Endophytic Colonization of *Vitis vinifera* L. by Plant Growth-Promoting Bacterium *Burkholderia* sp. Strain PsJN

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Patterns of colonization of *Vitis vinifera* L. cv. Chardonnay plantlets by a plant growth-promoting bacterium, *Burkholderia* sp. strain PsJN, were studied under gnotobiotic conditions. Wild-type strain PsJN and genetically engineered derivatives of this strain tagged with *gfp* (PsJN::*gfp*2x) or *gusA* (PsJN::*gusA*11) genes were used to enumerate and visualize tissue colonization. The rhizospheres of 4- to 5-week-old plantlets with five developed leaves were inoculated with bacterial suspensions. Epiphytic and endophytic colonization patterns were then monitored by dilution plating assays and microscopic observation of organ sections. Bacteria were chronologically detected first on root surfaces, then in root internal tissues, and finally in the fifth internode and the tissues of the fifth leaf. Analysis of the PsJN colonization patterns showed that this strain colonizes grapevine root surfaces, as well as cell walls and the whole surface of some rhizodermal cells. Cells were also abundant at lateral root emergence sites and root tips. Furthermore, cell wall-degrading endoglucanase and endopolygalacturonase secreted by PsJN explained how the bacterium gains entry into root internal tissues. Host defense reactions were observed in the exodermis and in several cortical cell layers. Bacteria were not observed on stem and leaf surfaces but were found in xylem vessels of the fifth internode and the fifth leaf of plantlets. Moreover, bacteria were more abundant in the fifth leaf than in the fifth internode and were found in substomatal chambers. Thus, it seems that *Burkholderia* sp. strain PsJN induces a local host defense reaction and systemically spreads to aerial parts through the transpiration stream.

In both natural and managed ecosystems, plant-associated bacteria play a key role in host adaptation to a changing environment (17, 56). Interactions between plants and beneficial bacteria can have a profound effect on crop health and yield and soil quality (27, 56). These microorganisms can presensitize plant cell metabolism, so that upon exposure to stress the presensitized or primed plants are able to respond more quickly and more efficiently than nonprimed plants and thus can better withstand the challenge (7, 63). The mechanisms by which beneficial microbes support plant growth and health include increasing nutrient availability, improving soil structure, inducing plant defense mechanisms, producing antibiotics, outcompeting pathogens, and providing growth-stimulating substances or enzymes (5, 14, 27, 30, 62). Despite the beneficial action of the microorganisms on plants, application of such microorganisms in the field is often hampered by inconsistent performance (57).

The ability to colonize roots has been considered the major factor that determines inoculum efficacy both for crop yield enhancement and for disease control (53, 64). This has led to an emphasis on selection of plant-beneficial bacteria that are rhizosphere competent (i.e., beneficial bacteria that effectively colonize the root system) (43). In addition, there is ample evidence that bacteria can also colonize internal tissues and thrive as endophytes in roots and/or shoots and leaves (8, 13, 17, 30, 65). Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil (55, 65). Moreover, plant growth promotion is often greater when it is induced by endophytes rather than by bacteria restricted to the rhizosphere and the root surface (4, 6). Therefore, a better understanding of the epi- and endophytic bacterial colonization patterns and the survival of introduced inocula both in the rhizosphere and in planta is a critical prerequisite for the development of effective ways to deliver and manage inocula.

Burkholderia sp. strain PsJN (unpublished results), which was originally designated *Pseudomonas* sp. strain PsJN (11), is an effective plant growth-promoting bacterium that was isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots (38). This bacterium promotes the growth of potatoes (11), vegetables (37), and grapevines (1) via reduction of the level of the inhibitory hormone ethylene by a high level of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase that is secreted (unpublished results). Strain PsJN also shows biocontrol activity against gray mold since it can effectively protect against in vitro and in vivo growth of *Botrytis cinerea* (2). Furthermore, during clonal multiplication of potato (40) and grapevine (1) via nodal explants taken from stock plants preinoculated with PsJN, the bacteria are transmitted through successive subcultures of plantlets with no reinoculation. This

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bacterium has been detected in roots and stems of potatoes (11) and tomatoes (42), as well as in grapevine leaves, following in vitro culture (1) and in grapevine roots under hydroponic conditions (unpublished results). However, the colonization pattern and the method used for translocation from the rhizosphere to internal plant tissues need to be clarified.

Recently, molecular techniques based on whole-cell hybridization methods have been used to detect and enumerate microorganisms in situ and on plant surfaces (3, 50, 67). Tagging with green fluorescent protein (GFP) (12, 58, 59) and β-glucuronidase (GUS) (47, 51, 66) gene markers has been particularly useful in following bacterial infection pathways for determination of tissue and organ colonization.

Thus, the objectives of this study were to construct *gfp*- and *gusA*-marked derivatives of *Burkholderia* sp. strain PsJN and to determine the epi- and endophytic patterns of colonization of *Vitis vinifera* L. under gnotobiotic conditions by using wild-type strain PsJN or genetically marked derivatives of this strain.

MATERIALS AND METHODS

Fluorescent labeling and GUS labeling of *Burkholderia* sp. strain PsJN. PsJN was tagged with the *gfp* and *gusA* marker genes by using mini-Tn5 systems, which form stable genomic insertions in a variety of bacteria (59, 60, 61, 67), according to the protocol described by Unge et al. (61). Briefly, wild-type strain PsJN was grown in King's B medium (26) in 5-ml cultures at 20°C until the optical density at 600 nm was 0.7. The bacterial cells were then pelleted by centrifugation (3,000 × *g*, 10 min, 4°C), washed three times with ice-cold distilled water, and resuspended in 500 μl of ice-cold glycerol. To each 100-μl cell suspension, 200 ng of delivery plasmid DNA was added; the plasmid used was either pUT*gfp*2x (61), in which two copies of the marker gene were constitutively expressed, or pCAM111 (67), in which *gusA* was under control of the *ptac* promoter. The mixture was then incubated for 15 min on ice and subsequently electroporated with a Gene Pulser Plus pulse controller (Bio-Rad, Richmond, Calif.) by using settings of 2.5 kV, 200 Ω, and 25 μF. Transformants carrying the *gfp* marker were selected on King's B medium containing 50 μg of kanamycin per ml (pUT*gfp*2x) or 50 μg of spectinomycin per ml (pCAM111). Colonies and cells of the *gfp*-marked strain were examined by using a fluorescence stereomicroscope (model MZ FLIII; Leica, Heerbrugg, Switzerland) equipped with a GFP 1 filter (Leica) and by using an optical microscope (model BH2; Olympus, Tokyo, Japan) equipped with a UV light source (BH2-RFL-T3; Olympus) and a 495-nm fluorescent filter (BP495; Olympus). The *gusA*-marked strain was grown for 4 days at 37°C on King's B medium amended with 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt; Sigma, St. Louis, Mo.) per ml. Then the bacteria were examined by using an optical stereomicroscope (model SZ-CTV; Olympus) and an optical microscope (model BH2; Olympus).

