

# Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: from the rhizosphere to inflorescence tissues

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Received 21 May 2007; revised 3 October 2007; accepted 4 October 2007.

First published online December 2007.

DOI:10.1111/j.1574-6941.2007.00410.x

Editor: Christoph Tebbe

## Keywords

*Burkholderia*; grape; microbial communities; inflorescence; *Vitaceae*.

## Abstract

The colonization pattern of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN was determined using grapevine fruiting cuttings with emphasis on putative inflorescence colonization under nonsterile conditions. Two-week-old rooted plants harbouring flower bud initials, grown in nonsterile soil, were inoculated with PsJN::*gfp2x*. Plant colonization was subsequently monitored at various times after inoculation with plate counts and epifluorescence and/or confocal microscopy. Strain PsJN was chronologically detected on the root surfaces, in the endorhiza, inside grape inflorescence stalks, not inside preflower buds and flowers but rather as an endophyte inside young berries. Data demonstrated low endophytic populations of strain PsJN in inflorescence organs, i.e. grape stalks and immature berries with inconsistency among plants for bacterial colonization of inflorescences. Nevertheless, endophytic colonization of inflorescences by strain PsJN was substantial for some plants. Microscopic analysis revealed PsJN as a thriving endophyte in inflorescence organs after the colonization process. Strain PsJN was visualized colonizing the root surface, entering the endorhiza and spreading to grape inflorescence stalks, pedicels and then to immature berries through xylem vessels. In parallel to these observations, a natural microbial communities was also detected on and inside plants, demonstrating the colonization of grapevine by strain PsJN in the presence of other microorganisms.

## Introduction

The interaction between plants and beneficial bacteria can have a profound effect on plant health, growth, development, yield as well as on soil quality (Welbaum *et al.*, 2004; Compant *et al.*, 2005a; Haas & Défago, 2005; Unno *et al.*, 2005). Plant growth-promoting rhizobacteria (PGPR) (Kloepper & Schroth, 1978) or plant growth-promoting bacteria (PGPB) (Bashan & Holguin, 1998) can colonize the plant interior and thrive as endophytes in various organs without harming their host (James & Olivares, 1998; Lodewyckx *et al.*, 2002; Berg *et al.*, 2004; Compant *et al.*, 2005b; Rosenblueth & Martinez-Romero, 2006). Bacterial endophytes have been found in different

vegetative parts of plants, such as roots, tubers, stem and leaves (Hallmann, 2001; Gray & Smith, 2005; Compant *et al.*, 2005b). A few studies have also reported bacterial endophytes in reproductive organs, such as flowers and fruits. This has been demonstrated mainly through the isolation of endophytes from their natural plant hosts (Samish *et al.*, 1961; Misaghi & Donndelinger, 1990; Hallmann, 2001; Bacon & Hinton, 2006). However, the colonization process of reproductive organs by endophytic bacteria, particularly by some PGPR, is unknown. An understanding of this process in plants of agricultural importance could potentially be used for enhancing plant production (Lodewyckx *et al.*, 2002; Welbaum *et al.*, 2004; Compant *et al.*, 2005a).

One of these endophytic PGPR, *Burkholderia phytofirmans* strain PsJN (Sessitsch *et al.*, 2005), has been isolated from surface-sterilized onion roots. Strain PsJN has been able to promote plant growth of nonnatural hosts differentially in addition to lowering biotic and abiotic stress. This bacterium can also thrive as an endophyte inside various plant hosts, including tomato (Pillay & Nowak, 1997; Sharma & Nowak, 1998) potato (Frommel *et al.*, 1991; Bensalim *et al.*, 1998), and grape (Ait Barka *et al.*, 2000, 2002, 2007; Compant *et al.*, 2005b). With grapevine, strain PsJN can form epiphytic as well as endophytic populations with *in vitro* plantlets (Compant *et al.*, 2005b). This was monitored mainly *via* the use of a *gfp* derivative of strain PsJN that allowed visualization of the bacterium both on and inside plants. Use of this tagged strain on grapevine plantlets has demonstrated bacterial colonization of the root surface and subsequent entrance into the endorhiza mainly through the 'root tip way', lateral root cracks or between rhizodermal cells *via* cell-wall-degrading enzyme secretions. Finally, strain PsJN colonized stem and leaves through xylem vessels, before thriving as an endophyte inside substomatal chambers of leaves after using the plant transpiration stream (Compant *et al.*, 2005b). Prior analysis of the colonization patterns of grapevine by strain PsJN was conducted under gnotobiotic conditions. The epi- and endophytic colonization behaviour of strain PsJN in plants, grown in nonsterile soil, has not been elucidated. Furthermore, nothing was known of inflorescence colonization by strain PsJN. However, in grapevine, inflorescences are of particular interest and putative reproductive organ colonization by strain PsJN needed to be studied. Therefore, the objective of the present study was to investigate epi- and endophytic colonization of grapevine by *B. phytofirmans* strain PsJN in plants grown in nonsterile soil with emphasis on putative inflorescence colonization, and to compare this pattern with the previous study on the colonization of *in vitro* plantlets.

