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Synergistic effects of water deficit and wood-inhabiting bacteria on pathogenicity of the grapevine trunk pathogen *Neofusicoccum parvum*

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Summary. Grapevine trunk diseases (GTDs), including Esca and Botryosphaeria dieback, are major factors limiting grapevine productivity and longevity in France. The influence of combined biotic and abiotic stress factors on GTD development is not well understood. This study evaluated individual and combined effects of a biotic factor (bacterium occurrence) and abiotic stress (water deficit), on the pathogenicity to grapevine of the GTD pathogen *Neofusicoccum parvum*. Co-inoculation of 46 different bacterium strains with *N. parvum* into growing grapevine-cuttings showed synergistic relationships between several of the strains and the pathogen. *Bacillus pumilus* (strain S35) and *Xanthomonas* sp. (strain S45), which cause canker lesions, were selected for testing bacterial and fungal interactions in grapevine, under individual and combined stress conditions. *In vitro*, each of the bacterium strains neither inhibited *N. parvum* nor the co-inoculated bacterium strain. None of the three microorganisms degraded lignin, but all three degraded cellulose and hemicellulose. In a greenhouse experiment, 9 months after microbial inoculations in plants under normal and water-restricted conditions, effects on canker formation of water deficit combined with the bacteria and *N. parvum* interactions were assessed and on *N. parvum* DNA contents. Synergistic effects of biotic and abiotic stresses were demonstrated. The bacterial infection stress influenced the grapevine/*N. parvum* interaction by increasing canker lesions and *N. parvum* DNA contents in plants co-inoculated with *B. pumilus* and/or *Xanthomonas* sp. qPCR assays showed that high contents of *N. parvum* DNA occurred in water-restricted potted vines inoculated with *N. parvum*, especially in the inoculation zones. These results provide insights into the relative roles of biotic and abiotic stress factors in *Botryosphaeriaceae* symptom expression, which could assist development of future GTD management.

Keywords. Esca, Botryosphaeria dieback, *Bacillus pumilus, Xanthomonas* sp.

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INTRODUCTION

Grapevine is simultaneously exposed to multiple biotic and abiotic stresses, which can limit productivity. Grapevine trunk diseases (GTDs), including Eutypa dieback, Esca and Botryosphaeria dieback, have increased considerably in recent decades (De La Fuente *et al*., 2016; Gramaje *et al*., 2018). While most GTDs are likely to be multi-factorial diseases (Darrieutort *et al*., 2007; Lecomte *et al.,* 2011; Bertsch *et al*., 2013), research has mainly concentrated on plant responses to biotic stresses, especially to determine the infection processes associated with the complex of fungal GTD pathogens (Bertsch *et al*., 2013; Gramaje *et al*., 2018; Brown *et al.,* 2019). Several studies have dealt with the infection of grapevine by one or combinations of different fungi involved in GTDs (Laveau *et al*., 2009; Rezgui *et al*., 2018; Brown *et al*., 2019). However, the involvement of other biotic stresses, such as bacterial infections, has received little attention (Bruez *et al.,* 2015; Haidar, 2016). Beside the microbial aspects of GTDs, abiotic stress factors are reported to be involved in the development of these diseases (Bertsch *et al*., 2009; Lecomte *et al*., 2011; Fischer and Kassemeyer, 2012), but the particularly complex effects of multiple and combined biotic and abiotic stress factors are not well understood.

Infection of grapevines by pathogens causing canker and/or dieback, such as *Botryosphaeriaceae*, has been reported to be affected by stressful cultural practices and environmental conditions, including unfavourable climate and soil moisture deficit (Lecomte *et al*., 2011; Amponsah *et al*., 2014; Lawrence *et al*., 2016; Kovács *et al*., 2017; Billones-Baaijens and Savocchia, 2018). *Neofusicoccum parvum* and other *Botryosphaeriaceae* species, such as *N. australe*, *N. luteum*, *Lasiodiplodia theobromae*, *Diplodia mutila*, *D. seriata* and *Botryosphaeria dothidea*, are important pathogens associated with GTDs. These fungi cause dieback, cankers and central wood necroses, and eventually vine death (van Niekerk *et al*., 2006; Úrbez-Torres, 2011; Gramaje *et al*., 2018; Mondello *et al*., 2018).

Key environmental stress factors such as drought or water deficit, especially associated with climate change, could predispose vines to infection by GTD pathogens (Slippers and Wingfield, 2007; Bertsch *et al.,* 2013; Songy *et al*., 2019). Interactions between abiotic stresses and pathogens can result in enhanced reduced host susceptibility to infection, depending on the stress and pathogen (Ramegowda *et al*., 2013; Ramegowda and Senthil-Kumar, 2015; Songy *et al*., 2019). For *Botryosphaeriaceae*, different physiological and histochemical changes have been associated with infections, including formation of tyloses and production of gums blocking host xylem vessels (Bertsch *et al*., 2013). In Eucalyptus, as in grapevine, the impacts of infections by *Botryosphaeriaceae* in relation to climatic conditions and stress factors have been reported previously (Chen *et al*., 2011; Barradas *et al*., 2016, 2018; Marsberg *et al*., 2017).

