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Activity of biocontrol agents against the grapevine pathogen *Fomitiporia mediterranea*

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Summary. Biological control agents (BCAs) have shown efficacy against several pathogens associated with Esca of grapevines, but their effects on the white rot pathogen *Fomitiporia mediterranea* (*Fmed*) have not been extensively studied. An assessment of several potential BCAs evaluated activity against *Fmed*. This included isolates of *Trichoderma simmonsii*, *T. citrinoviride*, *T. atroviride*, *Bacillus subtilis, B. amyloliquefaciens/velezensis* and *Pseudomonas koreensis*, all obtained from grapevines in Austria. Effects of the BCAs on *Fmed* growth were assessed in dual culture assays and in assays with fresh and autoclaved grapevine wood disks. In the dual culture assays, all the BCAs reduced growth of *Fmed* compared to experimental controls. In the *Trichoderma* experiments, *Fmed* growth only marginally exceeded the size of the initial mycelium plugs, and growth inhibition for all *Fmed* isolates and strains was 91 to 97%. Growth of *Fmed* was inhibited by 55 to 66% by *B. amyloliquefaciens/velezensis* isolates, by 41 to 49% by *B. subtilis* isolates, and by 55 to 66% by *P. koreensis*. In the wood disc assays, *Fmed* colonized fresh and autoclaved wood. All the *Trichoderma* isolates almost completely suppressed *Fmed* growth on fresh and autoclaved wood. Less but statistically significant inhibition was recorded for an isolate of *B. amyloliquefaciens/velezensis* and one of *P. koreensis*.

Keywords. *Trichoderma* spp., *Bacillus amyloliquefaciens/velezensis*, *Bacillus subtilis*, *Pseudomonas koreensis*, dual culture assays, wood disk assays.

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INTRODUCTION

Grapevine trunk diseases (GTDs) cause serious problems for viticulture. This disease complex is associated with many fungi affecting grapevine trunks, leading to wood decay and death of the plants. Esca in the GTD complex is present in vineyards in both world hemispheres (Fontaine *et al.,* 2016; Claverie *et al.,* 2020), and several disease symptoms have been included in the 'Esca' designation. Vascular symptoms, which likely result from blocking of host vessels by colonizing fungi combined with water stress, include longitudinal browning and necrosis of the young vessels below bark tissues.

More or less extensive white rot in the trunks of mostly old vines may impede their vital functions. Yellowing or drying of the leaf zones between the main veins results in striped appearance ("tiger stripes") of leaves. Most vines showing tiger stripes die some years after the first appearance of leaf symptoms. Esca is also associated with apoplexy, the sudden wilting of vines followed by a rapid death (Mugnai *et al.,* 1999; Lecomte *et al.,* 2012; Fontaine *et al.,* 2016; Ouadi *et al.,* 2019; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022).

Generally, it is assumed that the fungal species involved in this disease are endophytic, but have the potential of becoming pathogenic during the lives of infected vines. Several studies have provided evidence that the non-necrotic wood of grapevines showing "tiger stripe symptoms" and visually healthy grapevines hosted a more or less similar mycoflora (Bruez *et al.,* 2014, Elena *et al.,* 2018, Del Frari *et al.,* 2019). Under suitable conditions, potentially pathogenic fungi already colonizing the plants could become prevalent and lead to disease symptoms. Factors such as plant age, cultivar or pedo-climatic conditions probably influence the fungal communities within host plants (Bruez *et al.,* 2014; Bettenfeld *et al.,* 2021), and several field studies corroborate these assumptions. Climatic conditions, including high rainfall and cool temperatures in summer, have been shown to favour leaf symptoms (Calzarano *et al.,* 2018), whereas drought inhibited symptom development (Bortolami *et al.,* 2021). Vineyard soils, application of macro- and micronutrients, and plant age can also affect the disease (Kovács *et al.,* 2017; Calzarano *et al.,* 2023). No completely resistant host variety is known, but as reviewed by Beris *et al*., (2023) grapevine cultivars have different levels of tolerance or susceptibility to Esca.

Several reports indicate that the Ascomycetes *Phaeomoniella chlamydospora* (*Pch*) and *Phaeoacremonium minimum* (*Pmin*), and the Basidiomycetes *Fomitiporia mediterranea* (*Fmed*) and other *Fomitiporia* spp., are the main pathogens associated with Esca development. *Pch* and *Pmin* have predominantly been related to vascular disease symptoms, while *Fmed* and other *Fomitiporia* spp. are involved in wood decay (Mugnai *et al.,* 1999; Fischer and Garcia, 2015; Fontaine *et al.,* 2016; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022). These conclusions are strengthened by studies showing that *Pch* and *Fmed* prevail in the microbiome of Esca affected grapevines (Del Frari *et al.,* 2019; Bruez *et al.,* 2020). A wide range of other fungi have also been found in symptomatic grapevines, including *Stereum hirsutum, Eutypa lata*, *Cadophora luteo-olivacea*, and members of the *Botryosphaeriaceae*, but their roles in disease development are considered to be less relevant (Fischer, 2002; Fontaine *et al.,* 2016; Gramaje *et al.,* 2018; Fischer and Peighami-Ashnaei, 2019; Claverie *et al.,* 2020). Co-occurrence with *Fmed* and *Pch* of some bacterial species, such as *Sphingomonas, Mycobacterium* and *Paenibacillus,* possibly indicate their roles in disease development and wood degradation (Bruez *et al.,* 2020; Haidar *et al.,* 2021).

Eradication of the pathogens involved in Esca development is not possible. Therefore, control practices rely on disease prevention, or, if already present, mitigation of its effects. Vine training and pruning options considering an undisturbed sap flux may influence the Esca severity. Plants trained with long cordons were generally less affected by the disease than those with short or no cordons (Lecomte *et al.,* 2018). Surgery of infected vines to remove white rot affected wood has been shown to be effective for trunk remediation (Pacetti *et al.,* 2021).

Protection of pruning wounds, aiming to prevent infections by airborne pathogen spores, is likely one of the most effective GTD management practices. Treatments can include liquid or paste products forming barriers over pruning wounds, fungicides alone, fungicides in combinations with mechanical barriers, or biological control agents (BCAs). Fungicides, however, have the disadvantage, that the compounds remain effective for short periods, but pruning wounds remain susceptible to pathogens for several weeks to months. BCAs colonizing the pruning wounds may therefore be alternatives to chemical control methods or control by mechanical barriers. In addition, BCAs can increase resistance of host plants to biotic or abiotic stresses, and have potential to elicit systemic induced resistance. Regarding Esca management, BCAs have been evaluated as pruning wound protectants, for effects on pathogen spread during nursery processes, and for their general effects on plant growth, health, and resistance to the disease (for reviews see Gramaje *et al.,* 2018 and Mondello *et al.,* 2018).

