Application methods and modes of action of Pantoea agglomerans and Paenibacillus sp., to control the grapevine trunk disease-pathogen, Neofusicoccum parvum

Rana Haidar, Amira Yacoub, Jean Roudet, Marc Fermaud, Patrice Rey

Received: 1st November 2020 | Accepted: 10 June 2021 | Published: 2 July 2021
DOI:10.20870/oeno-one.2021.55.3.4530

Application methods and modes of action of *Pantoea agglomerans* and *Paenibacillus* sp., to control the grapevine trunk disease-pathogen, *Neofusicoccum parvum*

Rana Haidar[1,2*], Amira Yacoub[1], Jean Roudet[1], Marc Fermaud[1•] and Patrice Rey[1•]

[1] INRAE, UMR SAVE, Bordeaux Science Agro, ISVV, 33882 Villenave d'Ornon, France
[2] Tichreen University, Faculty of Science, Biology Department, Latakia, Syria

• These authors contributed equally to this work.

*corresponding author: haidarrana@gmail.com

Associate editor: Stephane Compant

ABSTRACT

Despite an increasing number of studies being carried out on the biocontrol of grapevine trunk diseases (GTDs), no commercial bacterial products have yet been developed to control GTDs. Knowledge of the precise modes of action (MOA) and the different application methods (AM) for biocontrol agents is crucial if they are to be successful in the field. In light of this, the present study aimed at selecting the most appropriate AM for eight bacterial strains with high potential for controlling *Neofusicoccum parvum*. These strains were applied on one-year-old grapevine in pots (grown from cuttings) using three methods: co-inoculation at stem level, preventive inoculation at stem level and preventive inoculation at the soil surface. The inhibitory activity of the bacterial strain against *N. parvum* was significantly dependent on the AM. Application of bacterial strains to stems, especially in a preventive way, was much more efficient than inoculation in the soil. When performing preventive inoculation on stems, the inhibition of *N. parvum* wood necrosis reached 50 and 65 % for *Pantoea agglomerans* (S1) and *Paenibacillus* sp. (S19) respectively. To decipher the underlying processes linked to fungal inhibition, the way in which several MOA affected the antagonistic capacity of these two strains was studied via *in vitro* and *in planta* assays. While *P. agglomerans* (S1) inhibited *N. parvum* by the secretion of antifungal volatile compounds, Paenibacillus sp. (S19) mainly inhibited this pathogen by antibiosis. In addition, both bacterial strains induced systemic defenses in grapevine. However, this affect tended to be higher at 15-dpi after inoculation with *P. agglomerans* (S1) than after innoculation with *Paenibacillus* sp. (S19) (three defense genes repressed versus five respectively). Finally, *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) were shown to be potential biocontrol candidates for fighting *N. parvum* in grapevine, due to the combination of direct control via their antifungal activity and indirect control via their ability to activate the grapevine defense system.

KEYWORDS

grapevine, *Botryosphaeriaceae*, *Pantoea agglomerans*, *Paenibacillus* sp.

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4530

INTRODUCTION

Grapevine trunk diseases (GTDs), including Esca and Botryosphaeria dieback, are currently considered as some of the most destructive diseases, as they markedly limit grapevine productivity and affect vineyard longevity (Gramaje *et al*., 2018; Claverie *et al*., 2020). Although several factors could be involved in GTD etiology, pathogenic fungi have been described as the main cause of these diseases. Until now, up to 133 fungal species belonging to 34 genera have been associated with GTDs (Gramaje *et al*., 2018). *Neofusicoccum parvum*, like other *Botryosphaeriaceae* species, are considered to be among the most pathogenic GTD-associated fungal species (Laveau *et al*., 2009; Úrbez-Torres and Gubler, 2009). Moreover, the *Botryosphaeria* species, which are widespread across different vineyards worldwide, may induce shoot dieback, cankers, central necroses in wood and/or grapevine dieback (Niekerk *et al*., 2006; Úrbez-Torres, 2011; Gramaje *et al*., 2018; Mondello *et al*., 2018). These fungi have been reported to be capable of colonising wood tissue, not only via pruning wounds, but also via mechanical or natural wounds (Molot *et al*., 2006; Phillips *et al*., 2013; Reis *et al*., 2019). *N. parvum* is known as a multi-host fungal pathogen associated with a wide spectrum of host plants (Phillips *et al*., 2013). On grapevine, several studies have shown that *N. parvum* is one of the most aggressive *Botryosphaeriaceae* species (Niekerk *et al*., 2004; Úrbez-Torres and Gubler, 2009; Pitt *et al*., 2013; Reis *et al.,* 2020, Spagnolo *et al.,* 2014). Despite the importance of *N. parvum* and the other fungal pathogens associated with GTDs, very little chemical products have been developed to limit their propagation, especially after the banning of sodium arsenite, the only registered fungicide which is efficient against these diseases - mainly Esca.

As regards biocontrol products, in the past decade in France, only two fungal products against Esca (Esquive® and Vintec) - based on two different strains of *Trichoderma atroviride* - have been registered. Therefore, the development of antagonistic microorganisms against GTD fungi is of prime importance. Recent studies have revealed high fungal and bacterial diversity in different types of wood tissue - necrotic or not - of both asymptomatic and esca-diseased grapevine (Bruez *et al*., 2015; Zarraonaindia *et al*., 2015; Berlanas *et al*., 2019; Kraus *et al*., 2019). The interactions of microbial communities could be beneficial for their host plant by, for example, increasing its resilience against certain pathogens (Compant *et al*., 2013; Pacifico *et al*., 2019). Therefore, the biocontrol potential against *N. parvum* of various isolated bacterial strains from vineyards has recently been explored (Haidar *et al*., 2016; Rezgui *et al*., 2016; Daraignes *et al*., 2018; Trotel-Aziz *et al*., 2019; Wu *et al.,* 2020). Although different degrees of protection against *N. parvum* have been obtained by using certain bacterial strains, less attention has been paid to the modes of action (MOA) and different application methods (AM) of the tested bacteria. This is a very important point to take into consideration, because the efficacy and the durability of biocontrol could be affected by several factors, including environmental conditions, as well as the AM and MOA of the biocontrol agents (Bardin *et al*., 2015; Köhl *et al*., 2019). If biocontrol agents are to be applied successfully in the vineyard in the future, an increased knowledge of the AM of bacteria and their different MOA, based on already available knowledge of *N. parvum*, will be required.

Table 1. The eight selected bacterial strains (Haidar *et al*., 2016a) tested in the present study.

Test code	Species identification	Bacterial origine from grapevine
S1	*Pantoea agglomerans*	Grape berries
S3	*Pantoea agglomerans*	Grape berries
S19	*Paenibacillus* sp.	Grapevine wood
S24	*Enterobacter* sp.	Grapevine wood
S27	*Brevibacillus reuszeri*	Grapevine wood
S28	*Brevibacillus reuszeri*	Grapevine wood
S32	*Bacillus pumilus*	Grapevine wood
S41	*Bacillus firmus*	Grapevine wood

S1 and S3 were isolated from Merlot cultivar and the otherstrains were isolated from Cabernet-Sauvignon cultivar.

The overall aim of the present study was therefore to select the best BCA bacteria for inhibiting *N. parvum* via *in vitro* and *in planta* assays; the second aim was to determine the MA and the MOA of the selected bacteria, via which they exercise their inhibitory effect.

Recently, 46 bacterial strains isolated from Bordeaux vineyards were screened for their antifungal activity against *N. parvum and Phaeomoniella chlamydospora* (Haidar *et al*., 2016a; Haidar *et al*., 2016b). Based on their fungal inhibition activity on one-year-old grapevine in pots (grown from cuttings), eight bacterial strains were selected in order to determine their biocontrol potential against *N. parvum.* Because the production of antifungal volatile compounds by some strains (*Paenibacillus* sp. (S19)) was reported in our last study (Haidar *et al.,* 2016b), in the present study, the biocontrol potential of the eight selected bacterial strains were compared by studying and comparing three methods of bacterial application: co-inoculation and preventive inoculations made in a hole perforated in the stem of one-year-old grapevine in pots (grown from cuttings) and preventive inoculation at the soil surface. We then focused on two specific strains, which were selected for their high antagonistic activity against *N. parvum*, by determining their direct (i.e., on the pathogen,) or indirect (i.e., on the plant) modes of action under laboratory conditions.

MATERIALS AND METHODS

1. Microorganisms and cultural conditions

1.1. Bacterial strains

Eight bacterial strains (Table 1) selected for their antagonistic activity against *N. parvum* were tested (Haidar *et al.*, 2016a). Depending on whether the assay was *in vitro* or *in planta*, the bacterial strains were grown on Trypto-Casein Soy Agar medium (TSA, Biokar diagnostics) and in Corning cell culture flask in Tryptic Soy Broth (TSB, Difco) respectively for 24 h at 28 °C. In the *in planta* bioassay, the bacterial cell concentration was adjusted to 10^8 CFU/mL.

1.2. *N. parvum*

The *N. parvum* isolate ('Cou 02') was selected from the INRAE-UMR 1065 SAVE collection, Bordeaux, France. This strain had been originally isolated from a Cabernet-Sauvignon cultivar in a vineyard near Bordeaux. It was sub-cultured on a Malt Agar (MA) medium and incubated at 27 °C (12 h light/12 h dark) for one week, before being used in the experiments.

2. *In planta* experimentation

2.1. Plant materials

The *in planta* bioassays were conducted on one-year-old grapevine in pots (grown from cuttings) of Cabernet-Sauvignon grapevine (Supplementary Figure 1) from the INRAE experimental vineyards near Bordeaux. The one-year-old grapevine in pots (grown from cuttings) were processed and prepared as described by Laveau *et al*. (2009).