In grapevine, previous studies of the effects of stress factors, such as drought or water deficit, on *Botryosphaeriaceae* disease expression, have produced variable results. Qiu *et al*. (2016) observed increased susceptibility to some species of *Botryosphaeriaceae,* including *B. dothidea*, *L. theobromae* and *N. parvum*, inoculated into water-restricted potted vines. Similarly, van Niekerk *et al*. (2011) reported that grapevine lesion lengths were greater in plants inoculated with some *Botryosphaeriaceae* species, when the vines were subjected to a low irrigation regime compared to well-irrigated vines. In contrast, Sosnowski *et al*. (2016) reported that water deficit did not increase grapevine susceptibility to *D. seriata*.

As GTD development may result from complex interactions *in natura*, there is a need to measure grapevine responses to different stress factors, tested singly or in combinations. Elucidation of the impacts of microorganisms and abiotic stress factors on grapevine responses is important for understanding the key factors that influence GTDs, and to develop effective management strategies for limiting GTD development.

The major objectives of the present study were as follows. Firstly, by inoculating grapevine plants with bacterial and fungal wood colonizing microorganisms, the combined effects were evaluated of two biotic stress factors on the expression of canker in cuttings. Two bacterium strains, *Xanthomonas* sp. (S45) and *B. pumilus* (S35) were used because previous research (Haidar *et al.,* 2016a) showed that wood necroses increased in young grapevines when they were co-inoculated with these two strains and *N. parvum*. Second, the consequences were assessed of grapevine exposure to inoculations with *N. parvum* and the two bacterium strains, combined with host abiotic stress (i.e. water deficiency). These biotic and abiotic stresses were tested individually and in combinations. Furthermore, by quantifying *N. parvum* DNA, the aim was to establish whether there was a link between symptom expression due to *N. parvum* infection and wood colonization by this pathogen. These results could give insights into the roles of biotic and abiotic stress factors in symptom expression, and provide new knowledge for developing management strategies for GTDs.

MATERIALS AND METHODS

Microorganisms and culture media

Neofusicoccum parvum *culture*

A *N. parvum* isolate (strain 'Cou 02') was selected from the INRAE-UMR 1065 SAVE collection, Bordeaux, France. This strain was originally obtained in 2008 from cv. Cabernet Sauvignon vines in an experimental INRAE vineyard, near Bordeaux, France. The isolate was characterized as very aggressive in previous studies (Laveau *et al*., 2009). The strain was subcultured on Malt Agar (MA) and incubated at 27°C (12 h light/12h dark) for 1 week, before being used in experiments.

Cultures of bacterium strains

Forty-six strains of bacteria were tested. These were from a previous study that included description of their origins and key features (Haidar *et al*., 2016a). For *in vitro* trials carried out with *B. pumilus* (S35) and *Xanthomonas* sp. (S45), the strains were grown for 24 h at 28°C on Trypto-Casein Soy Agar (TSA, Biokar Diagnostics). In two bioassays (see below), the bacterium preparations were made as described in Haidar *et al.* (2016a), with cell concentrations adjusted to 10^8 CFU mL^{-1}.

Stem disease bioassays

Plant material

Rooted cuttings of grapevine cv. Cabernet Sauvignon, originating from INRAE experimental vineyards near Bordeaux, were used in the bioassays. The cuttings were grown in an open greenhouse, and were processed and prepared as described by Laveau *et al.* (2009).

Bacterium and fungus inoculations

The bacterium and fungus inoculations were made as described by Haidar *et al.* (2016a). Briefly, for application of each bacterium strain, 40 µL of cell suspension was inoculated into a drilled hole in each stem cutting below the upper bud. Once the liquid inoculum had dried for 20 to 40 min at ambient temperature, the hole was inoculated with a mycelium plug of *N. parvum*. The treated wounds were covered with Parafilm® (Scellofrais film) to seal the inoculation zones.

Experimental design of the first bioassay (screening bioassay of 46 bacterium strains)

Forty-six bacterium strains were evaluated for their antagonistic activity against *N. parvum*. Two trials were conducted. The first included 26 bacterium strains (S1–S26), and the second included 20 strains (S27–S46). In the first trial, the experimental design was a completely randomized block, with two blocks, each of 224 plants. Twenty-eight treatments (with eight plants per treatment in each block) were applied, including the 26 bacterium strains co-inoculated with the *N. parvum* isolate and two experimental controls. In the second trial 252 plants were used. Eleven plants were tested for each bacterium strain co-inoculated with *N. parvum*. The experimental control treatments in both trials were; uninoculated uninfected control (UUC), where the plants were not inoculated with the fungus and not treated with bacteria; and an uninfected control (UC), where the plants were inoculated only with *N. parvum*.

Experimental design of the second bioassay (with the two selected bacterium strains)

The second bioassay was carried out between June 2017 and April 2018 with the two bacterium strains (S35 and S45) that gave the greatest synergistic effects with *N. parvum* in the first bioassay. The two strains were applied to grapevine cuttings by co-inoculation (as described above) to confirm their ability to enhance *N. parvum* symptoms in grapevine cuttings. To assess the impacts of water deficit on infection of the cuttings by *N. parvum*, the same experimentation was performed on well-watered (experimental control) or water-restricted grapevine cuttings. The experimental design was a randomized complete block with 30 cuttings per treatment and per watering condition. A total of 540 grapevine cuttings were used (270 plants for each watering condition). During the period of the experiment, the well-watered control grapevines received five times more water than the water-restricted grapevines (Lawrence *et al*., 2016). Each well-watered plant received water at 333.3 mL d^{-1}, while each water-restricted plant received 66.6 mL d^{-1}.

