Use of Endophytic and Rhizosphere Actinobacteria from Grapevine Plants To Reduce Nursery Fungal Graft Infections That Lead to Young Grapevine Decline

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ABSTRACT Endophytic and rhizosphere actinobacteria isolated from the root system of 1-year-old grafted *Vitis vinifera* plants were evaluated for their activities against fungi that cause grapevine trunk diseases. A total of 58 endophytic and 94 rhizosphere isolates were tested. Based on an *in vitro* bioassay, 15.5% of the endophytic isolates and 30.8% of the rhizosphere isolates exhibited antifungal activity against the fungal pathogen *Diplodia seriata*, whereas 13.8% of the endophytic isolates and 16.0% of the rhizosphere isolates showed antifungal activity against *Dactylonectria macrodidyma* (formerly *Ilyonectria macrodidyma*). The strains which showed the greatest *in vitro* efficacy against both pathogens were further analyzed for their ability to inhibit the growth of *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (formerly *Phaeoacremonium aleophilum*). Based on their antifungal activity, three rhizosphere isolates and three endophytic isolates were applied on grafts in an open-root field nursery in a 3-year trial. The field trial led to the identification of one endophytic strain, *Streptomyces* sp. VV/E1, and two rhizosphere isolates, *Streptomyces* sp. VV/R1 and *Streptomyces* sp. VV/R4, which significantly reduced the infection rates produced by the fungal pathogens *Dactylonectria* sp., *Ilyonectria* sp., *P. chlamydospora*, and *P. minimum*, all of which cause young grapevine decline. The VV/R1 and VV/R4 isolates also significantly reduced the mortality level of grafted plants in the nursery. This study shows that certain actinobacteria could represent a promising new tool for controlling fungal trunk pathogens that infect grapevine plants through the root system in nurseries.

IMPORTANCE Grapevine trunk diseases are a major threat to the wine and grape industry worldwide. They cause a significant reduction in yields as well as in grape quality, and they can even cause plant death. Trunk diseases are caused by fungal pathogens that enter through pruning wounds and/or the root system. Although different strategies have recently been developed to protect pruning wounds using antifungal compounds (natural or synthetic) or biocontrol agents, no tools are yet available for controlling soil pathogens that infect plants through their root system. This study shows that different actinobacterial isolates, when applied to grafts in a nursery, can significantly reduce the infection rate caused by fungal pathogens that enter through the root system. This is a new, promising, and green alternative for preventing the decline of young grapevines in nurseries and vineyards.

KEYWORDS young grapevine decline, trunk diseases, actinobacteria, rhizosphere, endophytes

Grapevines are one of the most important economic crops worldwide. Grapevine trunk diseases (GTD) are a major threat for the wine sector, causing serious economic losses to the wine industry (1, 2). GTD include different fungal diseases, the most relevant of which are *Botryosphaeria* dieback, esca, *Eutypa* dieback, Petri disease, and blackfoot. The incidence of GTD has increased over the last decades, primarily due to the lack of effective strategies for fighting these diseases (3, 4). Adult plant infections in mature vineyards can occur through the root system. This is the primary mode of colonization for pathogens belonging to the *Dactylonectria* or *Ilyonectria* genus (5), which are responsible for blackfoot disease. Other pathogens, such as species of the *Botryosphaeriaceae* family and *Eutypa lata*, mainly infect the plant through pruning wounds produced at the end of the growing season each year (6–8). Finally, other pathogens, like *Phaeoacremonium minimum* (formerly *Phaeoacremonium aleophilum*) or *Phaeomoniella chlamydospora*, can use both pathways to penetrate the plant, and these two microorganisms are the primary causal agents of Petri disease (9, 10). Young grapevine plants can be infected in the field by the same mechanisms described for adult plants. It is well known that planting material (grafted plants) produced in nurseries is frequently infected with fungal pathogens, especially those involved in blackfoot and Petri diseases. In fact, it has been widely reported that grafts can be infected at different stages of the propagation process that takes place in the nurseries (10, 11). GTD, particularly blackfoot disease and Petri disease, are also recognized as prevalent causes of young grapevine decline (YGD). Decline symptoms in young vineyards have dramatically increased all over the world since the early 1990s, and these plants are primarily infected through their root system (10).

Accordingly, in recent years, there have been many different studies aimed at controlling or diminishing fungal pathogen infection rates (6, 12–23). Most studies have focused on protecting pruning wounds by applying natural antifungal compounds (12) or different chemical fungicides. Benomyl and flusilazole are the most effective at controlling *Eutypa lata* infections (13, 14), thiophanate-methyl is used for treating both Petri disease and several *Botryosphaeriaceae* species (6) infections, and benzimidazole works effectively in protecting pruning wounds infected by *Diplodia seriata*, *P. chlamydospora*, or *Inocutis* sp. (15). The efficacy of several biological control agents (BCAs), including natural epiphytes and colonizers of pruning wounds (16–18), has been tested. The application of several strains of *Trichoderma* (19–22) has also been attempted.

Unfortunately, there is currently no treatment available to protect grafts in nurseries or grapevines in vineyards from phytopathogenic fungi that can infect plants through their root system. Nevertheless, some studies have been focused on the application of different *Trichoderma* strains to soils or the grapevine root system to prevent infection by fungi that cause GTD. Thus, in a nursery trial, Fourie and Hallen (23) reported that the application of *Trichoderma* strains had a growth-stimulating effect and reduced the infection rates of *Cylindrocarpon* sp., *Phaeoacremonium* sp., and *P. chlamydospora*. More recently, a study involving the application of *Trichoderma harzianum* at different vine growth stages in a nursery indicated that application during the rooting stage was the most effective in protecting against *P. chlamydospora* infections. Unfortunately, an increase in vine mortality was observed at the end of the growing season (19).

Actinobacteria, particularly streptomycetes, are a complex group of Gram-positive bacteria making up around 10% of the total soil microbiome (24). Although they are known as soil and rhizosphere bacteria, several reports indicate that they are also more intimately associated with plants, either as endophytic strains (25–27) or mycorrhiza-associated strains (28, 29). They are well known as important secondary metabolite producers, excreting antibiotic and antifungal compounds, and for their ability to control plant diseases (29–31).

Recently, bacterial communities associated with *Vitis vinifera* rhizospheres and their variations, including factors like geographical features (32) and vineyard management, have been characterized (33, 34). Although some reports indicate that grapevine rhizosphere-associated bacteria could have some beneficial effects on the plants, particularly in protecting them from reactive oxygen species (35), no special attention

has been focused on actinobacteria and streptomycetes as a potential group from which to isolate strains to control phytopathogenic fungi that cause GTD. Loqman and colleagues (36) isolated 142 actinobacterial strains from rhizospheres of *V. vinifera* plants cultivated in Moroccan soils, and some of these isolates were effective against different fungal pathogens not involved in GTD. This study further characterizes the endophytic and rhizosphere microbiota associated with the grapevine root system, particularly as a potential source of strains of interest in controlling the incidence of GTD produced by fungi that penetrate plants through their root system and which are therefore involved in YGD. The best-performing isolates were tested in a 3-year field trial with grafted vines in a commercial nursery in Spain between 2013 and 2015.