Trichoderma spp. have long been recognized as potential biocontrol agents for plant diseases. Their effects have been linked to production of antimicrobial compounds, induction of host resistance, mycoparasitism, and/or competition for nutrients and space (for review see Harman *et al.,* 2004). Numerous reports have indicated abilities of *Trichoderma* spp. to control several pathogens involved in the Esca complex, such as *Pch*, *Pmin* and *Botryospaeriaceae* (citations found within Mondello *et al.,* 2018). Promising results have led to the homologation of biopesticide products based on *Trichoderma* spp. for pruning wound protection in several European countries, e.g. for *T. atroviride* in Austria (BAES, 2023). Field studies on the effect of these pesti-

cides, however, have shown inconsistent efficacy. Experiments in Spain, including artificial inoculation of pruning wounds, detected no effects of *T. atroviride*-based treatments on infections by *Diplodia seriata* or *Pch* (Martínez-Diz *et al.,* 2021). In contrast, in recent studies in Italy *T. asperellum* and *T. gamsii* treatments reduced the ability of artificially inoculated *Pch* to colonize the vines (Di Marco et al., 2022). Under practical conditions in four vineyards in Northern Italy preventive *Trichoderma* applications over 9 years gave 66 to 90% reductions in Esca incidence (Di Marco *et al.,* 2022). These results were similar to those from another experiment (Bigot *et al.,* 2020), in which *T. asperellum* and *T. gamsii* applications over 7 years reduced incidence of infected grapevines by 22% in three 'Sauvignon blanc' vineyards in the Friuli Venezia Giulia region of North-eastern Italy.

Apart from antagonistic activity of *Trichoderma* spp. against GTD associated pathogens, other fungi, including the Ascomycetes *Clonostachys rosea* and *Epicoccum layuense* (Del Frari *et al.,* 2019; Silva-Valderrama *et al.,* 2021), and *Fusarium oxysporum* (Gkikas *et al.,* 2021), and the Oomycete *Pythium oligandrum* (Yacoub *et al.,* 2016), may also have antagonistic effects against GTD pathogens.

So far, bacterial BCAs have been less tested and research has predominantly focused on isolates from the *B. subtilis* group. *In vitro* studies indicated effects of *B. subtilis* against *Pch, Pmin* and *Lasiodiplodia theobromae* (Compant *et al.,* 2013). In the field, a *B. subtilis* isolate inoculated on pruning wounds reduced incidence of *Pch* (Kotze *et al.,* 2011). *Pseudomonas* spp. isolated from grapevine were effective against *Pch* and *Pmin* in dual culture assays (Niem *et al.,* 2020), and *Paenibacillus alvei* showed antagonistic activity against *Pch* (Gkikas *et al.,* 2021).

There are many reports of effects of BCAs on the Esca associated vascular pathogens, *Eutypa* and *Botryosphaeriaceae*. In contrast, studies on control of *Fmed* by BCAs have been few, although *Fmed* is considered to be the main white rot inducer in the Esca disease complex (Moretti *et al.,* 2021). The aim of the present study was to assess a range of BCAs for their inhibitory effects against *Fmed*. BCAs isolated from grapevines and a commercial BCA product were included in this research.

MATERIALS AND METHODS

Bacterial and fungal isolates

Potential bacterial and fungal antagonists included in this study are listed in Table 1. The isolated strains had been recognized as potentially antagonistic in several multiannual Esca experiments. They were recovered from trunks or dormant canes of old asymptomatic grapevines by placing wood pieces on malt extract agar (MEA, Roth), containing 20 g L^{-1} malt extract and 16 g L^{-1} agar (pH 6.8–7.2). The commercial BCA product *T. atroviride* (Vintec, Belchim, Schwechat, Austria) was also included in the experiments. The isolates *Fmed*_133 and *Fmed*_2395 were obtained from symptomatic grapevine trunks (Table 1).

For identification of bacterial isolates, suspensions of the bacteria in 0.01% Triton-X100 (Roth) were prepared and heated to 95°C for 7 min. Dilutions of the suspensions were then used directly for PCR. Fungal DNA was isolated with the DNeasy Plant Mini Kit as specified by the manufacturer (Qiagen). GoTaq G2 Green Master Mix (Promega) was used for all amplifications. Annealing temperature (Tm; Table 2) and elongation time at 72 °C were adjusted according to the target genes. All programs were run for 35 cycles. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen), and were sent to LGC (Berlin, Germany) for sequencing. The following markers were amplified and sequenced: small subunit rRNA (SSU), large subunit rRNA (LSU), intergenic region (IGS), internal transcribed spacer (ITS), DNA gyrase subunit B (gyrB), and translation elongation factor EF1α. As the isolates originated from different test series, different sets of markers were used for molecular identifications. Primers for amplification and sequencing of markers are summarized in Table 2.

Dual culture assays

Freshly growing cultures of the BCAs and *Fmed* were used for experiments. Three inoculation loops of each bacterial culture or *Trichoderma* conidium masses were suspended in 5 mL of sterilized PBS. Suspensions (each of 0.5 mL) were plated onto MEA plates and incubated at 28°C for 1 week. Cultures of *Fmed* were obtained by transferring three small pieces of a *Fmed* culture on MEA to each MEA plate, and the plates were then incubated for 10 d at 28°C. For dual culture assays, mycelial discs (12 mm diam.) were taken out from the *Fmed* cultures and placed in the centres of MEA culture plates (10 cm diam.). Identical discs were taken from potential antagonist cultures, cut into four quarters, and then were placed at the edge of each plate at regular intervals. The plates were incubated in the dark at 28°C. After 10 d the diameters of the *Fmed* cultures were measured and the radii less initial mycelium plugs were calculated. Petri dishes including *Fmed* cultures in the centres and MEA quarters at the edges served as experimental controls. Experiments including *Fmed*_133 were repeated 8 times.

Table 1. Bacterial and fungal isolates included in this study.

