (LB) medium and incubating at 25–28 °C. Experiments were performed when the bacterial culture was at the exponential growth phase. After centrifugation (5000×*g*, 10 min), the pellet was resuspended with a sterile 10 mM $MgSO_4$ medium adjusting the density to 10^6 colony-forming units (cfu) mL^{-1}.

2.2.2 Trichoderma atroviride *SC1*

Trichoderma atroviride SC1 (Vintec®, Certis Belchim B.V., Utrecht, the Netherlands; 10^{10} conidia per gram formulated product), therefore *Ta* SC1, was suspended in water at 2 g L^{-1} as indicated by the producer. The viability of the conidia in the commercial product was tested to be at a minimum of 85% before each trial, as described by Pertot *et al.*[13] A serial dilution of the conidia suspension was plated on PDA (Biokar-Diagnostics, Zac de Ther, France) and cfu counts were made after 24–48 h incubation at room temperature.

2.3 Nursery experiments

Grapevine propagating material (cuttings of 110 R rootstock subsequently grafted with Tempranillo cultivar) was treated with *Ta* SC1, *Bs* PTA-271 or the simultaneous application of both BCAs at

three stages of the grapevine propagating process as performed by Berbegal *et al.*[11]: (i) 24-h soak in suspension before grafting, (ii) 20-day application of suspension in sawdust at stratification, and (iii) 1-h soak of the basal parts of the plants in a BCA suspension before planting in the rooting field (Fig. 1). All propagation material underwent the three treatments, and the untreated control was performed with water at all stages. Two separate experiments were performed at different times and corresponding grafted plants were transferred to different rooting fields, using four replicates per treatment with 50 plants in each replicate. The two experiments were managed separately with plant material coming from different mother blocks (Fig. 1).

Grafted plants were transferred into two different nursery-rooting fields separated by >10 km (Experiment A was located at Llanera de Ranes and Experiment B was located at Rotglà i Corberà, both in Valencia province) on 19 May 2021 and 28 May 2021, respectively. Both fields were arranged in a randomized complete block design with four replicates per treatment (200 plants in total). Cultural practices were performed according to integrated pest management (IPM) guidelines and just copper compounds and wettable sulfur were applied at label amounts to control downy and powdery mildew, respectively, when required. Plants were uprooted in November 2021 and wrapped in individual perforated plastic bags to avoid cross-contamination, but also to prevent oxygen deprivation and fermentation, without exposing the cuttings to dehydration.

2.4 Fungal isolation at the end of the experiments

In each trial, 20 plants per treatment and replicate were randomly selected for fungal isolation analyses. Isolations were performed from 3-cm-long sections cut from three different areas: the grafting point, the basal end of the rootstock cuttings and the root system. Ten wood fragments per each type of area (five fragments per two Petri dishes) were analyzed (30 wood fragments per plant). The 3-cm sections were washed with tap water and surface-disinfested using 70% ethanol flamed, as described in Section 2.1. Then, ten internal wood fragments per section were placed on MEAS (five fragments per two Petri dishes). Plates were incubated for 10–15 days at 25 °C in the dark, and all developing colonies were transferred to PDA petri dishes. All GTD pathogens were identified using the techniques explained in Section 2.1, and a selection of *Trichoderma* colonies (10%) were analyzed using conventional PCR[44] to confirm the specific strain.

2.5 Quantification of biological control agents on plant material by qRT-PCR

From the 20 plants selected for fungal isolation, ten were randomly selected for qRT-PCR analysis of *Bs* PTA-271 and *Ta* SC1. Thus, after fungal isolation, the remaining wood tissues of the 3-cm-long sections from the rootstock base were ground in liquid nitrogen and stored at −20 °C. DNA was extracted from 200 mg powdered wood, according to the protocol from Alfonzo et al.[45] DNA quality was checked by agarose gel electrophoresis, and total DNA concentration was measured for each sample using the NanoDrop One spectrophotometer (Ozyme, Saint-Cyr-l'École, France) and adjusted to 50 ng μL^{-1}. Both *Ta* SC1 and *Bs* PTA-271 were tracked by qRT-PCR using specific primers. For *Ta* SC1, *endochitinase* gene (*ech42*) primers described in Savazzini et al.[46] was used. For *Bs* PTA-271, a set of primers were designed targeting the *DNA polymerase III* (*dnaE*) gene. The *DNA polymerase III* gene sequence of *Bs* PTA-271 full genome was aligned with other *Bacillus dnaE* sequences retrieved from GenBank nucleotide database using ClustalW (1.82) Multiple Alignment Program. A set of primers was designed (5′-TGGATGAAGCGAGACAGCAG-3′, 3′-TCTTCACTCAGGACAACGCC-5′), using NCBI primer designer tool.[47] A standard curve was prepared to calculate the efficiency of the primers. The curve was constructed using seven consecutive 1:5 dilutions from genomic DNA from a *Bs* PTA-271 culture in LB medium starting with 50 ng μL^{-1}. One milliliter from each bacterial concentration was subjected to DNA extraction by means of the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). Average threshold cycle (Ct) values (from duplicates) and standard deviations were calculated for each dilution. The Ct value was plotted against the log concentration of the template DNA, and the slope of the obtained regression line was used for calculating the efficiency (*E*) with the following equation: $E = [10(-1/\text{slope})] - 1 \times 100$.[48] The primers presented an efficiency of 99.8%.