2.2. Application of bacterial strains and fungal pathogen

The stem of each one-year-old grapevine in pots (grown from cuttings) was surface-sterilised by rubbing it with a paper towel soaked with 95 % ethanol. Below the upper bud of each stem, an artificial wound (hole) was made in the central part by drilling a hole in the wood (4 mm in diameter). The antagonistic bacterial effect of eight bacterial strains was evaluated using three different AM: i) co-inoculation (in the hole), ii) preventive inoculation in the stem (in the hole), and iii) preventive inoculation in the soil. For co-inoculation and preventive stem inoculation, the hole was drop-inoculated with 40 µL (4×10^6 CFU) of bacterial suspension immediately and four days respectively before *N. parvum* inoculation. For the preventive soil inoculation method, the collar of each plant was inoculated with 50 mL (5×10^9 CFU) of bacterial suspension four days before *N. parvum* inoculation. The holes in the samples of this latter method were made on the same day as the other bacterial treatments. The inoculation of *N. parvum* was done by inserting mycelium plug cut off (4 mm in diameter) from the margin of a fresh mycelial MA culture into hole drilled into the stem. All wounds were covered with Parafilm® (Scellofrais film) to protect the area of inoculation.

3. Effect of the method of application on bacterial antagonistic activity (first bioassay)

3.1. Experimental design

The experimental design was a randomised complete block with 20 plants for the pathogen control treatment (UC), and 15 one-year-old grapevines in pots (grown from cuttings) per treatment for all the other treatments. The experimental treatments consisted of plants inoculated with the bacterial strains and

N. parvum. These plants were: i) co-inoculated with the bacterial strains and *N. parvum* in the stem (Co-inoc), ii) treated with the bacterial strains in the hole and then, four days later, with *N. parvum* in the stem (Prev-inoc_hole), and iii) treated with bacterial suspension at the collar of each plant and then, four days later, with *N. parvum* in the stem (Prev-inoc_soil). The control treatments consisted of plants which were: i) mock inoculated to mimic the pathogen inoculation method (mock-control), ii) infected only with the *N. parvum* in the stem (pathogen-control), iii) treated in the stem with only the bacterial suspension (bacterial control), and iv) co-inoculated with a solution of the fungicide Fluazinam (Sekoya, Syngenta France SAS, 50 % a.i., 250 g a.i. 100 L) and *N. parvum* in the stem (fungicide control). Fluazinam was used because of its efficacy against some pathogens involved in GTDs, such as *P. chlamydospora* and *E. lata* (Halleen *et al*., 2010; Sosnowski *et al*., 2013, Haidar *et al*., 2016).

3.2. Evaluation of plant protection

After incubation periods of four months, we visually assessed the presence and the length of the external canker lesions which comprise brown-blackish sunken areas on the stems of the one-year-old grapevine in pots (grown from cuttings), spreading upwards and downwards from the point of inoculation. The stem of each plant was then cut longitudinally and the length of the internal wood necrosis lesions (vascular lesions in the wood tissue which develop from the point of inoculation) were measured with a ruler.

4. Study of the modes of action of two selected bacterial strains

Based on the efficacy of the bacterial strains in the last bioassay, *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) were selected to assess their potential MOA.

4.1. *In vitro* antagonism of selected bacterial strains against *N. parvum*

4.1.1. Effect of antifungal diffusible compounds produced by bacterial strains

The potential inhibition effect of *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) on the mycelial growth of *N. parvum* was assessed using the dual culture method as described by Rezgui *et al.* (2016).

4.1.2. Effect of antifungal volatile compounds produced by bacterial strains

The potential production of antifungal volatile metabolites by the *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) on the mycelial growth of *N. parvum* was determined according to Haidar *et al.* (2016 b) with minor modifications. Briefly, the bottom of two dishes were placed face-to-face with the TSA bacterial culture at the bottom and the MA culture of *N. parvum* on the top. The dishes were sealed with transparent adhesive tape and incubated at 27 °C. After 7 day of incubation, two perpendicular colony diameters were measured to calculate the percentage of mycelial inhibition (Haidar *et al*., 2016b). For the identification of the volatile compounds produced by *P. agglomerans*

TABLE 2. Genes involved in grapevine defense reaction (Dufour *et al.*, 2013, Dufour *et al.*, 2016)

Family	Genes	N° accession GeneBank
PR proteins	PR protein 1 *(VvPR1)*	AJ536326
	PR protein 10 *(VvPR10)*	AJ291705
	Chitinase class III *(VvCHIT3)*	Z68123
	b-1,3 glucanase *(VvGLU)*	AF239617
Cell wall reinforcement	Callose synthase *(VvCALS)*	AJ430780.1
Redox status	Glutathione S-transferase *(VvGST)*	AY156048.1
Indole and phenylpropanoid pathways	Antranilate synthase *(VvANTS)*	XM 002281597
	Stilbene synthase *(VvSTS)*	X76892.1
	Chalcone synthase *(VvCHS)*	X75969.1
	Phenylalanine ammonia lyase *(VvPAL)*	X75967

(S1), the same methodology as Haidar *et al.* (2016b) for volatile metabolites produced by *Paenibacillus* sp. (S19) was used.

4.2. Evaluation of grapevine defense induced by selected bacterial strains (second bioassay)

The ability of *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) to induce a grapevine defense reaction (Verhagen *et al.,* 2011; Haidar *et al*., 2016b) was tested on leaf samples from the one-year-old grapevines in pots (grown from cuttings) prepared as described above (2.1). The application of bacterial strains and fungal pathogen was performed as described above (2.2)

4.2.1. Experimental design of the second bioassay

The experiment was conducted using a randomized complete block design with 28 plants per treatment. The different treatments consisted of plants which were: i) co-inoculated with (S1 or S19) and *N. parvum,* ii) treated with S1 or S19, and four days later, inoculated with *N. parvum* in the stem (Prev-inoc_hole), iii) mock inoculated to mimic the pathogen inoculation method (mock-control), iv) infected only by the *N. parvum* in the stem (pathogen control), v) treated in the stem with the bacterial suspension alone (bacterial control), and vi) co-inoculated with a solution of the fungicide Fluazinam (Sekoya, Syngenta France SAS, 50 % a.i., 250 g a.i. 100L) and *N. parvum* in the stem (fungicide control).

4.2.2. Evaluation of plant protection

After incubation periods of three months the assessment of grapevine protection was performed as describe above (2.4)

4.2.3. Plant tissue sampling

Samples from this bioassay (except fungicide control) were analysed at molecular level. For each plant, one leaf (the third or fourth leaf from the apex) was collected at two time points: 2 hours (T0) and 15 days (T15) after pathogen inoculation. At each sampling time point, the leaves of six plants per treatment were sampled. The six samples were randomly grouped to obtain three biological repetitions. All samples were immediately frozen in liquid nitrogen and stored at -80 °C for further molecular analysis.

4.2.4. RNA extraction and cDNA preparation

The RNA extraction protocol was performed according to Reid *et al*. (2006). After being crushed in liquid nitrogen, 1 mL of an extraction buffer (300 mM Tris HCl, pH 8.0; 25 mM EDTA, 2 mM NaCl, 2 % CTAB, 2 % polyvinylpolypyrrolidone (PVPP), 0.05 % spermidine trihydrochloride and 2 % β- mercaptoethanol added extemporaneously), which had been preheated to 65 °C, was added to 200 mg of leaf powder. The mixture was stirred vigorously and incubated in a water bath at 65 °C for 10 min with regular stirring. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added and then centrifuged at 3500 g for 15 min (at 4 °C). The RNA extraction was done using the manufacturer's protocol for the Spectrum™ Plant Total RNA Kit as described by Haidar *et al.* (2016b). The obtained RNA were reverse-transcribed to cDNA using the M-MLV reverse transcriptase (Promega) following the manufacurer's instructions.

4.2.5. Quantitative polymerase chain reaction

The expression level of the 10 genes involved in the grapevine defense system was quantified using real-time quantitative PCR (Table 2). According to Dufour *et al*. (2013) and Yacoub *et al*. (2016), these genes are involved in PR protein production (PR protein1 (*VvPR1*), PR protein10 (*VvPR10*), Chitinase class III (*VvCHIT3*), ß-1,3 glucanase (*VvGLU*)), secondary metabolite biosynthesis (Phenylalanine ammonia lyase (*VvPAL*), Stilbene synthase (*VvSTS*), Chalcone synthase (*VvCHS*) and Antranilate synthase (*VvANTs*), redox status (Glutathione S-transferase (*VvGST*)) and cell wall reinforcement (callose synthase (*VvCALS*)). The γ -chain of Elongation Factor 1 (*VvEF1*γ) and glyceraldehyde-3-phosphate dehydrogenase genes (*VvGAPDH*, GenBank CB973647) were used as housekeeping genes.

