Biological Control of Grapevine Crown Gall Disease, Caused by Allorhizobium vitis , Using Paraburkholderia phytofirmans PsJN

Nguyen-Huu Trong, Jeanne Doré, Matthieu Gaucher, Cédric Jacquard, Nicolas Richet, Vincent Leclère, Essaïd Aït Barka, Marie-Noëlle Brisset, Isabelle Kerzaon, Céline Lavire, et al.

PhytoFrontiers™
An Open Access Journal from The American Phytopathological Society

Research

Biological Control of Grapevine Crown Gall Disease, Caused by *Allorhizobium vitis*, Using *Paraburkholderia phytofirmans* PsJN

Nguyen-Huu Trong[1] | Jeanne Doré[2] | Matthieu Gaucher[3] | Cédric Jacquard[1] | Nicolas Richet[1] | Vincent Leclère[1] | Essaïd Aït Barka[1] | Marie Noëlle Brisset[3] | Isabelle Kerzaon[2] | Céline Lavire[2] | Christophe Clément[1] | Ludovic Vial[2] | Lisa Sanchez[1,†] |

[1] Université de Reims Champagne-Ardenne, RIBP USC INRAE 1488, SFR Condorcet FR CNRS 3417, 51100 Reims, France

[2] Université Claude Bernard Lyon 1, CNRS, INRAE, VetAgro Sup, UMR Ecologie Microbienne, F-69622 Villeurbanne, France

[3] Institut de Recherche en Horticulture et Semences (IRHS), INRA Angers, Equipe ResPOM, 42 rue Georges Morel, F-49071 Beaucouzé cedex, France

[†] Corresponding author: L. Sanchez; lisa.sanchez@univ-reims.fr

Accepted for publication 15 June 2022.

Abstract

Controlling crown gall in grapevine (*Vitis vinifera* L.) caused by the pathogenic bacterium *Allorhizobium vitis* is a major challenge for global viticulture, as this pathogen is highly persistent in vineyards once infected. The bacteria can enter the plant through open wounds during pruning and then systemically colonize the plant. This study aimed at evaluating the potential of *Paraburkholderia phytofirmans* PsJN, a beneficial endophytic bacterium able to colonize the xylem of grapevine, the same ecological niche as *A. vitis*, to control grapevine crown gall disease. *P. phytofirmans* PsJN was root-inoculated on grapevine plantlets before infection by *A. vitis* S4 on shoots. *A. vitis* S4 level in planta, vitopine production, accumulation of lignin in tumors, and symptoms of crown gall were investigated on grapevine prebacterized or not with PsJN. The expression of 28 grapevine genes involved in defense mechanisms was also simultaneously determined by quantitative real-time PCR. Despite a direct antibacterial effect against *Av*S4, PsJN has no significant impact on the incidence of crown gall or disease severity. However, PsJN leads to a stronger accumulation of vitopine in tumors and significantly reduced the population level of the pathogen in planta.

Keywords: *Agrobacterium*/*Allorhizobium vitis*, biocontrol, crown gall disease, grapevine, opine, *Paraburkholderia phytofirmans* PsJN

In nature, grapevine (*Vitis vinifera* L.) usually suffers from attacks of a large range of pathogens that affect the plant health at every growth stage, leading to an important decrease in grape yield (up to 40% or even plant death) (Armijo et al. 2016). Crown gall disease on grapevine is caused by the tumorigenic bacterium *Allorhizobium vitis*, previously classified as *Agrobacterium vitis* (Mousavi et al. 2014; Ophel and Kerr 1990), and is becoming a huge challenge for global viticulture because this pathogen is persistent in vineyards once infected (Kuzmanović et al. 2018). The bacteria can enter the plant through wounds, caused by freezing temperatures or by pruning activities, and then systemically colonizes the host xylem vessels (Johnson et al. 2016). The tumor-inducing plasmid (pTi) determines the virulence of this pathogen

L. Vial and L. Sanchez contributed equally to this work.

Current address of N. Richet: Université de Reims Champagne-Ardenne, Plateau technique MOBICYTE, Reims, France.

Author contributions: All authors contributed to the (i) conception and design or acquisition and analysis of data, (ii) drafting or critically revising of the manuscript, and (iii) approval of the final submitted version.

Funding

This work was cofunded by Grand Reims, the European Union – "Europe invests in Champagne Ardennes," with the European Regional Development Fund (PhD grant, AGROVITIFREE project), EC2CO "Ecosphère Continentale et Côtière," and CNRS.

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

by encoding genes for the transfer and integration of transferred DNA (T-DNA) the plant genome. The oncogenes on T-DNA are subsequently expressed, leading to the excessive biosynthesis of auxins and cytokinins and consequently the uncontrolled proliferation of plant cells to form galls as an extensive ecological niche for the pathogen (Lacroix and Citovsky 2019). T-DNA also contains genes for the biosynthesis of opines, which serve as the specific nutrient source for *A. vitis* in the competition with other microbes (Dessaux and Faure 2018). In this research, we worked on *A. vitis* strain S4 (*Av*S4). This strain possesses opine synthase genes on its T-DNA, which generate the biosynthesis of vitopine and rideopine once integrated into the plant genome (Chilton et al. 2001).

To manage the disease, winegrowers can apply cultural practices including grafting on resistance rootstocks (Süle and Burr 1998), using transgenic disease-resistant plants (Galambos et al. 2013; Krastanova et al. 2010; Vidal et al. 2006), producing pathogen-free materials with hot water (Bazzi et al. 1991; Burr et al. 1989; Waite and Morton 2007; Wample et al. 1991), or in vitro tissue culture (Bisztray et al. 2012; Dula et al. 2007) coupled with PCR-based diagnostic methods (Johnson et al. 2016; Yepes et al. 2019). However, these cultural practices remain difficult to implement and have limited effectiveness in disease control (Kuzmanović et al. 2018). Some antibacterial compounds, such as copper products, are lethal to *Agrobacterium/Allorhizobium* (Alexander et al. 1999), but they are not efficient enough to fully control the disease (Burr et al. 1998). Copper is further deleterious to the environment and therefore cannot be considered a sustainable option. Hence, biological control has emerged as an alternative method that is safe for human health and eco-friendly, unlike chemical treatments. The well-known *Rhizobium rhizogenes* strain K84 and its derivative strain K1026 have been successfully shown to provide protection against crown gall disease caused by *Agrobacterium* (Kerr 2016). Unfortunately, they could not control the disease outbreak in grapevine caused by *A. vitis* (Burr et al. 1998). Therefore, many other studies have been conducted to identify other biocontrol agents (Filo et al. 2013; Habbadi et al. 2017; Kuzmanović et al. 2018).

Endophytic plant growth-promoting rhizobacteria (PGPR) are nonpathogenic microbes naturally associated with plant roots that colonize the internal spaces of plant tissues at a given period in their life cycles. They symbiotically increase the survival rate of their hosts through direct and/or indirect mechanisms (Glick 2012; Goswami et al. 2016). Among the PGPR, *Paraburkholderia phytofirmans* strain PsJN (Frommel et al. 1991a; Sessitsch et al. 2005) is able to endophytically colonize the xylem vessels of grapevine (Barka et al. 2002; Compant et al. 2008), the same ecological niche as *A. vitis* (Compant et al. 2005; Lehoczky 1968). In grapevine, this strain has been proven to stimulate the growth of root and aerial parts after inoculation (Barka et al. 2000). In addition, this bacterium showed a protection efficacy against gray mold disease caused by *Botrytis cinerea* (Barka et al. 2000; Miotto-Vilanova et al. 2016, 2019) and Pierce's disease caused by *Xylella fastidiosa* (Baccari et al. 2019). In grapevine plantlets grown in vitro, PsJN can migrate from the root tips up to aerial parts within 7 days after being inoculated through roots (Compant et al. 2005; Miotto-Vilanova et al. 2019). Moreover, PsJN was previously reported to inhibit gall development induced by pathogenic *Agrobacterium* on tomato plants (Toklikishvili et al. 2010). To control crown gall disease using biocontrol protocols, most studies used a method of coinoculation of both the biocontrol agent and pathogenic bacterium on shoots (Kawaguchi 2015; Kawaguchi et al. 2007). However, this method is impractical in vineyard management. As such, in this study, we chose a system where PsJN could be preintroduced to grapevine materials through roots before planting to prevent or mitigate potential crown gall disease outbreak. Therefore, we used grapevine plantlets prebacterized through the roots with PsJN before infecting the shoots with the pathogen *A. vitis* to evaluate the potential of this PGPR in controlling crown gall disease and to decipher the related mechanisms involved. On the basis of this interaction, we studied (i) the effect of PsJN on the population levels of *A. vitis* and the development of crown galls on grapevine stems, (ii) the direct antimicrobial effect of PsJN on *A. vitis* growth in vitro, (iii) the effect of PsJN and/or *A. vitis* on the expression of defense genes in grapevine, and (iv) the niche competition between these bacteria.

MATERIALS AND METHODS

Microorganisms

P. phytofirmans strain PsJN::GFP (PsJN) and *A. vitis* strain S4 (*Av*S4) were cultivated in King's B (KB) liquid medium supplemented with 50 μg ml^{-1} of kanamycin (KB_{K50}) and mannitol-glutamate (MG) liquid medium (Keane et al. 1970), respectively. The bacterial cultures were incubated at 28°C with agitation of 180 rotations per minute (rpm) overnight. The bacterial cells were collected by centrifugation (4,200 × *g* for 15 min at 4°C) and then washed twice with and resuspended in sterile phosphate-buffered saline (PBS, 10 mmol $liter^{-1}$, pH 6.5).

Antagonism between *P. phytofirmans* PsJN and *A. vitis* S4

Antagonism between PsJN and *Av*S4 was studied by coculturing both bacteria on the same MG medium in Petri dishes. One milliliter of the cell suspension of *Av*S4 after washing (OD_{600nm} = 0.02 in PBS) was spread evenly on the medium and allowed to dry. Then, three 10-μl drops of washed PsJN (10^9 CFU ml^{-1}, OD_{600nm} = 0.8 in PBS) were deposited on the medium. The incubation conditions were 28°C in the dark for 2 days. This experiment was performed twice in triplicate.