## Materials and methods

### Bacterial strains, growth conditions and inoculum preparation

The *gfp*-marked strain PsJN::*gfp2x* (Compant *et al.*, 2005b) was grown in 100 mL Luria-Bertani (LB) liquid medium in 250 mL Erlenmeyer flasks and incubated at 25 °C, on a shaker (150 r.p.m.) for 48 h as described by Compant *et al.* (2005b). Bacteria were collected by centrifugation (4500 g; 10 min) and washed twice with sterile phosphate buffer, pH 6.5 (PBS). The bacterial concentration of the inoculum was then adjusted with PBS, based on OD<sub>600 nm</sub> and confirmed with plate counting (Pillay & Nowak, 1997).

### Plant material and growth conditions

Three-node cuttings (20 cm long) of *Vitis vinifera* L. cv. Chardonnay were cane pruned from 6-year-old plants in 2005, 2006 and 2007 at the 'Moët et Chandon' vineyard (Epernay, France). Fruiting cuttings were then prepared according to Lebon *et al.* (2005) with the following modifications: the cuttings were treated with 0.05% Cryptonol<sup>®</sup> for 4 h and the distal node was covered with grafting wax (Agrochemie<sup>®</sup>, Germany) containing a fungicide (0.1% oxiquinoline) and plant growth regulators (0.00175% 2,5-dichlorobenzoic acid). Cuttings were then stored in the dark at 4 °C for at least 2 weeks. Before use and after 15 h of hydration at 28 °C, the two proximal nodes were removed and the cuttings were immersed for 30 s in indole-3-butyric acid solution (2 g L<sup>-1</sup>) to promote rhizogenesis. Each cutting was then placed in a plastic pot (9 cm × 9 cm × 10 cm) filled with one-part clay balls and four parts (v/v) potting soil (special Gramoflor, Gramoflor GmbH & Co. KG, Vechta, Germany) that was not sterilized in order to allow the growth of a natural microbial communities. The potted cuttings were irrigated daily with tap water and incubated in a growth chamber at 25/20 °C day/night temperature, 16 h photoperiod, under 500 μmol s<sup>-1</sup> m<sup>-2</sup> (Lumilux cool white L30W/840 and Fluora L30W/77, Osram, Germany) and 70% relative humidity. To avoid the beginning of vegetative growth and to facilitate development of inflorescences, leaves were removed daily according to Lebon *et al.* (2005). More than 400 fruiting cuttings were planted as only 25–75% of potted plants developed inflorescences.

### Plant inoculation and growth conditions

Fruiting cuttings with a root system and fully developed infructescence were inoculated with either 5 × 10<sup>7</sup> or 5 × 10<sup>8</sup> CFU of PsJN::*gfp2x* g<sup>-1</sup> of soil at stage 57 of the BBCH scale (last step of preflower bud development before anthesis, according to Meier, 2001).

### Preparation of plant samples for bacterial enumeration

The rhizoplane and endophytic colonization of roots and inflorescences by the *gfp*-marked strain were determined by plate counting of bacterial colonies under a fluorescence stereomicroscope (model MZ FLIII; Leica, Heerbrugg, Switzerland) equipped with a GFP 1 filter (Leica, Switzerland) after inoculation with 5 × 10<sup>7</sup> or 5 × 10<sup>8</sup> CFU g<sup>-1</sup> soil. Roots, stalks, and reproductive organs, such as preflower buds, flowers or young berries, were sampled at different time intervals (Table 1). Five replicates of 10 independent plating assays were used to determine the average colonization patterns. The base level of detection was between 0 and -1 log<sub>10</sub> CFU g<sup>-1</sup> fresh weight for each plant organ.

**Table 1.** Colonization of grapevine fruiting cuttings by *Burkholderia phytofirmans* strain PsJN after inoculation with  $5 \times 10^7$  CFU of PsJN::*gfp2x* g<sup>-1</sup> of soil

Weeks postinoculation	Rhizoplane	Endorhiza	Stalk	Reproductive organs
0	6.99 ± 0.43	ND	ND	ND (preflower buds)
1	5.57 ± 0.33	2.15 ± 0.93	ND	ND (flowers)
4	5.45 ± 0.36	2.26 ± 0.54	ND	ND (young berries)
5	5.42 ± 0.28	2.18 ± 0.61	1.08 ± 0.35	0.85 ± 0.65 (young berries)
6	5.54 ± 0.42	2.23 ± 0.45	0.99 ± 0.54	1.40 ± 0.60 (young berries)

Log numbers of plant populations of PsJN were determined on the rhizoplane, in the endorhiza as well as inside grape inflorescence stalks and reproductive organs. ND, not detectable. Base limit of detection was between 0 and  $-1 \log_{10}$  CFU g<sup>-1</sup> FW.

### Rhizoplane colonization

Root surface colonization by PsJN was determined at different times after soil inoculation. Plants were removed from the potting soil and roots were rinsed (four times) with sterile distilled water. Root samples (1 g each) were ground in a mortar and pestle, containing 1 mL PBS. The homogenates were serially diluted in microtitre plates, and 100 µL aliquots were plated on solid LB medium containing cycloheximide (100 µg mL<sup>-1</sup>) to inhibit fungal growth and kanamycin (100 µg mL<sup>-1</sup>; PsJN::*gfp2x* is resistant to kanamycin). Bacterial colonies were then counted under UV light 3 days after incubation at 30 °C. Rhizoplane colonization by the *gfp*-marked strain was determined by subtracting bacterial counts after surface sterilization from the total *gfp*-tagged bacteria enumerated without surface sterilization, as described in Compant *et al.* (2005b).