Gene expression levels were assessed using RT-qPCR according to the following protocol (Dufour *et al*., 2013). Briefly, PCR reactions were performed in a Stratagene Mx3005P qPCR thermocycler (Agilent technologies) and the SYBR Green dsDNA binding-die, 2 x MESA BLUE qPCR MasterMix Plus for SYBR® Assay Low ROX (Eurogentec), was used. The data were analysed with MxPro QPCR Software (Agilent technologies) to obtain for each sample/gene couple the cycle quantification number (Cq) which corresponds to the fluorescence signal of the amplified DNA intersected with the background noise. The Relative Expression (RE) was calculated using the $2^{-\Delta\Delta Cq}$ method, where $\Delta\Delta^{Cq}$ was the ΔCq between one sample and the control. The geometric mean of the two used reference genes was then used as an accurate normalisation

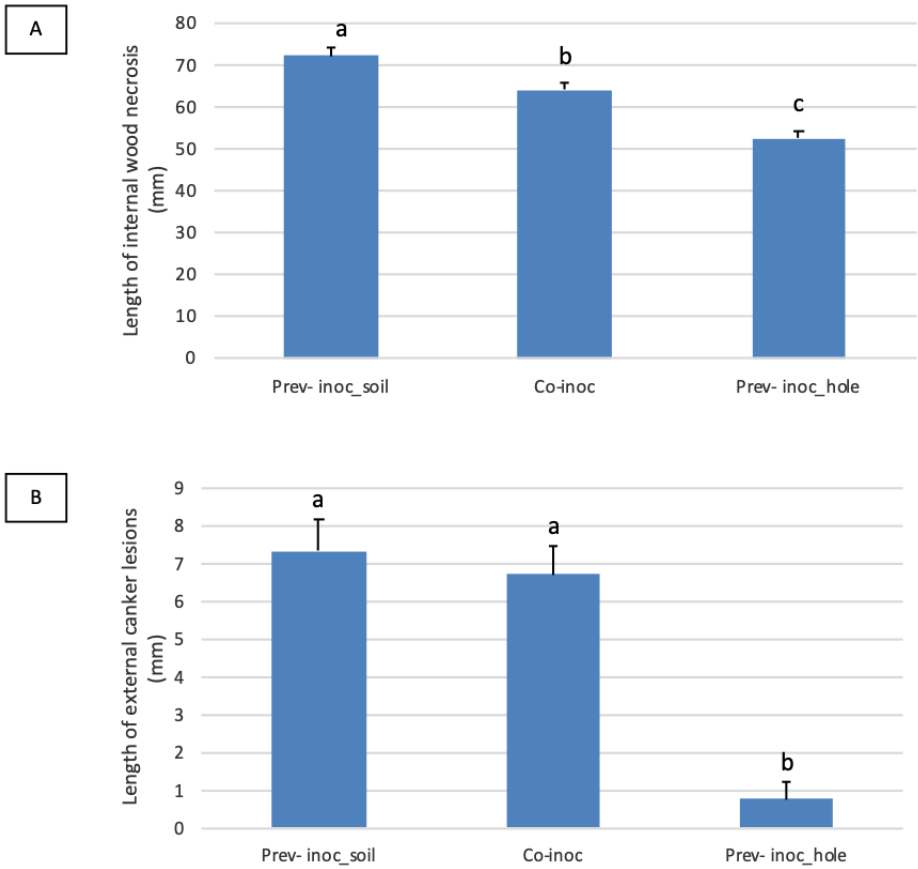

FIGURE 1. Main effect of the different modes of application of the 8 selected bacteria (applied individually) on the development of internal necrosis lesions (A) and external canker lesions (B) dues to *N. parvum*, in the first bioassay.
Co-inoc: plants co-inoculated with the bacterial strains and N. parvum in the stems. Prev-inoc_hole: treated with the bacterial strains in the hole and then, four days later, with N. parvum in the stem. Prev-inoc_soil: the collar of each plant was treated with bacterial suspension and then, four days later, with N. parvum on the stem. Different letters indicate significantly different at $P \leq 0.05$, according to the Newman and Keul's test after ANOVA. The error bar corresponds to the standard deviation of the mean.

factor to calculate the RE levels of the studied genes (Vandesompele *et al*., 2002).

5. Statistical Analyses

The experimental data (necrosis and canker lesions length from the *in planta* experiments and mycelial measurement from the *in vitro* experiments) were compared using an analysis of variance (ANOVA) followed by Newman-Keul's test ($P \leq 0.05$). These analyses were carried out with the StatBox software (Version 6.6, Grimmer© Logiciels, Paris). The statistical analysis of the expression levels of the genes was carried out using the non-parametric Kruskal-Wallis test ($P \leq 0.05$).

RESULTS

1. Reduction of *N. parvum* wood symptoms by the bacterial strains in the screening bioassay

The aim of this bioassay was to determine the most efficient method to apply the eight selected bacterial strains to the grapevine plants. Our results demonstrated that depending on the AM (either co-inoculation (Co-inoc), preventive inoculation in the hole (Prev-inoc_hole) or preventive inoculation at the soil surface (Prev-inoc_soil)), the mean internal necrosis lesions and the associated severity of the external symptom (i.e., the canker length) were significantly affected (Figure 1). However, the average length of the internal necrosis lesions obtained in the bacterial controls (BC) was 45 mm, corresponding to necrosis resulting from the inoculation method. No external canker lesions were observed in the bacterial controls.

1.1. Reduction of plant internal necrosis lesions due to *N. parvum* depends on bacterial strain and inoculation method

The comparison of the three methods of bacterial application (for all bacterial strains considered)

showed that the mean lengths of internal necrosis lesions due to *N. parvum* were 52.3, 63.8 and 72.0 mm for preventive inoculation at stem level, co-inoculation at stem level and inoculation at soil level respectively. The statistical analysis showed i) a significant interaction (P = 0.003) between the two main effects (i.e., bacterial effect and AM effect), and ii) that the two main effects were also significant, with the AM significantly affecting ($P \leq 0.05$) the mean internal necrosis lesion length, as well as the main effect of the bacterial strain (P = 0.007). All the corresponding quantified and ranked results are shown in Figures 2A, B and C, and Figure 1A. Compared with the fungal control inoculated with *N. parvum* alone, the application of bacteria to the stem (a few minutes or four days before inoculation with the pathogen) resulted in a significant reduction in the size of the internal necrosis lesions (Figures 2A and B).

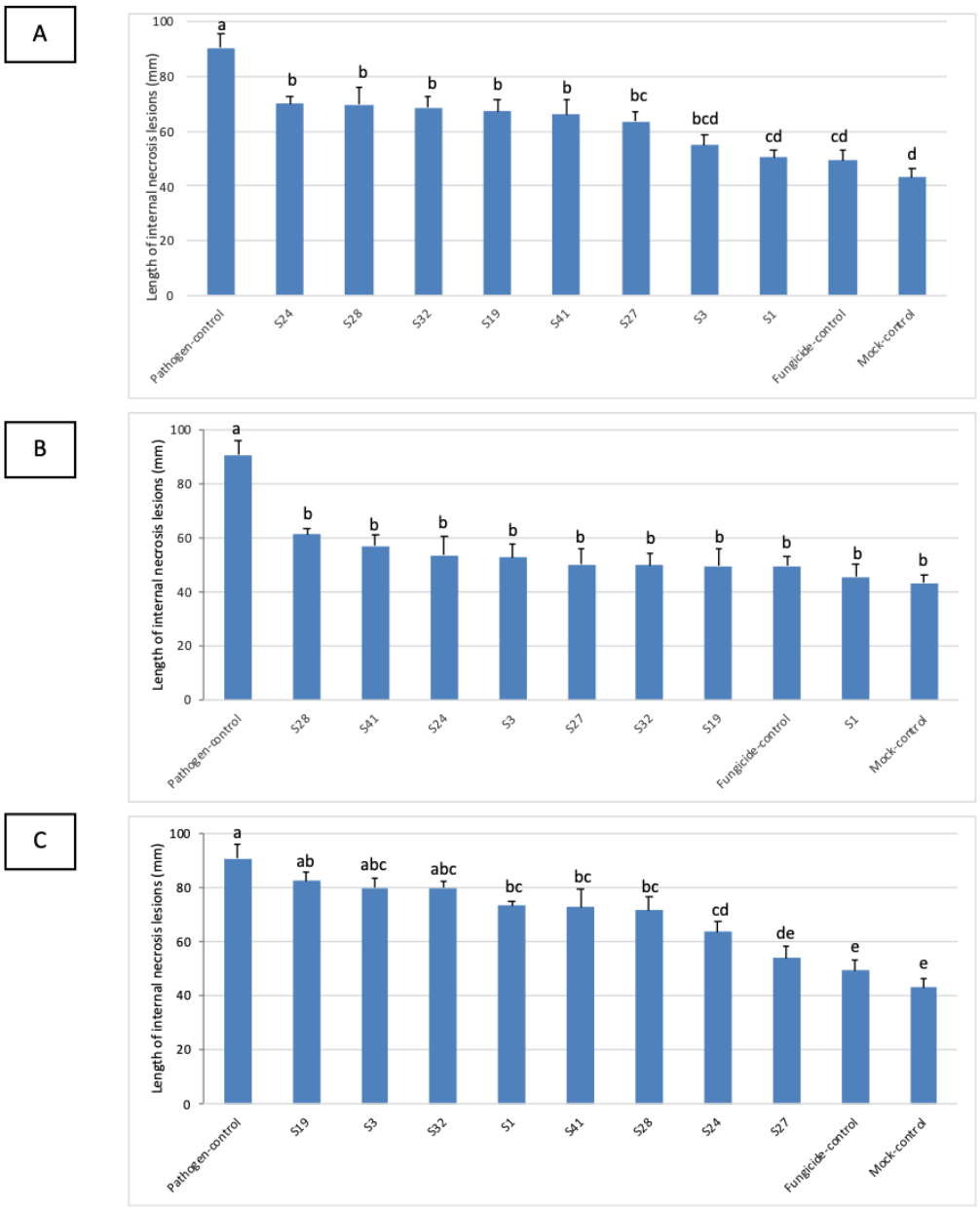

FIGURE 2. Antagonistic effect of the selected bacteria on the development of internal necrosis lesions due to *N. parvum* according to: A) Co-inoculation, B) Preventive inoculation in the stem, and C) Preventive inoculation in the soil in the first bioassay.

In the fungicide control, inoculation was at stem level. Each value represents the mean of 15 one-year-old grapevines in pots (grown from cuttings). Bars with the same letter are not significantly different at $P \leq 0.05$ according to the Newman and Keul's test after ANOVA. The error bar corresponds to the standard deviation of the mean.