Carbon source utilization of *P. phytofirmans* PsJN and *A. vitis* S4

The ability of the two bacterial strains to catabolize different carbon sources was characterized using Biolog microplates PM1 and PM2 (Biolog, Hayward, CA, U.S.A.) incubated at 30°C during 72 h and read using the OmniLog System as described in Le Quéré et al. (2017). These bioassays cover 152 carbon sources, including sugars, nucleosides, peptides, tweens, and amino acids. To investigate growth with phenolic compounds commonly found in grapevine but not included in Biolog microplates, the bacteria were grown in 96-well microplates, with each well containing 200 μl of bacterial suspension (OD_{600nm} = 0.05) in AB liquid medium (Chilton et al. 1974) supplemented with trans-coutaric acid (10 mmol $liter^{-1}$), coumaric acid (2.5 mmol $liter^{-1}$), ferulic acid (2.5 mmol $liter^{-1}$), or mannitol (10 mmol $liter^{-1}$) as positive controls. The microplates were incubated at 28°C, 180 rpm agitation for 68 h in a microplate reader (Infinite 200 Pro M Plex; Tecan, Männedorf, Switzerland) to monitor OD_{600nm} over time. This experiment was repeated twice.

Inoculation of in vitro plantlets with *P. phytofirmans* PsJN and infection with *A. vitis* S4

Plantlets of *V. vinifera* cultivar Chardonnay were micropropagated as described in Barka et al. (2006). Four-week-old in vitro

plantlets were inoculated through their root systems with 200 µl of PsJN inoculum suspension (10^9 CFU ml^{-1}, $OD_{600} = 0.8$ in PBS) or PBS as a control, deposited on the gelose, and incubated for 1 week in a growth chamber at 26°C with a photoperiod of 16 h/8 h day/night. These plantlets were then transferred to nutritional substrate (Gramoflor F05) in sterile magenta boxes (Dutscher, Bernolsheim, France). One week later, the stem segments between the fourth and fifth leaves were wounded with a sterile surgical blade and infected with 3 µl of *Av*S4 inoculum suspension at 10^5 CFU ml^{-1} (diluted from the 10^9 CFU ml^{-1} suspension, which corresponds with $OD_{600nm} = 1.0$ in PBS) or PBS as a wounding control. In addition, nonbacterized or PBS pretreated plantlets, as well as nonwounded plantlets, were included for a total of six modalities: Ctrl, Ctrl-PBS, Ctrl-S4, PsJN, PsJN-PBS, and PsJN-S4.

Evaluation of bacterial populations in planta

At 7 and 14 days postinfection (dpi) of *Av*S4, Ctrl-S4 and PsJN-S4 stem segments of 1 to 1.5 cm long around the infection points were collected. The *Av*S4 population levels in planta were quantified by the plate counting method. Three pools of three stem segments ($n = 9$) were weighed and ground in 1 ml of sterile PBS. The homogenates were then diluted 10-fold and spread on MG solid medium. The number of *Av*S4 colonies was counted after 2 days of incubation at 28°C. Three independent biological repetitions were done. Similarly, at 7 and 14 dpi, the population levels of PsJN in planta were also quantified by collecting three pools of three stem segments ($n = 9$) of PsJN, PsJN-PBS, and PsJN-S4 modalities and performing the plate counting method on KB supplemented with 100 µg ml^{-1} of kanamycin (KB_{K100}) solid medium. The number of PsJN colonies (PsJN tagged with GFP) was counted under UV light after 3 days of incubation at 28°C. S4 and PsJN populations were expressed per milligram of fresh weight.

Vitopine level evaluation in planta using a biosensor

At 14 dpi, three pools of 20 1 to 1.5 cm long stem segments ($n = 60$) of Ctrl-S4 and PsJN-S4 containing tumors were collected, immediately frozen in liquid nitrogen, freeze-dried for 24 h, and then ground with steel balls into powder. Opine extraction was performed from the total biomass of each pool (30 to 40 mg) as described in Padilla et al. (2021). Briefly, the dried powders were extracted twice with 1 ml of 80% MeOH with vortex homogenization, 10 min of sonication (Bransonic ultrasonic cleaner 2510EDTH) at room temperature, and centrifugation (14,500 × *g*, 10 min, 4°C). The combined extracts were evaporated to dryness at 30°C in a concentrator (CentriVap Concentrator, Labconco, Kansas City, MO, U.S.A.) overnight to constitute the dried crude tumor extracts. These extracts were then weighed and dissolved in distilled water at 10 mg ml^{-1}. Two independent biological repetitions were performed.

The biosensor was constructed based on the *avi_8321* gene encoding an enzyme involved in the vitopine catabolism in *Av*S4, whose transcription is induced by the presence of vitopine (Vial, unpublished data). The promoter region of *avi_8321* was PCR amplified with p8321F (5′-TACAAGCATAAAGCTGAACAT CCGCCATCACGTC-3′) and p8321R (5′-GTGGATCCCCCG GGCACCAACTGCTGTACCGATGA-3′) primers. The PCR fragment obtained was ligated into HindIII-PstI digested vector pOT1e containing gentamycin resistance and *egfp* fluorescence genes (Allaway et al. 2001) by the In-Fusion procedure (Takara, Kyoto, Japan) according to the manufacturer's instructions. Construction was confirmed by PCR with pOT1eF and pOT1eR primers (Allaway et al. 2001) and sequencing. Reporter construction was then introduced into *Av*S4 by electroporation. Gentamycin-resistant colonies were selected and verified again by PCR with pOT1eF and pOT1eR primers. The vitopine-based biosensor obtained was cultured overnight in liquid AB minimal medium supplemented with 10 mmol liter^{-1} of mannitol at 28°C and 180 rpm agitation. The bacterial biomass was washed twice and resuspended in sterile PBS to obtain $OD_{600nm} = 4$.

The biosensor tests were performed in 96-well microplates with three technical triplicates for each sample. Each well contained 190 µl of AB with 10 mmol liter^{-1} of mannitol, 5 µl of the biosensor at $OD_{600nm} = 4$ (final $OD_{600nm} = 0.1$), and 5 µl of tumor crude extracts at 10 mg ml^{-1} (final concentration 0.25 mg ml^{-1}). The microplate was then incubated in the dark at 28°C, 160 rpm agitation. After 16 h postincubation (hpi), the biosensor growth was assessed by measuring the absorbance at 600 nm, and emission of the green fluorescence signals was quantified at 485 nm after an excitation at 530 nm using the microplate reader TECAN. The fluorescence (in arbitrary unit) was calculated by dividing the value of fluorescence signals of each well by its corresponding OD_{600nm} value. All the samples were next normalized to Ctrl modality.

Chemotactic responses of *P. phytofirmans* PsJN

Three pools of three stem segments ($n = 9$) of Ctrl, Ctrl-PBS, and Ctrl-S4 modalities were weighed and ground in 1 ml of sterile PBS. The homogenates were then filtered through 0.22-µm syringe filters (ClearLine, Dutscher) to eliminate *Av*S4 cells. The chemotaxis assay of PsJN was performed as described in Mazumder et al. (1999). Briefly, a 13-mm Becton Dickinson sterile microlance needle with a 0.3-mm outer diameter was used as the chemotaxis capillary and was attached to a 1-ml polypropylene syringe (Terumo, Dutscher). The supernatant of plant homogenates was sucked into the syringe and progressively discharged until the syringe contained only 100 µl to ensure that the needle was thoroughly filled with the plant homogenates. One hundred and fifty microliters of the PsJN suspension (10^9 CFU ml^{-1}, $OD_{600} = 0.8$ in PBS) was pipetted into the 200-µl tip (Thermo Fisher Scientific, Waltham, MA, U.S.A.) as a holding chamber. Next, the needle-syringe capillary was inserted into the tip so that the needle was immerged into the bacterial cell suspension. After 1 and 3 h of incubation at 28°C in the dark, the needle-syringe was removed from the pipette tip, and the content was diluted 10-fold in sterile PBS and spread on KB_{K100} solid medium and incubated for 3 days at 28°C to quantify the levels of PsJN colonies under UV light. Two independent biological repetitions were carried out in triplicate.

RNA extraction and grapevine defense gene expression

For each plant modality, 10 1- to 1.5-cm stem segments were collected at 1 and 7 dpi and grounded in liquid nitrogen into fine powder. Total RNA from 50 mg of powder of each modality was extracted using Plant RNA purification reagent (Invitrogen, Waltham, MA, U.S.A.). RQ1 DNase enzyme (Promega, Madison, WI, U.S.A.) was used to eliminate genomic DNA traces. Next, in a total volume of 15 µl, 1 µg of RNA of each modality was used for reverse transcription with the Verso cDNA Synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions. The expression of 28 representative genes related in diverse defense pathways of grapevine (Supplementary Fig. S1) was analyzed with the quantitative RT-PCR microplate/DNA chip low density (qPFD patented tool, Brisset and Dugé de Bernonville et al. 2011). Relative changes in defense

gene expression ($\log_2$ ratio) were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) and three reference genes for normalization (Vandesompele et al. 2002). The Ctrl modality was used for the calculation of the $\log_2$ ratio of each defense gene. Three independent biological repetitions were used to the biplot of principal component analysis (PCA) of the defense gene expression profiles and the boxplots of the genes that contribute the most to the PCA results using the *FactoMineR* and *factoextra* packages in R.

Microscopic observation

At 16 dpi, the size of developing galls on Ctrl-S4 and PsJN-S4 plantlets were determined by measuring their surface with a 3D Keyence VHX-6000 microscope. Three independent biological repetitions were done, with 30 tumor measurements for each modality in each repetition.

To observe the lignification of grapevine shoots and crown galls from before infection until tumor development (at 1 day preinfection, 7 and 14 dpi), stems of Ctrl-S4 and PsJN-S4 were collected and hand cut into thin sections (~50 to 60 μm) with a razor blade. These sections were immediately immersed in the 1% (m/v) solution of phloroglucinol suspended in 95% ethanol for at least 5 min. Sections were then transferred into HCl 6N solution and subsequently observed under an optical microscope (Olympus Bx43). Lignified cell walls in shoot tissues would turn a cherry-red color (Speer 1987). The images of stained sections were captured with Infinity Analyze software (Teledyne Lumenera, Ottawa, Canada). Two independent biological repetitions were performed in duplicate.

Statistical analysis

All the results were statistically analyzed by the software R version 4.0.4. The Student's *t* test ($P < 0.05$) was used when comparing the means between two modalities. The Shapiro-Wilk test ($P < 0.05$) was used for the normality test. In the case of normal distribution, one-way ANOVA ($P < 0.05$) was applied, followed by a post hoc Tukey test to get the ranking. In contrast, when there was no normal distribution, the Kruskal-Wallis test ($P < 0.05$) was used with post hoc Fisher's least significant difference (package *agricolae*).

RESULTS

Significant reduction of the *A. vitis* S4 population and increase of the vitopine level in planta by *P. phytofirmans* PsJN

To determine the effect of PsJN on *A. vitis* S4 in planta, the pathogen levels were measured at 7 and 14 dpi. The presence of PsJN reduced significantly ($P < 0.05$) the *Av*S4 population in crown galls by three times (corresponding to 0.5 $\log_{10}$) at both 7 and 14 dpi (Fig. 1), indicating a significant effect of PsJN against *Av*S4.