### Endophytic colonization

To determine endophytic populations of the *gfp*-marked strain, roots, grape stalks, preflower buds and/or flowers or young berries were surface sterilized with 70% ethanol for 5 min, followed by a bath of 1% commercial bleach and 0.01% Tween 20 for 1 min. The samples were then washed three times with distilled water (1 min each) and ground and handled as described above. Bacterial colonies were counted under UV light and confirmed under a fluorescence stereomicroscope, 3–5 days after incubation at 30 °C.

### Evaluation of surface-sterilization methods

To determine the efficacy of the surface-sterilization procedure, roots were detached from five plants 1 week after soil inoculation with  $5 \times 10^8$  CFU g<sup>-1</sup> of soil, surface-sterilized as described and were observed them at  $\times 200$  and  $\times 1000$  under 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). Surface-sterilized roots were placed

on LB plates amended with 100 µg mL<sup>-1</sup> kanamycin and 100 µg mL<sup>-1</sup> cycloheximide for 1 min before crushing. Additionally, 100-µL aliquots of the last bath of surface-sterilization were plated on LB plates amended with 100 µg mL<sup>-1</sup> of each antibiotic and bacterial colonies were counted to be sure of the surface sterilization procedure.

To confirm systemic colonization from the rhizosphere to inflorescence tissues, 10 plants were sampled 6 weeks after soil inoculation with  $5 \times 10^8$  CFU g<sup>-1</sup> soil. Inflorescences were used without surface sterilization and ground in a mortar and pestle containing 1 mL PBS. The homogenates (100 µL aliquots) were plated on solid LB medium containing 100 µg mL<sup>-1</sup> cycloheximide and 100 µg mL<sup>-1</sup> kanamycin. Then, bacterial colonies were counted under UV light and confirmed under a fluorescence stereomicroscope, 3 days after incubation at 30 °C.

### Microscopy of epiphytic and endophytic colonization by PsJN

The epiphytic and endophytic colonization of roots, stalks and reproductive organs such as preflower buds and/or flowers or young berries by PsJN::*gfp2x* was monitored at different time intervals (as presented in Table 2) after soil inoculation with  $5 \times 10^8$  CFU g<sup>-1</sup> soil. The different parts were sampled separately and 10 longitudinal sections were made on independent samples of different plant parts taken from 20–60 plants (roots or inflorescences) before examination under an epifluorescence and/or a confocal microscope (MRC 1024; Biorad, Hercules, CA) equipped with a  $\times 63$  PlanApo objective.

### Statistical analysis

Microscopic observations were made on samples taken from independent experiments. Population density data (CFU) from experiments on fruiting cuttings were pooled over 3 years. These data were submitted to logarithmic transformation (Loper *et al.*, 1984) and statistically analysed using Student's *t* test.

**Table 2.** Colonization of grapevine fruiting cuttings by *Burkholderia phytofirmans* strain PsJN after inoculation with  $5 \times 10^8$  CFU of PsJN::*gfp2x*  $g^{-1}$  of soil

Weeks postinoculation	Rhizoplane	Endorhiza	Stalk	Reproductive organs
0	$7.41 \pm 0.01$	ND	ND	ND (preflower buds)
1	$5.72 \pm 0.37$	$2.3 \pm 1.0$	ND	ND (flowers)
4	$5.62 \pm 0.52$	$2.34 \pm 0.74$	ND	ND (young berries)
5	$5.63 \pm 0.45$	$2.35 \pm 0.65$	$1.56 \pm 0.41$	$1.36 \pm 0.32$ (young berries)
6	$5.77 \pm 0.51$	$2.4 \pm 0.62$	$1.66 \pm 0.22$	$1.89 \pm 0.31$ (young berries)

Log numbers of plant populations of PsJN were determined on the rhizoplane, in the endorhiza and inside grape stalks and reproductive organs as in Table 1. ND, not detectable. Base limit of detection was between 0 and  $-1 \log_{10}$  CFU  $g^{-1}$  FW.

## Results

### Density populations on the rhizoplane, in the endorhiza and inside inflorescences following soil inoculation

The rhizoplane of fruiting cuttings was colonized by PsJN::*gfp2x* cells immediately after soil inoculation, reaching  $6.99 \pm 0.43 \log_{10}$  CFU  $g^{-1}$  FW of root tissue within 0–15 h after inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil (Table 1) and  $7.41 \log_{10}$  CFU  $g^{-1}$  FW with  $5 \times 10^8$  CFU  $g^{-1}$  of soil (Table 2). The bacterial population subsequently stabilized at  $5.57 \pm 0.33$  and  $5.72 \pm 0.37 \log_{10}$  CFU  $g^{-1}$  FW 1 week after soil inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil (Table 1) or  $5 \times 10^8$  CFU  $g^{-1}$  of soil (Table 2), respectively. Then, these bacterial titre numbers remained almost unchanged over the 6-week duration of this study (Tables 1 and 2).