With co-inoculation, the highest level of protection was obtained with the *P. agglomerans* strains (S1 and S3), with a reduction in internal necrosis lesions by 44.2 and 39.1 % respectively (Figure 2A). With preventive inoculation in the stem, all bacterial strains resulted in a significant reduction in internal necrosis lesions (more than 40 %; Figure 2B). Inoculation in the soil was less effective in reducing *N. parvum* internal necrosis lesions than the application of bacteria to the stem.

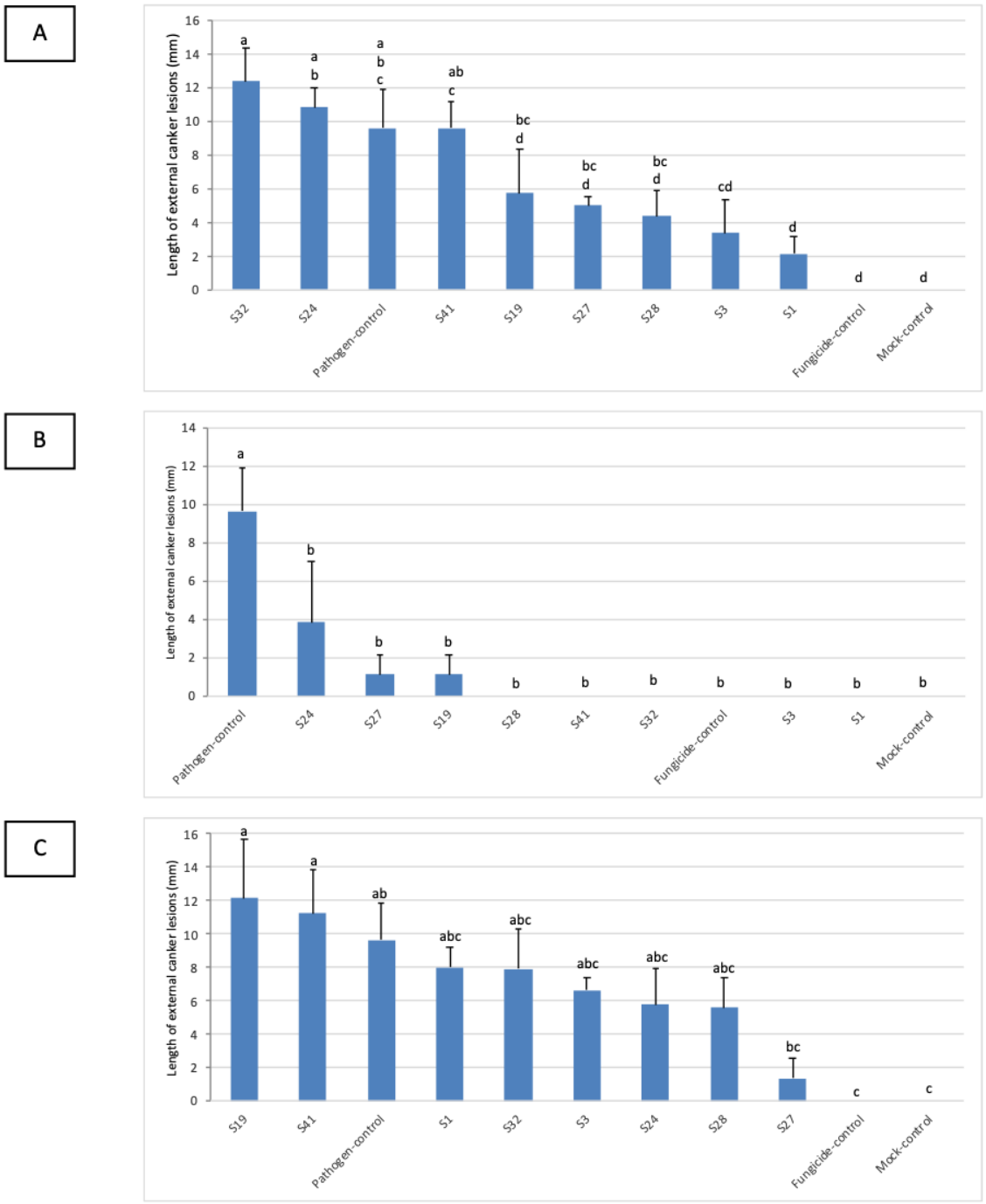

FIGURE 3. Antagonistic effect of the selected bacteria on the development of external canker lesions due to *N. parvum* according to: A) Co-inoculation; B) Preventive inoculation in the stem and C) Preventive inoculation in the soil, in the first bioassay.
The fungicide in the fungicide control was applied to the stem. Each value represents the mean of 15 one-year-old grapevines in pots (grown from cuttings). Bars with the same letter are not significantly different at $P \leq 0.05$ according to the Newman and Keul's test after ANOVA. The error bar corresponds to the standard deviation of the mean.

TABLE 3. Volatile compounds produced by *Paenibacillus sp*. (S19) and *P. agglomerans* (S1) (GC/MS analysis)

Bacterial strain	Volatile compound	Retention time (minute)	Molecular weight (g/mol)
P. agglomerans (S1)	phenylethyl alcohol	8.6	122.16
S19 (*Paenibacillus* sp.)	Compound of pyrazine type	12.8	-
(Haidar et al., 2016b)	2,6-Bis (2-methylpropyl) pyrazine	12.4	192.3
	1-Octen-3-ol	6.9	128.22

Five bacterial strains (*B. reuszeri* (S27), *Enterobacter* sp. (*S*24), *B. reuszeri* (S28), *B. firmus* (S41) and *P. agglomerans* (S1) significantly reduced the internal necrosis lesions length compared to the pathogen control (Figure 2C); only *B. reuszeri* (S27) reduced the length by more than 40 % (Figure 2C). The inoculation with the fungicide treatment (Fluazinam) resulted in a significant reduction of internal necrosis lesions, reaching 45 % (Figure 2).

1.2. The reduction of plant external canker lesions due to *N. parvum* depends on bacterial strain and inoculation method

Similar to the internal necrosis lesions, the reduction of external canker lesions was found to depend on the inoculation method of bacterial strains. The mean external canker lesion lengths of all the bacterial strains considered were 0.8, 6.7 and 7.8 mm for preventive inoculation in the stem, co-inoculation in the stem and inoculation in the soil respectively (Figure 1B). The statistical analysis showed i) a significant interaction ($P \leq 0.05$) between the two main effects (i.e., bacterial effect and application method effect), and ii) that the two main effects were also significant, with the application method significantly affecting the mean length of the internal necrosis lesions ($P = 0.001$), as well as the main effect of the bacterial strain ($P = 0.0006$). All the corresponding quantified and ranked results are shown in Figures 3A, B and C and Figure 1B. Figure 3 shows that the preventive inoculation with bacteria in the stem was the most efficient of the three AM in reducing external canker lesions. For the plants pretreated with bacteria in the stem, there was a significant reduction in external canker lesions for all bacterial strains. For instance, no canker was observed in the plants pretreated with the five following strains: *B. reuszeri* (S28), *P. agglomerans* (S1, S3), *B. pumilus* (S32) and *B. firmus* (S41) (Figure 3B). Interestingly, while *B. pumilus* (S32) and *B. firmus* (*S*41)) tended to increase the length of external canker lesions compared to the fungal control in co-inoculation and soil inoculation respectively, no plants pretreated at hole level showed values for external canker lesions higher than those observed in the fungal control (Figure 3).

In plants co-inoculated with *B. pumilus* (S32) and *Enterobacter* sp. (S24), external canker lesions tended to be larger than in the fungal control (Figure 3A). The same tendency was observed in plants treated with *B. firmus* (*S*41) and *Paenibacillus* sp. (*S*19) in the soil (Figure 3C).

2. *In vitro* evaluation of bacterial antagonism against *N. parvum*

When evaluated in dual cultures, *Paenibacillus* sp. (S19) was most effective in reducing mycelial growth of *N. parvum*, with more than 62 % inhibition compared to the pathogen control. However, *P. agglomerans* (S1) showed only 13 % inhibition. By producing volatile metabolites, *P. agglomerans* (S1) showed an inhibition rate of the mycelial growth of the pathogen which was greater than 20 %. In this last test, *Paenibacillus* sp. (S19) was ineffective in suppressing *N. parvum.*

3. Analysis of the volatile compounds produced by *Paenibacillus* sp. (S19) and *P. agglomerans* (S1)

As showed in Table 3, three volatile compounds were produced by *Paenibacillus* sp. (S19) (Haidar *et al.*, 2016b). However, the GC–MS analyses of the volatile compounds produced by *P. agglomerans* revealed phenylethyl alcohol to be the only volatile compound produced by *P. agglomerans* (S1).