The experiment treatments consisted of cuttings that were: (i) co-inoculated with S35 and *N. parvum*; ii) co-inoculated with S45 and *N. parvum*; or (iii) co-inoculated with both the bacterium strains and *N. parvum*. The control treatments consisted of plants that were: i) inoculated with *N. parvum*; ii) treated with S35; iii) treated with S45; iv) treated with S45 and S35; v) not inoculated with *N. parvum* nor treated with bacteria, but treated

with sterile bacterium and fungus growth media (mock control); or vi) not inoculated with *N. parvum*, nor treated with bacterium strain.

Effects of water deficiency on the root mass of cuttings in the second bioassay

After measurement of stem cankers (see below), six plants from each treatment were destructively harvested to provide measurements of root mass. The plants were removed from the soil, remaining soil particles were washed from the roots, and the roots were then weighed (Kamiloglu *et al*., 2014).

Evaluation of stem cankers

At 4 months post-inoculation in the first bioassay or 9 months in the second, length (mm) of stem canker was measured on each cutting.

Detection and quantification of Neofusicoccum parvum *within the wood of grapevine cuttings using quantitative PCR (qPCR)*

In the second bioassay, qPCR assays were performed to quantify *N. parvum* DNA in inoculated and uninoculated grapevine cuttings. For each treatment, five plants were sampled at the end of the experiment. After symptom (canker) measurement, two wood sections each of 15 mm were sampled from two stem zones of each plant, corresponding to zone (A), around the site of inoculation, and zone (B) 15 mm above the site of inoculation. Thus, four wood chips (each of 5 × 2 × 2 mm) were sampled from the stem of each plant after symptom evaluation. Overall, 180 wood samples were collected and stored at -80°C for DNA extractions.

DNA extractions from the wood of grapevine cuttings

DNA extracts from plants samples were obtained using the methods of Pouzoulet *et al*. (2013). Briefly, wood samples were lyophilized and subsequently ground at room temperature, using a Tissue Lyser II (Qiagen). Approximately 100 mg of wood powder was used for each DNA extraction, using the commercial kit DNeasy Plant Mini Kit (Qiagen), and the adapted protocol for grapevine wood (Pouzoulet *et al*., 2013) was implemented. The quality and quantity of DNA obtained were measured using nanodrop (ND-1000, Thermoscientific, Labtech), and diluted to 10 ng μL^{-1}.

DNA extraction from pure cultures of Neofusicoccum parvum

A pure culture of the *N. parvum* isolate was grown on MA at 25°C for 4 d. Mycelium was harvested and then freeze-dried overnight. The dried mycelia were ground with a small glass ball in a TissueLyserII (Qiagen) before DNA extraction with a cetyltrimethylammonium bromide (CTAB) procedure (Bruez *et al*., 2015). The DNA concentration was measured by the nanodrop (ND-1000, Thermoscientific, Labtech), and diluted to 50 ng μL^{-1}.

Quantitative real-time PCR analyses on the wood of grapevine cuttings

Each reaction, conducted in duplicate, contained 2 µL of sample DNA, 0.6 µL of each primer at 10 µM and 12.5 µL of 2× SYBR Green Quantitect Master Mix (Qiagen), and H_2O to 25 µL total volume. Experiments were conducted with Stratagene Mx3005P qPCR system (Agilent Technologies). The thermal cycling conditions were: an initial denaturation step at 95°C for 15 min; 40 cycles each of 15 s at 95°C (for denaturation) and 45 s at 62°C (for both annealing and extension); and an additional melting analysis for 40 min from 60°C to 95°C. Pure DNA from *N. parvum* cultures was used as the standard. Standard solutions, ranging in concentration from 750 ng μL^{-1} to 7.5×10^{-5} ng μL^{-1}, were performed. The sequence primers used were as follows: BpvQF, 5'-GCGCGAATGGCAATGGCTGA-3', and BpvQR, 5'-TACGTGTTTGTGCAATTAGTGAGAGAG-3' (Pouzoulet, 2012), and the primers were each used at a final concentration of 0.5 mM. For each sample, the amount of fungus DNA was calculated as described by Reid *et al*. (2006). Supplementary Figure S1 shows the relationship between Ct sample data and quantity of *N. parvum* DNA.

In vitro *microbial confrontations*

To assess the potential interactions between the bacteria S35 and S45, each strain was grown in 100 mL tryptic soy broth (TSB) at 28°C for 48h. Cell-free supernatants were prepared by centrifuging at 10,000 rpm for 30 min at 4°C. Appropriate agar medium plates (TSA or potato dextrose agar, (PDA; Biokar)) were each inoculated 100 µL of each strain, and wells (8 mm diam.) were cut and filled with 100 µL of the supernatants of tester strain. Plates were incubated at 28°C, for 2–3 days, and the inhibition zones around the inoculum wells were

then measured. The interaction between the two bacterium strains was also studied by a cross streak method (Lertcanawanichakul and Sawangnop, 2008). Each tester strain (S35 or S45) was inoculated by a single streak in the centre of an agar plate (PDA or TSA). After incubation for 2 d at 28°C, the respective receiver strain (S35 or S45) was then applied as a perpendicularly streak to the tester stain, and the plates were then incubated for 3 d at 28°C. The bacterium interactions were analyzed by observing development of inhibition zones. To determine if the bacterium strains S35 and S45 inhibited *N. parvum*, their direct and indirect *in vitro* effects on mycelium growth of *N. parvum* were assessed using the methods of Haidar *et al.* (2016b).