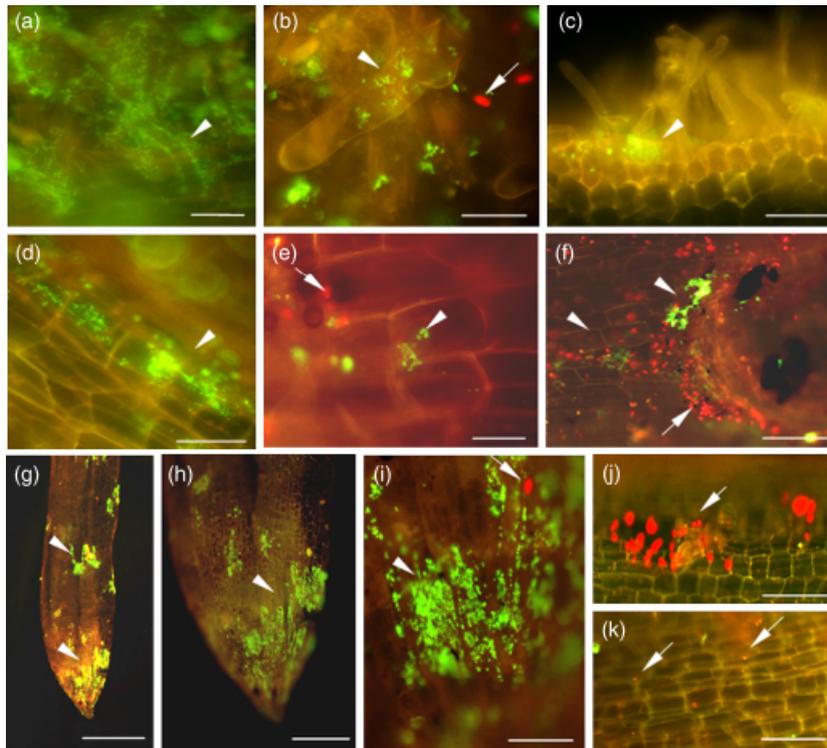
Following rhizoplane colonization, PsJN::*gfp2x* cells appeared in the endorhiza of all tested plants 2–3 days after soil inoculation and reached their highest level 1 week after soil inoculation ( $2.15 \pm 0.93 \log_{10}$  CFU  $g^{-1}$  FW after inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil and  $2.3 \pm 1.00 \log_{10}$  CFU  $g^{-1}$  FW with  $5 \times 10^8$  CFU  $g^{-1}$  of soil; Tables 1 and 2). Bacterial titre numbers stabilized at  $2.23 \pm 0.45 \log_{10}$  CFU  $g^{-1}$  FW after inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil and  $2.4 \pm 0.62 \log_{10}$  CFU  $g^{-1}$  FW with  $5 \times 10^8$  CFU  $g^{-1}$  of soil 2–6 weeks postinoculation (Tables 1 and 2).

In addition to these root analyses, endophytic subpopulation densities of strain PsJN in inflorescences were determined using plate counting. After soil inoculation with  $5 \times 10^7$  or  $5 \times 10^8$  CFU  $g^{-1}$  of soil, PsJN::*gfp2x* were not found in grape stalks, preflower buds and flowers during the first 2 weeks after inoculation (Tables 1 and 2). However, they were detected in grape inflorescence stalks and in young berries after 5 and 6 weeks postinoculation. Nevertheless, bacterial concentrations in these organs were low with  $1.08 \pm 0.35 \log_{10}$  CFU  $g^{-1}$  FW in grape stalks and  $0.85 \pm 0.65 \log_{10}$  CFU  $g^{-1}$  FW in young berries following inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil, and with  $1.56 \pm 0.41$  and  $1.36 \pm 0.32 \log_{10}$  CFU  $g^{-1}$  FW in stalks and berries, respectively, 5 weeks after  $5 \times 10^8$  CFU  $g^{-1}$  of soil (Tables 1 and 2).

Six weeks postinoculation, the densities of strain PsJN remained constant in inflorescence organs:  $0.99 \pm 0.54 \log_{10}$  CFU  $g^{-1}$  FW in grape stalks and  $1.40 \pm 0.60 \log_{10}$  CFU  $g^{-1}$  FW in young berries following soil inoculation with  $5 \times 10^7$  CFU  $g^{-1}$  of soil (Table 1),  $1.66 \pm 0.22 \log_{10}$  CFU  $g^{-1}$  FW in grape stalks and  $1.89 \pm 0.31 \log_{10}$  CFU  $g^{-1}$  FW in berries after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil (Table 2). However, even if these data represent the average colonization, a significant population ( $2 \log_{10}$  CFU  $g^{-1}$  FW) in stalks and berries of individual cuttings was only detected after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  but not with  $5 \times 10^7$  CFU  $g^{-1}$  of soil. Consequently, some cuttings yielded up to 2.12 and  $1.60 \log_{10}$  CFU  $g^{-1}$  FW in grape stalks and 1.90 and  $2.15 \log_{10}$  CFU  $g^{-1}$  FW in berries, 5 and 6 week postinoculation, respectively, after soil inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. Additionally, not all grape inflorescence stalks and berries from bacterized plants were colonized by strain PsJN: only 20–60% of grape stalks and berries, respectively, hosted the inoculated strain after  $5 \times 10^7$  CFU  $g^{-1}$  of soil and 13–60% of grape stalks and berries yielded strain PsJN tagged with the *gfp* gene after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil.