Determination of bacterial growth and inoculum preparation. The *gfp*- and *gusA*-marked strains and the wild-type strain were separately grown in King's B medium as described by Pillay and Nowak (42). Each bacterial inoculum was transferred to 100 ml of King's B liquid medium containing the appropriate antibiotic in a 250-ml Erlenmeyer flask and incubated at 20°C on a shaker (150 rpm) for 48 h. Bacteria were collected by centrifugation (4,500 × *g*, 15 min) and washed twice with phosphate-buffered saline (pH 6.5) (PBS). The concentration of the inoculum was then adjusted to approximately 3 × 10⁸ CFU/ml with PBS, based on the optical density at 600 nm, and was confirmed by plate counting as described by Pillay and Nowak (42).

Transformant stability and bacterial growth comparison. Transformant stability was determined by growing derivatives in King's B liquid medium without selection pressure for over 10 generations (doubling times) and then plating a dilution series on King's B medium with or without the appropriate antibiotic. Five replicates were included for each treatment. Furthermore, the colony and cell morphologies and growth patterns of the genetically derivatives were compared to those of the PsJN wild-type strain in King's B medium (26), Luria-Bertani medium, and M9 minimal medium with 0.4% glucose (49).

Plant material, growth conditions, and inoculation. Disease-free plantlets of *V. vinifera* L. cv. Chardonnay (i.e., plantlets free of visible bacterial or fungal contamination) were propagated by using nodal explants in 25-mm-diameter test tubes containing 15 ml of Martin medium (32). The cultures were grown in a growth chamber under white fluorescent light (200 μmol 52 m² · s⁻¹) with a 16-h photoperiod at 26°C (constant temperature).

Plant inoculation was monitored by spreading 200-μl aliquots of the bacterial inocula in PBS (wild-type strain PsJN and *gfp*- and *gusA*-marked strains) or PBS (control) on the surface of Martin medium in new test tubes under gnotobiotic conditions. Five-week-old rooted plantlets with five developed leaves were then delicately transferred into new test tubes previously inoculated with bacteria so that only the roots were in contact with the bacterial inoculum. The plantlets were then incubated in the culture chamber as described above.

Preparation of plant samples for determination of the plant growth-promoting effects. The effects of wild-type strain PsJN and the *gfp*- and *gusA*-marked strains on plant growth were compared to the effects of the control (PBS) 15 days after rhizosphere inoculation. Plantlets were removed from the growth medium, and shoot and root fresh weights and lengths were determined as growth parameters. There were four replicates for each treatment, and each replicate contained root and aerial systems from five plantlets.

Preparation of plant samples for bacterial enumeration. The rhizoplane and endophytic colonization of roots, shoots, and leaves by the *gfp*-marked strain was determined by plate counting, and bacterial colonies were counted by using a fluorescence stereomicroscope (model MZ FLIII; Leica) with a GFP 1 filter (Leica). Roots, the fifth internode, and the fifth leaf were sampled separately from 0 to 144 h after rhizosphere inoculation (see Fig. 2). For each plant part three samples were combined and weighed. Three or four replicates of three independent plating assays were used to determine the average colonization value. The lower limit of detection was between 0 and −1 log CFU/g (fresh weight).

(i) Rhizoplane colonization. Plantlets were removed from the agar, and roots were gently rinsed in sterile distilled water. The samples were then ground with a pestle in sterile Eppendorf microcentrifuge tubes containing 1 ml of PBS for approximately 1 min and shaken for 1 h (200 rpm) at the ambient temperature. The homogenates were vortexed for 5 s, 10-fold serially diluted, and cultured on King's B medium plates supplemented with kanamycin (50 μg/ml). Bacterial colonies were counted after 3 days of incubation at 30°C. Rhizoplane colonization by the *gfp*-marked strain was determined by subtracting the bacterial counts after surface sterilization from the total *gfp* bacterial counts determined without surface sterilization.

(ii) Endophytic colonization. To determine endophytic populations of the *gfp*-marked strain, samples were surface sterilized with 70% ethanol for 5 min (roots) or 3 min (fifth internode and fifth leaf), followed by 1% commercial bleach and a 0.01% Tween 20 solution for 1 min, and then washed three times in distilled water (1 min each time). The samples were then ground and handled as described above in order to determine the microbial populations inside surfaced-sterilized roots, internodes, and leaves. The bacterial colonies were counted after 3 days of incubation at 30°C.

Evaluation of surface sterilization methods for monitoring rhizoplane and endophytic populations. To determine the efficacy of the surface sterilization procedure, samples of sterilized roots, fifth internodes, and fifth leaves were taken 96 h after inoculation of the rhizosphere with the *gfp*-marked strain. The samples were observed with an epifluorescence microscope or placed on King's B medium plates containing kanamycin (50 μg/ml) and incubated for 1 min prior to crushing. The samples were then removed, and the plates were incubated at 30°C as described above. In addition, the wash solution from the last rinse was cultured on King's B medium plates amended with 50 μg of kanamycin per ml to determine the efficiency of sterilization.