In crown galls, opine is produced by the plant as the result of T-DNA integration and serves as a specific carbon source for *Av*S4 in competition with other microorganisms (Kuzmanović et al. 2018). The vitopine levels in tumors were estimated using a vitopine-based biosensor containing the fusion of the promoter of *avi_8321* gene (involved in the vitopine catabolism) of *Av*S4 with the *gfp* gene. On the basis of this bioassay, the results indicated a significantly higher level of vitopine in gall extracts from plants prebacterized with PsJN when compared with the controls at 14 dpi (Fig. 2).

Impact of PsJN on tumor size

The number of tumors that developed on grapevine shoots was counted (disease incidence), and their size was estimated by measuring their surface with a 3D microscope (disease severity). The bacterization with PsJN prior to infection with *Av*S4 was not able

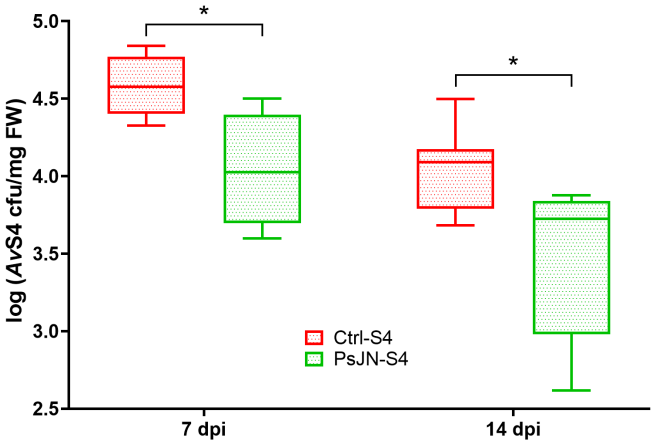

FIGURE 1
Impact of *Paraburkholderia phytofirmans* PsJN against *Allorhizobium vitis* S4 (*Av*S4) in grapevine plantlets. Quantification of the population levels of *Av*S4 in the presence (PsJN-S4) or absence (Ctrl-S4) of PsJN in planta. One of three repetitions with the same tendency is presented. Asterisks indicate significant differences at $P < 0.05$ (Student's *t* test).

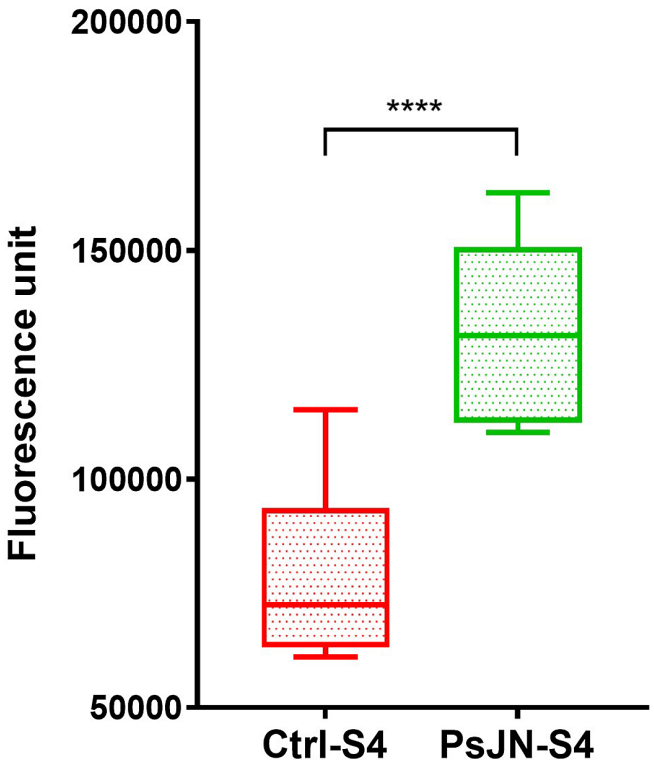

FIGURE 2
Evaluation of vitopine level in grapevine tumors caused by *Allorhizobium vitis* S4 at 14 days postinoculation (dpi) in the presence (PsJN-S4) or absence (Ctrl-S4) of *Paraburkholderia phytofirmans* PsJN. The experiment was performed twice in triplicate. Four asterisks indicate significative differences at $P < 0.001$ (Student's *t* test).

to reduce disease severity or incidence because neither the gall size nor the proportion of developed galls was significantly reduced between control and prebacterized plantlets (Fig. 3).

Direct antimicrobial effect of PsJN against *A. vitis* S4

To monitor whether the significant effect of PsJN on the *A. vitis* S4 population level observed could be due to a direct antagonism toward *Av*S4, the two microorganisms were cocultured on MG medium. After 2 days of incubation, weak inhibition zones were observed around the PsJN colonies (Fig. 4), indicating a restriction in the growth of the S4 strain. No increase of inhibition zones was observed after 2 days of incubation.

Competition for the same ecological niche

Colonization of *P. phytofirmans* PsJN and chemotaxis. We then monitored the population level of PsJN in plantlets over time to determine whether the direct antimicrobial effect exerted by PsJN against *Av*S4 could play a role in planta and might participate in the reduction effect on *Av*S4 populations (Fig. 1). For this purpose, the quantity of PsJN was compared in the shoots of three conditions (PsJN, PsJN-PBS, PsJN-S4). Interestingly, at 7 and 14 dpi, although the level of PsJN remained about 100 times lower than the level of AvS4 in planta (Fig. 1), the quantity of PsJN in planta was significantly higher, around 10 times (1 log10), in wounded plants than in the nonwounded ones (Fig. 5).

Because the wounding and the gall formation on grapevine shoots could attract or stimulate the PsJN populations in planta (Fig. 5), we tested if this attraction toward PsJN came from the metabolites of plants wounded and/or infected with *Av*S4. The chemotaxis results after 1 or 3 h of incubation indicated that the increase in PsJN population levels in wounded plants was not related to the metabolites produced by the host plant (Fig. 6).

Use of carbon sources by PsJN or *Av*S4. A potential competition between PsJN and *Av*S4 for nutrient sources in grapevine was also monitored. For that, the use of 152 carbon sources by PsJN or *Av*S4 was tested in vitro using Biolog microplates. Our

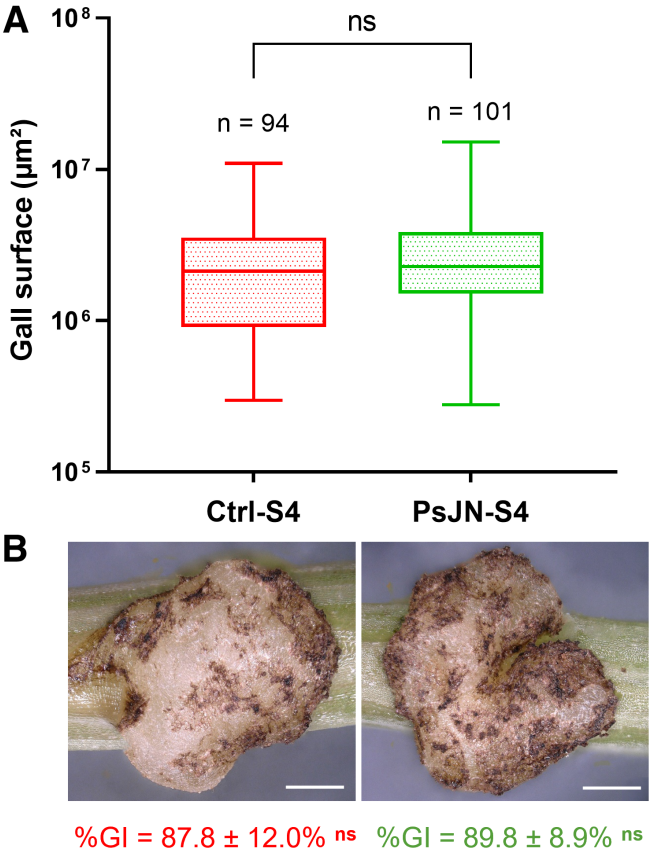

FIGURE 3
Impact on gall development caused by *Allorhizobium vitis* S4 in the presence (PsJN-S4) or absence (Ctrl-S4) of *Paraburkholderia phytofirmans* PsJN measured with a 3D microscope. **A,** Boxplot of the tumor surface measurements. **B,** Microscope observations of tumors. *n* = total number of galls measured from three independent biological repetitions. %GI = gall incidence, the number of tumors developed among infected plants. Bars = 500 µm. "ns" indicates no significant difference between two modalities at $P < 0.05$ (Student's *t* test).

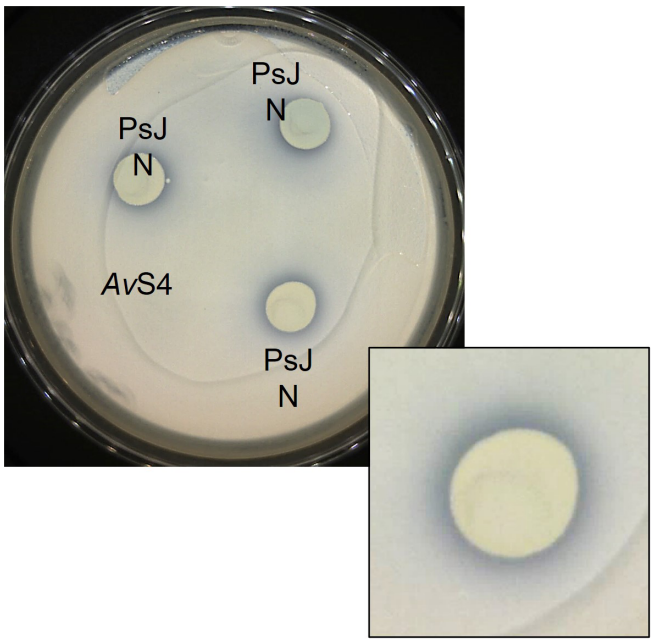

FIGURE 4
Antimicrobial effect of *Paraburkholderia phytofirmans* PsJN against *Allorhizobium vitis* S4 on mannitol-glutamate medium.