Species and isolate	Abbreviation	Source	SSU[a]	IGS/ITS	LSU	gyrB	EF1α
*Bacillus amyloliquefaciens/ velezensis*_624	*B. amylo_velez*_624	Unnamed grapevine cross, Langenzersdorf, A	OQ533503				
*B. amyloliquefaciens/ velezensis*_2143	*B. amylo_velez*_2143	'Grüner Veltliner', Langenzersdorf, A	OQ533504				
*B. amyloliquefaciens/ velezensis*_2277	*B. amylo_velez*_2277	'Pinot Noir', Langenzersdorf, A	OQ534377	OQ534377	OQ534377		
*B. subtilis*_224	*B. subtilis*_224	Unnamed grapevine cross, Langenzersdorf, A	OQ534529	OQ534529	OQ565287		
*B. subtilis*_230	*B. subtilis*_230	Unnamed grapevine cross, Langenzersdorf, A	OQ534530	OQ534530	OQ565288		
Pseudomonas koreensis subgroup 2273	*P. koreensis*_2273	'Pinot Noir', Langenzersdorf, A	OQ565286	OQ565286	OQ565289	OQ541843	
*Trichoderma citrinoviride*_232	*T. citrino*_232	Unnamed grapevine cross, Langenzersdorf, A		OQ534541	OQ534541		OQ541844
*T. simmonsii*_804	*T. simmonsii*_1056	Unnamed grapevine cross, Langenzersdorf, A		OQ534542	OQ534542		OQ541845
*T. simmonsii*_1056	*T. simmonsii*_804	'Saint Laurent', Langenzersdorf, A		OQ534543	OQ534543		OQ541846
T. atroviride SC1	*T. atro*_Vintec	Vintec, Belchim (Schwechat, A)					
*Fomitiporia mediterranea*_133	*Fmed*_133	'Roesler', Langenzersdorf, A		OQ534544	OQ534544		
*F. mediterranea*_2395	*Fmed*_2395	'Sauvignon blanc', Eppan, IT		OQ534545	OQ534545		OQ541847

[a] GenBank accession numbers for phylogenetic markers: SSU = small subunit rRNA gene; LSU = large subunit rRNA gene; IGS = intergenic spacer; ITS = internally transcribed spacer; gyrB = DNA gyrase subunit B; EF1α = translation elongation factor EF1α.

Table 2. Primers for amplification and sequencing of phylogenetic markers for bacteria and fungi.

Primer	Marker[a]	Direction	Sequence	Temperature	References
Bacteria					
16S0008F-YM	SSU	fwd	AGAGTTTGATYMTGGCTCAG	55°C	Frank *et al.,* 2008
16S0968F	SSU/IGS	fwd	AACGCGAAGAACCTTAC	55°C	Felske *et al.,* 1996
16S1512R	SSU	rev	ACGGTTACCTTGTTACGAC	55°C	Lane, 1991
pHr	IGS/LSU	fwd	TGCGGCTGGATCACCTCCTT	55°C	Massol-Deya *et al.,* 1995
p23SR01	LSU	rev	GGCTGCTTCTAAGCCAAC	55°C	Massol-Deya *et al.,* 1995
UP-1	gyrB	fwd	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	60°C	Yamamoto and Harayama, 1995
UP-2r	gyrB	rev	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCA	60°C	Yamamoto and Harayama, 1995
Fungi					
ITS1F	ITS/LSU	fwd	CTTGGTCATTTAGAGGAAGTAA	54°C	Gardes and Bruns, 1993
TW14	ITS/LSU	rev	GCTATCCTGAGGGAAACTTC	54°C	Setaro *et al,.* 2006
EF1-0728F	EF1α	fwd	CATCGAGAAGTTCGAGAAGG	50°C	Carbone and Kohn, 1999
EF1-1620R	EF1α	rev	GACGTTGAADCCRACRTTGTC	50°C	Stielow *et al.,* 2015

[a] SSU = small subunit rRNA gene; LSU = large subunit rRNA gene, IGS = intergenic spacer; ITS= internally transcribed spacer; gyrB DNA gyrase subunit B; EF1α= translation elongation factor EF1α.

To confirm the outcome of these experiments, a second isolate, *Fmed*_2395, was included and experiments with *Fmed*_2395 were repeated 4 times. Inhibition of mycelium growth (%) was calculated as follows:

C-T/C*100

where: C = radius of the fungal colony less radius (mm) of the initial mycelium plug in the control plates, and
T = radius of the fungal colony less initial mycelium plug in the BCA treatment.

Wood disc model

To confirm antagonistic effects observed in the dual culture assays a protocol was developed using grapevine wood sections. Young grapevine plants or rooted cuttings in pots were excluded because *Fmed* is a coloniser and degrader of (older) wood. Most research has cultivated *Fmed* only in agar plates, cultivation on dried and sterilized sawdust of grapevine trunks has been published recently (Schilling *et al.,* 2022). Cross sections of grapevine trunks placed on water agar were used in the present study. *Fmed* does not sporulate on agar plates (Fischer, 2002), so as with the dual culture assays (above), *Fmed* mycelium discs were used for *Fmed* inoculations. Healthy 10- to 15-year-old grapevines 'Rotburger' ('Zweigelt') in a vineyard in Langenzersdorf (Austria) were uprooted and their trunks were cut into approx. 4 cm thick cross sections. Initially and to keep the experiment similar to plant situations, the experiments used freshly cut trunk cross sections that were immediately used. However, sizes of *Fmed* colonies within the treatments, particularly from the treatments with bacterial BCAs, gave variable results (experiments in October and December 2021; Figures 1 and 2). In consequence, the experiments were enlarged using autoclaved wood discs. For autoclaving, all wood pieces required