In parallel, the fifth internodes and the fifth leaves taken from two sets of 10 plantlets were used without surface sterilization to determine if epiphytic colonization of these aerial plant parts occurred after rhizosphere inoculation.

Microscopy of rhizoplane colonization by PsJN. To determine colonization of the rhizoplane by the *gfp*-marked strain, approximately 20 plantlets were examined with the fluorescent stereomicroscope and the epifluorescence microscope, as describe above, 96 h after rhizosphere inoculation. Root surfaces were observed and photographed with an automatic photographic system (PM-CBSP; Olympus) or a numerical camera (C-4040; Olympus).

Similarly, 96 h after inoculation with the *gusA*-marked strain, 20 plantlets were used to localize the tagged strain. The *gusA*-tagged strain was stained by using the procedure described by Jefferson et al. (24). Fresh plant organs were individually immersed in the GUS staining solution in a desiccator connected to a pump. A vacuum was applied for 1 min to facilitate penetration of the substrate into the plant tissues. After 20 h of incubation at 37°C, the plant tissues were immersed in an ethanol bath. Then the samples were examined with a microscope and photographed as described above.

Microscopy of endophytic colonization by PsJN. Fresh plant organs (roots, fifth internodes, and fifth leaves) removed from six plantlets inoculated with either wild-type strain PsJN, genetically derivatives of this strain (*gfp*- or *gusA*-marked strain), or a control (PBS) were collected 96 h after inoculation. Samples

were then prepared for microscopy analysis as described by Gognies et al. (15), with some modifications. Briefly, plant organs were fixed for 24 h at room temperature in 2% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.24) with 2% (wt/vol) sucrose and 0.1% (vol/vol) Tween 20. After three rinses (5 min each) with the phosphate buffer containing 2% (wt/vol) sucrose, samples were fixed for 4 h in 1% (wt/vol) osmium tetroxide in phosphate buffer with 2% (wt/vol) sucrose. The samples were then dehydrated in an alcohol series, transferred to acetone, and embedded in araldite. Semithin sections (thickness, 1 μm) for different treatments were cut with a microtome (model Jung RM2055; Leica, Nussloch, Germany), collected on glass slides, stained with 0.1% toluidine blue, examined with a microscope (model BH2; Olympus), and photographed as described above.

In parallel, hand-cut transverse sections of different parts (roots, fifth internodes, and fifth leaves) of 20 plantlets inoculated with either PBS (control) or the *gfp*-marked strain were used to detect host defense reactions by visualizing autofluorescence and endophytic colonization by epifluorescence microscopy and were photographed as described above.

Additionally, samples taken from 20 plantlets inoculated with the *gusA*-marked strain were used to assess colonization of the fifth internode and leaf internal tissue after staining with the GUS substrate as described above. This method allowed us to visualize bacteria inside plant tissues without obtaining tissue sections.

Plate assays for endoglucanase and endopolygalacturonase activities. Enzyme activities were determined by the method of Reinhold-Hurek et al. (44), with some modifications. Briefly, plates containing KW (Kim-Wimpenny) solid medium (25), with or without D-glucose and with either 0.2% carboxymethyl cellulose (CMC) or 0.5% polygalacturonic acid, were spot inoculated with PsJN and incubated at 30°C for 3 days. Then the cells were removed from the plates, and the CMC-containing plates were stained with Congo red (0.1%) for 30 min; this was followed by several washes with 1 M NaCl to improve the contrast (44). Similarly, the polygalacturonic acid-containing plates were stained with ruthenium red (0.1%) and washed with 1 M NaCl (33). Endoglucanase (CMC-degrading cellulase) activity was determined by the appearance on a red background of clear yellowish halos around the points where the bacterium was inoculated (44). Endopolygalacturonase activity was determined by the appearance of intense purple-red halos on a colorless background at the point where the bacterium was inoculated (33).

Statistical analysis. Population densities estimated by using CFU were subjected to logarithmic transformation before data analysis (31). Data for plant growth and bacterial enumeration were statistically analyzed by using Student's *t* test.

RESULTS

Construction and stability of the *gfp*- and *gusA*-marked strains and comparison with the wild-type strain. The colony and cell morphologies and the growth patterns of the *gfp* and *gusA* genetic derivatives of *Burkholderia* sp. strain PsJN on King's B medium, Luria-Bertani medium, and M9 minimal medium (supplemented with 0.4% glucose) were similar to those of the wild-type strain (data not shown). Colonies and cells of the *gfp*-marked strain were strongly fluorescent under UV light, whereas the *gusA*-marked strain exhibited a blue color after application of the GUS substrate (data not shown). No white colonies appeared after several restreakings on King's B medium with or without the appropriate antibiotic, indicating that integrations of the mini-transposons into the bacterial chromosome were stable.

Comparison of the plant growth-promoting effects of *gfp*- and *gusA*-marked strains and the wild-type strain on grapevine plantlets. All inoculated plantlets survived rhizosphere bacterization and performed better that nonbacterized plantlets. Fifteen days postinoculation (p.i.) no significant differences ($P < 0.05$) in plantlet growth among the PsJN::*gfp*2x, PsJN::*gusA*11, and the wild-type treatments were found (Fig. 1). However, significant increases ($P < 0.05$) in the relative fresh weights of roots and aerial parts, as well as the lengths of

aerial parts, were observed for bacterized treatments compared to the nonbacterized control (Fig. 1A, B, and D). Root length was the only parameter that was not significantly different for the bacterized and control cultures ($P > 0.05$) (Fig. 1C).

Efficacy of surface sterilization protocols. To ensure that the endophytic colonization values determined in this study reflected only the numbers of cells in the interior of plant tissues, a specific surface sterilization method was developed. This sterilization method should have killed and/or washed away the surface bacteria while the internal bacteria survived. First, root samples were examined 96 h p.i. by epifluorescence microscopy for GFP-containing cells remaining on the plant surface after surface sterilization. No bacterial cells were ever observed when this technique was used. Second, no bacterial growth was observed after 3 days on King's B solid medium when surface-sterilized roots were added. Third, either no colonies or only a few (<10) colonies were observed 3 days after inoculation on plates inoculated with the last wash solution after surface sterilization. In the very few cases in which the last wash yielded colonies with GFP, the number of such colonies represented less than 0.001% of that the root endophytic populations occurring at 96 h p.i. Based on the results for these three controls, the endophytic colonization values presented here reflected only the numbers of cells within the tissues, as reported by Dong et al. (8). Thus, the surface sterilization treatment was efficient and could be used to determine the endophytic populations of root internal tissues.