qRT-PCR reactions were carried out in duplicate in 96-well plates in a 15 µL final volume containing Absolute Blue SYBR Green ROX mix including Taq polymerase ThermoPrime, dNTPs, buffer, and $MgCl_2$ (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 280 nM

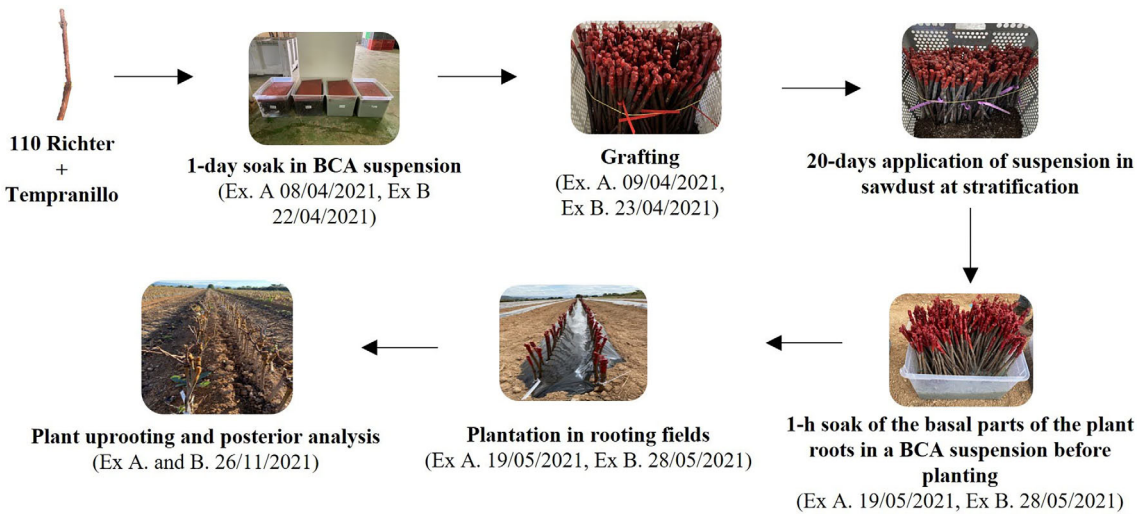

Figure 1. Process scheme of nursery experiments A and B, and BCA inoculations of all treatments (control, *Bs* PTA-271, *Ta* SC1, and both BCAs).

forward and reverse primers, and 10 ng μL^{-1} gDNA according to the manufacturer's protocol. Cycling parameters were 15 min of Taq polymerase activation at 95 °C, followed by 40 two-step cycles composed of 5 s of denaturation at 95 °C and 20 s of annealing and elongation at 60 °C. Melting curve assays were performed from 60 to 95 °C at 0.5°C s^{-1}, and melting peaks were visualized to check amplification specificity. Elongation factor *1-alpha* (*EF1*) gene was used as reference (5′-AACCAAAATATCCGGAGTAAAAGA-3′, 3′-GAACTGGGTGCTTGATAGGC-5′).[18] Relative gene expression was determined with the formula fold induction: 2(−11Ct), where 11Ct = [Ct TG (US) − Ct RG (US)] − [Ct TG (RS) − Ct RG (RS)], where the Ct value is based on the threshold crossing point of individual fluorescence traces of each sample, TG is the target gene, RG is the reference gene, US is an unknown sample and RS is reference sample. Integration of the formula was performed using CFX Manager 3.1 software (Bio-Rad, Hercules, CA, USA).

2.6 Statistical analyses

The isolation of pathogens belonging to the family Botryosphaeriaceae, causal agents of BOT disease, and *Cylindrocarpon*-like asexual morphs, causal agents of BF disease, from the grafted plants was expressed as the mean percentage of potentially infected plants and the mean percentage of positive fungal isolation from wood fragments per group of pathogens. Statistical analyses were carried out using JASP 0.16.1 (JASP team 2022). For treatment effect, mean values were analyzed using the Kruskal–Wallis test. When differences in the means were significant, Dunn's *post hoc* test ($\alpha = 0.05$) was applied to determine which treatments were significantly different from others.

