

Chapter 2

Rhizobial Biofertilizers for Ornamental Plants

Esther Menéndez, Rocío Escribano-Viana, José D. Flores-Félix,
Pedro F. Mateos and Raúl Rivas

Abstract Plant growth-promoting rhizobacteria (PGPR) are one of the most important groups of rhizospheric microorganisms, such as free-living bacteria, endophytes, and endosymbiotic bacteria. PGPRs colonize plant roots, conferring several benefits to the plants through different mechanisms. Despite its classical involvement in root nodule symbiosis with legumes, recent studies revealed the beneficial role of the genus *Rhizobium* as PGPR for several non-legume crops, supporting its inclusion in biofertilization schemes. However, studies based in the application of PGPRs as inoculants for ornamental crops are limited. Ornamental crops have a high economic importance for several regions, being Spain among the major producers of cut flowers. Due to their importance, we selected *Dianthus caryophyllus* (carnation) and a rhizobial strain for the evaluation of the PGPR effects in this plant. Selected strain was identified as *Rhizobium* sp. and presented PGPR mechanisms; phosphate solubilization, siderophores production, and IAA and precursors biosynthesis were positive. In vitro plant assays showed an increase in carnation development in early steps of plant development, when plants were inoculated respect to uninoculated plants. Moreover, effective root colonization was shown. Interestingly, an increase in root hairs redirections was observed in inoculated plants. To the best of our knowledge, this is the first study reporting the effects of *Rhizobium* in *D. caryophyllus*, showing effective root colonization and an improvement in early stages of plant development.

Keywords *Dianthus caryophyllus* · *Rhizobium* · Root colonization · Plant growth promotion · Biofertilizer

E. Menéndez (✉) · R. Escribano-Viana · J.D. Flores-Félix · P.F. Mateos · R. Rivas
Department of Microbiology and Genetics, University of Salamanca, Salamanca, Spain
e-mail: esthermenendez@usal.es

P.F. Mateos · R. Rivas
Associated Unit I+D University of Salamanca—CSIC ‘Plant-Microbe Interactions Group’,
Salamanca, Spain

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2.1 Introduction

Many rhizobacteria can promote plant growth through a wide range of mechanisms. These rhizobacteria are commonly called plant growth-promoting rhizobacteria (PGPR). Despite its classic role in legume symbiosis, the genus *Rhizobium* has increased its importance as PGPR, showing the ability of rhizobial strains to promote plant growth and development in several non-legume crops, such as lettuce, carrots, tomato, or pepper (Santillana et al. 2005; García-Fraile et al. 2012; Flores-Félix et al. 2013), and also increase yields in cereal crops, such as rice and maize (Yanni et al. 2001; Gutiérrez-Zamora and Martínez-Romero 2001).

Interestingly, some studies suggest that rhizobacteria can be implicated in the increase of other interesting compounds, which are beneficial not only for plant development but also for human health. For example, *Phyllobacterium endophyticum* is involved in the increase of vitamin C in strawberries (Flores-Felix et al. 2015). Moreover, other studies showed that *Rhizobium* species might promote the ripeness stage in fruits of inoculated pepper plants (Silva et al. 2014a, b).

Nevertheless, the application of rhizobial strains in other plants, such as ornamental plants, is still not common. Only a few examples of application of PGPR bacteria in ornamental plants have been described, such as the effects of some members of the genus *Pseudomonas* and *Bacillus* in *Euphorbia*, *Dahlia*, *Dianthus*, *Chrysanthemum*, and *Pelargonium* (Göre and Altin 2006; Sharma and Kaur 2010; Zulueta-Rodriguez et al. 2014) and of some *Azotobacter* and *Azospirillum* strains in *Gladiolus* or *Matthiola* (Srivastava and Govil 2007; Eid et al. 2009). However, there are no evidences of studies involving the application of Rhizobia in ornamental plants.

For that reason in this study, we will show the effects of a rhizobial strain in *Dianthus caryophyllus* (carnation), one of the most important ornamental plant in Spain, being the second cut flower in economical importance, after roses.

2.2 Materials and Methods

2.2.1 Bacterial Strain and Plant Material

Strain PEPV13 was isolated from an effective nodule of *Phaseolus vulgaris*, growing in a soil from Salamanca (NW, Spain) as described (Vincent 1970). As plant material, commercial variety of red carnation (*D. caryophyllus*, Vilmorin[®]) was selected among ornamentals for this study.