4. Reduction of internal necrosis lesions due to *N. parvum* by the two selected bacterial strains in the second bioassay

Based on the aforementioned results, two of the most efficient bacterial strains were selected for

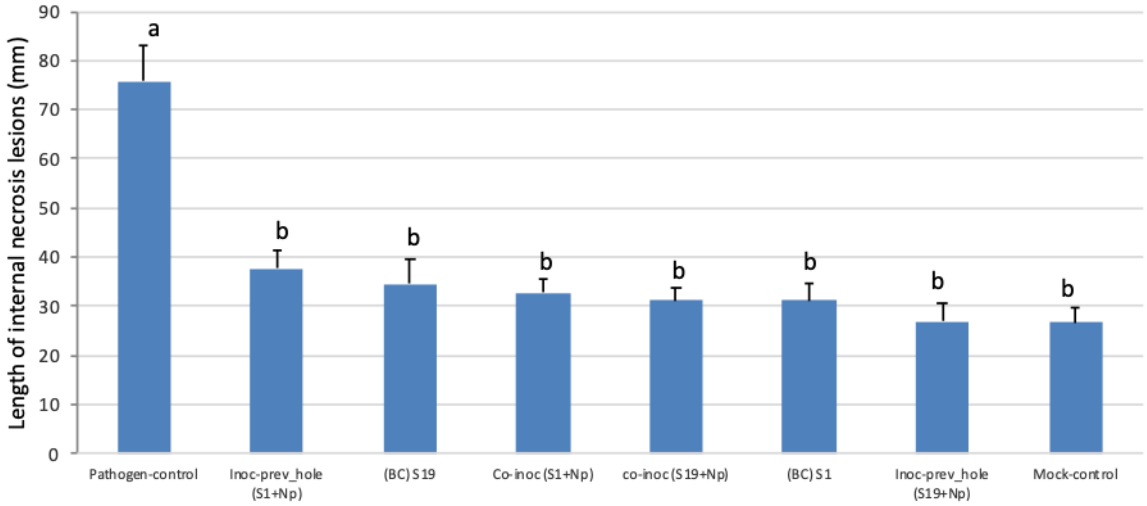

FIGURE 4. Antagonistic effect of S1 and S19 on the development of the internal necrosis lesions due to N. parvum according to A) Preventive inoculation at stem level and B) Preventive inoculation on the soil, in the second bioassay.

The two selected bacterial strains: *P. agglomerans* (S1) and *Paenibacillus sp*. (S19) were applied to control the development of internal necrosis lesions due to *N. parvum*. The pathogen-control was inoculated with *N. parvum* only. The bacterial control (BC) was treated with the bacteria only. The fungicide controls were treated with Fluazinam before *N. parvum* inoculation. The mock control was not treated with bacteria and not inoculated with the pathogen. Each value represents the mean of 16 one-year-old grapevines in pots (grown from cuttings). Bars with the same letter are not significantly different at $P \leq 0.05$, according to Newman and Keul's test after ANOVA. The error bar corresponds to the standard deviation of the mean.

the study of the MOA responsible for the inhibition of *N. parvum*. In this bioassay, we evaluated the effectiveness of these bacteria in inducing foliar grapevine defenses against *N. parvum* when applied to the stem (co-inoculation and preventive inoculation).

Compared to the pathogen control, which was inoculated with *N. parvum only*, the two bacterial strains (S1 and S19) significantly reduced the length of internal necrosis lesions due to *N. parvum*, regardless of bacterial method of application (Figure 4). When applied four days before the pathogen, *Paenibacillus* sp. (S19) induced the greatest reduction in *N. parvum* necrosis, which reached 65 % of inhibition. Importantly, a large reduction in necrosis (greater than 59 %) resulted from co-inoculation using *P. agglomerans* (S1). However, the bacterial controls (BC) S1 (inoculated with *P. agglomerans* (S1)) and (BC) S19 (inoculated with *Paenibacillus sp*. (S19)), showed internal necrosis lesions which reached 31 and 39 mm respectively; the formation of these necroses are a result of the inoculation method.

5. Evaluation of specific grapevine-defenses induced by *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) in response to *N. parvum* infection

The effect of the antagonistic bacteria on grapevine foliar defenses was assessed in response, or not, to *N. parvum* infection. The RE levels of 10 defense-related grapevine genes were evaluated at two different sampling time points; i.e., 2 hours and 15 days after *N. parvum* inoculation (T0 and T15) respectively (Table 4). For each selected bacterium, the effect of the AM (co-inoculation or preventive inoculation at stem level) was evaluated on plant defenses in the presence, or not, of the pathogen.

5.1. Evaluation of grapevine responses to *N. parvum* inoculation

For plants inoculated with *N. parvum* alone, the expression of all the studied genes were slightly repressed in response to the pathogen infection at T0 (Table 4A, B). Out of the 10 genes, the expression of two were significantly repressed compared to the mock control; i.e., *VvCHIT3* and *VvANTS* involved in PR proteins and Indole-Phenylpropanoid pathways respectively. Fifteen days after *N. parvum* inoculation, a few changes were observed. The expressions of three genes were significantly repressed compared to the mock control: *VvANTS* and *VvPAL* (involved in the Indole-Phenylpropanoid pathways) and *VvGST* (involved in Redox status). As for the encoding PR proteins studied genes, the expression of three out of four of the PR-proteins genes studied (i.e. *VvPR1*, *VvPR10* and *VvCHIT3*) were over-expressed but, only *VvPR1* showed a significant high over-expression (ER=25.3).

TABLE 4. Expression levels of 10 major defense-related genes with inoculation by the bacterial strain A) P. *agglomerans* (S1) and B) *Paenibacillus* sp. (S19).

A

		T0				T15			
		Np	Co-inoc S1	Prev-inoc_hole S1	S1	Np	Co-inoc S1	Prev-inoc_hole S1	S1
PR proteins	*VvPR1*	0.4 ±0.35	**4.8** ±1.24	**11.9** ±7.59	**11.4** ±7.0	**25.3** ±32.1	8.4 ±9.43	1.0 ±0.66	18.9 ±20.47
	VvPR10	0.6 ±0.13	**3.4** ±2.14	5.6 ±4.63	2.3 ±0.7	1.8 ±2.24	1.1 ±0.06	0.6 ±1.12	1.8 ±0.91
	VvCHIT3	**0.4** ±0.24	2.7 ±2.78	**3.8** ±1.94	**2.6** ±0.5	1.6 ±1.71	0.9 ±0.13	0.6 ±2.43	0.5 ±0.59
	VvGLU	0.7 ±0.19	2.1 ±0.85	**3.3** ±1.70	1.0 ±0.2	0.4 ±0.66	0.9 ±0.00	**0.1** ±0.78	1.7 ±1.35
Cell wall reinforcement	*VvCALS*	1.3 ±0.43	**6.4** ±4.52	**5.7** ±2.06	**3.5** ±2.0	0.7 ±0.31	**4.7** ±3.39	0.7 ±0.59	2.1 ±0.56
Redox status	*VvGST*	1.0 ±0.57	1.5 ±1.07	0.8 ±2.03	0.5 ±0.2	**0.3** ±0.04	6.6 ±8.17	1.5 ±1.60	1.8 ±0.33
Indole and phenylpropanoid pathways	*VvANTS*	**0.5** ±0.11	**0.2** ±0.17	**0.3** ±0.10	**0.3** ±0.0	**0.6** ±0.12	**1.6** ±0.06	**1.3** ±0.62	2.7 ±1.03
	VvSTS	0.6 ±0.18	**7.1** ±5.83	**3.7** ±2.02	1.4 ±0.8	1.6 ±1.93	**4.1** ±2.81	0.4 ±1.22	2.0 ±1.01
	VvCHS	0.6 ±0.35	0.6 ±0.23	0.8 ±0.75	0.9 ±0.6	1.9 ±1.4	2.5 ±1.93	0.6 ±0.12	0.8 ±0.36
	VvPAL	1.0 ±0.35	1.9 ±1.04	1.7 ±0.68	1.2 ±0.7	**0.3** ±0.19	1.3 ±0.39	**0.3** ±0.12	**0.4** ±0.15

B

		T0				T15			
		Np	Co-inoc S19	Prev-inoc_hole S19	S19	Np	Co-inoc S19	Prev-inoc_hole S19	S19
PR proteins	*VvPR1*	0.4 ±0.35	1.0 ±0.35	**20.7** ±11.81	**8.9** ±5.06	**25.3** ±32.1	2.0 ±1.80	1.5 ±0.4	2.8 ±3.03
	VvPR10	0.6 ±0.13	1.1 ±0.10	**2.2** ±0.26	**3.0** ±0.56	1.8 ±2.24	0.5 ±0.67	**0.2** ±0.16	**0.2** ±0.05
	VvCHIT3	**0.4** ±0.24	0.7 ±0.05	**6.0** ±2.71	**5.4** ±2.32	1.6 ±1.71	**0.2** ±0.08	0.8 ±0.13	**0.2** ±0.08
	VvGLU	0.7 ±0.19	0.7 ±0.16	2.3 ±1.88	2.6 ±1.29	0.4 ±0.66	**0.3** ±0.21	**0.0** ±0.02	**0.1** ±0.07
Cell wall reinforcement	*VvCALS*	1.3 ±0.43	**8.7** ±0.9	**5.1** ±0.70	**6.1** ±2.67	0.7 ±0.31	1.6 ±0.27	1.2 ±0.21	1.6 ±0.51
Redox status	*VvGST*	1.0 ±0.57	**2.9** ±0.91	**0.4** ±0.15	**3.0** ±1.04	**0.3** ±0.04	**0.2** ±0.15	0.5 ±0.37	1.9 ±0.53
Indole and phenylpropanoid pathways	*VvANTS*	**0.5** ±0.11	**0.1** ±0.06	**0.1** ±0.08	**0.2** ±0.0	**0.6** ±0.12	0.7 ±0.38	1.0 ±0.29	1.2 ±0.19
	VvSTS	0.6 ±0.18	**3.0** ±0.44	1.3 ±0.26	1.3 ±0.5	1.6 ±1.93	**0.3** ±0.18	**0.1** ±0.02	0.4 ±0.11
	VvCHS	0.6 ±0.35	0.7 ±0.15	**0.5** ±0.16	0.6 ±0.2	1.9 ±1.41	0.7 ±0.40	0.8 ±0.29	**4.4** ±1.48
	VvPAL	1.0 ±0.35	**2.0** ±0.26	1.3 ±0.26	1.4 ±0.4	**0.3** ±0.19	0.8 ±0.23	**0.3** ±0.08	0.8 ±0.37

< 0.1 — 1 — 5 <

The gene expressions were significantly induced ($P \leq 0.05$; bold and underlined) or repressed (bold and underlined) in leaves of grapevine one-year-old grapevines in pots (grown from cuttings) in the different treatments (pathogen control, bacterial control, co-inoculation and preventive inoculation in the hole), at 2 hpi (T0) and 15 dpi (T15) after pathogen inoculation compared to the mock control (not treated with bacteria and not inoculated with the pathogen).
The colour scale bars represent the ratio values corresponding to the mean of three independent samples. Up-regulated genes appear in shades of red, with relative expression level higher than 5 in bright red, while those down-regulated appear in shades of blue, with intensity lower than 0.1 in dark blue. VvPR1 = PR protein 1, VvPR10 = PR protein 10, VvCHIT3 = chitinase class III, VvPAL = phenylalanine ammonia lyase, VvSTS = stil bene synthase, VvCHS = chalcone synthase, VvANTS = antranilate synthase, VvCALS = callose synthase, VvGST = Glutathione S-transferase, and VvGLU = b-1,3 glucanase.