3 RESULTS

3.1 Pre-existing latent infection at the nursery entrance

In Experiment A, it was not possible to detect any infection by GTD pathogens on the 25 scions of cv. Tempranillo and the 25 cuttings of 110 Richter rootstocks collected before the grafting process. In Experiment B, only two rootstock cuttings showed infection by *N. vitifusiforme*, proving that the initial infection level was very low (data not shown).

3.2 *Ta* SC1 commercial viability, recovery from trial plants and compatibility with *Bs* PTA-271

In both trials, the percentage recovery of this strain was close to 100% (90–98%) on treated plants and wood fragments colonized by *Ta* SC1, and it was not recovered from untreated plants (data not shown). Moreover, the presence of *Bs* PTA-271 did not alter wood colonization by *Ta* SC1, because they were both >90% (data not shown).

3.3 Impact of BCA treatments on fungal pathogen recovery at the end of the nursery process

At the end of both experiments, fungal pathogens associated with BOT (Botryosphaeriaceae spp.) and BF (*Cylindrocarpon*-like asexual morphs) were recovered from the grafted plants. A list containing all isolated pathogens, as well as the percentage of plants infected with each pathogen is included in Supporting Information, Table S1. Pathogens associated with Petri disease were recovered only in very few plants, and thus they were considered insufficient for statistical analysis. Pathogens associated with BF were preferably found on the roots, whereas those associated with BOT were preferably found on the grafting point. Therefore, the statistical comparison of treated and untreated plants was performed grouping the results of the different pathogens associated with BOT and BF diseases and their preferred areas for infection.

3.4 Impact of BCA treatments on the percentage of potentially infected plants and percentage of fungal isolation from wood fragments at the end of the propagation process

3.4.1 *Experiment A*

At the end of the propagation process Botryosphaeriaceae isolated fungi were *Neofusicoccum parvum, Lasiodiplodia citricola, Diplodia seriata, Nelumbo lutea* and *Lasiodiplodia pseudotheobromae*, and isolated *Cylindrocarpon*-like asexual morphs were *Ilyonectria liriodendri, Dactylonectria novozelandica* and *Dactylonectria torresensis*. Data from the different pathogens were grouped according to the two main GTDs considered in Figs 2 and 3.

The percentage of plants potentially infected with BOT- (Fig. 2) and BF- (Fig. 3) associated fungi in untreated plants was 33.75% (BOT) and 18.75% (BF), in plants treated with *Ta* SC1 it was 13.75% and 11.25%, in plants treated with *Bs* PTA-271 37.5% and 7.5%, and in plants treated simultaneously with both BCAs 7.5% and 2.5%, respectively. Regarding the percentage of fungal isolation from wood fragments, untreated plants presented 13.75% (BOT) and 4.25% (BF), plants treated with *Ta* SC1 8.5% and 2.75%, plants treated with *Bs* PTA-271 20.25% and 0%, and plants treated simultaneously with both BCAs 3.75% and 0.75%, respectively (Figs 2 and 3). ANOVAs revealed a significant

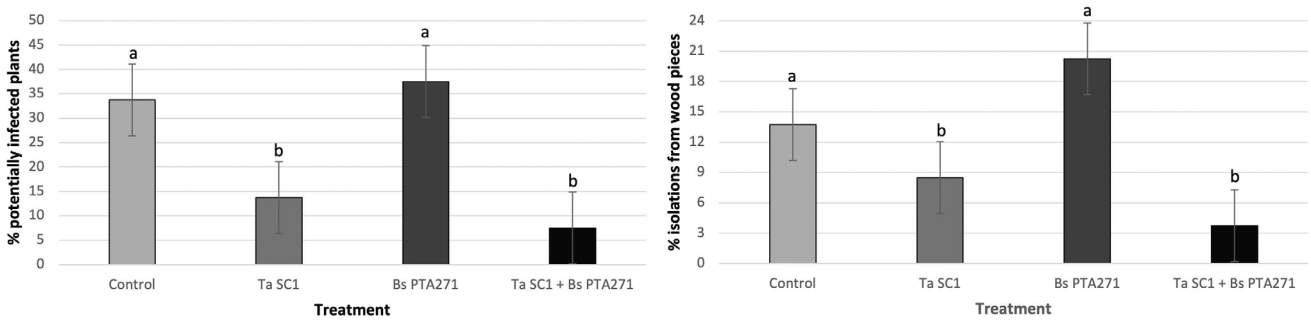

Figure 2. Percentages of plants potentially infected with Botryosphaeriaceae spp. ($P = 0.005$), and percentage of isolations of Botryosphaeriaceae spp. from wood fragments ($P = 0.007$) observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs and untreated plants (control) in the nursery experiment A. Mean percentages are based on four replicates of 20 plants per treatment and ten wood fragments per plant. Letters a and b represent significant differences ($P < 0.05$) between treatments.