Similar to the root tests, no bacterial colonies were found on stems and leaves in poststerilization wash tests performed with King's B medium amended with 50 μg of kanamycin per ml 3 days after plating. Furthermore, no bacterial colonies were observed on King's B medium amended with 50 μg of kanamycin per ml 3 days after we added the surface-sterilized aerial plant parts prior to crushing. Thus, the surface sterilization treatment was efficient and could be used to determine the populations of internal tissues of aerial plant parts.

In addition, PsJN::*gfp*2x cells were not found on the fifth internode and fifth leaf surfaces from zero time to 96 h after rhizosphere inoculation, as determined by fluorescence microscopy. Furthermore, there was no difference in the total PsJN::*gfp*2x bacterial populations colonizing aerial parts when samples taken from surface-sterilized and nonsterilized plantlets were compared (data not shown). This finding further demonstrated that the inner tissues of aerial organs could not have been contaminated by surface-colonizing bacteria.

Root surface colonization. The rhizoplane of grapevine plantlets was rapidly colonized by PsJN::*gfp*2x cells immediately after rhizosphere inoculation. The PsJN::*gfp*2x population peaked at 24 h p.i. and then remained stable at 9.15 log CFU/g (fresh weight) (Fig. 2A).

Microscopic observations of roots 96 h after PsJN::*gfp*2x inoculation revealed green fluorescence on both primary and secondary roots. PsJN::*gfp*2x cells congregated in high numbers at the sites of lateral root emergence on plantlets (Fig. 3A and B). At 96 h p.i., PsJN::*gfp*2x cells were also found close to the cell walls of the rhizodermis (Fig. 3C), as well as on the whole outline of some rhizodermal cells (Fig. 3D).

In the experiment with PsJN::*gusA*11 no blue color was detected on roots taken from nonbacterized plantlets after incubation in the GUS substrate (Fig. 3E). However, GUS expres-
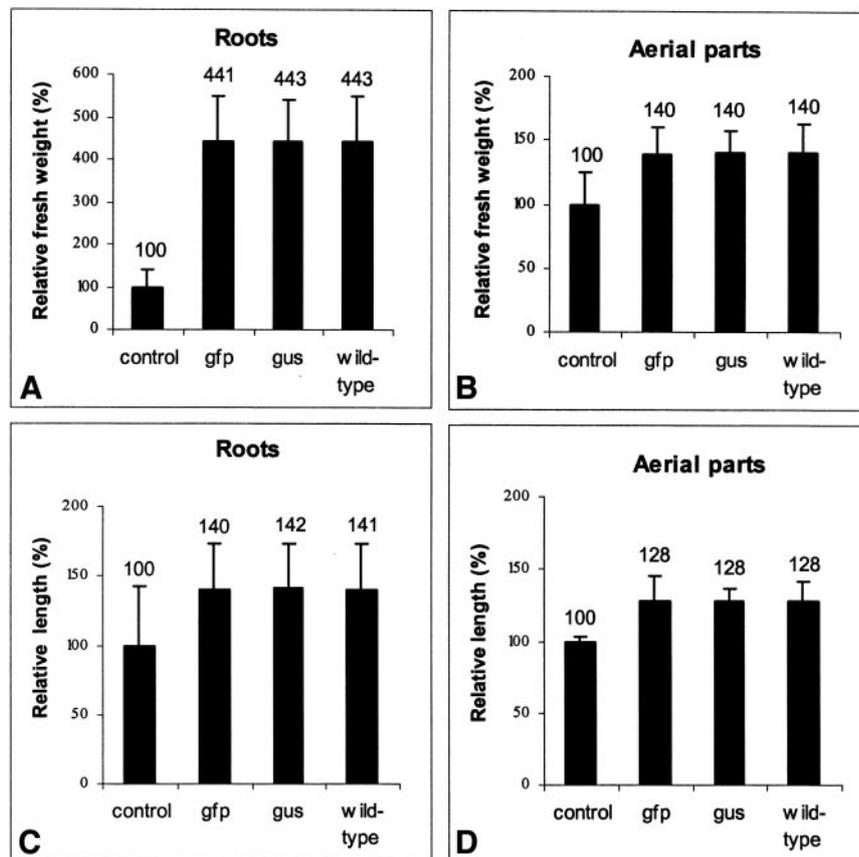
FIG. 1. Comparison of the plant growth-promoting effects of the PsJN::*gfp*2x, PsJN::*gusA*11, and wild-type PsJN strains on *V. vinifera* L. cv. Chardonnay plantlets 15 days after rhizosphere inoculation. (A and B) Percentages of relative fresh weight of the root system (A) and aerial parts (B). (C and D) Percentages of relative lengths of the root system (C) and aerial parts (D). The bars indicate means, and the error bars indicate standard deviations.

sion was detected on root tips at 96 h p.i. when the PsJN::*gusA*11 strain was applied (Fig. 3F and G).

Endophytic colonization of roots and host defense reactions. Colonization of the root interior by PsJN::*gfp*2x cells occurred between 1 and 3 h p.i., and the population remained at a level of 6.85 log CFU/g (fresh weight) for more than 48 h p.i. (Fig. 2B). Rhizodermis and endodermis layers, as well as xylem vessels of primary roots of control plantlets, exhibited yellow autofluorescence at 96 h p.i. (Fig. 3H) as a result of phenolic compounds in the cell wall which fluoresced under UV light (20). Yellow autofluorescence was also observed in primary roots of plantlets inoculated with PsJN::*gfp*2x (Fig. 3I). However, several cortical cell layers exhibited additional yellow fluorescence after inoculation with PsJN::*gfp*2x (Fig. 3I) compared to the nonbacterized control treatment (Fig. 3H). This enhancement indicated that there was accumulation of phenolic material corresponding to a host defense reaction. Furthermore, host defense reactions, which were correlated with a strengthening of some cell walls in the exodermis as well as cortical cells, were also observed in resin-embedded roots (Fig. 3J).