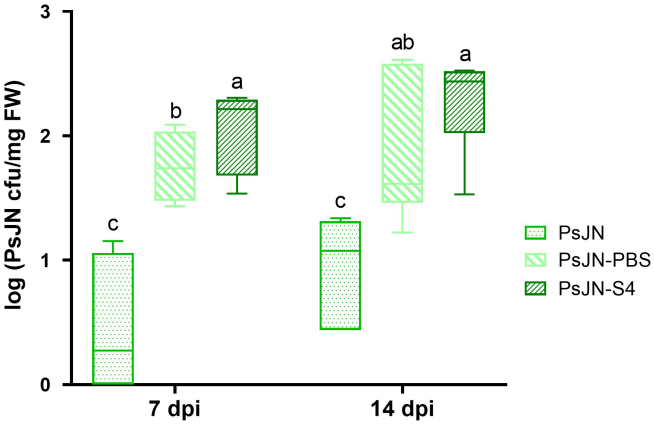

FIGURE 5
Population levels of *Paraburkholderia phytofirmans* PsJN in planta without wounding (PsJN), with wounding with phosphate-buffered saline (PsJN-PBS), or with wounding with *Allorhizobium vitis* S4 (PsJN-S4). One of the three biological repetitions with the same tendency is presented. Values with the same letters have no significant difference at $P < 0.05$ (ANOVA).

results showed no significant difference in the utilization of 67 substrates between PsJN and *Av*S4, whereas 37 other substrates were utilized more by PsJN and 48 by *Av*S4 (Table 1). In general, *Av*S4 can metabolize mono-, di-, and polysaccharides and aromatics better than PsJN can, whereas the PGPR has an advantage in catabolizing organic acids (sugar acids, carboxylic acids, amino acids). Moreover, some organic acids commonly found in grapevine (Singleton et al. 1986) were also tested because they are not included in Biolog microplates. Whereas coumaric acid and trans-coutaric acid were utilizable carbon sources for both bacteria, ferulic acid was barely catabolized by *Av*S4 and not by PsJN (Table 2).

Induction of plant immune responses

Little information is available on the effect of *Av*S4 on the expression of grapevine defense genes. PsJN was previously described to induce resistance against biotic and abiotic stresses, triggering an indirect mechanism via the plant host, in particular by priming of defense-related gene expression (Esmaeel et al. 2018). To explore the defense signaling pathways triggered by each bacterium in our conditions, we used a biomolecular tool to study the expression of 28 grapevine genes (Supplementary Fig. S1) (Brisset and Dugé de Bernonville 2011) at the stages of pathogen perception (1 dpi) and at the early stage of tumor development (7 dpi) on grapevine prebacterized or not with PsJN. Among these, three genes, cysteine lyase (*CSL*), farnesene synthase (*Far*), and pathogenesis-related protein (PR) *15* (*PR15*), were on the edge of detection (data not shown) and consequently were not included in the analysis. PCA of the gene expression data was performed. Genes were used as variables and plant treatment modalities as individuals.

At 1 dpi, the stem segments were collected when no symptom had developed yet (Fig. 7A). The first two principal components (PCs) described the largest variability (81.6% and 6.9% initial variation, respectively). PC1 enabled a strong separation between wounded/infected (Ctrl-PBS, Ctrl-S4, PsJN-PBS, PsJN-S4) and nonwounded conditions (Ctrl and PsJN) (Fig. 7B). A partial least squares discriminant analysis (PLS-DA) was also performed on the data and identified the eight most discriminant genes (variable importance for the projection, VIP score > 1): *PR1*, *PR3*, *PR4*, *PR5*, *PR8*, *PR10*, glutathione S-transferase (*GST*), and stilbene synthase (*STS*) (Fig. 7B). These genes were mostly upregulated in wounded plants (Ctrl-PBS, Ctrl-S4, PsJN-PBS, PsJN-S4) compared with the nonwounded ones (Ctrl and PsJN) (Fig. 7C). Chalcone synthase (*CHS*), dihydroflavonol reductase (*DFR*), peroxidase (*POX*), and polyphenol oxidase (*PPO*) were also induced following the wounding or the infection of *Av*S4 on plants (Supplementary Fig. S2). In addition, PC2, which explained 6.9% of variability compared with PC1, separated the control and PsJN conditions, proving a smaller effect of the PGPR on the expression of defense genes of grapevine (Fig. 7B). In fact, some genes, such as *CHS* and 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*), seemed to be less expressed, whereas cinnamyl alcohol dehydrogenase (*CAD*) tended to be overexpressed in the presence of the PGPR compared with the corresponding controls (Supplementary Fig. S2). None of the genes involved in the well-known defense pathways related to salicylic acid, jasmonate, or ethylene was significantly modulated by PsJN, except enhanced disease susceptibility 1 (*EDS1*), which tended to be more expressed than in the control plantlets (Supplementary Fig. S2).

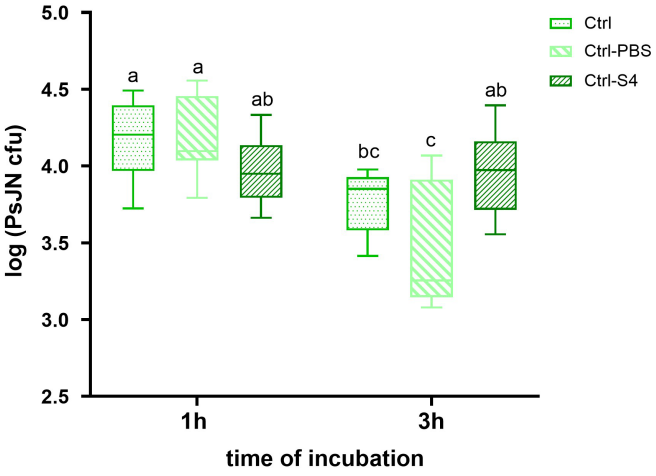

FIGURE 6
Chemotactic responses of *Paraburkholderia phytofirmans* PsJN toward the filtrate of plant homogenates of untreated (Ctrl), wounded (Ctrl-PBS), and *Allorhizobium vitis* S4-infected plants (Ctrl-S4). One of two repetitions with the same tendency is presented. The same letter indicates no significant difference (ANOVA, $P < 0.05$).

At 7 dpi, gall symptoms had started to develop on grapevine stems (Fig. 8A). The first two PCs explained 71% and 10% of total variance, respectively. Interestingly, compared with 1 dpi, the same eight discriminative genes *PR1*, *PR3*, *PR4*, *PR5*, *PR8*, *PR10*, *GST*, and *STS* were also highlighted by the PLS-DA (VIP score > 1). In all cases, infection *Av*S4 stimulated the upregulation of these genes (Fig. 8B and C). Few differences were observed in the expression profiles of other genes (Supplementary Fig. S3).

Overall, at both time points, none of the discriminant genes exhibited a stronger expression in PsJN-S4 plantlets compared with Ctrl-S4. This suggests that in response to subsequent infection of *Av*S4, PsJN did not prime the expression of any defense genes studied here.

Impact of *P. phytofirmans* PsJN on the accumulation of lignin in grapevine shoots and crown galls

From the results of defense gene expression at 1 dpi described above, the gene associated with the biosynthesis of lignin in grapevine *CAD* tended to be upregulated when the plantlets had been bacterized with the PGPR prior to the wounding/infection (Supplementary Fig. S2). Hence, histological studies of grapevine stem sections were carried out to determine the effect of PsJN and/or *Av*S4 at the anatomical level of the plant host (Fig. 9).

The hand-cut sections stained with acidified phloroglucinol at 1 day before infection (-1 dpi) showed a more intensive cherry-red color in plantlets prebacterized with PsJN compared with the controls, demonstrating the higher lignification level in plant vascular bundles in the presence of the PGPR (Fig. 9A and B). Later, as the plants and tumors grew, this difference was no longer obvious, with similar levels of cherry-red color observed in both modalities (Fig. 9C to F). The black color in the cross sections could indicate the necrosis or suberization parts of the plant tissue (Fig. 9C to F). Zooming in on the developing galls at 14 dpi, the staining also showed no lignification because no red color was observed in this part (Fig. 9E and F).

DISCUSSION

Crown gall disease in grapevine caused by *A. vitis* is extremely hard to control due to the systemic persistence of the pathogen inside plant xylem vessels (Lehoczky 1968;

Tarbah and Goodman 1987). Most studies on biocontrol against crown gall disease used a method of coinoculation on shoots. However, previous results obtained on PsJN-grapevine interaction showed that PsJN inoculated on the root system is able to migrate into the aerial part of plantlets (stems, leaves), conferring a resistance against abiotic as well as biotic stresses (Esmaeel et al. 2018). We chose a pathosystem more practical for farmers, where PsJN could be preinoculated in grapevine materials through roots before planting to prevent or mitigate potential crown gall disease outbreak later. In our conditions, the root inoculation with PsJN prior to *Av*S4 infection did not reduce the gall size in grapevine (Fig. 3). This result is in contrast with the study by Toklikishvili et al. (2010) on tomato, where, when inoculated through the root system, PsJN was able to reduce crown gall symptoms caused by *Av*S4 or *A. tumefaciens*. However, we demonstrated that the presence of PsJN significantly reduced the in planta population levels of *Av*S4 at 7 and 14 dpi (Fig. 1). This result was further strengthened by indirectly evaluating vitopine levels in galls with a biosensor. In the presence of PsJN, significantly higher levels of vitopine in crown galls compared with control plants were observed (Fig. 2), which could be correlated with the decrease of *Av*S4 populations (Fig. 1) and thus a decrease of opine consumption by *Av*S4 in crown galls.

In addition, we demonstrated a direct, minor antagonistic effect in vitro exerted by PsJN against *Av*S4 (Fig. 4). PsJN was previously described as an antagonist of the fungal pathogen *B. cinerea* on grapevine by inhibiting spore germination (Barka et al. 2002; Miotto-Vilanova et al. 2016) and having an impact on the physiology of *Drechslera teres* on barley (Backes et al. 2020), probably thanks to its cell wall degrading enzymes. However, PsJN was reported to have no antibacterial action against *Pseudomonas syringae* (Su et al. 2017) and *X. fastidiosa* (Baccari et al. 2019). To the best of our knowledge, this is the first study to describe any antimicrobial effect of PsJN against a phytopathogenic bacterium, although its precise mechanism remains unknown.

TABLE 2

Growth of *Paraburkholderia phytofirmans* PsJN and *Allorhizobium vitis* S4 in the presence of three different abundant organic acids in grapevine[a]

Substrate	PsJN	*Av*S4
Coumaric acid	+	+
Trans-coutaric acid	+	+
Ferulic acid	–	±

[a] + good, ± moderate, – no utilization.