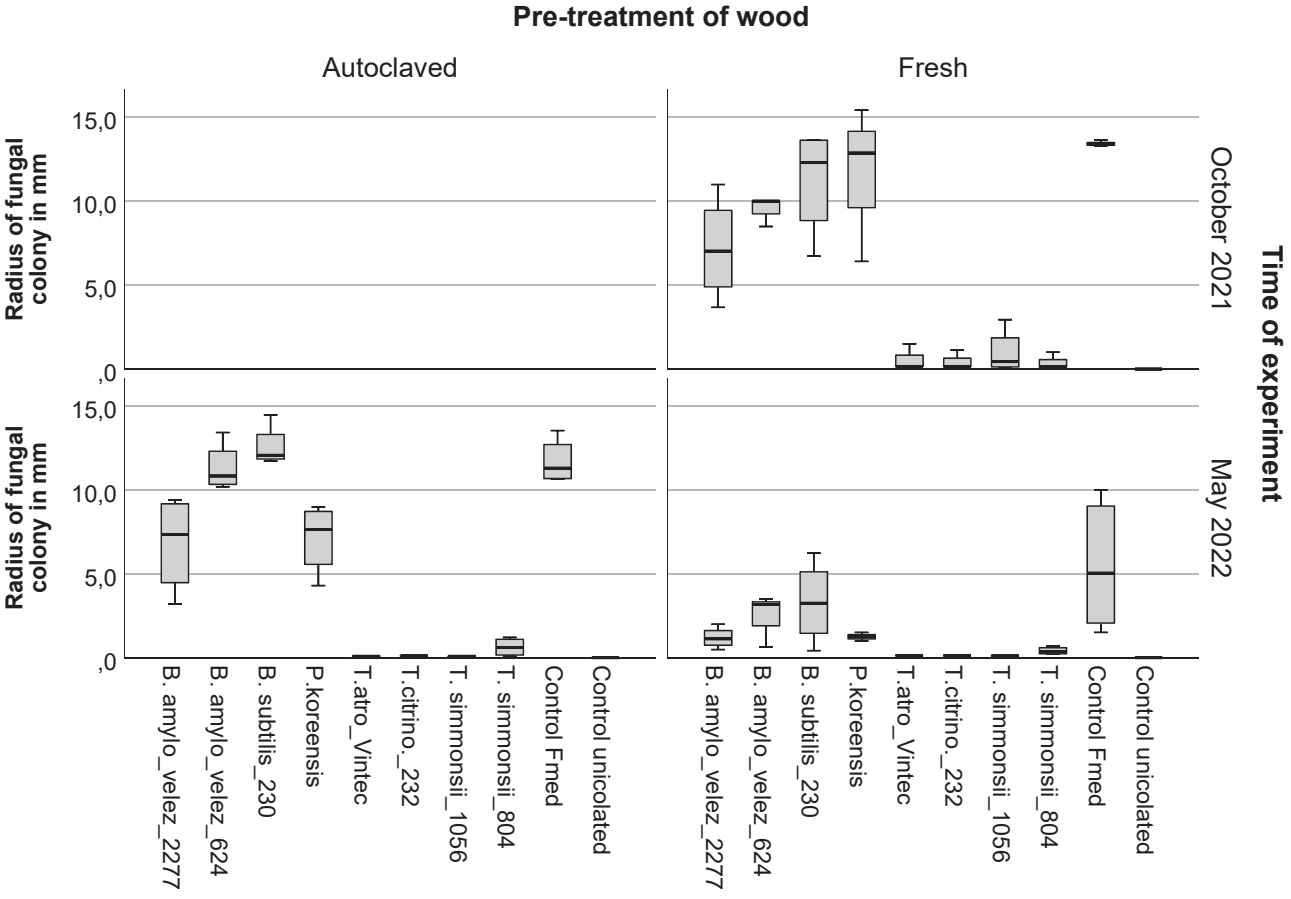

Figure 1. Data of radii of *Fomitiporia mediterranea* colonies on grapevine wood discs receiving different pre-treatments, in experiments carried out at different times of the year. Each boxplot shows a median value, and the box boundaries indicate the 25th and 75th percentiles of each distribution.

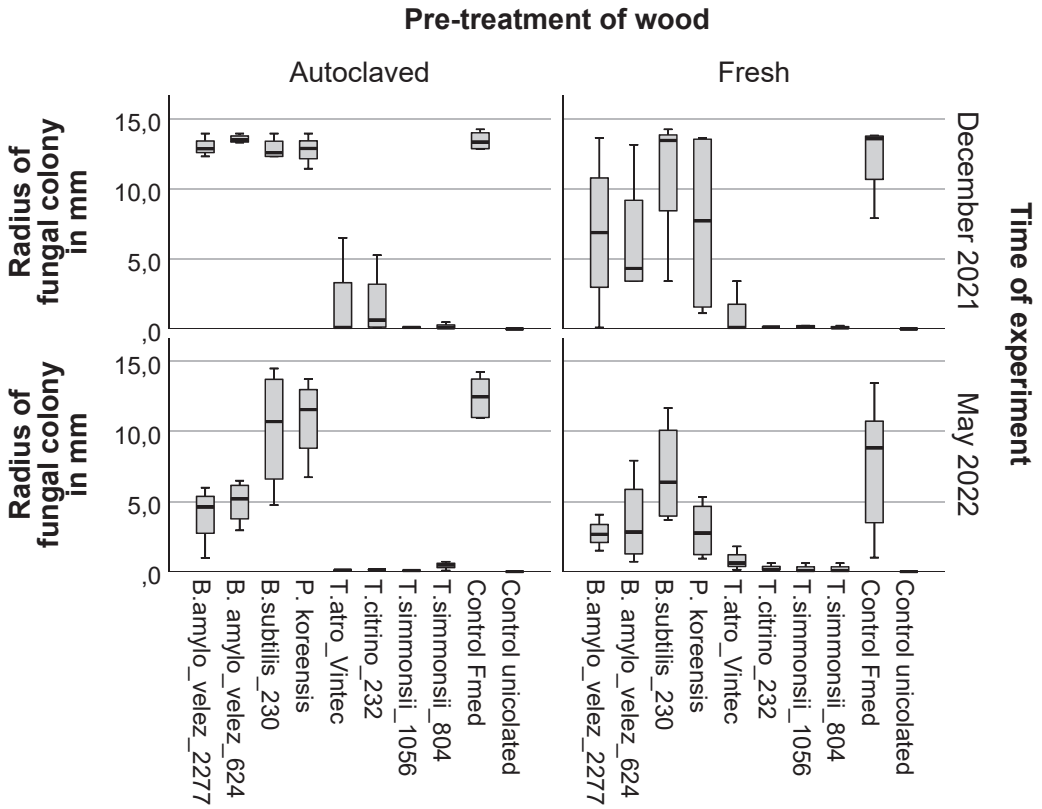

Figure 2. Data of radii of *Fomitiporia mediterranea* colonies on grapevine wood discs receiving different pre-treatments, in experiments carried out at different times of the year. Each boxplot shows a median value, and the box boundaries indicate the 25th and 75th percentiles of each distribution.

for one repetition of the experiment were together immersed in 500 mL of PBS, were autoclaved and then allowed to stand in PBS for approx. 1 week.