Wood cell component decomposition by Neofusicoccum parvum, Bacillus pumilus *S35 and* Xanthomonas *sp. S45.*

Wood component decomposition capabilities of bacterium strains and *N. parvum* were tested using three selective media: i) minimum medium [1 g K_2HPO_4; 1 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4.H_2O$; 0.5 g NaCl; 5 g carboxymethyl-cellulose sodium salt (Sigma), and 20 g agar per liter), with 5 g xylan from beechwood (Appolo Scientific) or carboxymethylcellulose (CMC, Sigma) as the sole carbon sources (Hervé *et al*., 2016); ii) WYA medium (1 g NaCl, 0.1 g yeast extract (Difco), 1.95 g MES (Sigma) and 20 g per liter agar, adjusted to pH 5, and containing 0.05% Remazol Brilliant Blue R (Sigma) (Hervé *et al*., 2016); or iii) PDA plates containing 0.02% Guaiacol.

Pure cultures of each microorganism strain were spot inoculated at the centres of Petri dishes (90 mm diam., 15 mL medium per plate). The Petri dishes were then incubated for 7 d at 28°C in the dark. Lignolysis was indicated by the Remazol Brilliant Blue R (RBBR) medium changing from blue to pale pink. Cellulolytic activity, on CMC medium, and xylanolytic activity on xylan medium, were detected using 0.1% Congo red (Sigma) for staining during 40 min. Staining was then followed by washing with 1M NaCl to counterstain the plates revealing clear zones where enzymatic activity occurred. Diameters of clear zones surrounding microbe colonies were measured to indicate the enzymatic activity.

Statistical analyses

The experimental data were compared using analysis of variance (ANOVA) followed by Newman-Keuls' test. For qPCR analyses, the comparison of grapevine watering conditions, sampling position and the effects of treatments, data were subjected statistical analyses using the non-parametric Kruskal-Wallis test. These analyses were carried out with the software packages StatBox (Version 6.6, Grimmer© Logiciels, Paris) and the Rcmdr package of the R software (64 3.0.1).

RESULTS

Effects of 46 different bacterium strains on Neofusicoccum parvum*-induced cankers*

In the control cuttings inoculated only with *N. parvum* (controls uninfected with bacteria: UC), the mean lengths of the canker lesions were 9.8 mm (Figure 1). No cankers were observed in the uninoculated uninfected controls (UUC) in which the samples were not inoculated with the fungus, nor treated with bacteria. Except for three bacterium strains, *P. agglomerans* (S1 and S3) and *B. reuszeri* (S27), all the stem cuttings co-inoculated with one tested bacterium strain and *N. parvum* exhibited canker lesions. On the other hand, co-inoculation of the fungus with five bacterium strains including *B. licheniformis* (S33 and S44), *Xanthomonas* sp. (S45), *B. pumilus* (S35) and *Bacillus* sp. (S43) increased ($P \leq 0.05$) canker size compared to the control plants inoculated with *N. parvum* alone (Figure 1).

Assessments of grapevine growth under two water regimes (second bioassay)

Plant growth was assessed visually between June and September 2017. The development of plants subjected to the water deficit regime was reduced compared to that of the normally irrigated plants, and was associated with external symptoms, including wilting, foliar chlorosis and dead leaves. A total of 52 plants had died by the end of the bioassay (Table S1), 40 of which were under the water deficit condition, and all but two (including one under the normal water condition) had been artificially infected. Of the 39 inoculated plants that died under the reduced-water regime, 12 had been inoculated with *B. pumilus* alone. The remaining 27 dead plants were distributed as follows: seven Bacil/Xanth, five Xanth, four Mock control, four Bacil/Xanth/Np, three Xanth/Np, two Np and two Bacil/Np.

Measurements of root mass (second bioassay)

As shown in Figure 2, the watering conditions affected root mass. The water deficient plants showed reduced root mass, which was reduced by half compared to