Due to the large bacterial population that developed on the rhizoplane, bacterial colonization of root internal tissues could not be confirmed microscopically by using hand-cut sections of plantlets inoculated with PsJN::*gfp*2x because cells could have

been introduced from the external root surface during sample preparation, as reported by Shishido et al. (54). However, microscopic analyses of resin-embedded roots demonstrated that cortical cells (Fig. 3J and K), the endodermis (Fig. 3L), and xylem vessels (Fig. 3M) were colonized by bacterial cells in an inter- and/or intracellular colonization pattern, in contrast to control samples, in which no bacteria were observed (data not shown).

Endophytic colonization of stems. No PsJN::*gfp*2x cells were detected in the fifth internode before 72 h p.i. Then the first bacteria appeared, the highest level was reached 84 h p.i., and the population remained at a level of 5.85 log CFU/g (fresh weight) (Fig. 2C).

No blue color was observed in the fifth internode of nonbacterized plantlets after incubation with the GUS substrate (Fig. 4A). However, a blue color appeared in the center of the fifth internode 96 h p.i. in the plantlets inoculated with the PsJN::*gusA*11 strain (Fig. 4B).

Green autofluorescence (epidermis), red autofluorescence (parenchyma), and yellow autofluorescence (vascular system) were detected in the fifth internode 96 h p.i. both in nonbacterized plantlets (Fig. 4C) and in PsJN::*gfp*2x-bacterized plantlets (Fig. 4D). Microscopic observations at high magnifications from the epidermis to xylem vessels revealed that PsJN::*gfp*2x cells or wild-type PsJN cells were found only in xylem vessels
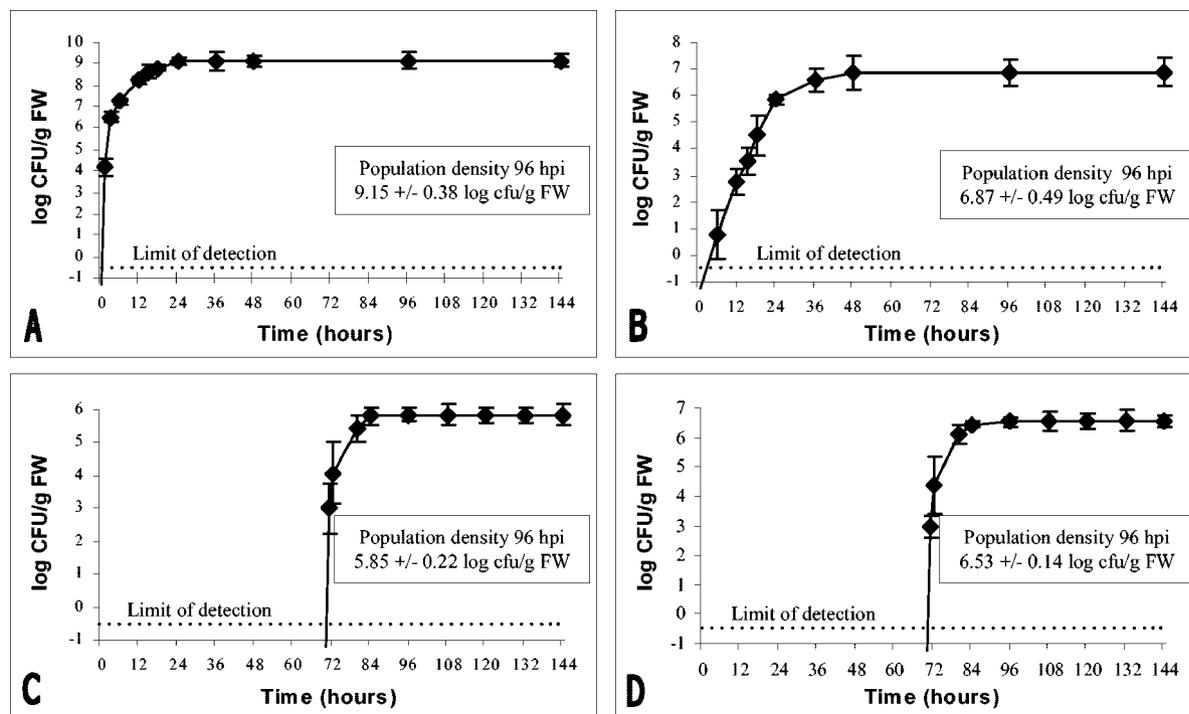
FIG. 2. Population dynamics of the PsJN::*gfp*2x strain for different parts of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation. (A) Rhizoplane; (B) root internal tissues; (C) fifth internode internal tissues; (D) fifth leaf internal tissues. The error bars indicate the standard deviations for mean log-transformed bacterial population sizes. The values are means and standard deviations. FW, fresh weight.

(Fig. 4E and F), in contrast to nonbacterized samples, in which no bacteria were observed (data not shown).

Endophytic colonization of leaves. No PsJN::*gfp*2x cells were detected in the fifth leaf until 72 h p.i. The highest population density occurred 84 h p.i., and the stationary-phase level was 6.53 log CFU/g (fresh weight) (Fig. 2D). The endophytic colonization of the fifth leaf was significantly greater than the endophytic colonization of the fifth internode ($P < 0.001$).

No blue color was observed for samples taken from nonbacterized plantlets after incubation with the GUS substrate (Fig. 4G). However, in the test conducted with the PsJN::*gusA*11 construct, a blue color was found in the primary and secondary veins 96 h p.i. (Fig. 4H).

As reported above for the fifth internode, the same autofluorescence was observed in the fifth leaf at 96 h p.i. both after control treatment and after PsJN::*gfp*2x treatment (Fig. 4I and J). PsJN::*gfp*2x or wild-type PsJN cells were found only in xylem vessels (Fig. 4K and L), in contrast to nonbacterized samples, in which no bacteria were observed (data not shown).