### Microscopic observations of the rhizoplane population of *B. phytofirmans* strain PsJN

During the first 4 weeks postinoculation, microscopic observations of the rhizoplane demonstrated that strain PsJN tagged with the *gfp* gene colonized the root surface after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. Strain PsJN was found mainly in the root hair zone, lateral root emergence sites and root tips. However, differences in the bacterial densities of *gfp*-tagged cells were observed in these different areas. The root hair zone was more highly colonized than other root zones with PsJN::*gfp2x* (Fig. 1a–c). Other rhizodermal cells were colonized differentially: some rhizoplane cells were filled with bacteria (Fig. 1d) whereas *gfp*-tagged cells were found closely attached to cell walls in other cells (Fig. 1d–e). In addition, some *gfp*-tagged cells were visualized in lateral root emergence sites and root tips (Fig. 1f–i). The root surface colonization pattern of strain



**Fig. 1.** Images under an epifluorescence microscope of roots of grapevine fruiting cuttings inoculated with PsJN::*gfp2x* 1–4 weeks after soil inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. *Gfp*-tagged bacteria (arrows) were visualized at the root hair zone (a–c) colonized root hairs (a–c), on other rhizodermal cells (d and e), at lateral root emergence sites (f) and at the root tip (g–i). A natural epiphytic microbial communities was also detected on the root surface of inoculated plants (arrows in b, e, f and i) as well as on the roots of control plants (arrows in j and k). Similar rhizoplane colonizations by strain PsJN were found from 1 to 4 weeks postinoculation. Scale bars: (a) 100  $\mu m$ , (b) 30  $\mu m$ , (c) 75  $\mu m$ , (d and e) 30  $\mu m$ , (f) 100  $\mu m$ , (g) 1 mm, (h) 500  $\mu m$ , (i) 250  $\mu m$  and (j and k) 100  $\mu m$ .

PsJN was also observed with native microbial communities. This included yellow-, green- (Fig. 1e and f) and/or blue-fluorescing bacteria that formed slight halos (data not shown) that were detected in control plants (Fig. 1k). Red-fluorescing algae were also detected under blue light in the rhizosphere of bacterized (Fig. 1b, f and i) or control plants (Fig. 1j) due to the use of nonsterile conditions and tap water. Some fungi (yeasts and mould), and even worms were detected on the root surface of these plants, in both control and inoculated plants (data not shown), indicating the diverse array of microorganisms in the rhizosphere.

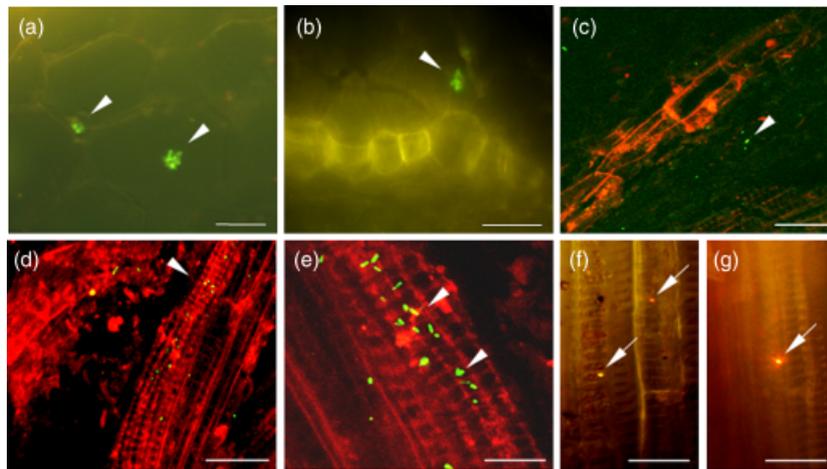
### Visualization of endorhiza colonization by *B. phytofirmans* strain PsJN

Following rhizoplane colonization, PsJN::*gfp2x* was detected inside the root cortex as well as in the central cylinder, both 1 and 4 weeks after soil inoculation (Fig. 2). *Gfp*-tagged bacteria were subsequently visualized in the exodermis, other cortical cell layers (Fig. 2a and b) and passing through the endodermis *via* an inter- and/or an intracellular pathway (Fig. 2c). Although the pericycle is expected to be the first cell layer within the central cylinder, it was not clearly defined under epifluorescence, making visualization of the bacterium in this tissue difficult. However, in the central cylinder, *gfp*-tagged bacteria were detected inside xylem vessels 4 weeks after inoculation (Fig. 2d and e). As with the root surface, other autofluorescent microorganisms