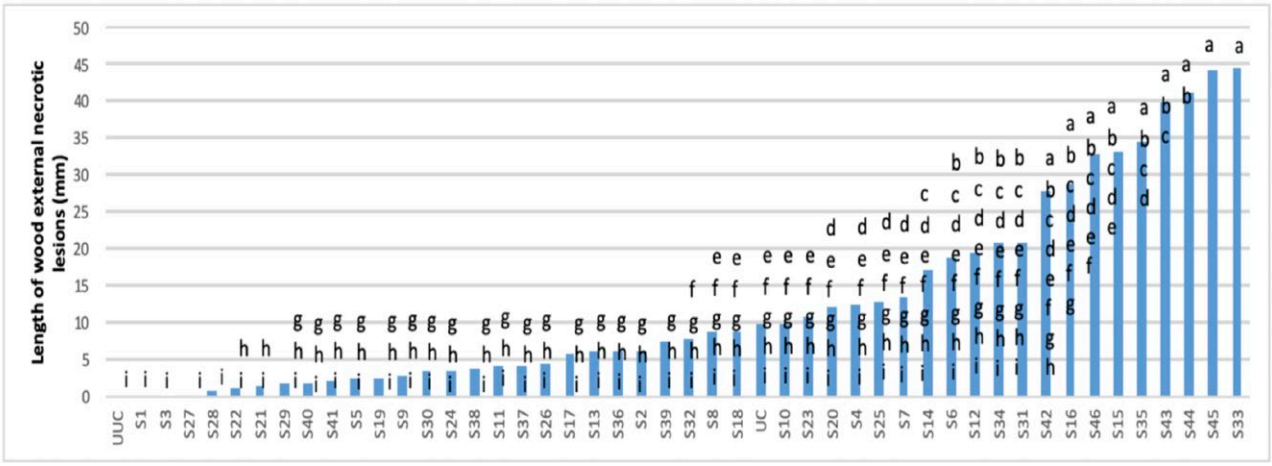

Figure 1. Mean wood canker lesion lengths (mm) in grapevine cuttings following inoculation with 46 different bacterium strains and co-inoculation with *Neofusicoccum parvum* in the first bioassay. Grapevine cutting stems (cv. Cabernet Sauvignon) were co-inoculated with bacteria and *N. parvum* before incubation in an open greenhouse for 4 months. The uninfected controls (UC) were inoculated only with *N. parvum*. The uninoculated, uninfected control (UUC) was not inoculated with the fungus and not treated with bacteria. Each value represents the mean of 16 or 11 cuttings, depending on the bioassay (Haidar *et al.,* 2016a). Means accompanied by the same letter are not significantly different ($P = 0.05$; Newman and Keuls' test after ANOVA).

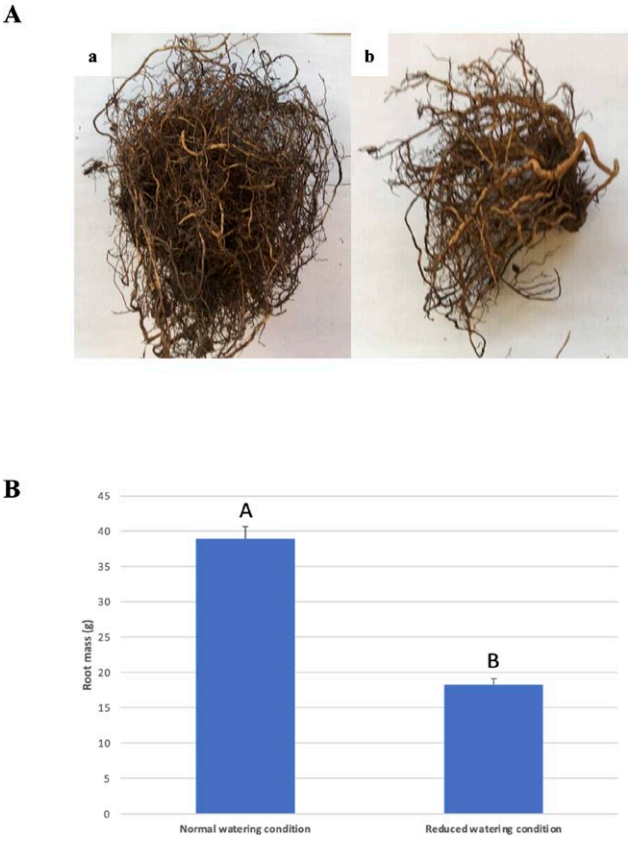

Figure 2. A. Mean (± SE; of 54 plants) root weights for potted grapevines in an open greenhouse. a) control plant roots. b) plant roots under water deficit. B. Mean root weights for potted grapevines in an open greenhouse. Different letters indicate differences ($P = 0.05$; Newman and Keuls' test after ANOVA)

that of normally irrigated plants. Under normal watering conditions, root mass reached a maximum mean of 46.1 g per plant, observed for the control plants, and a minimum of 31.9 g, observed in the plants co-inoculated with the three microorganisms.

Stem canker lesions in second bioassay

The results are presented in Figure 3. No cankers were observed when the fungus pathogen was not present (except for *B. pumilus* inoculation under the reduced water condition). All plants inoculated with *N. parvum* developed canker lesions regardless of the water regime applied. Furthermore, when the pathogen was inoculated alone or with one bacterium strain (*Xanthomonas* sp. (S45) or *B. pumilus* (S35)), there was no effect of water treatment on canker development. The data clearly showed that canker lesion length was affected by water regime in the plants co-inoculated with the three microorganisms. Under this multibiotic stress, mean lesion length was greater by a factor of two from the decreased irrigation treatment. On average, among the plants subjected to the three biotic stresses, mean lesion length was 12.3 mm from normal watering conditions and 26.2 mm from reduced watering. The enhancement of plant susceptibility to the pathogen due to these multiple stresses (especially reduced water supply) was probably responsible for the increased lesion lengths. Furthermore, within each watering condition (normal