RESULTS

Isolation and identification of culturable endophytic actinobacteria associated with the *Vitis vinifera* root system and their antifungal activity. A total of 58 endophytic strains were obtained from four different young *V. vinifera* plants using various culture media (Table 1). 16S rRNA gene sequencing of all the strains revealed their genus levels. *Streptomyces* was the most abundant genus (26 out of 58 strains), representing 44.8% of the total isolates. *Micromonospora* (15 out of 58) and *Saccharopolyspora* strains (10 out of 58) represented 25.9% and 17.2% of the total isolates, respectively (see Fig. S1 in the supplemental material). Microorganisms from other taxa were also isolated in smaller numbers, including the rare actinomycete taxa *Nonomuraea* (37), *Kribbella*, *Rathayibacter*, and *Corynebacterium* (4 out of 58 strains [6.9%]). These strains, 1 out of 58 in each case, represented 1.7% of the total isolates. Since the primary goal of this work was to evaluate the putative application of selected actinobacteria (able to infect plants through their root system in order to control fungal pathogens involved in GTD and YGD), we screened all the endophytic strains for antifungal activity. A bioassay-based *in vitro* evaluation showed that 15.5% (9 out of 58) and 13.8% (8 out of 58) of the endophytic isolates, respectively, exhibited antifungal activity against *D. seriata* and *Dactylonectria macrodidyma* pathogens (Table 1). These pathogens were initially selected for their high growth rate. The five strains showing higher inhibition (I) index values were also tested for their antifungal activities against *P. chlamydospora* and *P. minimum*. Three endophytic isolates, *Streptomyces* sp. VV/E1, *Streptomyces* sp. VV/E2, and *Streptomyces* sp. VV/E5, showed antifungal activities against all the pathogens tested. These were selected for field assays.

Antifungal activities of rhizosphere actinobacteria. A total of 94 rhizosphere strains were isolated. Bioassay-based *in vitro* screening showed that 30.8% (29 out of 94) and 16.0% (15 out of 94) of the isolates exhibited antifungal activity against *D. seriata* and *D. macrodidyma* pathogens, respectively (Table 2). Only those isolates exhibiting antifungal activity were identified by 16S rRNA sequencing, and all turned out to be members of the *Streptomyces* genus (Fig. S1). The five strains which showed the highest I index against *D. macrodidyma* were also tested for their antifungal activities against *P. chlamydospora* and *P. minimum*. Three rhizosphere isolates, *Streptomyces* sp. VV/R1, *Streptomyces* sp. VV/R4, and *Streptomyces* sp. VV/R5, showed antifungal activity against all the pathogens tested and accordingly were selected for field assays.

Multilocus sequence analysis of actinobacterial strains selected for field assays. As previously indicated, preliminary characterization of the selected endophytic and rhizosphere actinobacteria based on 16S rRNA sequencing led to their identification as species of the *Streptomyces* genus. To further characterize and more precisely identify them, we performed a multilocus sequence analysis (MLSA) study of five housekeeping genes (*atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*).

MLSA (Fig. S2) confirmed that isolates VV/E1 and VV/R4 are the same species (sharing an MLSA evolutionary distance of 0.000; Table 3), although they exhibit macroscopic differences when grown in different culture media (Fig. S3). Both strains are closely related to *Streptomyces albaduncus* (showing an MLSA distance of 0.028 with this species; Table 3). Isolates VV/R1 and VV/E5 were located in a well-delineated

TABLE 1 Antifungal activity and culture media for the isolation of culturable endophytic actinobacteria isolated from the root system of grafted grapevines

Isolate	I index[a]		Detection of antifungal activity[b]		Isolation medium	16S rRNA sequence accession no.
	D. seriata	*D. macrodidyma*	*P. chlamydospora*	*P. minimum*		
Streptomyces sp. VV/E1	53.47	33.91	+	+	SC	KY978646
Streptomyces sp. VV/E2	61.17	59.41	+	+	ISP2	KY978647
Saccharopolyspora sp. VV/E3	31.97	35.87	−	−	SC	KY978648
Streptomyces sp. VV/E4	46.95	46.62	−	+	SC	KY978649
Streptomyces sp. VV/E5	52.50	47.15	+	+	SC	KY978650
Streptomyces sp. VV/E6	49.57	33.86	ND	ND	DPA	KY978651
Saccharopolyspora pathumthaniensis VV/E7	36.81	—	ND	ND	ISP2	KY978652
Streptomyces sp. VV/E8	33.42	—	ND	ND	DPA	KY978653
Streptomyces sp. VV/E9	—	40.24	ND	ND	DPA	KY978654
Saccharopolyspora sp. VV/E10	29.97	—	ND	ND	ISP2	KY978655
Streptomyces sp. VV/E11	—	40.24	ND	ND	SAA	KY978656
Streptomyces atrovirens VV/E12	—	—	ND	ND	ISP2	KY978657
Streptomyces sp. VV/E13	—	—	ND	ND	ISP2	KY978658
Streptomyces sp. VV/E14	—	—	ND	ND	SAA	KY978659
Streptomyces sp. VV/E15	—	—	ND	ND	SC	KY978660
Streptomyces sp. VV/E16	—	—	ND	ND	SC	KY978661
Streptomyces sp. VV/E17	—	—	ND	ND	ISP2	KY978662
Streptomyces sp. VV/E18	—	—	ND	ND	SAA	KY978663
Micromonospora sp. VV/E19	—	—	ND	ND	ISP2	KY978664
Micromonospora sp. VV/E20	—	—	ND	ND	ISP2	KY978665
Micromonospora sp. VV/E21	—	—	ND	ND	SC	KY978666
Streptomyces sp. VV/E22	—	—	ND	ND	SC	KY978667
Streptomyces sp. VV/E23	—	—	ND	ND	DPA	KY978668
Streptomyces sp. VV/E24	—	—	ND	ND	SC	KY978669
Streptomyces sp. VV/E25	—	—	ND	ND	SC	KY978670
Streptomyces sp. VV/E26	—	—	ND	ND	DPA	KY978671
Micromonospora palomenae VV/E27	—	—	ND	ND	ISP2	KY978672
S. pathumthaniensis VV/E28	—	—	ND	ND	SC	KY978673
Streptomyces sp. VV/E29	—	—	ND	ND	ISP2	KY978674
Streptomyces sp. VV/E30	—	—	ND	ND	SC	KY978675
S. pathumthaniensis VV/E31	—	—	ND	ND	ISP2	KY978676
Streptomyces sp. VV/32	—	—	ND	ND	SC	KY978677
S. pathumthaniensis VV/E33	—	—	ND	ND	ISP2	KY978678
Rathayibacter caricis VV/E34	—	—	ND	ND	ISP2	KY978679
Nonomuraea kuesteri VV/E35	—	—	ND	ND	ISP2	KY978680
S. pathumthaniensis VV/E36	—	—	ND	ND	SC	KY978681
N. kuesteri VV/E37	—	—	ND	ND	SC	KY978682
S. pathumthaniensis VV/E38	—	—	ND	ND	ISP2	KY978683
Micromonospora sp. VV/E39	—	—	ND	ND	ISP2	KY978684
Micromonospora sp. VV/E40	—	—	ND	ND	SC	KY978685
Micromonospora sp. VV/E41	—	—	ND	ND	ISP2	KY978686
Micromonospora sp. VV/E42	—	—	ND	ND	ISP2	KY978687
Micromonospora sp. VV/E43	—	—	ND	ND	ISP2	KY978688
Streptomyces sp. VV/E44	—	—	ND	ND	SAA	KY978689
Micromonospora sp. VV/E45	—	—	ND	ND	SC	KY978690
Micromonospora sp. VV/E46	—	—	ND	ND	ISP2	KY978691
Micromonospora sp. VV/E47	—	—	ND	ND	ISP2	KY978692
Micromonospora sp. VV/E48	—	—	ND	ND	SC	KY978693
S. pathumthaniensis VV/E49	—	—	ND	ND	SC	KY978694
Micromonospora sp. VV/E50	—	—	ND	ND	ISP2	KY978695
Nonomuraea sp. VV/E51	—	—	ND	ND	SC	KY978696
S. pathumthaniensis VV/E52	—	—	ND	ND	ISP2	KY978697
Streptomyces sp. VV/E53	—	—	ND	ND	DPA	KY978698
Nonomuraea sp. VV/E54	—	—	ND	ND	ISP2	KY978699
Kribbella sp. VV/E55	—	—	ND	ND	SAA	KY978700
Corynebacterium sp. VV/E56	—	—	ND	ND	ISP2	KY978701
Micromonospora sp. VV/E57	—	—	ND	ND	ISP2	KY978702
Streptomyces sp. VV/E58	—	—	ND	ND	SAA	KY978703

[a]Inhibition index (I index) values shown are the average of three independent experiments. —, species not showing antifungal activity against *D. seriata* or *D. macrodidyma*.
[b]ND, not determined.