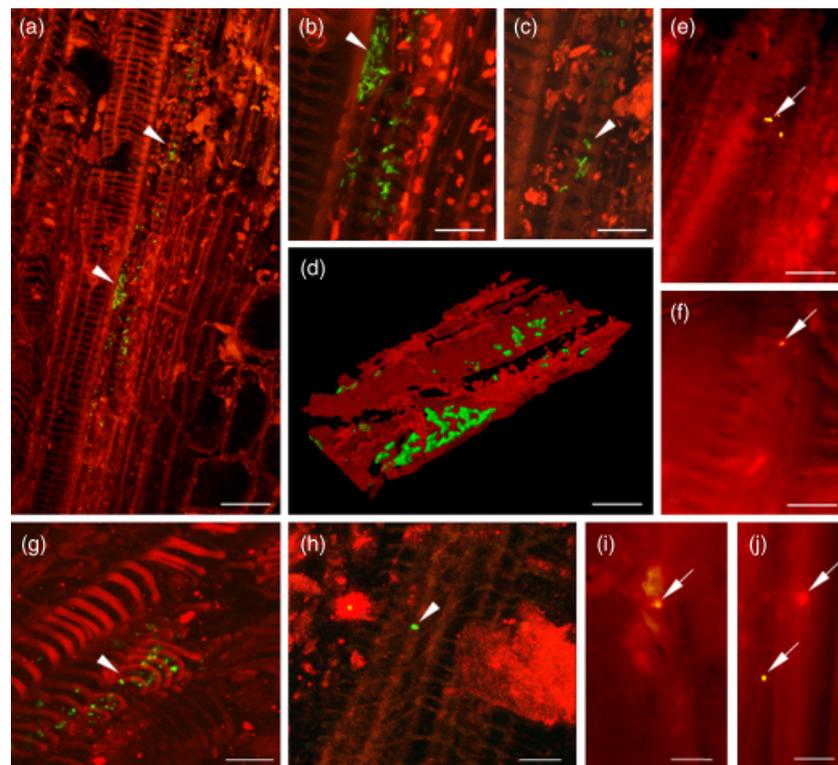
(mainly orange fluorescing bacteria) were observed in the endorhiza. These microorganisms were detected inside all root internal tissues as well as in xylem vessels of both control and bacterized plants (Fig. 2f and g).

### Endophytic subpopulations of *B. phytofirmans* strain PsJN in grape inflorescence stalks

Following endorhizal colonization, PsJN::*gfp2x* was detected inside xylem vessels of grape inflorescence stalks through epifluorescence or confocal microscopy 5 and 6 weeks postinoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. However, in some fruiting cuttings, *gfp*-tagged cells of PsJN were not present in the grape inflorescence stalk although many longitudinal sections were examined. Inflorescence stalk colonization by strain PsJN was visualized in only 10–60% of bacterized fruiting cuttings. In these plants harbouring *gfp*-tagged cells, PsJN::*gfp2x* was visualized within xylem vessels, exclusively in the lumen of the vascular bundles (Fig. 3a–d). However, xylem vessels were not replete with PsJN::*gfp2x*; only single or a few bacteria were detected inside the vascular bundles (Fig. 3a–d), mainly packaged within xylem elements (Fig. 3b and 3c). Again, a natural microbial communities was observed in the xylem vessels of stalks of bacterized plants (Fig. 3e) as well as control plants (Fig. 3f). This included orange (Fig. 3e and f) and also blue autofluorescent microorganisms (data not shown).



**Fig. 2.** Images from an epifluorescence and/or a confocal microscope of roots of grapevine fruiting cuttings inoculated with PsJN::*gfp2x* 1–4 weeks after soil inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil showing strain PsJN in the endorhiza. *Gfp*-tagged bacteria (arrow heads) were visualized in the cortex (a) near the endodermis barrier (b), passing this barrier (c) and colonizing xylem vessels (d–e). Other fluorescent microorganisms were visualized inside xylem vessels of inoculated cuttings (arrows in f) as well as of nontreated plants (arrows in g). Similar endorhiza colonizations by strain PsJN were visualized from 1 to 4 weeks postinoculation. Scale bars: (a and b) 30  $\mu m$ , (c) 50  $\mu m$ , (d) 30  $\mu m$ , (e) 15  $\mu m$  and (f and g) 20  $\mu m$ .



**Fig. 3.** Images under an epifluorescence and/or a confocal microscope of grape inflorescence organs of fruiting cuttings inoculated with PsJN::*gfp2x*, 5 weeks after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. PsJN cells tagged with the *gfp* gene (arrow heads) were detected inside xylem vessels of a grape stalk (a and c). (d) 3-D reconstruction of *gfp*-tagged cells inside a xylem vessel of a grape stalk showing PsJN::*gfp2x* in the lumen of this vascular bundle. Inflorescence stalks can contain other fluorescent microorganisms (e) as in control plants (f). PsJN::*gfp2x* (arrow heads) was also detected inside xylem vessels of pedicel (g) and young berries (h), together with other fluorescent microorganisms (arrows in i) similar to control plants (arrows in j). Scale bars: (a) 30  $\mu m$ , (b–e) 15  $\mu m$ , (f) 10  $\mu m$ , (g) 15  $\mu m$ , (h) 5  $\mu m$  and (i and j) 20  $\mu m$ .

### Endophytic colonization of grape berries

Strain PsJN has been detected as an endophyte within young berries following soil inoculation. This endophytic colonization of the fruit by *B. phytofirmans* strain PsJN followed the

same pattern shown in grape stalks. Strain PsJN was thus detected inside grape berries in 10–60% of fruiting cuttings in each of the six independent repetitions of this study after inoculation with  $5 \times 10^8$  CFU  $g^{-1}$  of soil. As described for grape stalk colonization, *gfp*-tagged cells were found

exclusively inside xylem vessels of pedicel and/or berries 5 and 6 weeks postinoculation. However, PsJN::gfp2x was not found inside all berries even when four to five longitudinal sections were examined for pedicels and berries. Some plants that demonstrated PsJN colonization in grape stalks did not host the inoculant strain in pedicels or in young berries. In addition, some plants hosted *gfp*-tagged cells in pedicels and young berries, despite their absence in grape stalks. Even in infructescences, only some of the young berries and their pedicels were colonized by strain PsJN. Some PsJN::gfp2x bacteria were thus detected in the pedicel and were visualized as singlets, doublets or clusters of a few cells inside some xylem elements (Fig. 3g). In some young berries, *gfp*-tagged cells were also visualized within xylem vessels (peripheral or central vascular elements), either as singlets (Fig. 3h) or doublets (data not shown). These *gfp*-tagged bacteria were visualized in the presence of a natural microbial communities, including mainly orange fluorescent microorganisms (Fig. 3i) as well as yellow and blue microorganisms (data not shown) that were also detected in control plants (Fig. 3j). This natural microbial communities was abundant inside young berries of some cuttings where *gfp*-tagged cells were not visualized. Strong defense reactions as well as necrotic tissues have been associated with some of these microorganisms (data not shown).