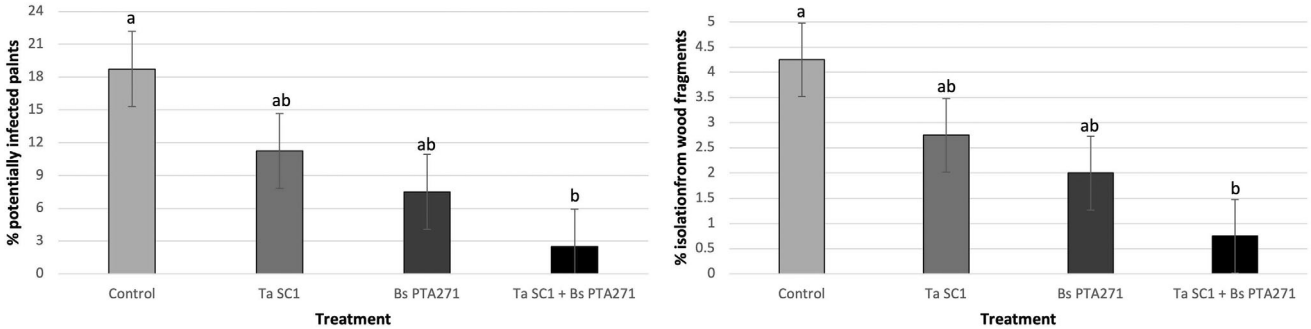

Figure 3. Percentages of plants potentially infected with *Cylindrocarpon*-like asexual morphs ($P = 0.003$), and percentage of isolations of *Cylindrocarpon*-like asexual morphs from wood fragments ($P = 0.011$) observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs, and untreated plants (control) in the nursery Experiment A. Mean percentages are based on four replicates of 20 plants per treatment and ten wood fragments per plant. Letters a and b represent significant differences ($P < 0.05$) between treatments.

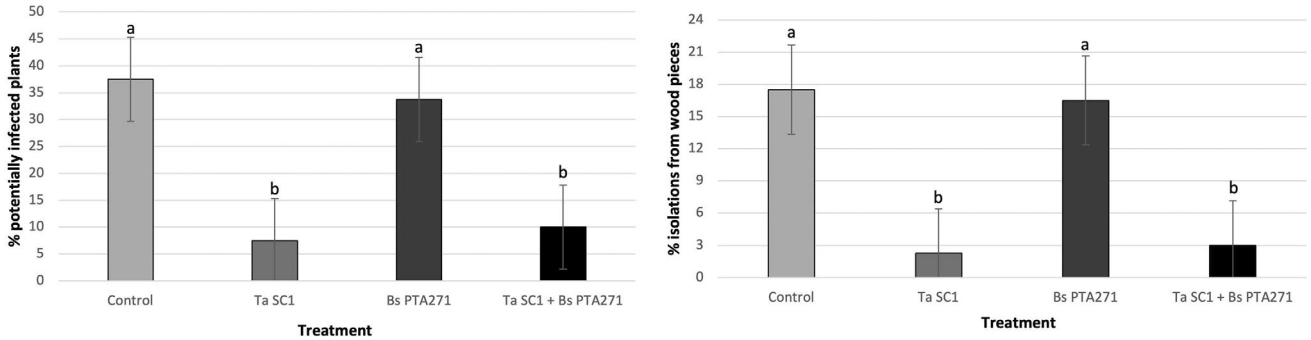

Figure 4. Percentages of plants potentially infected with Botryosphaeriaceae spp. ($P = 0.008$), and percentage of isolations of Botryosphaeriaceae spp. from wood fragments ($P = 0.01$) observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously, with both BCAs, and untreated plants (control) in the nursery Experiment B. Mean percentages are based on four replicates of 20 plants per treatment and ten wood fragments per plant. Letters a and b represent significant differences ($P < 0.05$) between treatments.

reduction of the percentage of potentially infected plants and the isolation from wood fragments of BOT-associated pathogens in plants treated with *Ta* SC1 and plants treated simultaneously with both BCAs (Fig. 2). Plants treated simultaneously with *Bs* PTA-271 and *Ta* SC1 presented the lowest percentage of BOT potentially infected plants and isolation from wood fragments, although this difference was not statistically different from plants treated with *Ta* SC1 alone. Plants treated with *Bs* PTA-271 showed no significant differences when compared to untreated plants. Regarding fungi associated with BF, plants treated simultaneously with both BCAs was the only treatment that showed significantly lower percentage of potentially infected plants and isolation from wood fragments, than untreated plants (Fig. 3). However, the values were not significantly different from the treatments with one BCA alone.