TABLE 2 Antifungal activity and culture media for the isolation of culturable rhizospheric actinobacteria isolated from the root system of grafted grapevines

Streptomyces isolate	I index[a]		Detection of antifungal activity[b]		Isolation medium	16S rRNA sequence accession no.
	D. seriata	D. macrodidyma	P. chlamydospora	P. minimum		
VV/R1	48.75	55.06	+	+	ISP2	KY978704
VV/R2	45.52	53.20	−	+	SC	KY978705
VV/R3	42.58	49.13	−	−	ISP2	KY978706
VV/R4	51.69	50.77	+	+	SC	KY978707
VV/R5	55.60	51.43	+	+	SC	KY978708
VV/R6	47.84	44.57	ND	ND	SC	KY978709
VV/R7	51.83	41.22	ND	ND	DPA	KY978710
VV/R8	51.04	44.40	ND	ND	SAA	KY978711
VV/R9	54.14	21.55	ND	ND	ISP2	KY978712
VV/R10	45.65	48.44	ND	ND	SC	KY978713
VV/R11	45.52	35.23	ND	ND	SC	KY978714
VV/R12	44.61	49.07	ND	ND	SC	KY978715
VV/R13	52.03	46.41	ND	ND	ISP2	KY978716
VV/R14	56.95	43.42	ND	ND	SC	KY978717
VV/R15	49.47	48.67	ND	ND	ISP2	KY978718
VV/R16	45.65	—	ND	ND	DPA	KY978719
VV/R17	40.48	—	ND	ND	SC	KY978720
VV/R18	62.27	—	ND	ND	SC	KY978721
VV/R19	48.66	—	ND	ND	SC	KY978722
VV/R20	44.67	—	ND	ND	SAA	KY978723
VV/R21	40.61	—	ND	ND	SAA	KY978724
VV/R22	55.21	—	ND	ND	ISP2	KY978725
VV/R23	48.27	47.02	ND	ND	SC	KY978726
VV/R24	40.71	—	ND	ND	ISP2	KY978727
VV/R25	80.50	—	ND	ND	ISP2	KY978728
VV/R26	62.87	—	ND	ND	DPA	KY978729
VV/R27	90.81	—	ND	ND	SC	KY978730
VV/R28	43.59	—	ND	ND	SC	KY978731
VV/R29	44.95	—	ND	ND	ISP2	KY978732

[a]Inhibition index (I index) values shown are the average of three independent experiments. —, species not showing antifungal activity against *D. seriata* or *D. macrodidyma*.
[b]ND, not determined.

subclade including *Streptomyces peucetius* and *Streptomyces xantholiticus* strains (Fig. S2). Both isolates share an MLSA distance of 0.108, indicating they are different species. Isolate VV/E5 was identified as *S. peucetius*, as it had MLSA distances smaller than 0.007 (Table 3) with the *S. peucetius* species. Strain VV/R1 matched between *S. peucetius* (0.108 MLSA distance) and *S. xantholiticus* (0.112 MLSA distance) (Fig. S2), suggesting that it could be a novel *Streptomyces* species. Isolate VV/E2 was located in a subclade including *Streptomyces albidochromogenes*, *Streptomyces flavidovirens*, and *Streptomyces helvaticus* (Fig. S2). MLSA distances with these strains were always higher than 0.007, indicating that isolate VV/E2 could be a novel species. Isolate VV/R5 is closely related to members of a subclade including *Streptomyces phaeochromogenes* and *Streptomyces umbrinus* (Fig. S2), although its MLSA distance with respect to both species clearly indicated that this isolate belongs to a different species.

Field assays. (i) Growth data and mortality rate. The application of actinobacteria to grafted plants did not have a significant effect, either positive or negative, on the growth of the surviving plants, as determined by measurement of total plant height [$F_{(6,413)} = 0.37$, $P = 0.90$] (Fig. 1A) or elongation of the seventh internode [$F_{(6,413)} = 0.12$, $P = 0.29$] (Fig. 1B).

The average mortality rate of noninoculated untreated (negative control) plants in the 3-year field study was 28.4% (64 dead plants out of a total of 225 plants) (Fig. 2). Significant differences in mortality rates were observed for two of the treatments, according to the chi-square tests. The mortality rates of plants inoculated with VV/R1 (19.1% [43 dead plants out of 225 plants]) [$\chi^2 (1) = 5.41$, $P < 0.05$] or VV/R4 (17.8% [40 dead plants out of 225 plants]) [$\chi^2 (1) = 7.20$, $P < 0.01$] strains were significantly lower (Fig. 2). Thus, we could conclude that the odds of plant survival were 1.68 times higher

TABLE 3 MLSA distances for strains phylogenetically near to our isolates

| No.	Strain[a]	MLSA (Kimura 2-parameter) distance																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	S. albaduncus NRRL B-3605T																		
2	S. albaduncus NRRL b-3605gT	0.000																	
3	**VV/E1**	0.028	0.028																
4	**VV/R4**	0.028	0.028	0.000															
5	S. umbrinus NRRL B-2572T	0.094	0.094	0.092	0.092														
6	**VV/R5**	0.096	0.096	0.091	0.091	0.040													
7	S. phaeochromogenes NRRL B-1248T	0.094	0.094	0.091	0.091	0.034	0.013												
8	S. phaeochromogenes subsp. phaeochromogenes NRRL B-3010T	0.094	0.094	0.091	0.091	0.034	0.013	0.000											
9	S. helvaticus NRRL B-12365T	0.124	0.124	0.130	0.130	0.121	0.124	0.126	0.126										
10	**VV/E2**	0.119	0.119	0.126	0.126	0.118	0.122	0.122	0.122	0.043									
11	S. albidochromogenes NRRL B-24308T	0.113	0.113	0.117	0.117	0.110	0.112	0.113	0.113	0.045	0.057								
12	S. flavidovirens subsp. flavidovirens NRRL B-2708T	0.113	0.113	0.113	0.113	0.106	0.113	0.111	0.111	0.067	0.055	0.048							
13	S. flavidovirens DSM 40150T	0.113	0.113	0.113	0.113	0.106	0.115	0.112	0.112	0.067	0.055	0.050	0.001						
14	S. peucetius subsp. peucetius NRRL B-3826T	0.104	0.104	0.105	0.105	0.111	0.113	0.108	0.108	0.131	0.128	0.131	0.124	0.124					
15	**VV/E5**	0.106	0.106	0.107	0.107	0.112	0.114	0.110	0.110	0.132	0.130	0.132	0.126	0.126	0.002				
16	S. peucetius CGMCC 4.1799T	0.122	0.122	0.105	0.105	0.111	0.113	0.111	0.111	0.131	0.128	0.131	0.124	0.124	0.000	0.002			
17	**VV/R1**	0.219	0.219	0.187	0.187	0.201	0.200	0.196	0.196	0.218	0.212	0.214	0.210	0.210	0.108	0.108	0.108		
18	S. xantholiticus NRRL B-12153T	0.106	0.106	0.108	0.108	0.113	0.114	0.111	0.111	0.130	0.125	0.128	0.124	0.124	0.012	0.014	0.112	0.112	

[a]Our isolates are in bold.

on VV/R1 treatment than on untreated control plants, whereas the odds of plant survival were 1.84 times higher on VV/R4 treatment than on untreated control plants.