Interestingly, PsJN::*gfp*2x cells were not found on the abaxial surface of the fifth leaf regardless of the time after inoculation. However, when a little pressure was applied by hand to a leaf placed between a glass slide and a coverslip, bacterial cells were observed around stomata 96 h p.i. (Fig. 4M), which indicates that they were present in substomatal chambers. This was confirmed in the PsJN::*gusA*11 treatment, in which blue bacterial cells were detected by leaf transparency under stomata at 96 h p.i. (Fig. 4N).

Analysis of cell wall-degrading enzyme activities. Qualitative plate assays demonstrated that PsJN secreted endoglucanase (Fig. 5A) and endopolygalacturonase (Fig. 5B) activities.

DISCUSSION

The present study clearly demonstrated that *Burkholderia* sp. strain PsJN can form sustaining endophytic populations in roots, stems, and leaves of *V. vinifera* L. plantlets. Following rhizosphere inoculation, colonization of the grapevine plantlets progressed in distinguishable stages. The initial step consisted of rhizoplane colonization of grapevine plantlets by PsJN, although this could be expected as the bacterial inoculum was in contact with the root system. Despite this, the presence of bacteria was not uniform on the entire root surface. The highest bacterial concentrations appeared on both primary and secondary roots, as well as at the base of the lateral roots and at root tips. Furthermore, PsJN cells were often closely attached to the rhizodermal cell walls and could follow the whole outline of epidermal cells. Hansen et al. (18) described a similar colonization behavior for *Pseudomonas fluorescens* DF57 on barley roots, suggesting that there is a common pattern for rhizoplane colonization by different bacteria. The extensive colonization of the rhizoplane can be explained by root exudate effects (for a review see reference 41).

Irrespective of the mode of action, a key feature of all plant-beneficial bacteria is efficient colonization of root surfaces (65). After this initial colonization step, certain bacteria are able to enter roots through cracks at root emergence sites (crack entry process) and/or by passing through root tips (root tip pathway) (reviewed in reference 45) or through the middle
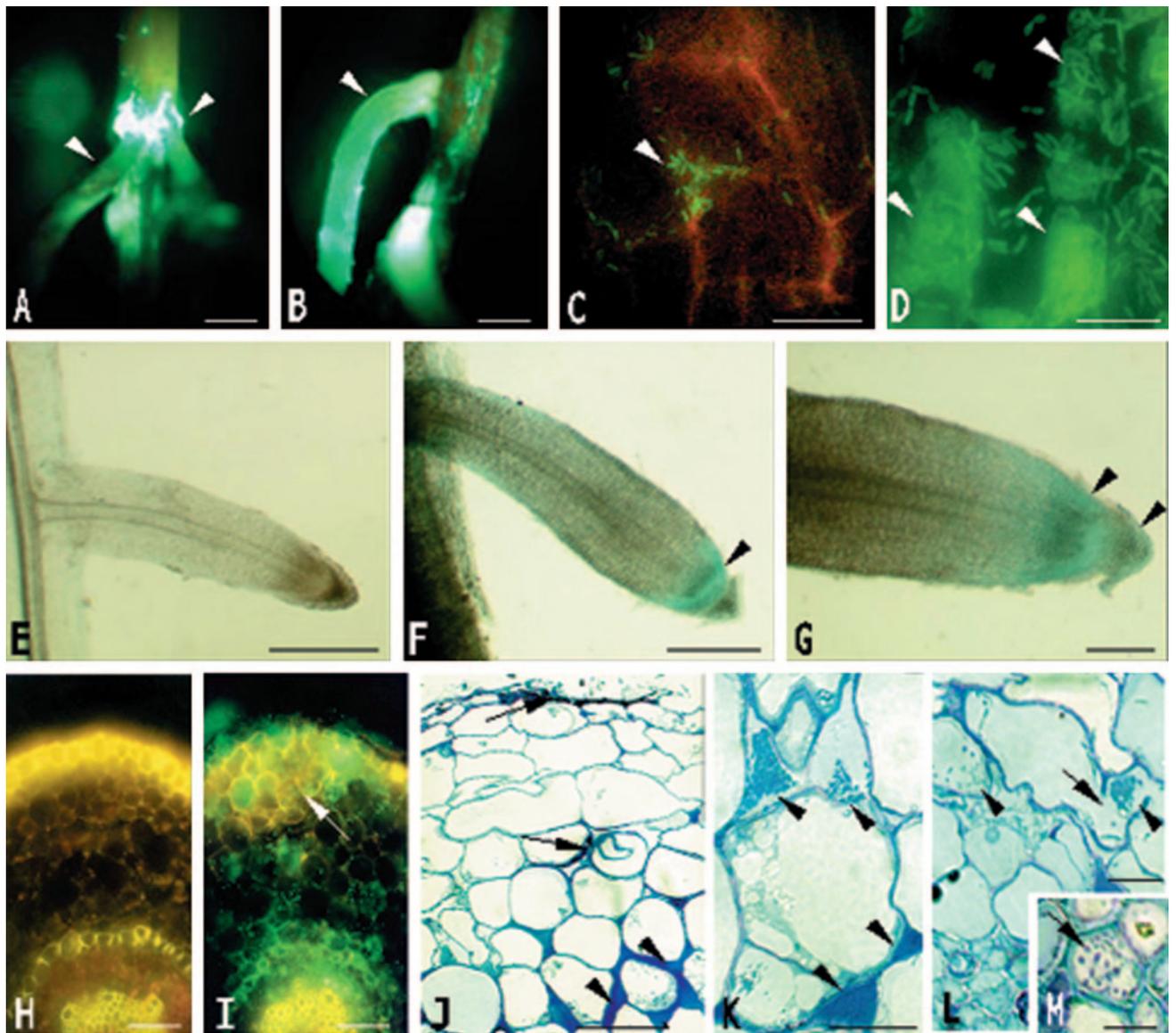
FIG. 3. Microphotographs of rhizoplane and root internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation with *Burkholderia* sp. strain PsJN. (A and B) Fluorescence stereomicroscope images of roots after inoculation of PsJN::*gfp*2x, showing strong fluorescence at the site of emergence of lateral roots of the primary root (A) and on a lateral root due to *gfp*-marked cells (A and B) (arrowheads). (C and D) Epifluorescence microscope images of rhizoplane, showing *gfp*-marked cells close to the cell walls (C) or around the whole outline of some rhizodermis cells (D) (arrowheads). (E to G) Light microscope images of a secondary root after PBS inoculation (E) and after inoculation with PsJN::*gusA*11 (F and G), showing a blue color due to *gusA*-marked cells at the root tips (arrowheads). (H and I) Epifluorescence microscope images of primary root internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after treatment with PBS (H) or after inoculation of the rhizosphere with PsJN::*gfp*2x (I), showing yellow fluorescence in several cortical cell layers corresponding to a host defense reaction (arrow). (J to M) Light microscope images of resin-embedded primary roots after inoculation with PsJN, showing host defense reactions (arrows) in the exodermis and cell wall of a cortical cell (J), intercellular colonization of cortical cells (J and K) (arrowheads), a break in the endodermis (arrow) caused by PsJN (L) (arrowheads), and PsJN in xylem vessels (M) (arrows). (A and B) Bars = 500 μm; (C) bar = 25 μm; (D) bar = 10 μm; (E and F) bars = 250 μm; (G) bar = 125 μm; (H and I) bars = 100 μm; (J, K, and L) bars = 50 μm; (M) bar = 20 μm.