3.4.2 *Experiment B*

At the end of the propagation process, Botryosphaeriaceae isolated fungi were *N. parvum, L. pseudotheobromae, D. seriata* and *Neofusicoccum algeriense*, and isolated *Cylindrocarpon*-like asexual morphs were *I. liriodendri, D. novozelandica* and *D. torresensis*. As for Experiment A, data from the different pathogens were grouped according to the two main GTDs considered in Figs 4 and 5.

The percentage of plants potentially infected with BOT- (Fig. 4) and BF- (Fig. 5) associated pathogens in untreated plants was 37.5% (BOT) and 10% (BF), in plants treated with *Ta* SC1 7.5% and 9.25%, in plants treated with *Bs* PTA-271 33.75% and 8.75%, and in plants treated simultaneously with both BCAs 10% and 6.25%, respectively. Regarding the percentage of fungal isolation from wood fragments, untreated plants presented 17.5% (BOT) and 2% (BF), plants treated with *Ta* SC1, plants treated with *Bs* PTA-271 16.5% and 1.75%, and plants treated simultaneously with both BCAs 3% and 2%, respectively (Figs 4 and 5). ANOVAs revealed significant reduction of the percentage of potentially infected plants and the fungal isolations from wood fragments of BOT associated fungi in plants treated with *Ta* SC1 and plants treated simultaneously with both BCAs. Plants treated with *Ta* SC1 presented the lowest percentage of BOT potentially infected plants and isolation from wood fragments. Plants treated with *Bs* PTA-271 showed no significant differences when compared to untreated plants (Fig. 4). Regarding fungi associated with BF, there was not a statistically significant reduction of the percentage of potentially infected plants or the percentage of fungal isolation from wood fragments with any treatment (Fig. 5).

3.5 BCA detection by qRT-PCR at the end of the propagation process

Because *Bs* PTA-271 is more often recovered from roots, and *Ta* SC1 more often recovered from the rootstock and grafting point, molecular analysis was performed with wood sections from the base of the rootstock, in order to facilitate the simultaneous detection of both BCAs. In both experiments, *Ta* SC1 and *Bs*

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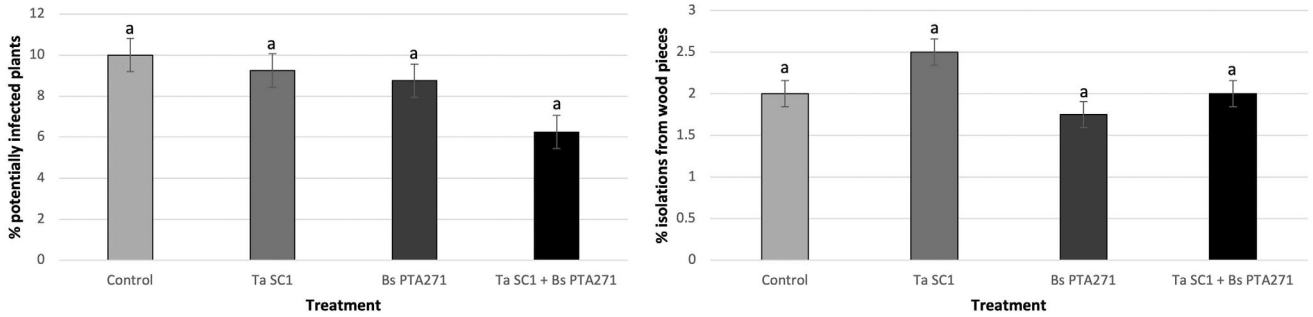

Figure 5. Percentages of plants potentially infected with *Cylindrocarpon*-like asexual morphs (*P*e = 0.819), and percentage of isolations of *Cylindrocarpon*-like asexual morphs from wood fragments ($P = 0.804$) observed in plants treated with *Bs* PTA-271, *Ta* SC1, simultaneously with both BCAs, and untreated plants (control) in the nursery Experiment B. Mean percentages are based on four replicates of 20 plants per treatment and ten wood fragments per plant. Letters a and b represent significant differences ($P < 0.05$) between treatments.