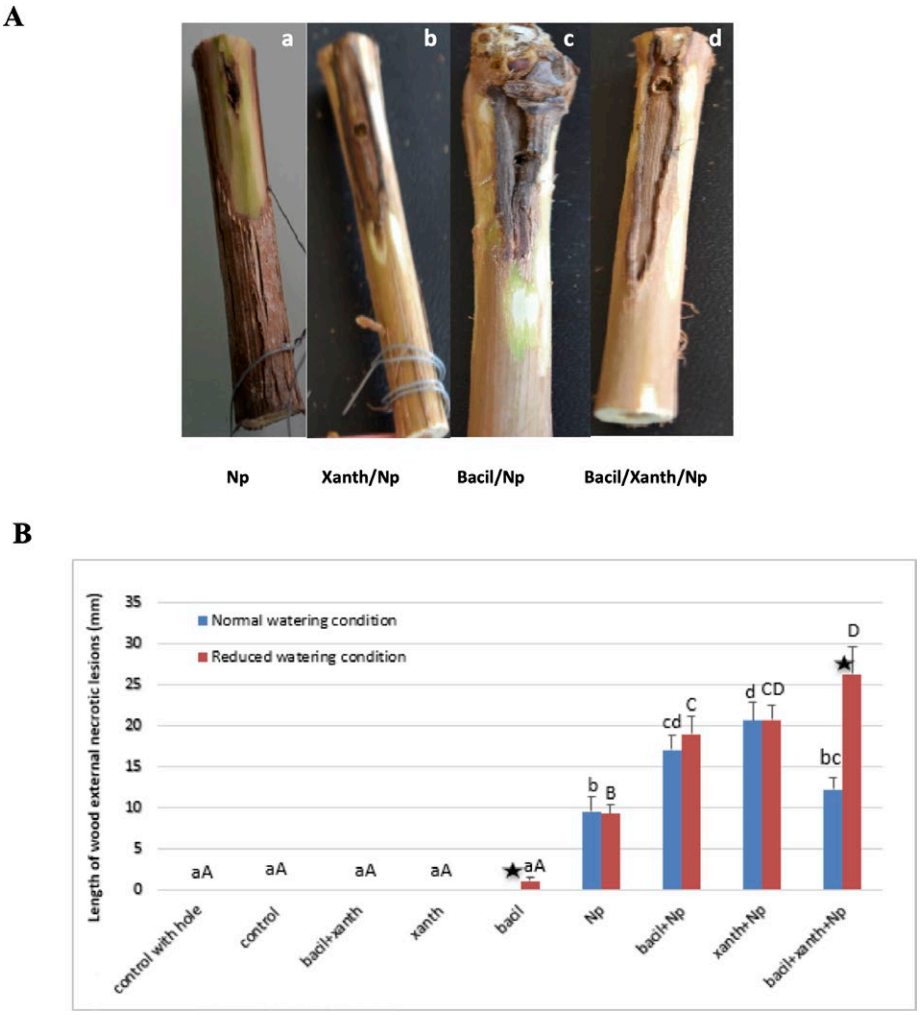

Figure 3. A. Mean wood canker lesion lengths on grapevine cuttings. (a) cuttings inoculated with *Neofusicoccum parvum* only; (b) cuttings co-inoculated with *N. parvum* and *Xanthomonas* sp. (S45); (c) cuttings co-inoculated with *N. parvum* and *B. pumilus* (S35); (d) cuttings co-inoculated with *N. parvum* and *Xanthomonas* sp. (S45) and *B. pumilus* (S35). B. Mean (±SE) wood canker lesion lengths from the different treatments in the second bioassay, under two water conditions, 9 months post-inoculation. Each value is the mean of 30 cuttings. Means accompanied by the same letter are not different ($P < 0.05$; Kruskal-Wallis test. indicates statistically significant interactions for the same modality under the two water conditions.

or reduced), a difference in canker length was detected between plants subjected to two or three stresses and those subjected to the stress caused by *N. parvum*. This difference was statistically significant in the reduced watering condition, with the plants co-inoculated with *N. parvum* and one or two bacterium strains exhibiting larger canker lesions than plants inoculated with *N. parvum* alone. This result clearly indicated potential synergetic interactions among the different microorganisms. Similarly, in the well-watered plants, the results also indicated a synergistic interaction, with plants subjected to two biotic stresses, i.e., *N. parvum* and either *B. pumilis* or *Xanthomonas* sp., developing larger lesions than those inoculated with *N. parvum* alone. However, the canker lesions were not significantly larger in the plants co-inoculated with the three microorganisms than in the control plants inoculated with *N. parvum* alone.

Quantification of Neofusicoccum parvum *DNA in the wood of grapevine cuttings*

The results of the quantification of *N. parvum* DNA are shown in Table 1. No amplification was detected in the treatments without *N. parvum* (data not shown). This confirmed that the primers used were specific to this pathogen and did not amplify nontarget DNA. In the

Table 1. Mean amounts (ng μL^{-1}; ±SE for five repetitions) of *Neofusicoccum parvum* DNA, measured in grapevine cuttings 9 months after the inoculation with different microorganisms, under two watering conditions. Means accompanied by different letters indicate that differences ($P < 0.05$) between modalities as determined by multiple comparison analyses (Kruskal-Wallis). Np: *Neofusicoccum parvum,* Bacill: *B. pumilus* (S35), Xanth: *Xanthomonas* sp. (S45).