Bacterial BCA isolates were cultivated on MEA at 28°C. After 1 week, three inoculation loops of bacterial colonies were transferred to 40 mL of liquid tryptic soy medium (trypticase peptone, 17 g L^{-1}; soy peptone, 3 g L^{-1}; NaCl, 5 g L^{-1}, K_2HPO_4, 2.5 g L^{-1}; glucose, 2.5 g L^{-1}, pH 7.2: chemicals supplied by Roth; US Food and Drug Administration, 2023), and the cultures were incubated at room temperature on a shaker for 18 h. The cultures were then centrifuged at 5400 g for 10 min. Resulting pellets were washed twice in PBS, resuspended in PBS, and the OD600 was adjusted to 0.15 0.2. *Trichoderma* species isolated during previous experiments were cultivated on MEA as described (above) for the dual culture assays. After 7 d, the Petri dishes were each covered with 10 mL of sterile PBS. *Trichoderma* conidia were released from the culture plates by aid of a Drigalski spatula. The resulting conidium suspensions were each filtered through a cheese cloth. Conidium counts were determined in duplicate using a Neubauer's chamber, and the inoculation suspensions were adjusted to 1×10^8 CFU mL^{-1}. *T. atroviride*_Vintec inoculation suspension was prepared according to the manufacturer's instructions (2 g L^{-1} (w/v) in tap water).

The tests followed two temporal sequences: *A. Preventative treatment – BCA inoculation of stem cross sections before inoculation with* Fmed. The wood pieces were each submersed in BCA suspension for a few seconds, run-off was allowed, and they were placed onto water agar. After incubation at 28°C for 7 d, a *Fmed* mycelium plug produced as described (above) for dual culture experiments was placed on the centre of each wood piece. After further incubation for 4 weeks, two perpendicular diameters of each developing *Fmed* colony were measured, and the colony radius (less the radius of the initial mycelium plug) was calculated. In rare cases, especially when fresh wood discs were used, two *Fmed* colonies grew on the wood discs. In these cases, the radii of both colonies were summed. The experiment in October 2021 (harvest of grapevine trunk on 6 October) comprised fresh wood discs only, while the experiment in May 2022 (harvest of trunk 31 May) included fresh

and autoclaved wood discs. Each experiment comprised four repetitions.

B. Curative treatment – Fmed *inoculation of trunk cross sections before antagonist application. Fmed* mycelium plugs were placed in the centres of the wood discs on water agar. After 7 days of incubation (as above), the *Fmed* agar plugs were removed. The wood discs were then immersed in antagonist conidium/spore suspensions for a few seconds, and placed on fresh water agar plates. After further incubation for 4 weeks, the sizes of the developed *Fmed* colonies were determined (as described above). Each experiment was repeated four times in December 2021 (trunk harvest 9 December) and in May 2022 (trunk harvest 24 May). Trunk cross sections treated with PBS only ("Control uninoculated") and cross sections inoculated with *Fmed* but not with BCAs ("Control *Fmed*") served as experimental controls. For each repetition of the experiments, wood from one grapevine was used.

Statistical analyses

Statistical analyses were carried out using the program SPSS 26.0 (SPSS, IBM, Vienna, Austria). All data were processed using generalised linear models, including the distribution and link function "gamma with log link" and the dependent variable "size of the *Fmed* colony".

For analysis of the dual culture assays, the model included the explanatory variables "treatment". For each *Fmed* isolate, an individual model was calculated (Table 3).

Data obtained for the wood discs treated according to the "preventative treatment" (BCA inoculation of trunk cross sections before inoculation with *Fmed*) in October 2021 or May 2022 were included in two separate models. The model for data from October 2021 comprised the explanatory variable "BCA treatment" only, and the model for data from May 2022 comprised the explanatory variables "BCA treatment" and "pre-treatment of wood" (autoclaved or fresh wood) (Table 4). The model for the wood discs from the "curative treatment" (*Fmed* inoculation before BCA application) included the explanatory variables "BCA treatment", "pre-treatment of wood" and "time of experiment" (December 2021 or May 2022) (Table 5). Main effects were assessed, as offered by the program. Differences in efficacy of the treatments were identified using Least significant difference (LSD) tests. The data sets for the wood disk models are illustrated as boxplots, each illustrating the median and the 25th and 75th percentiles of the distribution. Median *Fmed* colony radii are presented in the text.

RESULTS

Dual culture assays

As illustrated in Figure 1, all of the potential BCAs included in this study reduced ($P \leq 0.05$) growth of *Fmed* as compared to the untreated controls. The three *B. amyloliquefaciens/velezensis* isolates inhibited fungal growth by 55 to 66%, and the two *B. subtilis* isolates reduced growth by 41 to 49%. *P. koreensis*_2273 was more effective in experiments with *Fmed*_133, inhibiting growth of the pathogen by 66%, while for *Fmed*_2395 growth inhibition was 55%. In the *Trichoderma* experiments, *Fmed* growth only marginally exceeded the size of the initial mycelium plugs, giving growth inhibition of 91 to 97% for both *Fmed* strains from all the *Trichoderma* isolates. The generalised linear model indicated statistically significant effects of the factor "BCA treatment" for both *Fmed* strains (*Fmed*_133: Wald χ^2 = 378.99, df = 10; P = 0.000; *Fmed*_2395: Wald χ^2 = 251.60, df = 10, P = 0.000). The *Trichoderma* isolates were more effective ($P \leq 0.05$) against *Fmed* than the bacterial BCAs (Table 3).

Wood disc model

Placement of *Fmed* mycelium discs on the wood discs for 7 d allowed fungal colonisation of fresh and autoclaved wood. *Fmed* formed at the beginning white mycelia on the discs, which gradually turned yellow as the fungus developed.

Preventive treatment. BCA inoculation of the wood cross sections before inoculation with Fmed

In October 2021, the median radius of the *Fmed* cultures on the fresh control discs was 12.9 mm, and in the *Trichoderma* experiments the *Fmed* colonies measured between 0.1 and 0.45 mm. Median colony radii for the bacterial BCAs were from 7.0 mm (*B. amlyo_velez*_2277) to 12.9 mm (*P. koreensis*_2273). No fungal growth was observed on uninoculated wood discs (Figure 2). The generalised linear model indicated significant effects of the factor "BCA treatment" on *Fmed* colony size (Wald χ^2 = 148.97, P = 0.000, df = 8). All the *Trichoderma* isolates but none of the bacterial BCAs reduced ($P \leq 0.05$) fungal growth compared to the *Fmed* controls. (Table 4).

