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

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**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 inoculation 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., 2004; 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 (Esquiv® 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.

<table>
<thead>
<tr>
<th>Test code</th>
<th>Species identification</th>
<th>Bacterial origine from grapevine</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Pantoeca agglomerans</td>
<td>Grape berries</td>
</tr>
<tr>
<td>S3</td>
<td>Pantoeca agglomerans</td>
<td>Grape berries</td>
</tr>
<tr>
<td>S19</td>
<td>Paenibacillus sp.</td>
<td>Grapevine wood</td>
</tr>
<tr>
<td>S24</td>
<td>Enterobacter sp.</td>
<td>Grapevine wood</td>
</tr>
<tr>
<td>S27</td>
<td>Brevibacillus reuszeri</td>
<td>Grapevine wood</td>
</tr>
<tr>
<td>S28</td>
<td>Brevibacillus reuszeri</td>
<td>Grapevine wood</td>
</tr>
<tr>
<td>S32</td>
<td>Bacillus pumilus</td>
<td>Grapevine wood</td>
</tr>
<tr>
<td>S41</td>
<td>Bacillus firmus</td>
<td>Grapevine wood</td>
</tr>
</tbody>
</table>

S1 and S3 were isolated from Merlot cultivar and the other strains 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 10⁶ 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 x 10⁶ 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 x 10⁶ 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 in the stem 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)

<table>
<thead>
<tr>
<th>Family</th>
<th>Genes</th>
<th>N° accession GeneBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR proteins</td>
<td>PR protein 1 (VvPR1)</td>
<td>AJ536326</td>
</tr>
<tr>
<td></td>
<td>PR protein 10 (VvPR10)</td>
<td>AJ291705</td>
</tr>
<tr>
<td></td>
<td>Chitinase class III (VvCHIT3)</td>
<td>Z68123</td>
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<tr>
<td></td>
<td>b-1,3 glucanase (VvGLU)</td>
<td>AF239617</td>
</tr>
<tr>
<td>Cell wall reinforcement</td>
<td>Calloose synthase (VvCALS)</td>
<td>AJ430780.1</td>
</tr>
<tr>
<td>Redox status</td>
<td>Glutathione S-transferase (VvGST)</td>
<td>AY156048.1</td>
</tr>
<tr>
<td>Indole and phenylpropanoid pathways</td>
<td>Antranilate synthase (VvANTS)</td>
<td>XM 002281597</td>
</tr>
<tr>
<td></td>
<td>Stilbene synthase (VvSTS)</td>
<td>X76892.1</td>
</tr>
<tr>
<td></td>
<td>Chalcone synthase (VvCHS)</td>
<td>X75969.1</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine ammonia lyase (VvPAL)</td>
<td>X75967</td>
</tr>
</tbody>
</table>
(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 manufacturer’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), β-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 (VvEF1γ) 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-dye, 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-ΔΔCq method, where ΔΔCq 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.
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 ≤ 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 ≤ 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.
Five bacterial strains (B. reuszeri (S27), Enterobacter sp. (S24), 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 (S41) 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 (S41) and Paenibacillus sp. (S19) 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 (S1) 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

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

TABLE 3. Volatile compounds produced by Paenibacillus sp. (S19) and P. agglomerans (S1) (GC/MS analysis)
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. *VvPRI*, *VvPR10* and *VvCHIT3*) were over-expressed but, only *VvPRI* showed a significant high over-expression (ER=25.3).
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 PR-protein genes (*VvPR10*, *VvCHIT3* and *VvGLU*) was significantly down-expressed.

### 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).

<table>
<thead>
<tr>
<th>Gene</th>
<th>T0</th>
<th>T15</th>
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</thead>
<tbody>
<tr>
<td><strong>PR proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VvPR1</td>
<td>0.4 ± 0.35</td>
<td>0.2 ± 0.15</td>
</tr>
<tr>
<td>VvPR10</td>
<td>0.6 ± 0.13</td>
<td>0.0 ± 0.07</td>
</tr>
<tr>
<td>VvCHIT3</td>
<td>0.4 ± 0.34</td>
<td>0.0 ± 0.07</td>
</tr>
<tr>
<td>VvPAL</td>
<td>0.7 ± 0.19</td>
<td>0.0 ± 0.07</td>
</tr>
<tr>
<td>VvCALS</td>
<td>1.3 ± 0.48</td>
<td>0.7 ± 0.31</td>
</tr>
<tr>
<td><strong>Cell wall reinforcement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VvGST</td>
<td>1.0 ± 0.57</td>
<td>0.3 ± 0.09</td>
</tr>
<tr>
<td>VvSTS</td>
<td>0.6 ± 0.18</td>
<td>1.5 ± 0.97</td>
</tr>
<tr>
<td>VvCHS</td>
<td>0.6 ± 0.25</td>
<td>0.7 ± 0.31</td>
</tr>
<tr>
<td>VvPAL</td>
<td>1.0 ± 0.25</td>
<td>0.3 ± 0.12</td>
</tr>
<tr>
<td><strong>Indole and phenylpropanoid pathways</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VvPR1</td>
<td>0.4 ± 0.35</td>
<td>0.2 ± 0.15</td>
</tr>
<tr>
<td>VvPR10</td>
<td>0.6 ± 0.13</td>
<td>0.0 ± 0.07</td>
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<td>VvCHIT3</td>
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</tr>
<tr>
<td>VvPAL</td>
<td>1.0 ± 0.25</td>
<td>0.3 ± 0.12</td>
</tr>
</tbody>
</table>

The gene expressions were significantly induced (P ≤ 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 = stilbene synthase, VvCHS = chalcone synthase, VvANTS = antranilate synthase, VvCALS = callose synthase, VvGST = Glutathione S-transferase, and VvGLU = b-1,3 glucanase.

### 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-inoculated bacteria were very similar, with a repression of all studied genes, except for *VvPRI* 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-Garcia 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 supported by recent reports of bacteria which had been isolated from a vineyard having biocontrol activity, such as *Bacillus* sp., *Streptomyces* sp. and *Paenibacillus* sp., against GTD 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.

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