TABLE 1

List of the 85 carbon sources preferentially metabolized by either *Paraburkholderia phytofirmans* PsJN or *Allorhizobium vitis* S4, highlighted by Biolog microassays

Group of substrate	Carbon source	Utilized more by	Group of substrate	Carbon source	Utilized more by
Monosaccharides	D-galactose	PsJN	Sugar acids	D-glucosaminic acid	PsJN
	D-ribose	PsJN		D-saccharic acid	PsJN
	D-mannose	*Av*S4		D-gluconic acid	PsJN
	D-fructose	*Av*S4		m-tartaric acid	PsJN
	D-arabinose	*Av*S4		D-galacturonic acid	PsJN
	D-fucose	*Av*S4		D-glucuronic acid	PsJN
	N-acetyl-β-D-mannosamine	*Av*S4		N-acetyl-neuraminic acid	*Av*S4
	D-psicose	*Av*S4	Carboxylic acids	succinic acid	PsJN
	N-acetyl-D-galactosamine	*Av*S4		Bromo-succinic acid	PsJN
Substituted monosaccharides	3-methyl glucose	*Av*S4		D-malic acid	PsJN
	D-glucose-1-phosphate	*Av*S4		L-malic acid	PsJN
	β-methyl-D-glucoside	*Av*S4		α-ketoglutaric acid	*Av*S4
	β-methyl-D-xyloside	*Av*S4		Citric acid	*Av*S4
	α-methyl-D-mannoside	*Av*S4		Capric acid	*Av*S4
Disaccharides	D-trehalose	*Av*S4		Pyruvic acid	PsJN
	Sucrose	*Av*S4		β-hydroxybutyric acid	PsJN
	3-O-β-D-galactopyranosyl-D-arabinose	*Av*S4		γ-hydroxybutyric acid	PsJN
	D-maltose	*Av*S4		γ-amino-N-butyric acid	*Av*S4
	D-melibiose	*Av*S4		Propionic acid	PsJN
	D-cellobiose	*Av*S4		δ-amino-valeric acid	*Av*S4
	Gentiobiose	*Av*S4		Citramalic acid	PsJN
	D-turanose	*Av*S4		Citraconic acid	PsJN
	α-D-lactose	*Av*S4		Itaconic acid	PsJN
Polysaccharides	Dextrin	*Av*S4		Tricarballylic acid	PsJN
	Maltotriose	*Av*S4		Acetoacetic acid	*Av*S4
	Chondroitin sulfate C	PsJN		Oxalic acid	PsJN
	α-cyclodextrin	*Av*S4		Oxalomalic acid	PsJN
	γ-cyclodextrin	*Av*S4		D-aspartic acid	*Av*S4
	Laminarin	*Av*S4		D-serine	PsJN
	D-raffinose	*Av*S4		L-arginine	*Av*S4
	Stachyose	*Av*S4		L-asparagine	PsJN
Sugar alcohols	N-acetyl-D-glucosaminitol	*Av*S4	Amino acids	L-histidine	PsJN
	D-mannitol	PsJN		L-phenylalanine	PsJN
	L-arabitol	PsJN		L-leucine	PsJN
	Glycerol	PsJN		L-valine	PsJN
	D-lactitol	*Av*S4		L-glutamine	PsJN
	Maltitol	*Av*S4		L-threonine	PsJN
Nucleosides	Inosine	PsJN		N-acetyl-L-glutamic acid	*Av*S4
Peptides	Ala-Gly	*Av*S4	Tweens	Tween 20	PsJN
Aromatics	D-salicin	*Av*S4	Miscellaneous	D-galactonic acid-γ-lactone	PsJN
	Phenylethylamine	*Av*S4		L-alaninamide	*Av*S4
	p-hydroxyphenyl acetic acid	*Av*S4		D-ribono-1,4-lactone	*Av*S4
	Arbutin	*Av*S4			

Interestingly, we observed a higher population of PsJN in planta in wounded plantlets (PsJN-PBS, PsJN-S4) (Fig. 5). Previously, PsJN was reported as being attracted to *B. cinerea* on botrytized grapevine leaves surrounding the fungal mycelium (Miotto-Vilanova et al. 2016). Hence, the chemotaxis response of PsJN toward the filtrate of plant homogenates from plants unwounded (Ctrl), wounded (Ctrl-PBS), and with crown galls (Ctrl-S4) was then tested (Fig. 6). However, in our conditions, the results showed no difference among three conditions, indicating that response does not seem to be due to metabolites inside

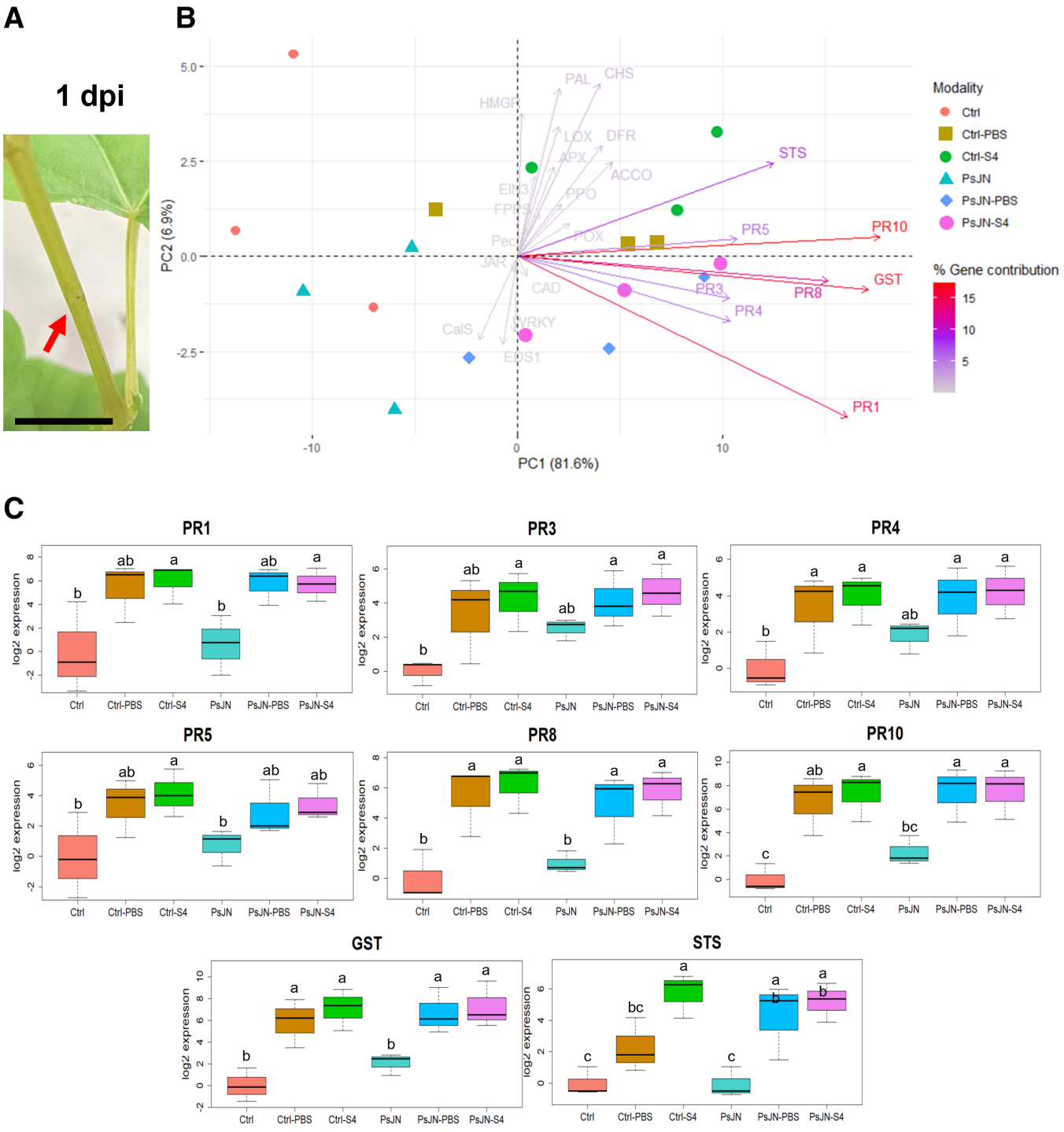

FIGURE 7

Grapevine defense gene expression at symptomless stage, one day postinoculation (1 dpi). **A,** Stem with the infection point (red arrow). Bar = 5 mm. **B,** Principal component analysis (PCA) biplot of principal components one (PC1) and two (PC2) showing the clustering of the 25 defense genes and the six plant modalities (Ctrl = nonpretreated/nonwounded; Ctrl-PBS = nonpretreated/wounded with phosphate-buffered saline [PBS]; Ctrl-S4 = nonpretreated/wounded with *Allorhizobium vitis* S4; PSJN = prebacterized with *Paraburkholderia phytofirmans* PsJN/nonwounded; PsJN-PBS = prebacterized with *P. phytofirmans*/wounded with PBS; PsJN-S4 = prebacterized with *P. phytofirmans*/wounded with *A. vitis* S4). **C,** The expression levels of the eight most discriminative genes according to partial least squares discriminant analysis results. Values with the same letters have no significant difference at $P < 0.05$ (Kruskal-Wallis test).

the plants as expected. Nevertheless, organic extracts from these plant tissues might be tested to further investigate the involved mechanisms. When no metabolite is implicated, the difference observed might be caused by a physical phenomenon where the open wounds on stems or the development of galls leads to more water loss, bringing up the water flow in the xylem containing PsJN to get to the wounding and/or gall sites. The water evaporation through young, fresh galls might cause the proliferation of PsJN at infection sites by moving up along with the xylem sap columns.