(ii) Isolation of pathogenic fungi. Two hundred days after planting (once the plants had completed their vegetative growth cycle and were in a dormant state), vines were carefully removed from the soil to obtain a root system that was as intact as possible. The incidence of fungal pathogens causing YGD (*P. chlamydospora*, *P. minimum*, and *Dactylonectria* sp.-*Ilyonectria* sp. group) and able to infect the plants through their root system was determined by collecting samples of wood taken from the root insertion point. All the selected actinobacteria led to a reduction in the number of fungal pathogens involved in YGD (Fig. 3) compared to the number of fungal pathogens isolated from control plants (raw data corresponding to this study are shown in Table S1).

In order to analyze the impact of every actinobacterial treatment on the number of each particular type of fungal pathogen involved in YGD, we used nested generalized linear models (Poisson distribution, with intercept versus intercept plus treatment as dependent variables into the model). The existence of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria and the number of pathogens isolated from control plants was determined using chi-square tests. Thus, in accordance with the nested generalized linear models, significant associations between the nature of the treatment and the number of fungal pathogens isolated were found in the *Dactylonectria* sp.-*Ilyonectria* sp. group and for *P. chlamydospora* ($P < 0.05$ in the chi-square tests conducted to compare the nested models) for all the actinobacteria assayed. On the other hand, no significant association ($P > 0.05$ in the chi-square tests) between the nature of the treatment and the number of fungal pathogens isolated was detected for *P. minimum*, probably due to the low number of individuals of this species isolated.

After that, chi-square and Fisher's tests were performed to establish the existence of significant differences between treatments. The results are shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated (Fig. 4). Surprisingly, despite the data shown by nested generalized linear models, significant differences ($P < 0.05$) between the negative control and the three treatments (VV/E1, VV/R1, and VV/R4) were found with respect to *P. minimum* (Fig. 3). However, these significant associations were very close
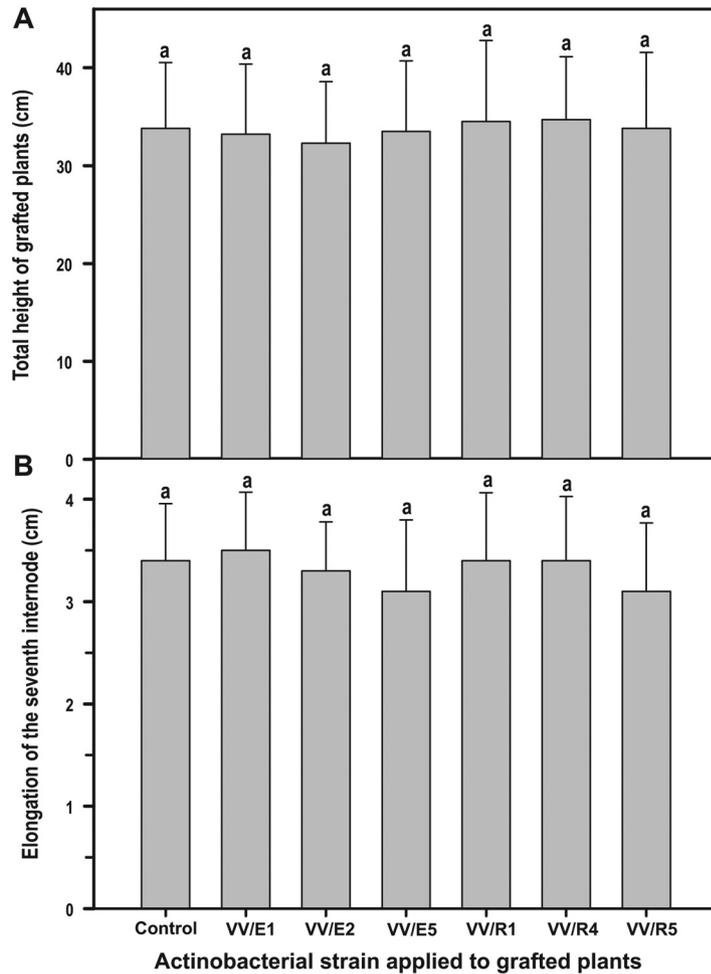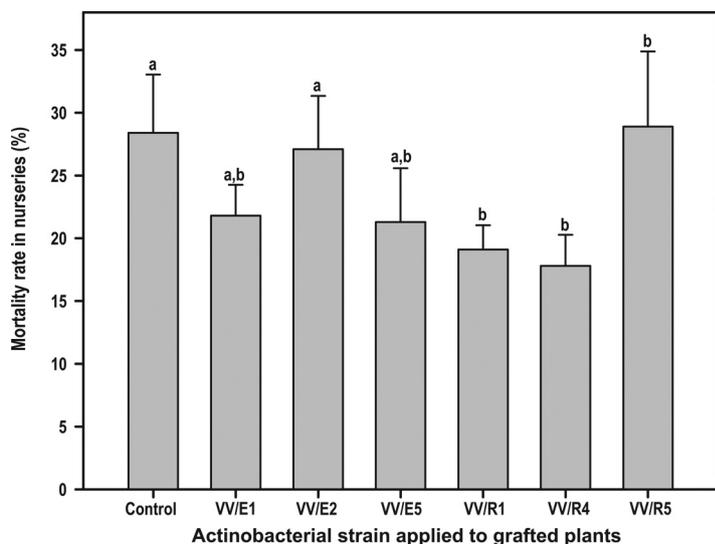
FIG 1 Plant growth in the experimental nurseries (2013, 2014, and 2015 growing seasons) as determined by total height (A) or length of the 7th internode (B) in grafted plants treated with different endophytic (VV/E1, VV/E2, or VV/E5) or rhizosphere (VV/R1, VV/R4, or VV/R5) actinobacteria compared to untreated control plants. Each bar corresponds to the arithmetic mean of the values determined for the total number of surviving plants in the three seasons tested and their corresponding standard error (SE). Bars marked with the same letter are not significantly different ($P \geq 0.05$).

to the edge of the significance threshold (0.05). It may be that the nested model tests are more conservative than individual tests performed between treatments.

In the cases related to the *Dactylonectria* sp.-*Ilyonectria* sp. group isolation, and solely focusing on those highly significant associations between the nature of the treatment and the number of fungal pathogens isolated ($P < 0.01$ and <0.001), the following results should be highlighted: control and VV/E1 [$\chi^2 (1) = 46.4$, $P < 0.001$], control and VV/R4 [$\chi^2 (1) = 12.0$, $P < 0.001$], control and VV/R1 [$\chi^2 (1) = 7.15$, $P < 0.01$], and control and VV/R5 [$\chi^2 (1) = 5.91$, $P < 0.01$] (Fig. 4). If we focus on *P. chlamydospora*, the following highly significant associations were detected: control and VV/R4 [$\chi^2 (1) = 15.8$, $P < 0.001$], control and VV/E1 [$\chi^2 (1) = 10.8$, $P < 0.01$], control and VV/E2 [$\chi^2 (1) = 10.8$, $P < 0.01$], control and VV/R1 [$\chi^2 (1) = 10.8$, $P < 0.01$], and control and VV/R5 [$\chi^2 (1) = 9.43$, $P < 0.01$] (Fig. 4).

(iii) Rootstock colonization by selected actinobacteria. The ability of the selected actinobacteria to colonize rootstocks at the root insertion point was analyzed 10 and 200 days after their application. As shown in Table 4, 10 days after application, all the actinobacteria were successfully recovered from rootstocks at percentages ranging between 11.43% (*Streptomyces* sp. VV/R5) and 36.43% (*Streptomyces* sp. VV/E1), indicating that the application method was effective enough to allow actinobacterial

FIG 2 Average mortality rates (%) and their corresponding SE values of grafted grapevine plants observed in the experimental nurseries (2013, 2014, and 2015 growing seasons) after treatment with different endophytic (VV/E1, VV/E2, or VV/E5) or rhizosphere (VV/R1, VV/R4, or VV/R5) strains compared to untreated control plants. Bars marked with the same letter are not significantly different ($P \geq 0.05$).

penetration into the rootstock. The differences were significant in all cases with respect to the isolation values for the same strain from control plants.