Sampling position	Treatment	Normal watering condition	Reduced watering condition
Zone A (around the site of inoculation)	Np	$7.03 \times 10^{-3} \pm 2.03 \times 10^{-3}$ **b**	$1.43 \times 10^{-2} \pm 2.42 \times 10^{-3}$ **c**
	Bacil/Np	$8.54 \times 10^{-1} \pm 6.02 \times 10^{-1}$ **c**	$1.12 \pm 5.26 \times 10^{-1}$ **c**
	Xanth/Np	$3.48 \times 10^{-3} \pm 7.28 \times 10^{-4}$ **b**	$9.37 \times 10^{-3} \pm 3.98 \times 10^{-3}$ **b**
	Bacil/Xanth/Np	$4.53 \times 10^{-3} \pm 1.34 \times 10^{-3}$ **b**	$4.91 \times 10^{-3} \pm 1.33 \times 10^{-3}$ **b**
Zone B (above the site of inoculation)	Np	0 **a**	$4.91 \times 10^{-3} \pm 4.14 \times 10^{-4}$ **b**
	Bacil/Np	$9.93 \times 10^{-2} \pm 6.41 \times 10^{-2}$ **c**	$9.19 \times 10^{-2} \pm 3.99 \times 10^{-2}$ **c**
	Xanth/Np	$2.79 \times 10^{-3} \pm 1.58 \times 10^{-4}$ **a**	$1.11 \times 10^{-2} \pm 2.04 \times 10^{-3}$ **c**
	Bacil/Xanth/Np	0 **a**	$3.44 \times 10^{-3} \pm 7.18 \times 10^{-5}$ **b**

different treatments, depending on the sampling position and water supply condition, the amounts of fungus DNA were different within the same treatment. While *N. parvum* DNA was detected in all treatments with pathogen inoculation under the water deficit condition, no amplification of pathogen DNA occurred in samples from the zone above the site of inoculation in the plants inoculated with *N. parvum* alone or with the three microorganisms under the normal water condition. An increase in *N. parvum* DNA quantity was observed in the presence of *B. pumilus* (S35). However, plants co-inoculated with *N. parvum* and *B. pumilus* differed in quantity of *N. parvum* DNA from the control plants inoculated with *N. parvum* alone at both sampling zones under the normal watering condition. In contrast, under the reduced water condition, this difference was recorded only in the zone above the site of inoculation. This result indicates a favourable impact of reduced watering on *N. parvum* development.

In vitro *microbial interactions*

The susceptibility of each of the two selected bacterium strains to the antimicrobial substances produced by the other strain was tested using two *in vitro* methods (involving diffusible or volatile metabolites). These tests demonstrated that the two selected bacterium strains did not inhibit each other's growth. For the bacterium–*N. parvum* interactions, the results from the two *in vitro* experiments demonstrated that the two tested bacterium strains, S45 (*Xanthomonas* sp.) and S35 (*B. pumilus*), did not suppress growth of *N. parvum* (Figure S2).

Wood component decomposition ability

For ligninolytic activity, neither *N. parvum* nor the bacteria were able to degrade the Remazol Brilliant Blue R (RBBR) (data not shown). In contrast, all the tested microorganisms showed cellulase and xylanase activity halos, suggesting their potential capacities for grapevine wood degradation (Figure S3).

DISCUSSION

In vineyards, grapevines are exposed to multiple abiotic and biotic stresses. The impacts of combined stresses on plants may differ depending on the individual stresses involved. As combined stresses elicit complex plant responses, previous research on GTDs has mainly concentrated on grapevine responses to only individual biotic stresses, mostly those from fungus infections (Laveau *et al*., 2009; Spagnolo *et al*., 2017; Rezgui *et al*., 2018; Brown *et al*., 2019). In the present study, the aims were first assess grapevine responses to two combined biotic stresses (bacterium and fungus inoculation) using grapevine cuttings under open greenhouse conditions.

In a previous study, Haidar *et al*. (2016a) observed that eight bacterium strains, i.e., *B. pumilus* (S35), *Xanthomonas* sp. (S45), *B. licheniformis* (S33), *Paenibacillus polymyxa* (S15), *Curtobacterium* sp. (S42), *Bacillus* sp. (S43), *Bacillus* sp. (S46) and *Paenibacillus* sp. (S16), increased inner necrotic lesion lengths compared to that in control plants inoculated only with *N. parvum*. In the present study, four of these eight strains (*B. licheniformis* (S33), *Xanthomonas* sp. (S45), *Bacillus* sp. (S43) and *B. pumilus* (S35)) increased *N. parvum* canker lesion lengths (Figure 1). This result contributes to the growing

evidence that bacteria play roles in host wood decomposition and that the bacteria inhabiting wood could promote the ability of fungi to colonize and decompose wood structures (Hervé *et al*., 2014, 2016; Johnston *et al*., 2016, 2018; Válková *et al*., 2017; Probst *et al*., 2018). Detailed studies of bacterium/fungus interactions can increase understanding of the complex etiology of GTDs. Accordingly, in the present study, *B. pumilus* (S35) and *Xanthomonas* sp. (S45) were selected to test the effects of multiple stresses on grapevine plants. These bacteria were selected because *B. pumilus* and strains of *Xanthomonas* have been reported to be plant pathogenic bacteria (Galal *et al*., 2006; Bathily *et al*., 2010; Yan and Wang, 2011).