2.2.2 16S rRNA Gene Identification of Bacterial Strain

DNA extraction, PCR reactions, and PCR conditions were performed according to REDExtract-N-Amp Plant Kit (Sigma), with minor modifications. For 16S rRNA

gene amplification, primers 27F and 1522R were used (Rivas et al. 2002). PCR product was purified using GeneJet Purification kit (Thermo-Fisher) according to the manufacturer's specifications. Partial sequencing was performed on an ABI PRISM sequencer (Applied Biosystem®, EEUU) using a "Dye Terminator Cycle Sequencing Ready Reaction Kit" (Sequencing Service, University of Salamanca). Sequence was compared with type strain by using EzBiocloud Server (Chun et al. 2007).

2.2.3 Determination of Plant Growth Promotion Mechanisms (PGPM)

To determine PEPV13 strain PGPR abilities, three mechanisms were analyzed: (i) siderophores production, (ii) indol acetic acid (IAA) biosynthesis, and (iii) phosphate solubilization. Siderophores production was tested using M9-CAS agar medium (Alexander and Zuberer 1991). IAA and precursors biosynthesis was determined using modified JMM medium (O'Hara et al. 1989; Khalid et al. 2004), measuring the absorbance at 550 nm after the addition of Salkowski reagent and incubation during 15 min. Pikovskaya medium (Pikovskaya 1948), containing CaHPO_4 as source of inorganic P, was used for phosphate solubilization determination.

2.2.4 Plant Assays in Axenic Conditions

Red carnation seeds were surface-disinfected with 70 % ethanol for 30 s and 2 % NaClO for 5 min. Subsequently, several washes with sterile distilled water were performed. Seeds were placed in 1 % inverted agar plates and incubated in dark for four days at room temperature. Then, seedlings were transferred to 1 % agar square plates overlaid with sterile filter paper (five seedlings per plate). Seedlings were inoculated with 250 μl plant⁻¹ of a bacterial suspension with a turbidity of 6 in McFarland Standards (1.5×10^6 CFU ml⁻¹). Plates were incubated vertically in a growth chamber and observed at 5, 7, and 12 days post-inoculation. An uninoculated treatment was included as negative control. The root length was measured.

2.2.5 Colonization Assays

For colonization events observation, a GFP-tagged derivative of PEPV13 strain was obtained by biparental mating with *Escherichia coli* S17.1, carrying pHC60 plasmid (Cheng and Walker 1998) as described in García-Fraile et al. (2012). Tetracycline was added to the media when required (10 $\mu\text{g/mL}$).

The colonization assays were performed in axenic conditions, set similarly to the assays described in Sect. 2.2.4. Colonization events and root hair deformations were observed at 5, 7, and 12 days post-inoculation under NIKON Eclipse 80i epifluorescence microscope.

2.2.6 *Glasshouse Assays*

For the evaluation of plant development, microcosms assays were performed under glasshouse conditions. Red carnation seeds were surface-disinfected as described in Sect. 2.2.4. Then, four-day-old seedlings were transferred to pots (1 seedling per pot) with a mix of non-sterile peat:vermiculite (3:1) soil. Seven days after the sow, each plant was inoculated with 5 ml of a PEPV13 strain suspension with a turbidity of 6 in McFarland Standards (1.5×10^6 CFU ml⁻¹). Uninoculated plants were also included as negative control. Red carnation plants were harvested 73 days post-inoculation. Shoot and root length and weight (fresh weight and dry weight) were measured in each case.

2.2.7 *Statistical Analysis*

Data obtained from each experiment were analyzed with one-way ANOVA using a post hoc test (Fisher's PSLD $p \geq 0.05$). Statview 5.0 software (SAS Institute Inc.) was used for these analyses.

2.3 Results and Discussion

2.3.1 *Identification of PEPV13 Strain*

We obtained a partial 16S rRNA gene sequence of the strain PEPV13 (up to 400 nt). This strain was classified into the genus *Rhizobium*, showing 100 % of identity with two *Rhizobium* type strains, *R. leguminosarum* and *R. laguerreae*. Further sequencing reactions will be necessary in order to confirm the species; however, strain PEPV13, isolated from common bean nodules, belongs to the genus *Rhizobium*, which is considered a safe microorganism for agriculture and human health (García-Fraile et al. 2012).