At the end of the trials (200 days after planting), only four strains could be reisolated from the plants, and these were always at lower levels than those detected 10 days after application. Significant differences compared to the control were observed for *Streptomyces* sp. VV/E1 and *Streptomyces* sp. VV/E2 strains. These data showed that with the passage of time, the degree of colonization tends to decrease.

DISCUSSION

It is well established that grapevine plants are mainly infected by fungal pathogens causing GTD through pruning wounds or the root system, with a reduced number of
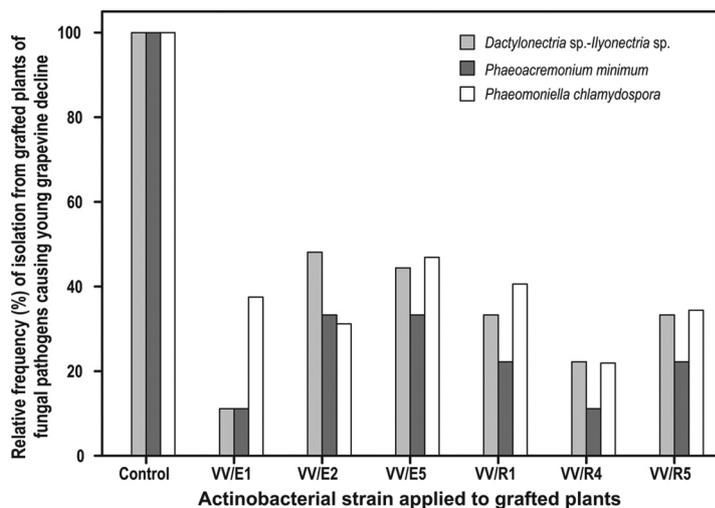
FIG 3 Relative isolation frequency (%) of fungal pathogens involved in young grapevine decline from grafted plants treated with different endophytic and rhizosphere actinobacteria with respect to untreated plants. A value of 100 was arbitrarily assigned to the total number of fungal pathogens of a particular species (*P. chlamydospora* or *P. minimum*) or *Dactylonectria* sp.-*Ilyonectria* sp. group analyzed once the plants had been removed from the field. The relative frequency for each type of pathogen analyzed in the plants treated with the different actinobacteria was calculated as a percentage (%) compared against the value of 100 assigned to the control plants. Data shown correspond to the average of 3 batches of 25 plants in 3 experimental nurseries (2013, 2014, and 2015 growing seasons).
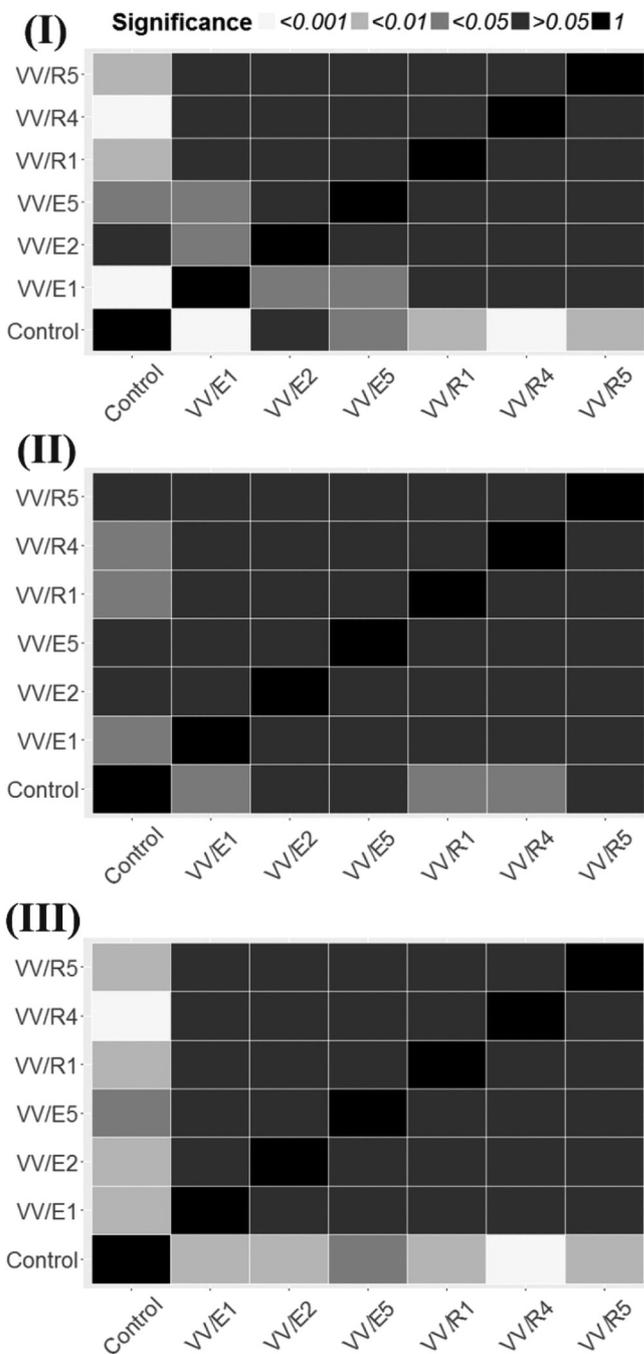
FIG 4 Analysis of significant differences between the number of fungal pathogens isolated from plants treated with the different actinobacteria versus the number of pathogens isolated from control plants. The analysis is shown as heatmaps of independence assumptions between the type of actinobacterial treatment and the number of fungal pathogens isolated. Color key corresponds to the significance of chi-square or Fisher's test results (according to expected cell values). (I) Data relative to *Dactylonectria* sp.-*Ilyonectria* sp. group; (II) data relative of *P. minimum*; (III) data relative to *P. chlamydospora*. Data shown correspond to 3 batches of 25 surviving plants in 3 experimental nurseries (2013, 2014, and 2015 growing seasons).

fungal strains that can use both routes of entry. There have been many different studies to test pruning wound protection with chemical antifungals (6, 13–15), natural antifungal compounds (12), or BCAs (16–18). Given the many years that a plant remains in the ground and the consequent extent and depth of root development and soil penetration, it is clear that the protection of grapevines from fungi that penetrate

TABLE 4 Rootstock colonization rates by selected actinobacteria at 10 and 200 days after their application

Actinobacterium isolated by batch of plants	% of isolated actinobacteria after[a]:	
	10 days	200 days
Control plants		
VV/E1	6.43 (9)	4.29 (6)
VV/E2	1.43 (2)	0.71 (1)
VV/E5	4.29 (6)	2.14 (3)
VV/R1	3.57 (5)	ND (0)
VV/R4	2.86 (4)	3.57 (5)
VV/R5	ND (0)	0.71 (1)
Treated plants		
VV/E1	36.43 (51)	13.57 (19)
VV/E2	27.14 (38)	7.86 (11)
VV/E5	16.43 (23)	4.29 (6)
VV/R1	30.00 (42)	ND (0)
VV/R4	20.71 (29)	7.14 (10)
VV/R5	11.43 (16)	ND (0)

[a]ND, not detected. The percentage of each particular strain was calculated as the number of clones growing from wood chips and identified by sequencing of 16S rRNA against a number of 140 chips tested under each condition (7 chips isolated from two stubs per 10 plants analyzed). Values are the average of two independent experiments. Raw data (number of each particular actinobacterium isolated from 140 chips) are indicated in parentheses.

through the root system is much more complicated. Current studies have not gone much beyond the application of BCAs to the root system, mainly different strains of *Trichoderma* sp. (19, 23). Thus, it seems evident that development of technologies that can limit the infection of vine plants through their root systems is of great interest for the grape and wine industry. Thus, we decided to investigate the putative application of certain actinobacteria that might help mitigate this problem.