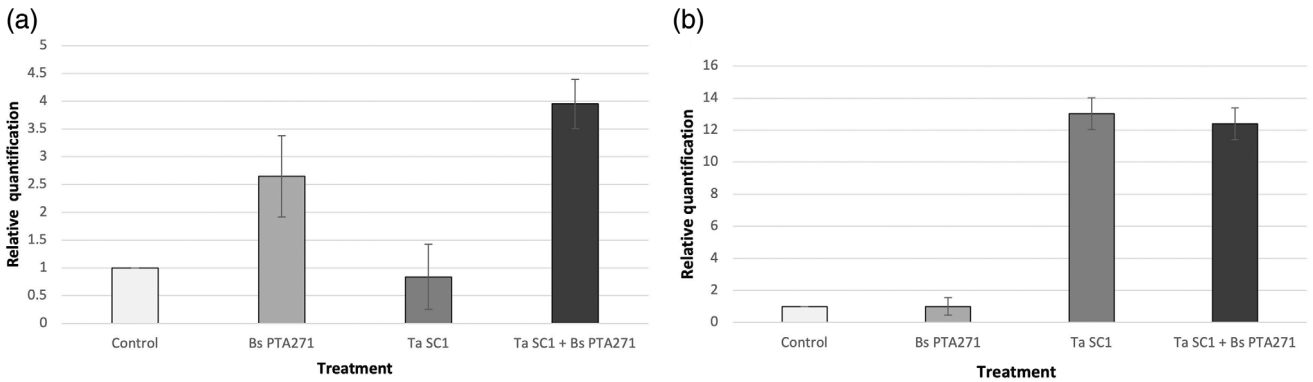

Figure 6. Relative quantification of *Bs* PTA-271 (a) and *Ta SC1* (b), in Experiment A on plants treated with *Bs* PTA-271, *Ta* SC1, and simultaneously with both BCAs. The quantification of each BCA is in comparison with untreated plants (control) that represents 1×.

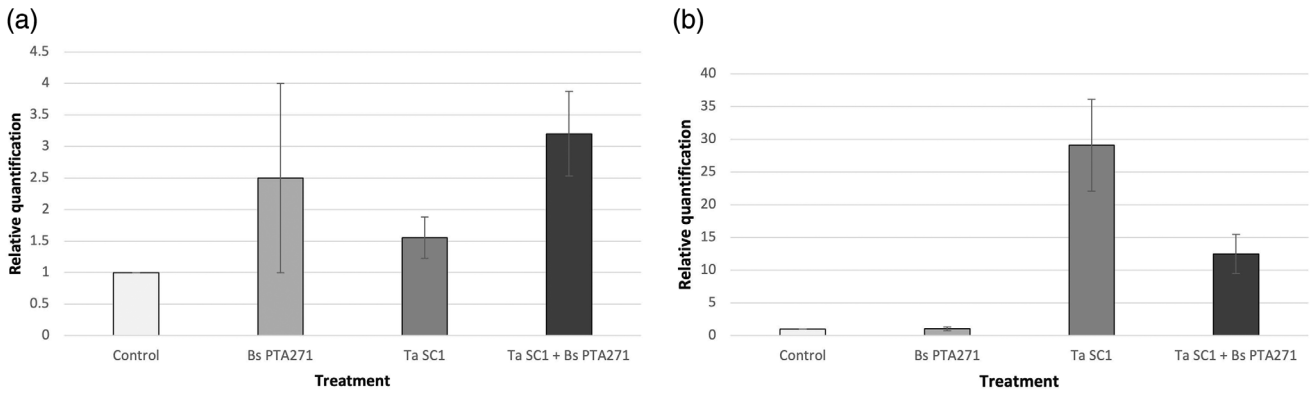

Figure 7. Relative quantification of *Bs* PTA-271 (a) and *Ta SC1* (b), in Experiment B on plants treated with *Bs* PTA-271, *Ta* SC1, and simultaneously with both BCAs. The quantification of each BCA is in comparison with untreated plants (control) that represents 1×.

PTA-271 were detected in every treated plant, and in untreated plants, neither *Ta* SC1 nor *Bs* PTA-271 were detected (Figs 6 and 7).

3.5.1 *Experiment A*

In Experiment A (Fig. 6), plants treated with *Bs* PTA-271 presented 2.65-fold more *Bs* PTA-271 than the untreated plants, and plants treated simultaneously with both BCAs presented 3.96-fold more *Bs* PTA-271 than the untreated plants. Plants treated only with *Ta* SC1 showed a similar quantity of *Bs* PTA-271 to that of the control, showing no signs of cross-contamination. Regarding *Ta* SC1, plants treated with *Ta* SC1 presented 13.1-fold more *Ta* SC1 than the untreated plants, and the plants treated simultaneously with both BCAs showed 12.2-fold more *Ta* SC1 than the untreated plants. Plants treated with only *Bs* PTA-271 showed a similar quantity of *Ta* SC1 to that of the control, showing no signs of cross-contamination.