Combined stresses, including abiotic stresses, trigger responses in plants at physiological, biochemical, and molecular levels (Suzuki *et al*., 2014; Pandey *et al*., 2017). For *Botryosphaeriaceae* species, studies have shown that water deprivation increases the susceptibility of woody plants to these fungi (van Niekerk *et al*., 2011; Sherwood *et al*., 2015; Qiu *et al*., 2016). The present study is the first on effects of water deficit on the interactions between two xylanolytic and cellulolytic bacterium strains and *N. parvum*. Although multistress bioassays may include environmental conditions that do not perfectly simulate real vineyard conditions, in the present study the grapevines growing under normal water conditions exhibited developed greater root masses than vines growing under the reduced irrigation regime. These results are consistent with previous results from other plant hosts, showing that water-deficient plants were smaller to well-watered plants (Souza and Cardoso, 2003; Granda *et al*., 2011; Correia *et al*., 2014; McKiernan *et al*., 2014). In the present study, the large decrease in root mass (by a factor of two) under the reduced irrigation regime confirmed that this watering condition was stressful for the plant cuttings.

Under both watering conditions, the lengths of the canker lesions were greater in the plants subjected to two biotic stresses in combination, compared to plants subjected to *N. parvum* infection alone. Indirect effects of microbial community interactions, such as interactions of mutualistic or competing species with fungus pathogens, could be one of the reasons for this phenomenon (Wargo, 1996; Desprez-Loustau *et al*., 2006; Frey-Klett *et al*., 2011). A statistically significant effect of abiotic stress on the canker lesions was evident only when the plants were affected by three biotic stresses simultaneously (i.e., inoculated with three microorganisms). Our current hypothesis is that this phenomenon may have been caused by strong competition among the pathogenic microorganisms for water in the plants. Stated otherwise, this observation could depend on the amount of water available in the plants. However, watering limitation and water availability in the soil have quantitative effects on water available within plants. Further studies are needed to test this hypothesis. Furthermore, pathogens can produce primary and secondary compounds that affect plant growth and development (Berger *et al*., 2007). Therefore, the application of multiple types of stresses, as in the present study, as well as watering limitation, could increase the susceptibility of plants to pathogens. In addition, the present study showed the cellulolytic and xylanolytic activities of the different microorganisms tested. These activities could explain why more lesions were observed when the bacterium strains were present (co-inoculated) along with *N. parvum*.

In recent years, GTD fungus inoculum has been successfully quantified using quantitative real-time PCR (qPCR) for some major grapevine trunk pathogens, including *P. chlamydospora, P. aleophilum, D. seriata, E. lata* and several species of *Botryosphaeriaceae* (Pierron *et al*., 2016; Pouzoulet *et al*., 2017; Billones-Baaijens and Savocchia, 2018). This research was implemented with grapevine wood samples that had natural or inoculated infections from these pathogens. We detected *N. parvum* in all plants inoculated with this pathogen under both water conditions. In the plants inoculated only with the pathogen, in each watering condition, greater amounts of *N. parvum* were detected when sampling was conducted around the inoculation sites than from the zone just above the inoculation sites. This indicates a clear, effect of sampling location on the amount of *N. parvum* DNA measured. In the plants inoculated only with *N. parvum*, a significant effect of water condition was also observed, with *N. parvum* DNA detected more frequently under the reduced water condition than under the normal water condition. Similarly, *B. dothidea* (*Botryosphaeriaceae* family) was reported to preferentially colonize wounded and stressed tissues in *Eucalyptus* (Smith *et al*., 1996). McPartland and Schoeneweiss (1984) also found that *B. dothidea* hyphae in vessels of birch (*Betula alba*) stems were large and rectilinear in drought-stressed stems but were thin and contorted in unstressed stems. They also reported greater numbers of swollen (*vs.* intact) hyphal tips in unstressed stems than in stressed stems. The quantity of *N. parvum* DNA increased in the presence of the bacterium strains for plants tested under the two watering conditions.

In plants co-inoculated with *B. pumilus*, *N. parvum* DNA was detected in large amounts. Bacteria and fungi have been reported to influence the survival and colonization of their interacting partners (Frey-Klett *et al.,*

2011). Indirect effects of the modification of wood environments on bacterium-fungus interactions could also explain this result. For example, changes in pH were reported to influence the structure of microbial communities by either promoting or inhibiting the growth of particular organisms in different environments (Frey-Klett *et al*., 2011).

In summary, the present study, where up to four abiotic or biotic stresses were applied in combinations to grapevines, showed that strong synergistic effects occur between bacteria and fungus, and that positive interactions favour the *N. parvum* pathogenic process. Bacterium infection positively influenced the grapevine–*N. parvum* interaction as indicated by increased canker lengths. Further investigations are required to better understand bacterium–fungus interactions in GTDs, and the mechanisms associated with abiotic stress that may affect grapevine susceptibility to biotic infections. In addition, the present study has provided an example of the implementation of an experimental multifactorial model for study of combined stresses and their interactions in a grapevine pathosystem.

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