We also evaluated the ability of PsJN or *Av*S4 to use different carbon sources. Among 152 compounds tested in Biolog, *Av*S4

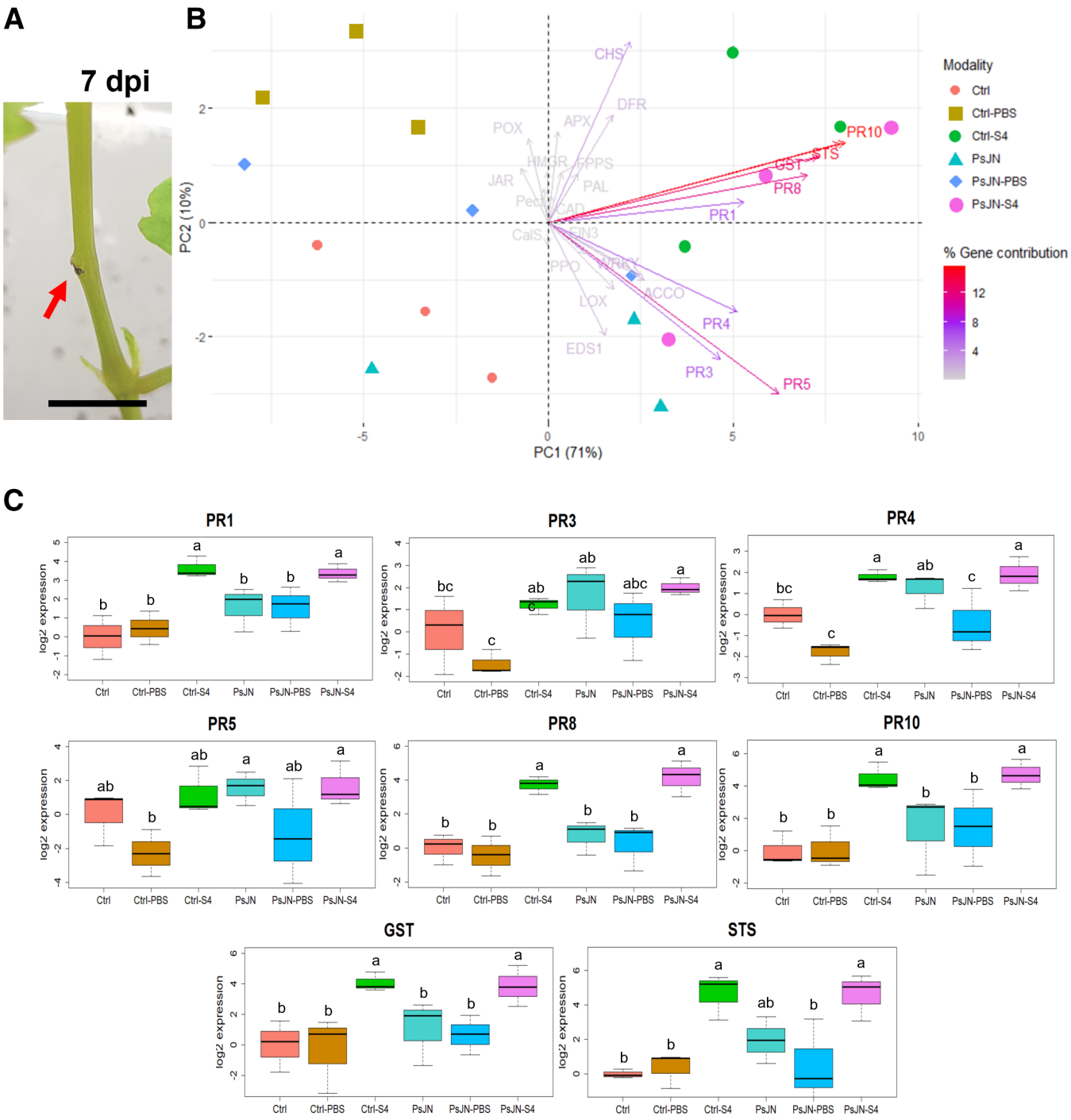

FIGURE 8

Grapevine defense gene expression at the early stage of gall development, 7 days postinoculation (7 dpi). **A,** Stem with a newly developed gall (red arrow). Bar = 5 mm. **B,** Principal component analysis (PCA) biplot of principal components one (PC1) and two (PC2) showing the clustering of the 25 defense genes and the six plant modalities (Ctrl = nonpretreated/nonwounded; Ctrl-PBS = nonpretreated/wounded with phosphate-buffered saline [PBS]; Ctrl-S4 = nonpretreated/wounded with *Allorhizobium vitis* S4; PSJN = prebacterized with *Paraburkholderia phytofirmans* PsJN/nonwounded; PsJN-PBS = prebacterized with *P. phytofirmans*/wounded with PBS; PsJN-S4 = prebacterized with *P. phytofirmans*/wounded with *A. vitis* S4). **C,** The expression levels of the eight most discriminative genes according to partial least squares discriminant analysis results. Values with the same letters have no significant difference at $P < 0.05$ (Kruskal-Wallis test).

metabolized 48 carbon sources better than PsJN did, whereas the latter utilized only 37 sources better than the former (Table 1). Many of these carbon sources have been described as abundant in grapevine (Ali et al. 2010; Kliewer 1966), such as saccharides (fructose, sucrose, maltose, raffinose), which are metabolized more by *Av*S4, and organic acids (tartaric acid, malic acid, succinic acid), which are favored by PsJN. The utilization of both bacteria toward coutaric, coumaric, and ferulic acid was additionally tested, and a difference was only observed in the case of ferulic acid. This last compound is an intermediate in phenylpropanoid pathways with antimicrobial activity (Ou and Kwok 2004) and, hence, probably toxic for PsJN. *Av*S4, however, could slowly metabolize ferulic acid (Table 2), which is also a strong *vir* gene inducer (Brencic et al. 2004). On the other hand, PsJN better catabolized a large range of nitrogen sources, such as amino acids and nucleosides, than *Av*S4 did (Table 1). Nevertheless, this might not confer an advantage to the PGPR in the competition with *Av*S4 because the pathogenic bacteria can utilize vitopine, which is abundantly produced by plant cells after T-DNA integration (Burr et al. 1998). Here, we hypothesize that PsJN is not able to utilize vitopine because no reduction of its levels in tumor was observed in the presence of the PGPR (Fig. 2), and no gene involved in opine metabolism was found in this strain. In general, each bacterium prefers different nutrient sources, and none of them showed a clear competitive advantage over the other. Taken together with the difference in bacterial abundance in planta between these bacteria, we suppose that their direct interactions might not be the main mode of action to explain the significant decrease of *Av*S4 populations in the presence of PsJN in planta.

Previously, PsJN was reported as triggering induced resistance in *Arabidopsis thaliana* against *P. syringae* (Su et al. 2017; Timmermann et al. 2019), in grapevine against *B. cinerea* (Miotto-Vilanova et al. 2016) and *X. fastidiosa* (Baccari et al. 2019), and in tomato against *Fusarium oxysporum* (Frommel et al. 1991b). Therefore, we hypothesized that the impact of PsJN against *Av*S4 could be explained via the plant host. In fact, grapevine possesses a variety of defense mechanisms against pathogens, beginning with the recognition of microbes, then signaling transcription that leads to the subsequent expression of defense genes (Héloir et al. 2019). Herein, we used a biomolecular tool developed by Brisset and Dugé De Bernonville (2011) to screen the expression of 28 genes of grapevine involved in different defense pathways (Supplementary Fig. S1) at 1 and 7 dpi. At both time points, almost all the *PR* genes were strongly expressed, together with *GST* and *STS* (Figs. 7 and 8), by the pathogen. At the early perception stage, however, these upregulations seemed to be mainly due to the physical damage caused by the inoculation procedure, as they were also observed in

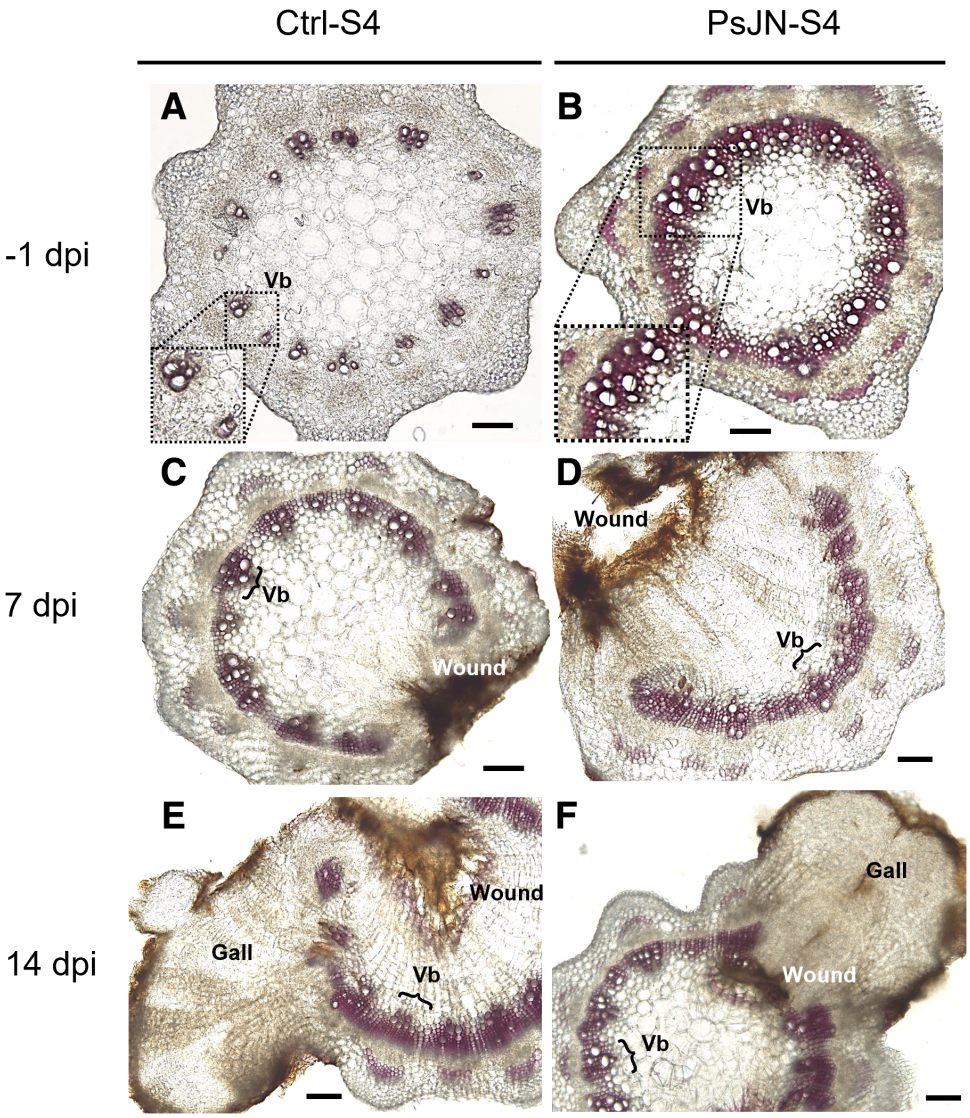

FIGURE 9
Lignification of grapevine shoots at 1 day prior to inoculation (–1) and 7 and 14 days postinoculation (dpi) with *Allorhizobium vitis* S4. The plants were prebacterized with *Paraburkholderia phytofirmans* PsJN **(B, D, F)** or pretreated with phosphate-buffered saline as the control **(A, C, E)** 2 weeks prior to the infection of *A. vitis* S4. Lignified plant tissues stained with phloroglucinol-HCl showed cherry-red color. Vb = vascular bundle. Bars = 100 µm.