3.5.2 *Experiment B*

In Experiment B (Fig. 7), plants treated with *Bs* PTA-271 presented 2.5-fold more *Bs* PTA-271 than the untreated plants, and plants treated simultaneously with both BCAs presented 3.2-fold more

Bs PTA-271 than the untreated plants. Plants treated only with *Ta* SC1 showed a similar quantity of *Bs* PTA-271 to that of the control, showing no signs of cross-contamination. Regarding *Ta* SC1, plants treated with *Ta* SC1 presented 29.09-fold more *Ta* SC1 than the untreated plants, and the plants treated simultaneously with both BCAs showed 12.48-fold more *Ta* SC1 than the untreated plants (Fig. 7). Plants treated only with *Bs* PTA-271 showed a similar quantity of *Ta* SC1 to that of the control, showing no signs of cross-contamination (Fig. 7).

4 DISCUSSION

The aim of the current study was to investigate the biocontrol effect of *Ta* SC1 and *Bs* PTA-271, in single and simultaneous application, against BOT- and BF-associated pathogens, during the grapevine propagation process. Leal *et al.*[20] already evaluated the biocontrol effect of *Ta* SC1 and *Bs* PTA-271, in single and simultaneous application, against *N. parvum* Bt67 in Chardonnay and Tempranillo grapevines in controlled glasshouse conditions. Although the layout of both works can be somewhat related, nursery experiments enabled us to work in much more realistic environmental conditions, including natural infections with GTD pathogens, and additional microorganism interactions, delivering much more accurate results.

It is well-known that the grapevine grafting process leads to an increased risk of infection by GTDs pathogens as a consequence of the several cuts and wounds produced during this process, by the use of grafting machines and scissors infected with pathogen spores, and during callusing (subjected to high temperature and humidity) and field rooting phases.[2,7,9] It also is during the propagation process that grapevine acquires the first GTD pathogen infections, that will progress in the plant, leading to the appearance of GTD symptoms years later.[49] For this reason, when performing experiments with nursery grapevines, it is not possible to detect GTD symptoms because the infections are only recently acquired.[9] In fact, in our nursery experiments, before the grafting process, the natural infection levels of GTDs in the plant material were zero or almost negligible, with only one pathogen, *N. vitifusiforme* found in Experiment B. However, at the end of the propagation process, the diversity of GTD pathogens recovered from untreated plants increased significantly. Gramaje *et al.*[50] studied the temporal dynamics of the fungal microbiome in rootstocks during the propagation process, and concluded that during the process, the abundance of plant pathogens largely increases, and genus *Neofusicoccum* was found as a persistent taxon in different plant material, in agreement with our results. In both experiments, BOT- and BF-associated pathogens were prevalent, emphasizing the importance of trying to contain these early infections in the propagation process, to avoid BOT and BF symptoms- developing later in the vineyard; BOT and BF have been reported as some of the most common GTDs worldwide.[2,3,9,51]

The colonization of the BCAs *Ta* SC1 and *Bs* PTA-271, alone or simultaneously, in treated plants, was successful in both experiments, with higher quantities of the BCAs in treated plants than in untreated plants. This was concluded using, for the first time in nursery field experiments, qRT-PCR methodology applied to wood samples collected from the rootstock base. Unlike the traditional PCR technique, qRT-PCR has the sensitivity and accuracy necessary to detect specific bioaugmented strains of microorganismsand can be used to monitor BCA population changes over a period of time, representing the future of pathogens and BCA detection.[33,34]

In both experiments, plants treated with *Ta* SC1 presented significantly lower percentages of potentially infected plants and of fungal isolations from wood fragments, of BOT-associated pathogens, compared with untreated plants. Berbegal *et al.*.[11] also proved this effect during the propagation process. Concerning BF-associated pathogens, plants treated with *Ta* SC1 showed no significant differences in neither the percentage of potentially infected plants nor the percentage of isolation from wood fragments, when compared to the untreated plants. In Experiment B, this was most likely caused by the initial lower percentage of potentially infected plants (untreated plants 10%, compared with 18% in Experiment A). However, it is known that *Trichoderma* spp., like any other BCA, shows inconsistencies in its biocontrol effect, depending not only on the types of pathogens, but also on the environmental conditions (temperature, relative humidity, surface wetness, gases present and air movement), plant cultivar metabolism, and other microorganisms present. In fact, several studies have shown the lack of effect of *Trichoderma* spp. against BF-associated pathogens.[11,19,52,53]

Focusing now on *Bs* PTA-271 effect, several authors previously have pointed out a positive effect of *B. subtilis* to control infections by BOT-associated fungi,[1,33,54,55] More specifically, Trotel-Aziz *et al.*[18] and Leal *et al.*[20] had already shown the beneficial effect of *Bs* PTA-271 in reducing the symptoms of one BOT-associated fungus (*N. parvum*) in grapevine cv. Chardonnay, but could not detect the same positive effect in cv. Tempranillo. Corroborating these previous findings, treatments with *Bs* PTA-271 did not reduce either the percentage of potentially infected plants or the percentage of fungal isolation from wood fragments of BOT-associated fungi.