5.2. Evaluation of grapevine responses to *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) inoculations

We also determined the ability of *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) to enhance grapevine immunity in the plants inoculated with these strains. Overall, grapevine responses to each bacterial inoculation are slightly similar at the first sampling time point. (Tables 4A and 4B). At T0, compared to the mock control, the results showed a significant over-expression of two PR-protein genes (*VvPR1* and *VvCHIT3*) and the gene involved in cell wall reinforcement (*VvCALS*) for *P. agglomerans* (S1). Compared to the mock control at T15, the expression of nine of the studied genes was either over- or down-expressed, but not significantly, except for the *VvPAL* gene (Indole-Phenylpropanoid pathway), which was significantly repressed.

A relatively similar trend was observed for *Paenibacillus* sp. (S19) at T0, with the expression of four PR-proteins genes being significantly over-expressed, together with *VvCALS* (cell wall reinforcement) and *VvGST* (Redox status). The expression of four genes from the Indole-Phenylpropanoid pathways was neither over-expressed nor repressed, except for the *VvANTS* gene (Indole-Phenylpropanoid pathway), which was significantly repressed. This trend was not observed at T15, because the expression of three of PR-protein genes (*VvPR10*, *VvCHIT3* and *VvGLU*) was significantly down-expressed.

5.3. Evaluation of grapevine responses to bacterial inoculation and *N. parvum* inoculations

In order to focus on the effect of bacterial strains on grapevine responses to pathogen infection, the

RE levels of the studied genes were analysed in the plants pre-treated or co-inoculated with bacterial strains and *N. parvum* at each sampling time point.

Relatively similar responses were observed at T0 in plants co-inoculated or pre-treated with *P. agglomerans* (S1) and *N. parvum* (Table 4). Compared to the mock control, the expression of four and five of the ten studied genes were significantly over-expressed in the plants– co-inoculated and pre-treated respectively with *P. agglomerans* (S1). Only the expression of *VvANTS* gene was significantly repressed in the two conditions compared to the mock control. Fifteen days after *N. parvum* inoculation, in the plants co-inoculated with the two microorganisms, only the expressions of two genes (*VvCALS,* and *VvSTS)* were significantly over-expressed, and the expression of the *VvANTS* gene was now significantly over-expressed. In contrast to T0, the expression of all the genes in S1 pre-treated plants at T15 were reduced, with the activity of the two genes *VvANTS* and *VvPAL* being significantly reduced.

As shown in Table 4B, the responses at T0 of grapevine pre-treated with *Paenibacillus* sp. (S19) and then infected with *N. parvum* tend to be different from those observed when the two microorganisms were co-inoculated (all treatments were compared to mock control). In particular, this trend could be observed with the four studied PR-protein genes which were over-expressed in pre-treated S19 plants, but not expressed in the co-inoculated S19 grapevines. The expression of the gene involved in cell wall reinforcement (*VvCALS*) was significantly over-expressed in the pre-treated and co-inoculated plants. Inversely, the expressions of genes involved in Redox status (*VvGST*) and indole-phenylpropanoid pathways (*VvSTS* and *VvPAL*) were significantly up-regulated in plants co-inoculated with S19 and *N. parvum* and repressed, or not expressed at all, in plants inoculated with bacteria four days before pathogen inoculation. At T15, plant responses to *N. parvum* in the presence of either pre-treated or co-inoculaed bacteria were very similar, with a repression of all studied genes, except for *VvPR1* and *VvCALs* which were slightly over-expressed.

DISCUSSION

Despite the high potential of biocontrol as a relevant solution to reduce GTDs, its implementation is currently limited by the lack of efficient and commercially available microbial control agents. In order to optimise biocontrol, it is important to test the effects of different AM on the biocontrol capacity of different bacteria. Our result confirms that the appropriate AM of a potential BCA-bacterial strain will significantly improve its efficacy.

First, our results are consistent with previous findings reporting that bacterial efficiency depends highly on the bacterial strain and AM (Magnin-Robert *et al*., 2007; Parikh *et al*., 2018). We have demonstrated for the first time that the preventive application of bacteria to the stem has better potential for reducing *N. parvum* wood necroses compared to bacterial application at the soil surface. All the tested bacterial strains applied in the stem significantly reduced the length of internal necrosis lesions due to *N. parvum*, the higher values being obtained when the bacteria were applied four days before the pathogenic fungus. It should be noted that this result seems to be pathogen-dependent, because in a previous study with another GTD pathogen (*P. chlamydospora)* AM was not found to affect bacterial efficacy (Haidar *et al*. 2016b). To explain these differences, it can be suggested that the mechanisms underlying the plant perception of *P. chlamydospora* and *N. parvum* are different, as reported by Zeilinger *et al*. (2016) for other pathogens. Presumably, and depending on the AM, various microbial interactions also took place. These AM differences do not seem to affect the *P. chlamydospora* control, but only the *N. parvum* control.

The bacterial preventive application to the stem was the most appropriate AM for protecting grapevine against *N. parvum* attack. With this AM, no external canker lesion developed in the plants inoculated with 5 of the 8 tested strains: *P. agglomerans* (S1, S3), *B. pumilus* (S32), *B. firmus* (S41) and *B. reuszeri* (S28). It can be hypothesised that these bacterial strains had efficiently colonised the wood tissues before fungal inoculation. This may enhance the BCA control efficacy, as recently shown by González-García *et al*. (2019): when they analysed the colonisation of *Stryptomonas* in uprooted grapevine pretreated with bacteria injected into the rootstock, the highest rate of bacterial colonisation was obtained in the wood of these treated plants. Accordingly, and because we used ungrafted plant material, a future area of research could be to test preventive treatment on grafted grapevine material in nurseries.

When bacteria were applied to the stems four days before the pathogen, the greatest inhibitory effect of *N. parvum* necrosis was obtained at the rate of

49.7 and 45.4 % when using *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) respectively.

In this AM, the inhibitory efficacy of these two strains was similar (S19) or greater (S1) to that exhibited by the fungicide Fluazinam. Under field conditions, the active ingredient Fluazinam has been found to be one of the most effective fungicide treatments for pruning wounds, inhibiting *Eutypa lata* and *Botryosphaeria* canker formation (Sosnowski *et al*., 2008; Pitt *et al*., 2012; Gramaje *et al.*, 2018). To our knowledge, there are no other reports of *Paenibacillus* antagonism of *N. parvum,* but we have previously shown that the same strain of *Paenibacillus* sp. (S19) is an excellent biocontrol candidate against *P. chlamydospora* (Haidar *et al.,* 2016b). In terms of whole bacterial communities, Bruez *et al*., 2015 and Bruez *et al.*, 2020 were the first to show that specific complexes of bacteria colonise the different tissues (necrotic and non-necrotic) of grapevine expressing, or not, Esca-foliar symptoms. If we put together our findings on *P. agglomerans* and *Paenibacillus* sp., and the bacteria communities colonising the grapevine wood, it can be suggested that grapevine tissues comprise a reservoir of beneficial bacteria with potential biocontrol properties against GTD pathogens; this is supportedby recent reports of bacteria which had been isolated from a vineyard having biocontrol activity, such as *Bacillus* sp., *Streptomyces* sp. and *Paenibacillus* sp., against GTDs pathogens (Pacifico *et al*., 2019; Trotel-Aziz *et al*., 2019).

It is possible that the mechanisms behind the potential biocontrol activity of *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) are linked to the production of various antifungal substances. This assumption is based on results of the present study which showed that, in dual interaction, *Paenibacillus* sp. (S19) reduced mycelial growth of *N. parvum* by 62 % and *P. agglomerans* (S1) by 13 %, suggesting that the diffusible antifungal substances involved (e.g., antifungal secondary metabolites commonly produced by *Paenibacillus* species (Keswani *et al*., 2019)) are different. The same bacteria also act differently depending on the pathogens; for instance, *Paenibacillus* sp. (S19) strongly inhibited *N. parvum* mycelia development via antibiosis, but Haidar *et al.* (2016) showed there was no direct inhibition of *P. chlamydospora*. Thus, the diffusible antifungal substances produced by *Paenibacillus* sp. (S19) are pathogen-targeted. In agreement with this, Bruisson *et al*. (2019) reported that both *Botrytis cinerea* and *Phytophthora infestans* pathogens may react differently to the secondary metabolites of BCAs. Regarding the volatile molecules, phenylethyl alcohol, an antifungal substance (Prakash *et al*., 2015; Wan *et al*., 2008, Boukaew and Prasertsan, 2018), dominated the volatile profile of *P. agglomerans* (S1) and is likely involved in the inhibition effect on *N. parvum*.