lamella of the epidermal layer (19). In our study, the presence of PsJN at lateral root emergence sites suggested that crack entry colonization occurred in grapevine plantlets, similar to the phenomenon previously observed with the same strain and potato (39). Moreover, the occurrence of a blue color at root tips after PsJN::*gusA*11 inoculation also supports the possibility of entry via root tips. Furthermore, PsJN cells colonized rhizodermal cells, the inter- and intracellular spaces of cortical cells, the endodermis, and xylem vessels. This indicated that this bacterium can invade root internal tissues by passing between epidermal and cortical cells and can permeate the central cylinder by breaking the endodermis barrier. This conclusion is supported by the production of the cell wall-degrading enzymes endoglucanase and endopolygalacturonase.

It has been reported that the production of cell wall-degrading enzymes by endophytic bacteria is usually linked to localized host
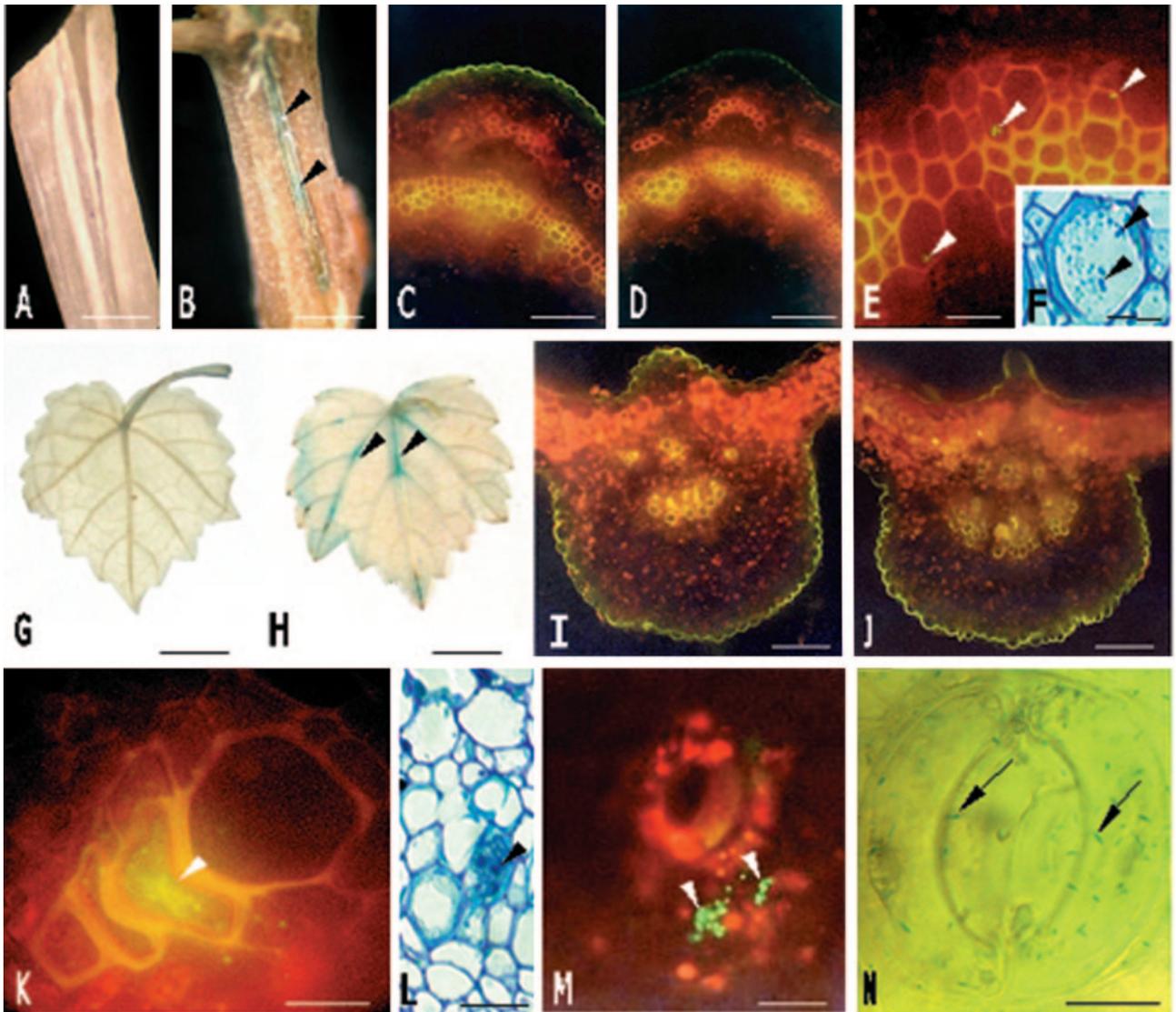
FIG. 4. Photographs of the fifth internode and leaf internal tissues of *V. vinifera* L. cv. Chardonnay plantlets after rhizosphere inoculation with PsJN. (A and B) Light stereomicroscope images of the fifth internode cut longitudinally with a razor after treatment with PBS (A) or after inoculation with PsJN::*gusA*11 (B), showing the blue color (arrowheads). (C to E) Epifluorescence microscope images of hand-cut sections of the fifth internode after treatment with PBS (C) or after inoculation with PsJN::*gfp*2x (D and E), showing PsJN::*gfp*2x cells in xylem vessels (E) (arrowheads). (F) Light microscope image of resin-embedded stem after inoculation with wild-type strain PsJN, showing PsJN cells in xylem vessels (arrowheads). (G and H) Light micrographs of the fifth leaf after treatment with PBS (G) or after inoculation with PsJN::*gusA*11 (H), showing the blue color in veins due to *gusA*-marked cells (arrowheads). (I to K) Epifluorescence microscope images of hand-cut sections of the fifth leaf after treatment with PBS (I) or after inoculation with PsJN::*gfp*2x (J), showing PsJN::*gfp*2x cells in xylem vessels (K) (arrowhead). (L) Light microscope image of resin-embedded leaf after inoculation with wild-type strain PsJN, showing PsJN cells in xylem vessels (arrowheads). (M and N) Epifluorescence (M) and light (N) microscope images of the fifth leaf after inoculation of PsJN::*gfp*2x (M), showing bacteria exiting from stomata (arrowheads), or after inoculation of PsJN::*gusA*11 (N), showing blue cells (arrows) under stomata. (A and B) Bars = 500 μm; (C and D) bars = 150 μm; (E) bar = 30 μm; (F) bar = 30 μm; (G and H) bars = 500 μm; (I and J) bars = 150 μm; (K) bar = 20 μm; (L) bar = 50 μm; (M) bar = 10 μm; (N) bar = 5 μm.