Actinobacteria are an integral part of soil microbial communities, making up around 10% of the total soil microbiome (24), and they stand out, particularly streptomycetes, for their ability to produce a wide range of secondary metabolites, including antifungal compounds (29–31). Several studies indicate that actinobacteria can enter root tissues and establish an endophytic lifestyle with plants (26–30). As reported in other plants, the grapevine root system contains many different endophytic actinobacteria. *Streptomyces* and *Micromonospora* were the most frequently isolated genera, representing up to 70.7% of the total isolates. Similarly, when the population of endophytic actinobacteria in *Artemisia annua* was analyzed, *Streptomyces* and *Micromonospora* represented 57.0% of the total identified isolates (26). Other minority genera found in grapevine plants, like *Nonomuraea* and *Kribbella*, were also detected in *A. annua* (26). However, *Corynebacterium* and *Saccharopolyspora*, genera isolated from *V. vinifera*, were not detected in *A. annua* (26). These data confirm that actinobacteria are frequent endophytes in the root systems of both plants.

Streptomyces species identification is a great challenge, further complicated by factors, such as the fact that there are a great number of species, many of which are poorly defined. The insufficient resolution of 16S rRNA as a phylogenetic marker, difficulties in effectively addressing phenotypic differences, and the absence of rapid molecular identification methods applicable to a high number of species also hinder the accurate identification of species of *Streptomyces* (38, 39). Since 16S rRNA sequencing does not provide sufficient resolution for species-level identification of streptomycetes, we performed an MLSA study of the six isolates used in field trials by partially sequencing five housekeeping genes. MLSA has proven to be an efficient molecular tool for the improved taxonomic resolution of *Streptomycetaceae* family (39), as well as for the right identification of *Streptomyces* species for the *S. albidoflavus* (40), *S. griseus* (41), and *S. hygroscopicus* (38) clades. These clades include some of the more widely studied species in the genera, many of which produce antibiotics and other industrially and agronomically important secondary metabolites (38). In our case, the MLSA al-

lowed us to identify the isolate VV/E5 as belonging to the *S. peucetius* species. Unfortunately, this study did not lead to an accurate identification of the rest of the strains at the species level, probably due to a poor characterization of those closely related phylogenetic strains. This setback makes it difficult to establish whether these strains are unique to the root system of grapevines or to assess their ability to produce known antifungal compounds. We cannot even rule out that some of them are new species. The case of *Streptomyces* sp. VV/E1 and VV/R4 is particularly intriguing, since the MLSA study indicated that the two isolates belong to the same species. In fact, these strains share 99.5% homology at the 16S rRNA level. However, they exhibit quite different macroscopic traits when growing on various culture media, as shown in Fig. S3. Remember that *Streptomyces* sp. VV/E1 was isolated as an endophytic strain, whereas *Streptomyces* sp. VV/R4 was isolated from the rhizosphere environment. We can speculate that the observed macroscopic differences could be a putative adaptation to two different lifestyles. However, currently, we do not know for certain whether a particular strain present in the rhizosphere might also develop an endophytic lifestyle. Works under way for the development of molecular markers for the specific detection of these strains, as well as genome sequencing of the VV/E1 strain, should enable us in the future to address many of these issues that remain unanswered for now.

Recently, there have been several attempts to characterize the endophytic bacterial microbiota of grapevine plants. Andreolli et al. (42) analyzed the diversity of bacterial endophytes in 3- and 15-year-old plants. In 3-year-old plants, *Actinobacteria* was the second dominant class (26%), and the genera detected were *Nocardioides*, *Curtobacterium*, *Microbacterium*, *Brachybacterium*, *Kocuria*, and *Micrococcus*. None of these genera were detected in our study, and surprisingly, Andreolli and colleagues did not detect any *Streptomyces* sp. strains. In 15-year-old plants, the population of endophytic actinobacteria was reduced to 5%, with only 2 strains detected, belonging to the *Microbacterium* and *Curtobacterium* genera. Also recently, Rezgui et al. (43) analyzed the population of endophytic bacteria that inhabit the wood tissues from grapevines of Tunisian vineyards. Actinobacteria were hardly detected, and *Curtobacterium* was the only genus found. These differences can be attributed to many different factors. First, in both studies, endophytes were isolated from vine stems and mature arms, which are aerial habitats far from the ground. In fact, it has been reported that the population of actinobacteria decreases along the plant axis from 20% in the root, to 5% in the graft union, and just 2% in the cane samples (44). Second, the observed differences may be attributed to some extent to the different isolation culture media used, and even to the different molecular techniques used in the two works (43, 44).

Recently, different studies have focused on the characterization of the grapevine microbiome (45, 46) and vineyard soils (32, 34). After *Proteobacteria*, *Actinobacteria* are the second most abundant microorganisms in vineyard soils, representing averages of 9.72% and 20.48% of the total microorganisms detected in vineyards of Argentina (34) and California (32), respectively. In New York vineyards, *Actinobacteria* were present at 5.1% in grapevine roots, whereas their relative abundance was lower in all aboveground samples (grapes, leaves and flowers) tested (45). *Actinobacteria* are also a prominent group detected in both rhizosphere (around 44%), and roots (around 36%) of plants from Austrian vineyards (46). Unfortunately, most of these studies dealing with soil and root microbiomes do not delve deeply into characterization of the actinobacteria detected beyond the taxonomic class level. Future studies, focused more specifically on the population of actinobacteria detected in the root system of grapevine plants at the genus or even species level, should shed light on many issues that remain unresolved.

Actinobacteria were also easily isolated from young grapevine plant rhizospheres, and a screening based on antifungal activity against fungi causing GTD and YGD resulted in the identification of a total of 29 isolates, all belonging to the genus *Streptomyces*. Furthermore, Loqman and colleagues (36) have isolated strains of *Streptomyces* with antifungal activity against *Botrytis cinerea* from a grapevine rhizosphere in Morocco. Together, these data indicate that both the rhizosphere and the interior of the

grapevine root system are highly interesting sources for actinobacteria which may be potential BCAs for the treatment of different vineyard pathologies.

Blackfoot and Petri diseases, recognized as prominent causes of YGD, along with failure of planting material, are some of the most worrisome problems affecting the wine grape industry since the 1990s (10). Evaluations of declining young vineyards has revealed that many factors are involved. Among all the diseases, the grafted plant root system infections by fungal trunk pathogens in nurseries are the most relevant (10). The main fungal trunk pathologies associated with YGD are Petri disease, primarily produced by *P. chlamydospora* and different *Phaeoacremonium* species, and blackfoot disease, mainly produced by species of *Campylocarpon*, *Dactylonectria*, and *Ilyonectria*. These pathogens can infect a significant portion of all the grafted plants produced every year in nurseries all around the world. They are frequently found infecting the rootstock mother vines (10, 23). They can also infect grafts once they are planted in an open-root field nursery.

This step seems to be particularly critical, since it is supposed that the pathogens present in the soil can infect grafts through the incipient root system that the vines must produce to survive and develop in soil. Thus, when Halleen and colleagues analyzed the presence of fungal pathogens involved in YGD in nurseries during the different steps of the propagation process, they found that less than 1% of the plants were infected with *Cylindrocarpon* spp. before planting in the nursery, whereas 50% or more of the plants were infected at the end of the season (47). This result is highly significant, since it demonstrates that most of the grafted plants in the nurseries can be infected through the roots once they are planted in an open-root field nursery. Our study shows that the application of actinobacteria, selected for their *in vitro* antifungal properties against fungal pathogens which cause YGD, is highly effective in controlling the infection at this step. Application of all the selected actinobacteria resulted in a clear reduction in the isolation of fungal pathogens involved in YGD from surviving plants. Furthermore, the application of actinobacterial strains VV/E1, VV/R1, and VV/R4 led to a statistically significant decrease ($P < 0.05$) in the presence of three types of pathogenic fungi analyzed, *Dactylonectria* sp.-*Ilyonectria* group, *P. chlamydospora*, and *P. minimum*. None of the actinobacteria had a significant effect, either positive or negative, on the growth of the surviving plants. However, whereas the average mortality rate of untreated (negative control) plants in the 3-year field study was 28.4%, the mortality rate was significantly reduced when the actinobacteria VV/R1 and VV/R4 were applied to the grafts. Graft failure is a serious problem for nurseries since, on average, it results in the failure of 30 to 60% of the grafts taken to the field. Many different factors can contribute to graft failure, including abiotic causes, such as improperly healed rootstock, disbudding sites and graft unions, or improper storage and management of the plant material used for obtaining grafts (10).