## Discussion

The present study clearly demonstrates the intimate association between *B. phytofirmans* strain PsJN and *V. vinifera* using fruiting cuttings grown in nonsterile soil, with emphasis on colonization of inflorescence organs. Following soil inoculation, grapevine fruiting cutting colonization by *B. phytofirmans* strain PsJN proceeded in distinct steps. First, the root surface was rapidly colonized in a nonuniform manner. The highest concentration occurred in the root hair zone compared with the emerging lateral roots and root tips. This colonization pattern was comparable to that observed under a gnotobiotic system (Compant *et al.*, 2005b). However, *in vitro* grapevine plantlets do not develop root hairs when grown on agar (unpublished results). Similar rhizoplane colonization patterns have been described for other plant–bacterial interactions (Hansen *et al.*, 1997; Gamalero *et al.*, 2004) where rhizosphere bacteria colonize exudate-rich zones such as root hairs, emerging lateral roots or root tips that provide them with nutrients (Walker *et al.*, 2003; Bais *et al.*, 2006). However, rhizoplane colonization by strain PsJN has been monitored in the present study with plants grown in nonsterile soil and thus in the presence of a natural microbial communities that may have competed for nutrients with the inoculated strain. Accordingly, this epiphytic population of strain PsJN declined 1 week after inoculation. However, strain PsJN survived in the presence of these

others microorganisms and continued to colonize the root surface, demonstrating its rhizosphere competence.

Following root surface colonization, strain PsJN colonized the endorhiza environment. To enter the root system, endophytic PGPR may secrete cell wall-degrading enzymes (James *et al.*, 2001; James *et al.*, 2002; Lodewyckx *et al.*, 2002; Compant *et al.*, 2005a; Rosenblueth & Martínez-Romero, 2006). This has been demonstrated for *B. phytofirmans* strain PsJN that secretes endoglucanase and endopolygalacturonase (Compant *et al.*, 2005b), as well as endo- $\beta$ -D-cellobiosidase and exo- $\beta$ -1,4-glucanase (unpublished results), which may facilitate its entrance into the endorhiza. Following its entry inside roots, strain PsJN progressed from the rhizodermis to the exodermis and then to the cortical cell layers intercellularly. Then, strain PsJN was detected passing the barrier of the endodermis. Using a gnotobiotic biosystem the authors have reported previously on the colonization of the peripheral cylinder by strain PsJN and the endodermis barrier can be broken following the progression of strain PsJN in the endorhiza (Compant *et al.*, 2005b). However, in this study, performed under nonsterile conditions, passage of strain PsJN through the endodermis could also be attributed to breaches opened by others microorganisms observed in nonsterilized fruiting cuttings. Alternatively, the fact that the endodermis was broken can also be attributed to the emergence of secondary roots (Hallmann, 2001). However, passage of strain PsJN through the endodermis was detected even without the presence of secondary root growth. Following this progression, strain PsJN has been detected inside the central cylinder mainly inside xylem vessels and even in the presence of other microorganisms. This colonization of xylem vessels by strain PsJN was similar to the authors previous study conducted under gnotobiotic conditions (Compant *et al.*, 2005b), demonstrating the possibility to colonize these vascular bundles even under nonsterile conditions.

Xylem vessel colonization at the root level by strain PsJN allowed it to spread inside plants. Following colonization of the endorhiza, strain PsJN was subsequently found in the vascular bundles of grape inflorescence stalks, pedicels and young berries. However, strain PsJN was not found in xylem elements of preflower buds and of flowers of the same plants. This can be attributed to the slow systemic spread of strain PsJN, requiring 5 weeks to reach the inflorescence following soil inoculation. Endophytic colonization of aerial plant parts by strain PsJN required more time compared with the *in vitro* system in a previous study, in which only 72 h were required for the bacterium to spread systemically inside plants. This difference could be attributed to the difference of the physiology between the two systems. The xylem network of an *in vitro* plantlet is considerably smaller, and only a primary xylem must occur in *in vitro* plantlets in comparison with cuttings as suggested by Thorne *et al.*

(2006). This can explain the differences in colonization between the two models, together with the fact that cell-wall degrading enzymes activities of strain PsJN are probably required to allow passage from one vessel element to another and thus bacterial progression inside small plantlets and cuttings is different. However, Thorne *et al.* (2006) and Chatelet *et al.* (2006) have recently demonstrated that xylem structures and connectivity of grapevine provide passive mechanisms for a bacterial spread inside plants without the need for cell-wall-degrading enzyme secretion. This also explains why the nonpathogenic endophyte *B. phytofirmans* strain PsJN could colonize aerial plant parts even under *in vivo* conditions without harming its host. This does not explain, however, all the differences in the colonization processes between the two models. In fruiting cuttings, some xylem elements may be occluded, restricting passage between xylem elements through pit membrane degradation, thereby lengthening the time required for strain PsJN to reach the aerial plant parts. However, the present study demonstrated that some cuttings did not yield strain PsJN in the inflorescences. Thus, problems with xylem connectivity can be envisaged. Some microorganisms from the natural microbial communities detected inside xylem vessels may be neutral, beneficial or even pathogenic strains that impact the physiology of xylem in cuttings. Alternatively, competition between strain PsJN and other microorganisms in the roots, in the endorhiza or during xylem colonization, may have delayed systemic spread inside plants and contributed to differences between the two models.