plant defense (17). In the present study, we also observed a host defense reaction that coincided with localized accumulation of phenolic compounds in several cortical cells following colonization by PsJN. This is not surprising since it is well established that phenolic compound accumulation is associated with a plant defense mechanism (35). We concluded that PsJN cells can induce a host defense response in roots of grapevines. Strengthening of the cell walls in the exodermis, as well as in some cortical cells,

was also observed, as reported previously for tomato root colonization by *P. fluorescens* strain WCS417r (9).

Following colonization of the root interior, PsJN colonizes stems and leaves. Other studies have also detected endophytes within aerial plant parts, including stems, leaves, and flowers (28, 34, 46, 52). It has been suggested that bacteria can be transported in xylem vessels through the transpiration stream (16, 19, 22, 23, 54) or by colonizing intercellular spaces from
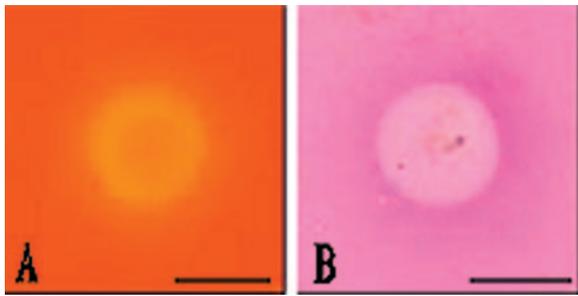
FIG. 5. Photographs of endoglucanase (A) and endopolygalacturonase (B) activities of PsJN. Bars = 3 mm.

roots to aerial parts (10, 16). Our results demonstrate that PsJN systemically spreads to aerial parts through the transpiration stream from root xylem vessels.

In this study, we observed significantly higher numbers of PsJN cells in leaves than in stems. Similar results were reported for the colonization of tomato plants by *P. fluorescens* SE34r (70). The differences between stem and leaf populations that were observed can be explained by accumulation of bacterial cells in the leaf, which can be considered a sink, whereas the stem serves only for transition. However, in this study we also detected PsJN in substomatal chambers of grapevine leaves because PsJN cells exited from stomata after a little pressure between a glass slide and a coverslip was exerted on a leaf. Furthermore, PsJN was not detected on the leaf surface but was found inside substomatal chambers with *gus*-tagged bacteria after rhizosphere inoculation. This demonstrated that PsJN cells can reach substomatal chambers of leaves after spreading within the plant. Substomatal chambers, interstices, and trichomes are preferred habitats for bacterial survival and multiplication due to their relatively protected hydrophilic environments (29, 36, 68). Thus, stems and leaves that are colonized differently may also be explained by considering substomatal chambers of grapevines microhabitats for PsJN where multiplication may occur. However, the lack of PsJN cells in substomatal chambers in resin-embedded leaves may indicate that there was a low number of bacteria in this habitat which could not be detected. Furthermore, multiplication of endophytic bacteria inside plant tissues is difficult to demonstrate (17), and work is needed to confirm this hypothesis. Despite this, all rhizosphere bacteria are not capable of establishment in this habitat. Several studies have shown that endophytic populations of strains of various nonpathogenic bacterial species cannot become established following infiltration into leaves (48, 69). To our knowledge, only James et al. (21, 23) have reported colonization of substomatal chambers by plant-beneficial bacteria, but there was the possibility of epiphytic bacterial propagation on the phylloplane (21, 23) and/or spreading within the plant (23). Our finding of PsJN cells in substomatal chambers after an initial root colonization step confirmed that substomatal chambers can be colonized by bacteria after they spread within the plant. Such knowledge could ultimately lead to a better understanding of the plant-endophytic bacterium interactions. Although our experiments were based on early events, an agar-based system and substomatal chamber colonization by PsJN need to be demonstrated by

using a soil-based system or hydroponic conditions under which PsJN can form endophytic populations in grapevine (unpublished results).

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