2.3.2 In Vitro Plant Growth Promotion Assays

In first place, we analyzed the in vitro PGPR ability of PEPV13 strain. This strain was able to produce siderophores, as revealed by the appearance of a yellow orange halo surrounding the colony. Moreover, this strain was able to solubilize phosphate, producing a clear halo around the colony. IAA and precursors biosynthesis was also evaluated. PEPV13 strain showed a moderate production of this auxin (0,077 mg l⁻¹). Several studies reported that strains of *Rhizobium*, mostly *R. leguminosarum*, produce siderophores such as cathecolate, vivibactin, or schizokinen (Patel et al. 1988; Dilworth et al. 1998; Storey et al. 2006). Moreover, this genus was described to produce IAA (Spaepen and Vanderleyden 2011). Regarding to phosphate solubilization, the genus *Rhizobium* is shown to be a phosphate solubilizer bacteria (Sharma et al. 2013), being in agreement with our results.

Then, we preformed an in vitro assay to test the potentiality of this strain to promote plant growth in early stages of development. We measured root length in *D. caryophyllus* plants at 5, 7, and 12 days after inoculation (Table 2.1). Our results showed that PEPV13 inoculated plants presented significative differences in root length respect to uninoculated plants.

These findings suggest that this strain is a good candidate as PGPR for in vitro culture of *D. caryophyllus* and with potentiality to promote growth in natural conditions.

2.3.3 Colonization of *Dianthus caryophyllus* Roots

Due to the positive interaction observed before, next step was to test colonization ability of the selected strain. For that, we obtained a GFP-labeled transformant of PEPV13 and inoculated carnation (Fig. 2.1). Root colonization was positive and bacterial cells were disposed along the root surfaces. Moreover, typical 3D structures or biofilms were observed. Also, bacterial cells formed aggregates in the root emergence zones and occupying intercellular spaces. This behavior is typical of rhizobial strains, which colonize legumes (Fujishige et al. 2006) and non-legumes different than ornamentals (Chabot et al. 1996; Antoun et al.1998; García-Fraile et al. 2012; Flores-Félix et al. 2013).

Table 2.1 Effect of PEPV13 strain in plant root length (mm) in in vitro conditions

	5 DAI	7 DAI	12 DAI
Uninoculated	4.20 ± 0.69 a	10.00 ± 0.49 a	17.90 ± 0.55 a
PEPV13	16.14 ± 1.84 b	16.66 ± 0.50 a	22.00 ± 0.65 b

Each value represent the mean (±S.E.) of at least 12 plants of three different assays. Values followed by the same letter in each treatment are not significantly different (*p* ≥ 0.05) according to Fisher’s Protected LSD test (Least Significant Differences). *DAI* days after inoculation