In this study, it was demonstrated that the endophytic populations of strain PsJN were low ( $< 2 \log_{10}$  CFU g<sup>-1</sup> FW) in inflorescence tissues after soil inoculation with  $5 \times 10^7$  CFU g<sup>-1</sup>, practically nonexistent from a biological viewpoint. Similar results were obtained after soil inoculation with  $5 \times 10^8$  CFU g<sup>-1</sup>. However, some cuttings contained more than  $2 \log_{10}$  CFU g<sup>-1</sup> FW and berries of fruiting cuttings yielded up to  $2.15 \log_{10}$  CFU g<sup>-1</sup> FW after soil inoculation with  $5 \times 10^8$  CFU g<sup>-1</sup>. This demonstrates that the berries of grapevine can be colonized by the bacterium after inoculation at a high density. However, the inconsistency of colonized grape inflorescences and the low density of endophytic populations support previous hypotheses of plant colonization by endophytic bacteria: i.e., endophytes are low or absent in generative organs like flowers and fruits (Hallmann, 2001). With *gfp* molecular markers, the colonization of inflorescence parts by endophytes can be monitored, even when a low endophytic colonization occurs. Strain PsJN tagged with the *gfp* gene was visualized inside inflorescences, in particular inside the lumen of xylem vessels of grape stalks, pedicels and young berries. This indicates bacterial progression from xylem vessels of the root system to the grape inflorescence stalks, pedicels and then to the berries. Consequently, stem stalks

may serve only as a pathway for bacteria to reach berries. However, this bacterial progression may sometimes be blocked inside plants. This may be related to the xylem structure or the natural microbial communities as discussed above. However, this microbial communities which may have derived from the rhizosphere, is unlikely to have a strong impact on strain PsJN colonization, as in parallel to this study, the same average numbers of endophytic subpopulations were detected using sterilized soil and surfaces of fruiting cuttings (unpublished results). Plant defence responses can influence and regulate endophytic colonization (Iniguez *et al.*, 2005; Miché *et al.*, 2006) in addition to their potential to protect the plant against phytopathogen invasion and disease (Maurhofer *et al.*, 1994; Wang *et al.*, 2005). Although this assumption has to be tested in grapevine–strain PsJN association, at both root and inflorescence levels, and against invasion of inflorescences by a pathogen (S. Compant *et al.*, in preparation), in the present study, it was demonstrated that strain PsJN can colonize roots epiphytically and thrive as an endophyte in the endorhiza, inside grape inflorescence stalks and subsequently inside young berries. Further work will be needed to determine whether strain PsJN persists as an endophyte during berry ripening and maturity.

Although endophytic subpopulations of strain PsJN are low in inflorescences of grapevine plants, they can be monitored using a *gfp* molecular marker. This technique and others have been used to monitor epi- and/or endophytic colonization of beneficial bacteria inside different plant hosts (Gamalero *et al.*, 2004; Chi *et al.*, 2005; Götz *et al.*, 2006). However, studies on colonization by rhizosphere-derived bacteria have mainly focused on vegetative plant parts such as roots, stems and leaves. In this study, additional characteristics of plant colonization by PGPR have been elucidated through colonization of grapevine inflorescences by strain PsJN. Such knowledge will lead to a better understanding of plant–endophytic bacteria interactions, in particular in cultivated plants with which some beneficial strains are currently or will be used for agricultural improvement.

Data from the present study relate to an old debate on plant colonization by microorganisms. It has been strongly argued by some plant anatomists that only pathogens can be transported via the xylem vessel lumen and that endophytes colonize only nonfunctional vessels or move through the apoplast to reach aerial plant parts (McCully, 2001). Although this has been argued for monocotyledons, it should also apply to dicotyledons. According to this hypothesis, the migration of strain PsJN from the endorhiza to inflorescence organs of grapevine would use nonfunctional vessels or strain PsJN should in fact be considered a phytopathogen. However, as strain PsJN can promote plant growth, and protect its host against biotic and abiotic stress,

it is a PGPR and unlikely to be a pathogen. Furthermore, xylem vessels are open conduits that allow bacterial progression within plants (Chatelet *et al.*, 2006; Thorne *et al.*, 2006) and strain PsJN was detected in the lumen of xylem vessels. The present study provides a substantial contribution to this debate, with particular relevance to inflorescence colonization by an endophytic PGPR, on a woody plant for which the most valuable part is the infructescence.

## Acknowledgements

This work was supported by a Ph.D. grant from Europól-Agro (Reims, France). The authors are grateful to Dr Richard E. Veilleux (Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg) and Dr Guy Abell (Department of Bioresources, Austrian Research Centers GmbH, Seibersdorf, Austria) for critically reviewing the manuscript.

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