Besides the direct antagonism of *N. parvum*, and the production of diffusible and volatile antifungal substances by *P. agglomerans* (S1) and *Paenibacillus* sp. (S19) respectively, the two bacterial strains were able to stimulate grapevine defenses against the GTD-pathogen. The RE levels of 10 major grapevine defense genes clearly showed the ability of the two bacterial strains to activate the plant defense genes. This was more significant at T0 than at T15, corresponding to 4 and 19 days after bacterial inoculation (note that bacteria were inoculated 4 days before pathogen inoculation). At T0, the responses of grapevines pre-treated with the bacteria or co-inoculated were more up-regulated than those in plants inoculated with *N. parvum* only. This tendency was not maintained 15 days after the pathogen inoculation, except for plants co-inoculated with S1 and *N. parvum* always showing a high expression of certain genes (*VvCALS*, *VvGST*, *VvSTS* and *VvCHS)* when compared to the mock control. The same trend was observed with *B. pumilus* (S32) when it was co-inoculated with *P. chlamydospora* (Haidar *et al*., 2016b). Thus, whatever the method of application, grapevine defenses tended to be more induced in plants treated with the two microorganisms than those infected with the pathogen only, especially at T0. These findings suggest that bacterial strains induce the responses of grapevine challenged by *N. parvum* at a very early stage in the infection process. This hypothesis could explain, in part, the reduction of *N. parvum* necrosis obtained at the end of the experiment. Few studies have shown the capacity of BCA bacterial strains to induce systemic grapevine defenses against GTD-pathogens (Haidar *et al*., 2016b; Mondello *et al*., 2018; Trotel-Aziz *et al*., 2019). Interestingly, the RE level of the callose synthase gene (*VvCALS*) was higher with all bacterial treatment than that observed in the *N. parvum* treatment. Additionally, the greatest RE level of this gene was observed in the presence of bacteria at T0. Since the *VvCALS* gene is involved in cell wall reinforcement, the bacterial wood colonisation by the BCA bacterial strain could enhance the ability of the plant to reinforce its cell walls via callose accumulation. This phenomenon has been reported to be of

importance by Luna *et al.* (2011), who observed that the cell wall is an important component of innate plant defense priming. This defense mechanism was also observed by Duke *et al*. (2017), who demonstrated that plants pretreated with *Pseudomonas chlororaphis* after inoculation with *Sclerotinia sclerotiorum* exhibited changes in the expression of genes related to cell wall architecture.

CONCLUSION

By combining and focusing on the interactions between pathogen, host-grapevine and two antagonistic bacteria, *P. agglomerans* (S1) and *Paenibacillus* sp. (S19), this study showed that under controlled conditions the latter strains were able to protect young vine plants from infection by *N. parvum*. The inhibitory effect of these potential BCA strains was clearly related to both direct (i.e., via the production of antifungal compounds) and indirect (i.e., via the induction of grapevine defense) effects. The protective effect obtained in the plants pretreated with bacteria at wood level makes this AM a very promising method for future application in nurseries and/or vineyards.

Acknowledgements: This work is supported by the French Ministry of Agriculture (V1302 Casdar research project on Grapevine Trunk Diseases) and the Industrial Chair GTDfree funded by ANR (French National Research Agency) and the Hennessy Company. The authors are also indebted to the following INRAE UMR-SAVE staff: Alain Deschamps, Marie Cécile Dufour, Marie France Corio-Costet, Olivier Bonnard and Sébastien Gambier for their valuable help with laboratory and greenhouse assays.

REFERENCES

Bardin, M., Ajouz, S., Comby, M., Lopez-Ferber, M., Graillot, B., Siegwart M., & Nicot, P. C. (2015). Is the efficacy of biological control against plant diseases likely to be more durable than that of chemical pesticides? *Frontiers in Plant Science*, 6. https://doi.org/10.3389/fpls.2015.00566

Berlanas, C., Berbegal, M., Elena, G., Laidani, M., Cibriain J. F., Sagües, A. & Gramaje, D. (2019). The Fungal and Bacterial Rhizosphere Microbiome Associated With Grapevine Rootstock Genotypes in Mature and Young Vineyards. *Frontiers in Microbiology*, 10, 1142. https://doi.org/10.3389/fmicb.2019.01142

Boukaew, S., & Prasertsan, P. (2018). Inhibitory effects of acelophenone or phenylandhyl alcohol as fumigant to protect soybean seeds against two aflatoxin-producing fungi. *Journal of Food Science and Technology*, 55(12), 51235132. https://doi.org/10.1007/s13197-018-3458-6

Bruez, E., Haidar, R., Alou M., T., Vallance, J., Bertsch, C., Mazand, F., Fermaud, M., Deschamps, A., Guerin-Dubrana L., Compant, S., & Rey, P. (2015). Bacteria in a wood fungal disease: Characterization of bacterial communities in wood tissues of esca-foliar symptomatic and asymptomatic grapevines. *Frontiers in Microbiology*, 6. https:// https://doi.org/10.1111/1462-2920.15180

Bruez, E., Vallance, J., Gautier, A., Laval, V., Compant, S., Maurer, W., Sessitsch, A., Lebrun, M.H., & Rey, P. (2020). Major changes in grapevine wood microbiota are associated with the onset of esca, a devastating trunk disease. *Environmental Microbiology*. https://doi:10.1111/1462-2920.15180

Bruisson, S., Zufferey, M., L'Haridon, F., Trutmann, E., Anand, A., Dutartre, A., De Vrieze, M., &Weisskopf, L. (2019). Endophytes and Epiphytes From the Grapevine Leaf Microbiome as Potential Biocontrol Agents Against Phytopathogens. *Frontiers in Microbiology*, 10, 2726. https://doi.org/10.3389/fmicb.2019.02726

Claverie, M., Notaro, M., Florence, F., & Wéry, J. (2020). Current knowledge on Grapevine Trunk Diseases with complex etiology: A systemic approach. *Phytopathologia Mediterranea,* 59(1), 29-53. https://doi.org/10.14601/Phyto-11150

Compant, S., Brader, G., Muzammil, S., Sessitsch, A., Lebrihi, A., & Mathieu, F. (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *BioControl*, 58(4), 435455. https://doi.org/10.1007/s10526-012-9479-6

Daraignes, L., Gerbore, J., Yacoub, A., Dubois, L., Romand, C., Zekri, O., Roudet, J., Chambon, P., & Fermaud, M. (2018). Efficacy of *P. oligandrum* affected by its association with bacterial BCAs and rootstock effect in controlling grapevine trunk diseases. *Biological Control*, 119, 5967. https://doi.org/10.1016/j.biocontrol.2018.01.008

Dufour, M. C., Lambert, C., Bouscaut, J., Mérillon, J. M., & Corio-Costet, M. F. (2013). Benzothiadiazole-primed defence responses and enhanced differential expression of defence genes in *Vitis vinifera* infected with biotrophic pathogens *Erysiphe necator* and *Plasmopara viticola*. *Plant Pathology*, 62(2), 370382. https://doi.org/10.1111/j.1365-3059.2012.02628.x

Dufour, M.C., Magnin, N., Dumas, B., Vergnes S. and Corio Costet M.F. (2016). High-throughput gene-expression quantification of grapevine defense responses in the field using microfluidic dynamic arrays. *BMC Genomics* 17, 957. https://DOI: 10.1186/s12864-016-3304-z.

Duke, K. A., Becker, M. G., Girard, I. J., Millar, J. L., Dilantha Fernando, W. G., Belmonte, M. F., & de Kievit, T. R. (2017). The biocontrol agent *Pseudomonas chlororaphi*s PA23 primes Brassica napus defenses

through distinct gene networks. *BMC Genomics*, 18(1), 467. https://doi.org/10.1186/s12864-017-3848-6

González-García, S., Álvarez-Pérez, J. M., Miera, L. E. S., Cobos, R., Ibañez, A., Díez-Galán, A., Garzón-Jimeno, E., & Coque, J. J. R. (2019). Developing tools for evaluating inoculation methods of biocontrol *Streptomyces* sp. Strains into grapevine plants. *PLOS ONE*, 14(1), e0211225. https://doi.org/10.1371/journal.pone.0211225

Gramaje, D., Úrbez-Torres, J. R., & Sosnowski, M. R. (2018). Managing Grapevine Trunk Diseases With Respect to Etiology and Epidemiology : Current Strategies and Future Prospects. *Plant Disease*, 102(1), 1239. https://doi.org/10.1094/PDIS-04-17-0512-FE

Haidar, R., Deschamps, A., Roudet, J., Calvo-Garrido, C., Bruez, E., Rey, P. & Fermaud, M. (2016a). Multi-organ screening of efficient bacterial control agents against two major pathogens of grapevine. *Biological Control*, 92, 5565. https://doi.org/10.1016/j.biocontrol.2015.09.003

Haidar, R. (2016). Caractérisation, criblage et mise en oeuvre de souches bactériennes issues du vignoble bordelais pour la lutte biologique contre les champignons impliqués dans la Pourriture grise et l'Esca de la vigne [These de doctorat, Bordeaux]. http://www.theses.fr/2016BORD0155

Haidar, R, Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., Gautier, T., Deschamps, A., & Fermaud M. (2016b). Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. *Microbiological Research*, 192, 172184. https://doi.org/10.1016/j.micres.2016.07.003

Keswani, C., Singh, H. B., Hermosa, R., García-Estrada, C., Caradus, J., He, Y.-W., Mezaache-Aichour, S., Glare, T. R., Borriss, R., Vinale, F., & Sansinenea, E. (2019). Antimicrobial secondary metabolites from agriculturally important fungi as next biocontrol agents. *Applied Microbiology and Biotechnology*, 103(23), 92879303. https://doi.org/10.1007/s00253-019-10209-2