However, despite the large number of studies showing the presence of fungal pathogens in nursery plants at the end of the propagation process, to date, no clear data are available that correlate the presence of pathogens with mortality levels. In fact, after analyzing the presence of fungal pathogens in plants with failed graft unions, Rumbos and Rumbou concluded that those pathogens were not the cause of YGD (48). Our data suggest a putative relationship between the number of fungal pathogens and the mortality rate, since the application of actinobacterial strains VV/R1 and VV/R4 led to both a significant reduction in the number of fungal pathogens detected and a significant reduction in the mortality rate. Interestingly, the dead plants at the end of the field trials showed a very small, almost nonexistent root system. We can speculate that the early infection with fungi causing YGD may interfere with the development of a potent root system, resulting in a premature graft death. Clearly, graft failure is a complex problem with many different factors, but in view of these results, it seems that the presence of fungal pathogens could significantly contribute to this problem.

Finally, in recent years, it has become clear that endophytic microorganisms, particularly actinobacteria, can be a significant reservoir of genetic diversity and an important source for the discovery of novel bioactive secondary metabolites (27) that

can help control many different crop pathologies. This work represents one of the first reports on the characterization of the endophytic actinobacterial population in the root system of young grapevines at the levels of genus and species. Several of the isolates tested had a beneficial impact when applied to grafts in nurseries, resulting in a significant decrease in both the mortality and infection rates by fungal pathogens involved in YGD. Therefore, the application of endophytic and/or rhizosphere actinobacteria to grafts in nurseries is a novel, green, and promising technology that can reduce the incidence of grapevine plant fungal infections through the root system.

MATERIALS AND METHODS

Isolation of culturable endophytic and rhizosphere actinobacteria from the grapevine root system. A total of four 7-month-old *Vitis vinifera* cv. Tempranillo plants grafted on Richter 110 (110R) rootstock were collected from an open-root field nursery (Viveros Villanueva Vides S.L.) located in Larraga (Spain) at 350 m above sea level (a.s.l.) (01°48′45.8″W and 42°32′12.9″N). Actinobacteria associated with the root system were isolated from root-adjacent soil (rhizosphere) and from interior root tissue (endophytic strains). Roots and soil were placed in sterile containers to be taken to the lab. Two different approaches were used to isolate the culturable actinobacterial population associated with the root system. Rhizosphere strains were isolated from 1 g of root-adjacent soil samples obtained from every plant collected. Soil samples were suspended in a final volume of 10 ml of sterile water and homogenized using a vortex (1 min). Tenfold serial dilutions were made in sterile water, and 0.1-ml aliquots of each dilution were inoculated on starch-casein (SC) (49), ducitol-proline agar (DPA) (26), International *Streptomyces* Project 2 (ISP2) (50), and sodium succinate-asparagine agar (SAA) media (26), supplemented with cycloheximide (150 μg · ml^{-1}) and nalidixic acid (25 μg · ml^{-1}) (Sigma-Aldrich, Madrid, Spain) in order to prevent fungal and Gram-negative bacterial growth, respectively. Plates were incubated at 28°C for 3 to 7 days.

Endophytic strains were isolated from root tissue samples. Two different sections of root tissue were used: 2-cm stubs corresponding to the root insertion point, and root fragments corresponding to the 6-cm apical ends. Wood from both samples was mixed, and microorganisms were extracted as indicated below. Roots were washed in sterile phosphate-buffered saline (PBS) and sonicated (160 W; 2 min) to dislodge soil and organic matter from the sample surface. After drying at room temperature (RT), roots were cut into 2.0-cm-long fragments. Root fragments were surface-sterilized by immersing in 20 ml of Tween 20 (0.1%) (Sigma-Aldrich) for 30 s, followed by sodium hypochlorite (1%) for 6 min and then Na$_2$S$_2$O$_3$ (2.5%) (Panreac, Barcelona, Spain) for 10 min to remove the residual chlorine. After that, samples were washed three times with sterile water. Next, the root fragments were submerged in 70% (vol/vol, in sterile water) ethanol for 6 min, followed by three washes with sterile water and air-dried (in petri plates) in a laminar flow hood (Telstar AV-100; Telstar, Terrassa, Spain). To confirm the effectiveness of the surface disinfection process, 0.2 ml of liquid from the final washing step was spread onto ISP2 medium and incubated at 28°C. Two different methods were used to isolate endophytic actinobacteria from the disinfected root fragments. The first method involved the disruption of 5 g of root material using a mortar and pestle. A volume of 20 ml of 0.9% (wt/vol) NaCl was added to the samples, and they were incubated at 28°C with strong agitation (220 rpm) to favor microorganism extraction. The second method entailed disruption of the samples (0.1 g) in 2-ml tubes containing glass beads (0.5 mm diameter) with a FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA) at 4,000 oscillations per min for 8 s, for a total of 10 times. Samples from both methods were diluted 10-fold, and 0.1-ml aliquots were plated on SC, DPA, ISP2, and SAA media. Plates were incubated at 28°C for 3 to 7 days.

Preliminary identification of actinobacteria by 16S rRNA sequencing. Different isolates were selected according to their morphological and cultural characteristics, including colony properties, presence/absence of aerial mycelia, spore mass color, distinctive reverse colony color, and production of diffusible pigments. Isolates were routinely cultivated and maintained on ISP2 medium at 4°C. Spore-producing isolates were maintained as spore suspensions at −20°C in glycerol (40%). Endophytic and rhizosphere strains exhibiting antifungal activity were identified by 16S rRNA sequencing. Briefly, genomic DNA extraction was performed as described by Hopwood et al. (51). 16S rRNA genes were amplified using the oligonucleotides 27F and 1492R (52). Isolates were identified by comparing them to corresponding sequences of the type strain found on the EzTaxon-e database (53) (http://www.ezbiocloud.net/eztaxon/identify). Sequence alignment and generation of phylogenetic trees were performed using the MEGA 6.0 software (http://www.megasoftware.net/) using a neighbor-joining (NJ) algorithm. Evolutionary distance was calculated using the Kimura two-parameter (K2P) model for nucleotide sequences (54).

Identification of the actinobacteria selected by field assays using multilocus sequence analysis. Multilocus sequence analysis (MLSA) was carried out using five housekeeping genes, namely, *atpD* (ATP synthase F1, beta subunit), *gyrB* (DNA gyrase B subunit), *recA* (recombinase A), *rpoB* (RNA polymerase, beta subunit), and *trpB* (tryptophan synthase, beta subunit) (38). PCR amplification of housekeeping genes was carried out using the primers and under the amplification conditions previously described by Guo et al. (41) and Rong et al. (40). The GenBank accession numbers of DNA sequences from the housekeeping genes are listed in Table 5. Phylogenetic trees were constructed from a concatenation of the five housekeeping genes. All the sequences were concatenated by joining them head to tail. DNA sequences were manually trimmed at the same position before being aligned using MEGA 6.0 software with sequences from type strains obtained from the ARS Microbial Genomic Sequence Database server

TABLE 5 GenBank accession numbers of DNA sequences from the endophytic and rhizosphere *Streptomyces* sp. strains isolated from the root system of grapevine plants used in the MLSA

Genus/species	Isolate	GenBank accession no.				
		atpD	*gyrB*	*recA*	*rpoB*	*trpB*
Streptomyces sp.	VV/E1	MF437320	MF437326	MF437332	MF437338	MF437344
Streptomyces sp.	VV/E2	MF437321	MF437327	MF437333	MF437339	MF437345
Streptomyces peucetius	VV/E5	MF437322	MF437328	MF437334	MF437340	MF437346
Streptomyces sp.	VV/R1	MF437323	MF437329	MF437335	MF437341	MF437347
Streptomyces sp.	VV/R4	MF437324	MF437330	MF437336	MF437342	MF437348
Streptomyces sp.	VV/R5	MF437325	MF437331	MF437337	MF437343	MF437349

(http://199.133.98.43). Phylogenetic trees were constructed using the maximum likelihood method with the Kimura two-parameter model (54). MLSA evolutionary distances were calculated using MEGA 6.0 by calculating the K2P distance. Strain pairs having ≤0.007 MLSA evolutionary distance were considered conspecific based on the guideline empirically determined by Rong and Huang (38).