In May 2022, *Fmed* median colony radius on autoclaved wood in the control treatments was 11.3 mm, and for treatments with the *Trichoderma* isolates was from 0.1 to 0.6 mm. In treatments with the bacterial BCAs mean

Table 3. Activity of BCAs in reducing growth of two *Fomitiporia mediterranea* isolates (*Fmed*_133 or *Fmed*_2395) in dual culture assays. Outcomes of the generalised linear models calculated for each isolate and mean colony dimensions of *F. mediterranea*. Means accompanied by different letters are different ($P \leq 0.05$).

Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug.						
Factor: BCA treatment	*Fmed*_133			*Fmed*_2395		
	Wald χ^2 = 378.99, P = 0.000, df = 10			Wald χ^2 = 251.60, P = 0.000, df = 10		
	Mean radius	SD	% Inhibition	Mean radius	SD	% Inhibition
*B. amylo_velez*_2143	6.1bc	0.7	58.6	7.5b	0.5	60.5
*B. amylo_velez*_2277	5.4b	0.2	63.2	6.4b	1.6	66.5
*B. amylo_velez*_624	5.8b	0.6	60.7	8.5b	1.0	55.3
*B. subtilis*_224	8.7d	2.8	40.6	9.6b	1.1	49.3
*B. subtilis*_230	8.4cd	1.1	42.7	9.7b	1.0	48.7
*P. koreensis*_2273	5.0b	0.8	66.0	8.4b	0.6	55.9
*T. citrino*_232	1.3a	2.3	91.0	0.6a	0.4	96.7
*T. atro*_Vintec	0.8a	0.7	94.9	0.9a	0.3	95.4
*T. simmonsii*_1056	0.9a	0.4	93.7	1.0a	0.6	94.7
*T. simmonsii*_804	0.5a	0.5	96.6	1.3a	0.7	93.4
Control *Fmed*	14.6e	2.0		19.0c	0.3	

colony radii were 7.3 mm from *B. amlyo_velez*_2277, and 12.1 mm from *B. subtilis*_230. On fresh wood, in May 2022, median size of *Fmed* colonies in the controls was 5.0 mm, from all *Trichoderma* treatments was 0.1 to 0.35 mm, and from the bacterial BCAs was from 1.1 mm (*B. amlyo_velez*_2277) to 3.2 mm (*B. subtilis*_230) (Figure 1). No *Fmed* growth was detected on the uninoculated wood discs Statistical analyses confirmed effects ($P \leq 0.05$) of the factors "BCA treatment" (Wald χ^2 = 592.18, P = 0.000, df = 8) and "pre-treatment of wood" (Wald χ^2 = 20.92, P = 0.000, df = 1) on *Fmed* growth. All the *Trichoderma* isolates and *B. amylo_velez*_2277 and *P. koreensis*_2273 reduced ($P \leq 0.05$) *Fmed* growth, compared to the *Fmed* control. *Fmed* grew more rapidly on autoclaved than on unautoclaved wood pieces (Table 4).

Curative treatment. Inoculation of wood discs by Fmed *before BCA application.*

In December 2021, the median size of *Fmed* colonies in the control treatment on autoclaved wood was 13.3 mm, and on fresh wood 13.6 mm. Median colony sizes for the *Trichoderma* treated wood discs were between 0.1 and 0.6 mm on autoclaved wood, and were 0.1 mm for all isolates on fresh wood. On autoclaved wood, median

Table 4. Preventive activity of biocontrol agents on growth of *Fomitiporia mediterranea* (*Fmed*_133) on grapevine wood disks. Outcomes of the generalised linear models and estimated marginal mean colony dimensions of *F. mediterranea*. Values accompanied by different letters are different ($P \leq 0.05$).

Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug		
Factor	Variant	Estimated marginal mean radius
October 2021		
BCA treatment Wald χ^2 = 148.97, P = 0.000, df= 8.	*B. amylo_velez*_2277	7.17b
	*B. amylo_velez*_624	9.49b
	*B. subtilis*_230	11.23b
	*P. koreensis*_2273	11.88b
	*T. atro*_Vintec	0.49a
	*T. citrino*_232	0.40a
	*T. simmonsii*_1056	0.99a
	*T. simmonsii*_804	0.35a
	Control *Fmed*	13.43b
May 2022		
BCA treatment Wald χ^2 = 592.18, P = 0.000, df = 8.	*B. amylo_velez*_2277	3.24cd
	*B. amylo_velez*_624	6.03de
	*B. subtilis*_230	6.76de
	*P. koreensis*_2273	3.62cd
	*T. atro*_Vintec	0.12a
	*T. citrino*_232	0.13a
	*T. simmonsii*_1056	0.10a
	*T. simmonsii*_804	0.51b
	Control *Fmed*	7.93e
Pre-treatment of wood Wald χ^2 = 20.92, P = 0.000, df = 1.	Autoclaved wood	1.61a
	Fresh wood	0.08b

Fmed colony sizes on wood discs treated with bacterial BCAs ranged from 12.6 to 13.5 mm, and on fresh wood, from 4.3 mm (*B. amylo_velez*_624) to 13.4 mm (*B. subtilis*_230). In May 2022, median *Fmed* colony radii on autoclaved wood discs were 12.5 mm and on fresh control discs 8.8 mm. Median radii of *Fmed* colonies on *Trichoderma* treated discs in no case exceeded 0.6 mm, on autoclaved and on fresh wood. For the bacterial BCAs, *Fmed* colony radii on autoclaved wood varied from 4.6 mm (*B. amylo_velez*_2277) to 11.6 mm (*P. koreensis*_2273), and on fresh wood from 2.7 mm (*B. amylo_velez*_2277) to 6.4 mm (*B. subtilis*_230). No *Fmed* growth was observed on uninoculated control discs (Figure 2). Statistical analyses showed significant effects of the factors "BCA treatment" (Wald χ^2 = 500.24, P = 0.000, df = 8), "pre-treatment of wood" (Wald χ^2 = 8.14, P = 0.004, df = 1), and "time of the experiment"

Table 5. Curative activity of biocontrol agents on growth of *Fomitiporia mediterranea* (*Fmed*_133) on grapevine wood disks. Outcomes of the generalised linear models and estimated marginal mean colony dimensions of *F. mediterranea*. Values accompanied by different letters are different ($P \leq 0.05$).