Köhl, J., Kolnaar, R., & Ravensberg, W. J. (2019). Mode of Action of Microbial Biological Control Agents Against Plant Diseases : Relevance Beyond Efficacy. *Frontiers in Plant Science*, 10, 845. https://doi.org/10.3389/fpls.2019.00845

Kraus, C., Voegele, R. T., & Fischer, M. (2019). Temporal Development of the Culturable, Endophytic Fungal Community in Healthy Grapevine Branches and Occurrence of GTD-Associated Fungi. *Microbial Ecology*, 77(4), 866876. https://doi.org/10.1007/s00248-018-1280-3

Laveau, C., Letouze, A., Louvet, G., Bastien, S., & Guerin-Dubrana, L. (2009). Differential aggressiveness of fungi implicated in esca and associated diseases of grapevine in France. *Phytopathologia Mediterranea*, 48(1), 3246. https://doi.org/10.14601/Phytopathol_Mediterr-2873.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., & Ton, J. (2011). Callosedeposition: a multifaceted plant defense response. *Molecular Plant-Microbe Interactions,* 24, 183–19. https://doi.org/10.1094/MPMI-07-10-0149

Magnin-Robert, M., Trotel-Aziz, P., Quantinet, D., Biagianti, S., & Aziz A. (2007). Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and β-1,3 glucanase activities under field conditions. *European Journal of Plant Pathology*, 118(1), 4357. https://doi.org/10.1007/s10658-007-9111-2

Molot, B., Larignon, P., & Coarer, M. (2006). Black dead arm. De nouvelles pistes. Paper presented at the Colloque Mondiaviti, Bordeaux, 29–30 novembre 2006, 15–17.

Mondello, V., Songy, A., Battiston, E., Pinto, C., Coppin, C., Trotel-Aziz, P., Clément, C., Mugnai, L., & Fontaine, F. (2018). Grapevine Trunk Diseases : A Review of Fifteen Years of Trials for Their Control with Chemicals and Biocontrol Agents. *Plant Disease*, 102(7), 11891217. https://doi.org/10.1094/PDIS-08-17-1181-FE

Niekerk, J. M. van, Fourie P. H., Halleen F. & Crous P. W. (2006). *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathologia Mediterranea*, 45, S43S54. https://doi.org/10.1071/AP08045

Niekerk, J. M., Crous, P. W., Groenewald, J. Z., Fourie, P. H., & Halleen, F. (2004). DNA Phylogeny, Morphology and Pathogenicity of *Botryosphaeria* Species on Grapevines. *Mycologia*, 96(4), 781798. https://doi.org/10.2307/3762112

Pacifico, D., Squartini, A., Crucitti, D., Barizza, E., Lo Schiavo, F., Muresu, R., Carimi, F., & Zottini, M. (2019). The Role of the Endophytic Microbiome in the Grapevine Response to Environmental Triggers. *Frontiers in Plant Science*, 10. https://doi.org/10.3389/fpls.2019.01256

Parikh, L., Eskelson, M. J., & Adesemoye, A. O. (2018). Relationship of in vitro and in planta screening : Improving the selection process for biological control agents against Fusarium root rot in row crops. *Archives of Phytopathology and Plant Protection*, 51(34), 156169. https://doi.org/10.1080/03235408.2018.1441098

Phillips, A.J.L, Alves, A., Abdollahzadeh, J., Slippers, B., Wingfield, M.J., Groenewald, J.Z., & Crous, P.W. (2013). The *Botryosphaeriaceae*: genera and species known from culture. *Studies in Mycology*, 76(1), 51–167. https://doi.org/10.3114/sim0021 PMID: 24302790

Pitt, W. M., Huang, R., Steel, C. C., & Savocchia, S. (2013). Pathogenicity and epidemiology of Botryosphaeriaceae species isolated from grapevines in Australia. *Australasian Plant Pathology*, 42(5), 573582. https://doi.org/10.1007/s13313-013-0221-3

Pitt, W., Sosnowski, M., Huang, R., Qui, Y., Steel, C., & Savocchia, S. (2012). Evaluation of Fungicides for the Management of Botryosphaeria Canker of Grapevines. *Plant Disease*, 96, 13031308. https://doi.org/10.1094/PDIS-11-11-0998-RE

Prakash, B., Singh, P., Goni, R., Raina, A. K. P., & Dubey, N. K. (2015). Efficacy of Angelica archangelica essential oil, phenyl ethyl alcohol and α- terpineol against isolated molds from walnut and their antiaflatoxigenic and antioxidant activity. *Journal of Food Science and Technology*, 52(4), 22202228. https://doi.org/10.1007/s13197-014-1278-x

Reid, K. E., Olsson, N., Schlosser, J., Peng, F., & Lund, S. T. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology*, 6, 27. https://doi.org/10.1186/1471-2229-6-27

Reis, P., Gaspar, A., Alves, A., Fontaine, F., Lourenço, I., Saramago, J., Mota, M., & Rego, C. (2020). Early Season Symptoms on Stem, Inflorescences and Flowers of Grapevine Associated with *Botryosphaeriaceae* Species. *Plants* 9, 1427. https://doi.org/10.3390/plants9111427

Rezgui, A., Ben Ghnaya-Chakroun, A., Vallance J., Bruez, E., Hajlaoui, M. R., Sadfi-Zouaoui N., & Rey, P. (2016). Endophytic bacteria with antagonistic traits inhabit the wood tissues of grapevines from Tunisian vineyards. *Biological Control*, 99, 2837. https://doi.org/10.1016/j.biocontrol.2016.04.005

Sosnowski, M. R., Creaser, M.L., Wicks, T. J., Lardner, R., & Scott E. S. (2008). Protection of grapevine pruning wounds from infection by *Eutypa lata*. *Australian Journal of Grape and Wine Research*, 14(2), 134142. https://doi.org/10.1111/j.1755-0238.2008.00015.x

Spagnolo, A., Magnin-Robert, M., Alayi, T.D., Cilindre, C., Schaeffer-Reiss, C., van Dorsselaer, A., Clément, C., Larignon, P., Ramirez-Suero, M., Chong, J., Bertsch, C., Abou-Mansour, E. & Fontaine, F. (2014). Differential responses of three grapevine cultivars to Botryosphaeria dieback. *Phytopathology.* 104, 10211035. https://doi: 10.1094/PHYTO-01-14-0007-R. PMID: 24724741.

Trotel-Aziz, P., Abou-Mansour, E., Courteaux, B., Rabenoelina, F., Clément, C., Fontaine, F., & Aziz, A. (2019). *Bacillus subtilis* PTA-271 Counteracts Botryosphaeria Dieback in Grapevine, Triggering Immune Responses and Detoxification of Fungal Phytotoxins. *Frontiers in Plant Science*, 10, 25. https://doi.org/10.3389/fpls.2019.00025

Úrbez-Torres, J. R. & Gubler, W. D. (2009). Pathogenicity of *Botryosphaeriaceae* Species Isolated from Grapevine Cankers in California. *Plant Disease*, 93(6), 584592. https://doi.org/10.1094/PDIS-93-6-0584

Úrbez-Torres, J. R. (2011). The status of *Botryosphaeriaceae* species infecting grapevines. *Phytopathologia Mediterranea*, 50, S5S45. https://doi.org/10.14601/Phytopathol_Mediterr-9316

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3(7). https://doi.org/10.1186/gb-2002-3-7-research0034

Verhagen, B., Trotel-Aziz, P., Jeandet,P., Baillieul, F. & Aziz, A (2011). Improved resistance against *Botrytis cinerea* by grapevine-associated bacteria that induce a prime oxidative burst and phytoalexin production. *Phytopathology*. 101(7), 768-77. https://doi: 10.1094/PHYTO-09-10-0242. PMID: 21425931.

Wan, M., Li, G., Zhang, J., Jiang, D., & Huang, H.C. (2008). Effect of volatile substances of S*treptomyces platensis* F-1 on control of plant fungal diseases. *Biological Control*, 46(3), 552559. https://doi.org/10.1016/j.biocontrol.2008.05.015

Wu, H., Spagnolo, A., Marivingt-Mounir, C., Clément, C., Fontaine, F., & Chollet, J.F. (2020) Evaluating the combined effect of a systemic phenylpyrrole fungicide and the plant growth-promoting rhizobacteria *Paraburkholderia phytofirmans* (strain PsJN::gfp2x) against the grapevine trunk pathogen *Neofusicoccum parvum*. *Pest Management Science,* **76**, 38383848. https://doi.org/10.3389/fmicb.2018.02093

Yacoub, A., Gerbore, J., Magnin, N., Chambon, P., Dufour, M.C., Corio-Costet, M.F., Guyoneaud, R. & Rey, P. (2016). Ability of *Pythium oligandrum* strains to protect *Vitis vinifera* L., by inducing plant resistance against *Phaeomoniella chlamydospora*, a pathogen involved in Esca, a grapevine trunk disease. *Biological Control*, 92, 716. https://doi.org/10.1016/j.biocontrol.2015.08.005

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., Bokulich, N. A., Mills, D. A., Martin, G., Taghavi, S., Van der Lelie, D., & Gilbert, J. A. (2015). The Soil Microbiome Influences Grapevine-Associated Microbiota. *MBio*, 6(2), e02527-14. https://doi.org/10.1128/mBio.02527-14

Zeilinger, S., Gupta, V. K., Dahms, T. E. S., Silva, R. N., Singh, H. B., Upadhyay, R. S., Gomes E. V., Tsui, C. K.-M., & Nayak S. C. (2016). Friends or foes? Emerging insights from fungal interactions with plants. *FEMS Microbiology Reviews*, 40(2), 182207. https://doi.org/10.1093/femsre/fuv045.