Evaluation of antifungal activity. All isolates were tested for antifungal activity using an *in vitro* antifungal assay, as described by Lamsal et al. (55), with minor modifications. Briefly, isolates were inoculated on potato dextrose agar (PDA) plates (Sigma-Aldrich) in a 1.0-cm² area (4 isolates per plate inoculated at 1 cm from the edge of the plates). An agar plug (0.7 mm) containing *D. seriata* CBS 112555 or *Ilyonectria macrodidyma* CBS 120170 (now reclassified as *Dactylonectria macrodidyma*) was placed in the middle of the plates. The plates were incubated up to 12 days at 25°C to detect growth inhibition areas. Growth was quantified by calculating the inhibition index (I index) according to the following formula: I index (%) = $[1 - (Ra - R)/(Rc - R)] \times 100$, where Ra is the radius of the fungal colony opposite the bacterial colony, Rc is the maximum radius of the fungal colony (farther away from the bacterial effect), and R is the radius of the agar plug containing the fungi (3.5 mm). This assay was performed in triplicate for each actinobacterium tested (Fig. S4A).

Strains showing antifungal activity against both pathogens were also screened for their ability to inhibit the growth of *P. chlamydospora* and *P. minimum* in PDA, Czapek Dox (Sigma-Aldrich), or ISP5 (50) agar plates as follows (Fig. S4B): fungal pathogens were inoculated on an agar plate forming a circle 2 cm from the periphery of the petri dish. Actinobacterial strains were inoculated in 2.25-cm² patches located 5 mm from the edge of the fungal strain. Plates were incubated at 25°C for up to 20 days to detect the effect of growth inhibition.

Field evaluation in nurseries. Field assays were conducted in three experimental open-root field nurseries of grafted *Vitis vinifera* cv. Tempranillo plants that were grafted on Richter 110 (110R) rootstock in 2013, 2014, and 2015. Every experimental nursery consisted of 525 plants. Grafts were subdivided into 7 batches of 75 units (3 replicas of 25 units per batch), each of which was treated with one of the 6 selected actinobacteria, 3 endophytic (VV/E1, VV/E2, and VV/E5) and 3 rhizosphere (VV/R1, VV/R4, and VV/R5) isolates. A negative-control batch (grafts not treated with actinobacteria) was also included in the study. The biomass production of each strain was obtained from 200-ml liquid cultures of tryptic soy broth (TSB) medium (Sigma-Aldrich) incubated at 220 rpm and 28°C for 3 days in 500-ml Erlenmeyer flasks that were inoculated with 8×10^8 spores. Cells were centrifuged (20 min, 12,000 rpm), washed with saline solution (0.9% [wt/vol]), and resuspended in a final volume of 50.0 ml of saline solution. Actinobacterial isolates were applied to a batch of 75 grafts by partially immersing the grafts (up to a depth of 10 cm) in a rooting hormone solution (indole-3-butyric acid; 0.2 mg · ml⁻¹) containing the selected actinobacteria (10^7 CFU · ml⁻¹) for 24 h at RT. Whole sets of plants were planted (during the month of May in 2013, 2014, and 2015) in an open-root field nursery. After 60 days in the field (month of July), several parameters were measured: mortality rate, total plant height, and elongation of the 7th internode in surviving plants. Two hundred days after planting (month of December), once the plants had completed their cycle of vegetative growth and were in a dormant state, they were carefully removed from the soil to obtain a root system as intact as possible to be analyzed for the presence of fungal pathogens as described below. A total of 75 live plants per batch (corresponding to three replicates of 25 plants) were analyzed.

Analysis of rootstock colonization by selected actinobacteria. In order to check rootstock colonization by the selected actinobacteria, we analyzed the graft interiors. A total of 10 grafts inoculated with each actinobacteria were analyzed 10 days and 200 days (at the time of uprooting the plants) after their application. The wood was surface-sterilized by immersion in 70% ethanol (1 min), 4% sodium hypochlorite (2 min), and then again in 70% ethanol (1 min). Two 1-cm-long stubs (isolated from 2 and 5 cm from the lower end of the rootstock) were split longitudinally, and samples were taken from 7 tissue sections (wood chips of 3 by 2 by 2 mm obtained using a sterile scalpel). Wood samples were plated on ISP2 agar medium containing cycloheximide (200 μg · ml⁻¹) (Sigma-Aldrich) to prevent fungal growth and nalidixic acid (25 μg · ml⁻¹) to inhibit the growth of Gram-negative bacteria. Plates were incubated at 28°C, and actinobacteria growing from the chips were identified by amplification and sequencing of 16S rRNA genes, as described above.

Fungal isolation from wood and pathogen identification. Plants were removed from the soil and analyzed for the presence of pathogenic fungi. A 1-cm stub was cut 1 cm above the root insertion point. The wood was surface-sterilized as described above, and inner tissue sections were obtained as previously indicated and placed on PDA plates supplemented with chloramphenicol (150 μg · ml⁻¹) to

prevent bacterial growth. Plates were incubated at 25°C for up to 4 weeks, subculturing where necessary. Pathogenic fungi were identified by their cultural-morphological traits as *P. chlamydospora*, *P. minimum*, *Dactylonectria* sp., and *Ilyonectria* species. Subsequently, their identities were confirmed by sequencing their internal transcribed spacer 1 (ITS1)-5.8S-ITS2 regions using ITS1 and ITS4 primers under the PCR conditions described by White et al. (56). Other isolates were identified by sequencing the same region. DNA from wood samples was isolated using the REDExtract-N-Amp kit (XNAP) (Sigma-Aldrich).

Statistical data analysis. Plant growth data were tested for univariate normality using the Shapiro-Wilk test. They were subjected to univariate analysis of variance using the general linear means procedure to determine if there were significant differences between treated and untreated plants. Previously, total height and length of the 7th internode data were tested for univariate normality using the Shapiro-Wilk test and then subjected to univariate analysis of variance using the general linear means procedure. In the case of grafted grapevine mortality data, we used a chi-square to test the null hypothesis of no differences in mortality rates against a two-tailed alternative hypothesis that the difference in mortality rates was not zero. When the expected frequencies were less than 5, Fisher's exact test was used. The chances of plant survival versus untreated control plants were calculated as the ratio between the odds of vine survival between a particular treatment and the control (the odds ratio for a particular treatment is the quotient between the number of live and dead plants). To research the treatment efficacy on the number of fungal pathogens isolated, we used the Poisson distribution (comparing the results achieved with nested models) to construct generalized linear models. The null hypothesis of the independence assumption between the frequency of fungal pathogen isolation and treatments was checked using either the chi-square or Fisher's exact test, depending on the nature of the data. Statistical analyses were performed using R Core Team (3.3.0) software (http://www.R-project.org/).

Accession number(s). The accession numbers for the 16S RNA sequences in this article are presented in Tables 1, 2, and 5.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01564-17.

SUPPLEMENTAL FILE 1, PDF file, 4.7 MB.

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