mock-inoculated plants (Fig. 7B and C). At 7 dpi, when the pathogen was well established and the disease symptoms started to develop, the effect of *Av*S4 on the expression profile of all the genes studied was obviously strong and covered the effect of the beneficial bacteria (Fig. 8B and C). Pathogenesis-related genes encoding for PR proteins were reported to be mainly induced in response to infection by pathogens and sometimes by stress, mimicking the effect of pathogen infection (Malik et al. 2020). *GST* encodes for glutathione S-transferases, which play an important role in mediating oxidative stress and biotic stress in planta (for review, see Gullner et al. 2018). *STS* genes encode stilbenes synthases, which are highly inducible in response to mechanical damage (Chiron et al. 2000) and pathogenic infection (Liswidowati et al. 1991). It was previously reported that wounding and infection by agrobacteria could cause an oxidative burst in plant cells and trigger programmed cell death (León et al. 2001; Xu and Pan 2000). Wounding alone has been demonstrated to trigger the expression of some defense genes in grapevine, including *PR10* and *STS* (Pierron et al. 2016). *PR10* encodes for a protein acting directly against pathogens, including *A. tumefaciens* (Flores et al. 2002; Liu and Ekramoddoullah 2006; Oliver et al. 2009). *PR1* and *PR4* have been described to be upregulated when grapevine is challenged with *A. tumefaciens* (Asghari et al. 2020). In *Arabidopsis thaliana*, *PR1*, *PR3*, and *PR5* were overexpressed following *tumefaciens* infection, which, in turn, can induce *GST* to prevent H_2O_2 accumulation and mitigate the plant defense reaction (Lee et al. 2009). This could explain the overexpression of these genes during the pathogen perception on grapevine in our research. Our results showed that PsJN did not prime the resistance of grapevine against the crown gall causal agent, unlike some endophytic strains of *Pseudomonas* and *Pantoea* spp. that induce systemic resistance to protect grapevine from *A. tumefaciens* (Asghari et al. 2020). This could be due to the fact that the plants were inoculated with PsJN up to 2 weeks prior to the infection and that the low population levels of the PGPR could not efficiently prime the plant defenses. In contrast, in previous studies using PsJN (Baccari et al. 2019; Frommel et al. 1991b; Miotto-Vilanova et al. 2016; Su et al. 2017; Timmermann et al. 2019), inoculation with PsJN up to 2 weeks before the pathogen infection did not occur. Hence, we can hypothesize that priming of resistance to *Av*S4 by PsJN would be relatively short-lived, similar to what Baccari et al. (2019) proposed in the case with *X. fastidiosa*. Shortening the time interval between PGPR inoculation and pathogen infection, or reinoculating with PsJN, might be consequently needed to optimize the protection effect of this biocontrol agent against *Av*S4 in grapevine.

Among various defense pathways, lignification plays an important role in mediating microbe-host interactions because lignin is one of the major components of the cell wall, which is one of the first lines of defense against invasive pathogens (Bhuiyan et al. 2009). PsJN has been reported to enhance the lignin content of potato (Frommel et al. 1991a), *Arabidopsis* (Poupin et al. 2013), and grapevine shoots (Miotto-Vilanova et al., unpublished data). The expression profile of *CAD*, a gene involved in the lignin biosynthesis pathway, was also shown to be upregulated in the plantlets bacterized with PsJN compared with the controls (Supplementary Fig. S2). Therefore, by using the staining technique on cross sections, we visualized the higher lignification of grapevine shoots prebacterized with PsJN at the beginning of infection (Fig. 9A and B). In our conditions, this increased lignification was not sufficient to stop the establishment of the pathogenic bacteria. This mechanism could be efficient against external pathogens, making their progression within plant tissues more difficult, but it does not seem able to stop a bacterium from colonizing the plant through the xylem vessels.

CONCLUSION

We tested the potential protective effect of PsJN against the pathogenic agent of grapevine crown gall disease. In our conditions, PsJN could not control the crown gall incidence and disease severity, but the PGPR still had a significant effect on the population levels of the pathogen in vitro and in planta. Reducing the pathogen load could reduce the risks of contamination in the field and of dissemination by contaminated plants. Considering the common endophytic and systemic lifestyle of both PsJN and S4, an optimization of the research model exploiting a potential rhizospheric competition between the two strains by coinoculation on roots can be considered to fully exploit the potential of PsJN as a biocontrol agent against crown gall disease in grapevine.

LITERATURE CITED

Alexander, E., Pham, D., and Steck, T. R. 1999. The viable-but-nonculturable condition is induced by copper in *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. Appl. Environ. Microbiol. 65: 3754-3756.

Ali, K., Maltese, F., Choi, Y. H., and Verpoorte, R. 2010. Metabolic constituents of grapevine and grape-derived products. Phytochem. Rev. 9: 357-378.

Allaway, D., Schofield, N. A., Leonard, M. E., Gilardoni, L., Finan, T. M., and Poole, P. S. 2001. Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. Environ. Microbiol. 3:397-406.

Armijo, G., Schlechter, R., Agurto, M., Muñoz, D., Nuñez, C., and Arce-Johnson, P. 2016. Grapevine pathogenic microorganisms: Understanding infection strategies and host response scenarios. Front. Plant Sci. 7:382.

Asghari, S., Harighi, B., Ashengroph, M., Clément, C., Aziz, A., Esmaeel, Q., and Barka, E. A. 2020. Induction of systemic resistance to *Agrobacterium tumefaciens* by endophytic bacteria in grapevine. Plant Pathol. 69: 827-837.

Baccari, C., Antonova, E., and Lindow, S. 2019. Biological control of Pierce's disease of grape by an endophytic bacterium. Phytopathology 109: 248-256.

Backes, A., Hausman, J., Renaut, J., Barka, E. A., Jacquard, C., and Guerriero, G. 2020. Expression analysis of cell wall-related genes in the plant pathogenic fungus *Drechslera teres*. Genes (Basel). 11:300.

Barka, E. A., Belarbi, A., Hachet, C., Nowak, J., and Audran, J. C. 2000. Enhancement of *in vitro* growth and resistance to gray mould of *Vitis vinifera* co-cultured with plant growth-promoting rhizobacteria. FEMS Microbiol. Lett. 186:91-95.

Barka, E. A., Gognies, S., Nowak, J., Audran, J. C., and Belarbi, A. 2002. Inhibitory effect of endophyte bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. Biol. Control. 24:135-142.

Barka, E. A., Nowak, J., and Clément, C. 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. Appl. Environ. Microbiol. 72:7246-7252.

Bazzi, C., Stefani, E., Gozzi, R., Burr, T. J., Moore, C. L., and Anaclerio, F. 1991. Hot-water treatment of dormant grape cuttings: Its effects on *Agrobacterium tumefaciens* and on grafting and growth of vine. Vitis 30:177-187.

Bhuiyan, N. H., Selvaraj, G., Wei, Y., and King, J. 2009. Role of lignification in plant defense. Plant Signal. Behav. 4:158-159.

Bisztray, G. D., Civerolo, E. L., Dula, T., Kölber, M., Lázár, J., Mugnai, L., Szeged, E., and Savka, M. A. 2012. Grapevine pathogens spreading with propagating plant stock: Detection and methods for elimination. Pages 1-86 in: Grapevines: Varieties, Cultivation and Management. Nova Science Publishers, New York, NY.

Brencic, A., Eberhard, A., and Winans, S. C. 2004. Signal quenching, detoxification and mineralization of *vir* gene-inducing phenolics by the VirH2 protein of *Agrobacterium tumefaciens*. Mol. Microbiol. 51: 1103-1115.

Brisset, M. N., and Dugé de Bernonville, T. 2011. Device for determining or studying the state of stimulation of the natural defences of plants or portions of plants. U.S. patent 9,290,788.

Burr, T. J., Bazzi, C., Süle, S., and Otten, L. 1998. Crown gall of grape: Biology of *Agrobacterium vitis* and the development of disease control strategies. Plant Dis. 82:1288-1297.

Burr, T. J., Ophel, K., Katz, B. H., and Kerr, A. 1989. Effect of hot water treatment on systemic *Agrobacterium tumefaciens* Biovar 3 in dormant grape cuttings. Plant Dis. 73:242-245.

Chilton, M., Currier, T., Farrand, S., Bendicht, A., Gordont, M., and Nester, E. W. 1974. *Agrobacterium tumefaciens* DNA and PS8 Bacteriophage DNA not detected in crown gall tumors. Proc. Nat. Acad. Sci. U.S.A. 71:3672-3676.

Chilton, W. S., Petit, A., Chilton, M., and Dessaux, Y. 2001. Structure and characterization of the crown gall opines heliopine, vitopine and ridéopine. Phytochemistry 58:137-142.

Chiron, H., Drouet, A., Lieutier, F., Payer, H. D., Ernst, D., and Sandermann, H. J. 2000. Gene induction of stilbene biosynthesis in Scots pine in response to ozone treatment, wounding, and fungal infection. Plant Physiol. 124:865-872.

Compant, S., Kaplan, H., Sessitsch, A., Nowak, J., Barka, E. A., and Clément, C. 2008. Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: From the rhizosphere to inflorescence tissues. FEMS Microbiol. Ecol. 63:84-93.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., and Barka, E. A. 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl. Environ. Microbiol. 71:1685-1693.

Dessaux, Y., and Faure, D. 2018. Niche construction and exploitation by *Agrobacterium*: How to survive and face competition in soil and plant habitats. Pages 55-86 in: Agrobacterium Biology. Current Topics in Microbiology and Immunology. S. B. Gelvin, ed. Springer, Cham.

Dula, T., Kölber, M., Lázár, J., and Szegedi, E. 2007. Production of healthy grapevine propagating material: Pathogens and methods.

Esmaeel, Q., Miotto-Vilanova, L., Rondeau, M., Leclère, V., Clément, C., Jacquard, C., Sanchez, L., and Barka, E. A. 2018. *Paraburkholderia phytofirmans* PsJN-plants interaction: From perception to the induced mechanisms. Front. Microbiol. 9:2093.

Filo, A., Sabbatini, P., Sundin, G. W., Zabadal, T. J., Safir, G. R., and Cousins, P. S. 2013. Grapevine crown gall suppression using biological control and genetic engineering: A review of recent research. Am. J. Enol. Vitic. 64: 1-14.

Flores, T., Alape-Girón, A., Flores-Díaz, M., and Flores, H. E. 2002. Ocatin. A novel tuber storage protein from the Andean tuber crop oca with antibacterial and antifungal activities. Plant Physiol. 128: 1291-1302.

Frommel, M. I., Nowak, J., and Lazarovits, G. 1991a. Growth enhancement and developmental modifications of *in vitro* grown potato (*Solanum tuberosum* spp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol. 96:928-936.