By contrast, it is noteworthy to highlight that *Bs* PTA-271 showed promising effects against BF-associated fungi. Indeed, in Experiment A, plants treated with *Bs* PTA-271 presented a reduced percentage of potentially infected plants, and percentage of isolation from wood fragments of BF-associated fungi, when compared with untreated plants, although in Experiment B it was not possible to detect any significant difference. Other studies also have reported the effect of endophytic bacteria from genus *Streptomyces* reducing infection rates by BF pathogens, both in propagation material and in young grapevines.[19,56] These results are promising when considering that BF pathogens are particularly difficult to manage as a result of the many parameters influencing their development, such as soil nature, but also environmental and plant stress factors such as soil mineral deficiency, poor drainage, soil compaction, heavy crop loads on young plants, and poorly prepared soil.[57] More importantly, during the grapevine propagation process and especially at the time of planting, the susceptible basal ends of most of the nursery cuttings are partly or even fully exposed, because the young callus roots easily break during the planting process, resulting in additional wounds susceptible to infection by soilborne pathogens.[58] The grapevine roots therefore are an area of intense infection where BF soil-borne pathogens establish a parasitic relationship with the plant. But to succeed in infecting the root tissues, BF pathogens must compete with the beneficial microorganisms that make up the complex rhizosphere microbiome for nutrients and ecological niches,[59] explaining why this disease is so unpredictable and hard to control.[60] Currently, no curative control measures are available to eradicate BF pathogens in nurseries, and the management of this disease involves improving the quality of grapevine planting material not only by maintaining a healthy microbiome, but also by ensuring good hygiene and wound protection. During the last decades, several studies have been carried out to find effective strategies to manage

BF pathogens in nursery and established vineyards,[19,51,61–63] and a treatment with *Bs* PTA-271 could be a potential effective way to reduce the infection by BF-associated fungi during the grapevine propagation process.

For both BOT- and BF-associated pathogens, one BCA (*Ta* SC1 or *Bs* PTA-27), depending on the disease, could be the basic and simplest way to reduce infections caused by these fungi. However, both BCAs presented inconsistencies, and only showed a beneficial effect against Botryosphaeriaceae spp. (BOT) (*Ta* SC1) or *Cylindrocarpon*-like asexual morphs (BF) (*Bs* PTA-271). The simultaneous application of *Ta* SC1 and *Bs* PTA-271 presented a significant decrease in the percentage of potentially infected plants and the percentage of isolation from wood fragments, for both BOT and BF, when compared to untreated plants, but only observed in Experiment A. Simultaneous applications of BCAs already have been indicated as a possible effective strategy to overcome inconsistent control of plant pathogens in vineyards,[19,64,65] and the literature has been reporting that the interaction of more than one BCA can improve specific disease management and promote plant development and therefore resistance.[20,65–69] However, further research is necessary to recommend the benefits of the simultaneous applications of *Ta* SC1 and *Bs* PTA-271 in grapevine nurseries.

5 CONCLUSION

The results obtained in this study showed a promising biocontrol potential of treatments with *Bs* PTA-271 and *Ta* SC1 against BOT and BF pathogen infections during the grapevine propagation process of cv. Tempranillo. Alone, *Ta* SC1 confirmed its effectiveness to reduce the percentage of potentially infected plants with BOT-associated pathogens and the percentage of isolation from wood fragments, and *Bs* PTA-271 demonstrated, for the first time, effectiveness in reducing infections caused by BF-associated pathogens during the propagation process. In one of the experiments, the simultaneous application of both BCAs caused a significant decrease in the number of potentially infected plants and the number of infected wood fragments for both groups of pathogens.

In our study, qRT-PCR techniques were used for the first time in grapevine nursery experiments, enabling accurate quantification of the inoculated BCAs, confirming the successful colonization by BCAs in treated plants, and the lack of cross-contaminations between treatments.

Because the pathogen inoculum of GTD pathogens can be strongly affected by environmental factors, further research is still needed to elucidate the effectiveness of *Bs* PTA-271 and the benefits of simultaneous application with *Ta* SC1 for the control of GTD pathogens in nurseries. These biological treatments may be a relevant component of an integrated approach, using complementary management strategies to limit infection by GTD pathogens. Moreover, a long-term follow-up to evaluate the behavior of young plants treated by both BCAs once they are planted in the vineyard appears to be necessary.

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CONFLICT OF INTEREST

Trichoderma atroviride SC1 is commercialized by the company Belchim Crop Protection, which partially financed the research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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