Dependant variable: Radius (mm) of fungal colony less radius of mycelium plug		
Factors	Variant	Estimated marginal mean radius
BCA treatment Wald $\chi^2 = 500.24$, $P = 0.000$, df = 8.	*B. amylo_velez*_2277	6.38b
	*B. amylo_velez*_624	5.88bc
	*B. subtilis*_230	9.89bc
	*P. koreensis*_2273	7.81bc
	*T.atro*_Vintec	0.84a
	*T.citrino*_232	0.47a
	*T.simmonsii*_1056	0.16a
	*T.simmonsii*_804	0.23a
	Control *Fmed*	11.45c
Pre-treatment of wood Wald $\chi^2 = 8.14$, $P = 0.004$, df = 1.	Autoclaved wood	2.47a
	Fresh wood	1.61b
Time of experiment Wald $\chi^2 = 8.52$, $P = 0.004$, df = 1.	December 2021	2.48a
	May 2022	1.60b

(Wald $\chi^2 = 8.53$, $P = 0.004$, df = 1). A significant effect on *Fmed* growth as compared to the control was proven for all *Trichoderma* isolates and for the bacterial BCA *B. amylo_velez*_2277. *Fmed* grew faster on autoclaved wood and in the experiment in December 2021 (Table 5).

DISCUSSION

In the first step of our experiments, the dual culture assays on MEA, all of the *Trichoderma* isolates were highly effective. They reduced mycelium growth of the *Fmed* isolates by more than 90%, and in all cases overgrew the pathogen colonies (data not shown). Effects on growth of *Fmed* in dual culture assays were previously reported for *T. asperellum* and *T. gamsii* where growth inhibition up to 65% was reported. *T. asperellum* completely overgrew *Fmed* at 18 and 23°C (Di Marco *et al.,* 2022). Reasons for the higher *Fmed* growth inhibition in the present study are unclear, but may be due to differences in efficacy of the tested BCA strains, the sensitivity of the *Fmed* isolates, or in the experimental methods (e.g. different temperatures and/or culture media).

The dual culture experiments recorded biocontrol activity for all the bacterial BCAs assessed. Compared to previous reports, growth inhibition rates of approx. 60% recorded in the present study for *B. amyloliquefaciens/velezensis* and *P. koreensis* appeared promising. Haidar *et al.* (2021) tested 59 bacterial species from various taxa, isolated from grapevines, for interactions with *Fmed* in co-cultures on agar plates. Only six of the tested isolates inhibited fungal growth at rates greater than 50%. Of these, two *Bacillus* sp. isolates gave mean inhibition of 55.7%, and one for one *Pseudomonas* sp. isolate was 52.7%. Efficacy of bacterial BCAs in laboratory assays has also been reported against other Esca associated pathogens. Several strains of *Pseudomonas poae* and *P. moraviensis* induced growth inhibition of up to 70% for *Pch*, but only up to 26% for *Pmin* (Niem *et al.,* 2020). In a study including potential bacterial BCAs isolated from Bordeaux vineyards, 46 isolates were screened for effects against *Pch* using dual inoculations of grapevine stem cuttings. Reductions of stem necroses between 31% and 39% were recorded for *Paenibacillus, Enterobacter, Pantoea* and *Bacillus* isolates (Haidar *et al.,* 2016).

Developmental conditions on MEA strongly differ from the situation in grapevines, so a protocol closer to the situation *in planta* was developed to test the antifungal potential of BCA strains beyond dual culture assays. This included autoclaved and fresh grapevine wood, and *Fmed* mycelium discs, which colonised the wood within 7 d. The observed developing *Fmed* colonies were white and later yellow, as previously reported for *Fmed* cultivation on sawdust (Schilling *et al.,* 2022). At the beginning of the present experiments, fresh wood discs were used to keep experimental conditions as close as possible to the situation in grapevines. However, as illustrated in Figures 1 and 2, the size of *Fmed* colonies in control experiments and treatments with bacterial BCAs showed high variability, although each experiment contained wood discs from the same grapevine trunk and an identical procedure was used.

Pathogens and other microorganisms occurring in natural ecosystems (including host plants) are parts of complex microbial communities. Members of each community interact with one another and with host plants. Likewise, host plants depend on their microbiomes for survival and defence from pathogen attack. Development of pathogens or BCA agents depends on several factors, such as host genotype and nutrient status, abiotic and other environmental stresses, and microbial interactions (Brader *et al.,* 2017). Grapevine wood microbiota is particularly in rich in species, interactions within the microbial community and between the microbiota and vine physiology can strongly affect the pathogen devel-

opment (Hofstetter *et al.,* 2012; for review see Claverie *et al.,* 2020).

The current data for fresh grapevine wood discs indicated that growth of the *Fmed* colonies and establishment of bacterial BCAs were both strongly influenced by the natural microbiome in the wood. In consequence, the test design was expanded to include autoclaved wood discs. The results of these subsequent tests confirmed the presumption that the grapevine microbiome reduced growth of *Fmed*. Over all experiments, the fungus developed more rapidly on autoclaved than on non-autoclaved wood (Tables 4 and 5), and use of autoclaved wood discs to some extent reduced variability of colony growth in experimental controls and on wood discs treated with bacterial BCAs (Figures 1 and 2). Therefore, the methods used gave conditions to assess interactions between BCAs and *Fmed* and effects of natural grapevine microbiomes on interaction between BCAs and *Fmed*.

Apart from pre-treatment of wood (fresh or autoclaved), *Fmed* growth on the wood discs depended on the factor "time of the experiment". In both experimental types (models for preventive or curative treatments), *Fmed* grew more rapidly on trunk cross sections harvested in autumn or early winter than on trunks harvested in May (data for preventive treatment not presented, data for curative treatment Table 5). Previous reports have indicated declines in macro nutrients in grapevine perennial structures from bud-burst to flowering, and increases during post-harvest periods when nutrients are stored for the next growing season (Holzapfel *et al.,* 2019). Bruez *et al.,* (2014) attributed changes within fungal communities in grapevine trunks to seasonal nutrient dynamics. It therefore seems possible that fluctuating nutrient dynamics in the grapevine trunks accounted (at least partly) for the seasonal differences in *Fmed* growth observed in the current study.

The observed variability of *Fmed* growth data on wood discs within identical experiments, in several cases lacking normal distributions and the significant impacts of experimental season and pre-treatment of wood, led to the decision to waive calculation of growth inhibition rates. Instead, the results were presented as box plots allowing insights into the data sets. Aiming to consider the interlinked factors, statistical analyses of the data were carried out using multifactor generalised models.