Frommel, M. I., Nowak, J., and Lazarovits, G. 1991b. Plant growth stimulation and biocontrol of *Fusarium* wilt by co-inoculation of tomato seeds with *Serratia plymuthica* and *Pseudomonas* sp. Fitopatologia 26: 66-73.

Galambos, A., Zok, A., Kuczmog, A., Oláh, R., Putnoky, P., Ream, W., and Szegedi, E. 2013. Silencing *Agrobacterium* oncogenes in transgenic grapevine results in strain-specific crown gall resistance. Plant Cell Rep. 32:1751-1757.

Glick, B. R. 2012. Plant growth-promoting bacteria: Mechanisms and applications. Scientifica (Cairo). 2012:963401.

Goswami, D., Thakker, J. N., and Dhandhukia, P. C. 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food Agric. 2:1127500.

Gullner, G., Komives, T., Király, L., and Schröder, P. 2018. Glutathione S-transferase enzymes in plant-pathogen interactions. Front. Plant Sci. 9:1836.

Habbadi, K., Benkirane, R., Benbouazza, A., Bouaichi, A., Maafa, I., Chapulliot, D., and Achbani, E. H. 2017. Biological control of grapevine crown gall caused by *Allorhizobium vitis* using bacterial antagonists. Int. J. Sci. Res. 6:1390-1397.

Héloir, M., Adrian, M., Brulé, D., Claverie, J., Cordelier, S., Daire, X., Dorey, S., Gauthier, A., Lemaître-Guillier, C., Negrel, J., Trdá, L., Trouvelot, S., Vandelle, E., and Poinssot, B. 2019. Recognition of elicitors in grapevine: From MAMP and DAMP perception to induced resistance. Front. Plant Sci. 10:1117.

Johnson, K. L., Cronin, H., Reid, C. L., and Burr, T. J. 2016. Distribution of *Agrobacterium vitis* in grapevines and its relevance to pathogen elimination. Plant Dis. 100:791-796.

Kawaguchi, A. 2015. Biological control agent *Agrobacterium vitis* strain ARK-1 suppresses expression of the *virD2* and *virE2* genes in tumorigenic *A. vitis*. Eur. J. Plant Pathol. 143:789-799.

Kawaguchi, A., Inoue, K., and Nasu, H. 2007. Biological control of grapevine crown gall by nonpathogenic *Agrobacterium vitis* strain VAR03-1. J. Gen. Plant Pathol. 73:133-138.

Keane, P. J., Kerr, A., and New, P. B. 1970. Crown gall of stone fruit II. Identification and nomenclature of *Agrobacterium* isolates. Aust. J. Biol. Sci. 23:585-596.

Kerr, A. 2016. Biological control of crown gall. Australas. Plant Pathol. 45: 15-18.

Kliewer, W. M. 1966. Sugars and organic acids of *Vitis vinifera*. Plant Physiol. 41:923-931.

Krastanova, S. V., Balaji, V., Holden, M. R., Sekiya, M., Xue, B., Momol, E. A., and Burr T. J. 2010. Resistance to crown gall disease in transgenic grapevine rootstocks containing truncated *virE2* of *Agrobacterium*. Transgenic Res. 19:949-958.

Kuzmanović, N., Puławska, J., Hao, L., and Burr, T. J. 2018. The ecology of *Agrobacterium vitis* and management of crown gall disease in vineyards. Curr. Top. Microbiol. Immunol. 418:15-53.

Lacroix, B., and Citovsky, V. 2019. Pathways of DNA transfer to plants from *Agrobacterium tumefaciens* and related bacterial species. Annu. Rev. Phytopathol. 57:231-251.

Lee, C., Efetova, M., Engelmann, J. C., Kramell, R., Wasternack, C., Ludwig-Müller, J., Hedrich, R., and Deeken, R. 2009. *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. Plant Cell Online 21:2948-2962.

Lehoczky, J. 1968. Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine, after natural infection. J. Phytopathol. 63:239-246.

León, J., Rojo, E., and Sánchez-Serrano, J. J. 2001. Wound signalling in plants. J. Exp. Bot. 52:1-9.

Le Quéré, A., Tak, N., Gehlot, H. S., Lavire, C., Meyer, T., Chapulliot, D., Rathi, S., Sakrouhi, I., Rocha, G., Rohmer, M., Severac, D., Filali-Maltouf, A., and Munive, J. A. 2017. Genomic characterization of *Ensifer aridi*, a proposed new species of nitrogen-fixing rhizobium recovered from Asian, African and American deserts. BMC Genomics 18:85.

Liswidowati, Melchior, F., Hohmann, F., Schwer, B., and Kindl, H. 1991. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta 183:307-314.

Liu, J., and Ekramoddoullah, A. K. M. 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiol. Mol. Plant Pathol. 68: 3-13.

Malik, N. A. A., Kumar, I. S., and Nadarajah, K. 2020. Elicitor and receptor molecules: Orchestrators of plant defense and immunity. Int. J. Mol. Sci. 21:963.

Mazumder, R., Phelps, T. J., Krieg, N. R., and Benoit, R. E. 1999. Determining chemotactic responses by two subsurface microaerophiles using a simplified capillary assay method. J. Microbiol. Methods. 37: 255-263.

Miotto-Vilanova, L., Courteaux, B., Padilla, R., Rabenoelina, F., Jacquard, C., Clément, C., Comte, G., Lavire, C., Barka, E. A., Kerzaon, I., and Sanchez, L. 2019. Impact of *Paraburkholderia phytofirmans* PsJN on grapevine phenolic metabolism. Int. J. Mol. Sci. 20:5775.

Miotto-Vilanova, L., Jacquard, C., Courteaux, B., Wortham, L., Michel, J., Clément, C., Barka, E. A., and Sanchez, L. 2016. *Burkholderia phytofirmans* PsJN confers grapevine resistance against *Botrytis cinerea* via a direct antimicrobial effect combined with a better resource mobilization. Front. Plant Sci. 7:1236.

Mousavi, S. A., Österman, J., Wahlberg, N., Nesme, X., Lavire, C., Vial, L., Paulin, L., de Lajudie, P., and Lindström, K. 2014. Phylogeny of the *Rhizobium-Allorhizobium-Agrobacterium* clade supports the delineation of *Neorhizobium* gen. nov. Syst. Appl. Microbiol. 37:208-215.

Oliver, J. P., Castro, A., Gaggero, C., Cascón, T., Schmelz, E. A., Castresana, C., and Ponce de León, I. 2009. *Pythium* infection activates conserved plant defense responses in mosses. Planta 230:569-579.

Ophel, K., and Kerr, A. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol. 40: 236-241.

Ou, S., and Kwok, K. 2004. Ferulic acid: Pharmaceutical functions, preparation and applications in foods. J. Sci. Food Agric. 84:1261-1269.

Padilla, R., Gaillard, V., Le, T. N., Bellvert, F., Chapulliot, D., Nesme, X., Dessaux, Y., Vial, L., Lavire, C., and Kerzaon, I. 2021. Development and validation of a UHPLC-ESI-QTOF mass spectrometry method to analyze opines, plant biomarkers of crown gall or hairy root diseases. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1162: 122458.

Pierron, R., Pouzoulet, J., Couderc, C., Judic, E., Compant, S., and Jacques, A. 2016. Variations in early response of grapevine wood depending on wound and inoculation combinations with *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. Front. Plant Sci. 7:268.

Poupin, M. J., Timmermann, T., Vega, A., Zúñiga, A., and González, B. 2013. Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS One 8:e69435.

Schmittgen, T. D., and Livak, K. J. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3:1101-1108.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G., and Nowak, J. 2005. *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. Int. J. Syst. Evol. Microbiol. 55:1187-1192.

Singleton, V., Zaya, J., and Trousdale, E. 1986. Caftaric and coutaric acids in fruit of *Vitis*. Phytochemistry 25:2127-2133.

Speer, E. O. 1987. A method of retaining phloroglucinol proof of lignin. Stain Technol. 62:279-280.

Su, F., Villaume, S., Rabenoelina, F., Crouzet, J., Clément, C., Vaillant-Gaveau, N., and Dhondt-Cordelier, S. 2017. Different *Arabidopsis thaliana* photosynthetic and defense responses to hemibiotrophic pathogen induced by local or distal inoculation of *Burkholderia phytofirmans*. Photosynth. Res. 134:201-214.

Süle, S., and Burr, T. J. 1998. The effect of resistance of rootstocks to crown gall (*Agrobacterium* spp.) on the susceptibility of scions in grape vine cultivars. Plant Pathol. 47:84-88.

Tarbah, F., and Goodman, R. N. 1987. Systemic spread of *Agrobacterium tumefaciens* biovar 3 in the vascular system of grapes. Phytopathology 77:915. http://www.apsnet.org/publications/phytopathology/backissues/Documents/1987Abstracts/Phyto77_915.htm

Timmermann, T., Poupin, M. J., Vega, A., Urrutia, C., Ruz, G. A., and González, B. 2019. Gene networks underlying the early regulation of *Paraburkholderia phytofirmans* PsJN induced systemic resistance in *Arabidopsis*. PLoS One 14:e0221358.

Toklikishvili, N., Dandurishvili, N., Vainstein, A., Tediashvili, M., Giorgobiani, N., Lurie, S., Szegedi, E., Glick, B. R., and Chernin, L. 2010. Inhibitory effect of ACC deaminase-producing bacteria on crown gall formation in tomato plants infected by *Agrobacterium tumefaciens* or *A. vitis*. Plant Pathol. 59:1023-1030.

Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D., and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. Vol. 3:research0034.1.

Vidal, J. R., Kikkert, J. R., Malnoy, M. A., Wallace, P. G., Barnard, J., and Reisch, B. I. 2006. Evaluation of transgenic "Chardonnay" (*Vitis vinifera*) containing magainin genes for resistance to crown gall and powdery mildew. Transgenic Res. 15:69-82.

Waite, H., and Morton, L. 2007. Hot water treatment, trunk diseases and other critical factors in the production of high-quality grapevine planting material. Phytopathol. Mediterr. 46:5-17.

Wample, R. L., Bary, A., and Burr, T. J. 1991. Heat tolerance of dormant *Vitis vinifera* cuttings. Am. J. Enol. Vitic. 42:67-72.

Xu, X. Q., and Pan, S. Q. 2000. An *Agrobacterium* catalase is a virulence factor involved in tumorigenesis. Mol. Microbiol. 35:407-414.

Yepes, L. M., Burr, T. J., Reid, C. L., and Fuchs, M. 2019. Elimination of the crown gall pathogen, *Agrobacterium vitis*, from systemically infected grapevines by tissue culture. Am. J. Enol. Vitic. 70:243-248.