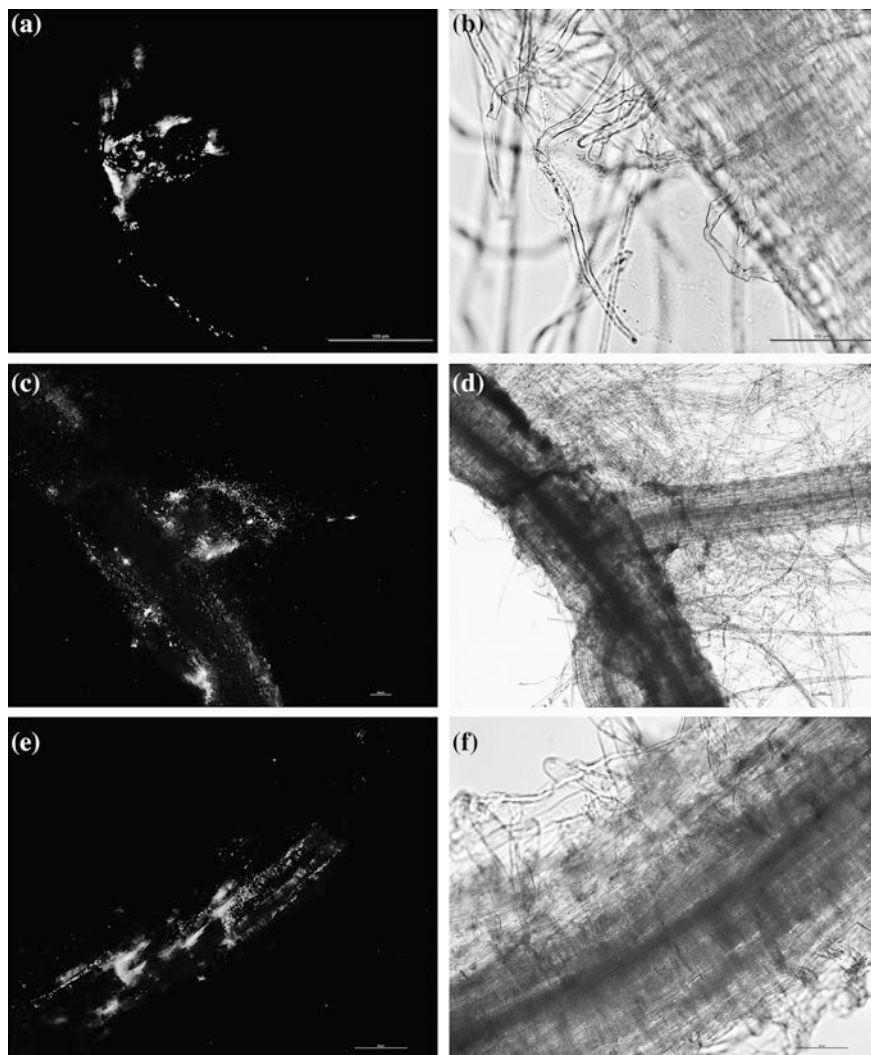


Fig. 2.1 Scanning micrographs of colonization events in carnation seedling roots 12 days after inoculation with GFP-tagged PEPV13 strain. Images **a**, **c**, and **e** were obtained by epifluorescence microscopy. Images **b**, **d**, and **f** were obtained by bright field microscopy, Bar: 100 μ m

Interestingly, we observed an increased number of root hair deformations, and redirection in PEPV13 inoculated roots, in contraposition to the root hairs of uninoculated plants. This effect is in concordance with the effects observed in rice, when is inoculated with a rhizobial strain (Perrine-Walker et al. 2007). Also, similar observed deformations and polar redirections in root hairs of legumes inoculated with cellulase-overproducing strains were described before (Robledo et al. 2011). However, these phenotypes should be analyzed in depth in the near future.

2.3.4 Plant Assays: Analysis of Plant Growth Parameters Under Glasshouse Conditions

Finally, microcosms assays were performed under glasshouse conditions. Plants were harvested at 63 days after inoculation and several measures were obtained, such as shoot length and fresh and dry weight of the aerial parts. The results were not statistically significant in any of the obtained data, being PEPV13 inoculated plants slightly less developed than plants of the uninoculated treatment (data not shown). Fresh and dry weight of inoculated plants also presented lower values than non-inoculated ones.

Several studies addressed the competitiveness events among soil microorganisms. In legumes, competition for nodulation determines the ability of *Rhizobium* strains to nodulate a legume host in competition with other rhizospheric strains close to those legume roots (Dowling and Broughton 1986). Specifically, Duodu et al. (2009) reported the difference in the competitiveness of two *R. leguminosarum* strains to nodulate white clover, showing that they were able to nodulate, despite of their limited survival rates in soil. In non-legumes, such as cereals and horticultural crops, rhizobial strains can compete for root colonization in different soils (Flores-Félix et al. 2013). However, plants can also secrete antimicrobial molecules and other compounds or have other antibacterial mechanisms (De Coninck et al. 2015), affecting bacterial colonization and competitiveness. Rosenblueth and Martinez-Romero (2004) described that a highly competitive rhizobial strain belonging to *Rhizobium etli* has resistance to antimicrobial molecules synthesized by maize roots. Moreover, other soil microorganisms have antimicrobial activity, which might make difficult the survival of other rhizobacteria (Sharma and Kaur 2010). Despite the promising PGPR results that strain PEPV13 showed, our result showed that PEPV13 strain is not able to compete with other soil microorganisms by itself, suggesting the occurrence of any of the events cited above that may affect rhizobial competitiveness. Further assays should be performed in order to elucidate the cause of this result.

2.4 Conclusion

Despite the results of PEPV13 strain inoculation in microcosm assays, our conclusion is that this strain might be considered as PGPR for the initial steps of plant development.

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