Despite the *Fmed* growth variations, the wood disc models improved insight into the multitrophic interactions between *Fmed*, the tested BCAs, the trunk microbial community and the host plant physiology. For all of the T*richoderma* treatments, similar results were obtained, regardless of the mode of treatment (preventive or curative), the time of the experiment, or pre-treatment of the wood discs. *Fmed* growth hardly exceeded the size of the initial mycelial plugs (Figures 1 and 2). Development of the *Trichoderma* isolates was not affected by the microbiome within the fresh wood discs, and was, at maximum, slightly affected by the season in which the trunks had been harvested. Comparable to our dual culture experiments and previous assays on agar plates (Di Marco *et al.,* 2022), all the *Trichoderma* isolates completely overgrew the *Fmed* mycelia on the initial mycelial plugs (data not presented). Overall, present and previous laboratory data indicate that that the *Trichoderma* spp. efficiently supressed *Fmed*.

Field studies (Bigot *et al.,* 2020, Di Marco *et al.,* 2022) have indicated prominent roles of *Trichoderma* pruning wound protection for reducing Esca disease indices. In addition to *Fmed*, *Pch* and *Pmin* are important pathogens involved in Esca (Mugnai *et al.,* 1999; Fischer and Garcia, 2015; Fontaine *et al.,* 2016; Claverie *et al.,* 2020; Moretti *et al.,* 2021; Kassemayer *et al.,* 2022). *Pch* and *Pmin* penetrate grapevine wood in several ways. They colonize (pruning) wounds (Mugnai *et al.,* 1999), and frequently spread during plant propagation processes (Aroca *et al.,* 2010; Gramaje and Armengol, 2011). In addition, vineyard or nursery soils might be inoculum sources for *Pch* and *Pmin* (Agusti-Brisach *et al,* 2013; Saccà *et al.,* 2018). In contrast, for *Fmed*, infections of (pruning) wounds by basidiospores or transmitted inoculum from pruning tools, are considered to be the only modes of pathogen entry to host plants (Mugnai *et al.,* 1999; Moretti *et al.,* 2021). The results of previous and the present *in vitro* studies indicate a high efficacy of *Trichoderma* for protecting pruning wounds from *Fmed* infections. In the field *Trichoderma* pruning wound protection might largely prevent *Fmed* infections and this way strongly contribute to the reduction of Esca incidence in treated vineyards.

Beyond protection of pruning wounds, the curative effects of treatments against *Fmed* observed in the present study, and the ability of *Trichoderma* spp. to overgrow *Fmed* (Di Marco *et al.,* 2022), indicate that *Trichoderma* spp. could also have curative efficacy against *Fmed* infections in grapevines. *Trichoderma* conidia could be infiltrated into white rot zones of diseased vines or applied in nanomaterials (Spasova *et al.,* 2022). However, *Trichoderma* spp. have not only been isolated from asymptomatic grapevines but to an even higher extent from diseased vines. In a 10-year-old 'Cabernet Sauvignon' vineyard, *Trichoderma* spp. were isolated from 75% of asymptomatic plants and 93% of Esca symptomatic plants (Bruez *et al.,* 2014). Future field experiments are required to determine if *Trichoderma* treatments can be successful curative Esca treatments.

Several *Trichoderma* isolates from the trunks in the experimental vineyard at Langenzersdorf were identified as *T. citrinoviride*, which can grow at human body temperature (Jaklitsch, 2011) and is classified as risk group 2 fungus (TRBA 460, 2016). Grapevine trunk temperatures can regularly reach temperatures above 30°C, which selects for fungi that are adapted to these temperatures such as *Fmed* (Fischer, 2002) or possibly also *T. citrinoviride*. As an opportunistic human pathogen, *T. citrinoviride* cannot be applied as a BCA, but the results from the present study demonstrate that other *Trichoderma* spp. have potential to control growth of *Fmed*.

For the bacterial BCAs, the tests using grapevine wood discs confirmed the results obtained in the dual culture assays, but only to some extent. Figures 1 and 2 show the varied results between the different experimental protocols, and also within each protocol. Efficacy of the treatments was probably influenced by the multilateral interactions between the BCAs, grapevine microbiomes, trunk nutrient status, and pathogen development (Brader *et al*., 2017*)*. Over all the experiments, the isolate *B. amylo_velez*_2277 was the most effective in reducing *Fmed* growth on wood discs, and *P. koreensis* had some effects. *B. subtilis*_230 and *B. amylo_velez*_624 gave results that were not significantly different from the control treatments in any of the experiments (Tables 4 and 5). In previous field experiments including artificial infections of pruning wounds by *Pch*, a *B. subtilis* isolate showed some effect against the pathogen, but efficacy of *Trichoderma* sp. was superior (Kotze *et al.,* 2011). Studies in Greece, including several grapevine cultivars and different vineyards, showed positive correlations of *Bacillus* and *Streptomyces* with asymptomatic vines and negative co-occurrence of these bacteria with *Pch* and *Pmin* (Beris *et al.,* 2022). Protective effects of a *B. velezensis* isolate added to pruning wounds, from infections with *Neofusicoccum parvum* and *Diplodia seriata* were recorded by Langa-Lomba *et al.* (2023).

Experiments on the effects of BCAs on *Fmed* have rarely been reported (Moretti *et al.,* 2021), and previous studies have been carried out using agar plate assessments. Therefore, the present study adds new knowledge on the potential of BCAs for control of diseases caused by the main white rot pathogen in the Esca disease complex. Inconsistent results of BCA treatments in the field have been linked to the fact that many commercial products do not originate from the plant species or plant part they are applied to (Bruisson *et al.,* 2019). In the present study, all the BCAs except *T. atroviridae*_Vintec were isolated from grapevine wood. This and the observed efficacy against *Fmed* encourage further evaluation of the two *T. simmonsii* isolates _804 and _1056 for preventive Esca control in the field. Future efforts towards the development of suitable BCA applications within trunk tissues could allow assessment of their efficacy as curative *Fmed* treatments. The present study has also demonstrated significant efficacy of bacterial BCAs for control of *Fmed* infections, though their activity was weaker than observed for *Trichoderma* spp. Nevertheless, particularly the isolate *B. amylo_velez*_2277, may be worth further examination, in a first step for practical protection of grapevine pruning wounds.

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