Use of *Rhizobium leguminosarum* as a potential biofertilizer for *Lactuca sativa* and *Daucus carota* crops

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**Abstract**

Microbial biofertilizers are becoming an effective tool for sustainable agriculture by means of the reduction of the use of chemical fertilizers. However, the knowledge of each specific plant–microorganism interaction is essential for a correct application. In this study, we analyzed the *in vitro* plant-growth-promotion mechanisms of a *Rhizobium leguminosarum* strain named PEPV16 isolated from *Phaseolus vulgaris* nodules. This strain was able to produce siderophores and indole acetic acid and to solubilize phosphate. Confocal microscopy showed that this strain was able to colonize the roots of two horticultural crops, *Lactuca sativa* L. (lettuce) and *Daucus carota* L. (carrot). Strain PEPV16 was also able to promote the plant growth of both plant species increasing the dry matter of shoots and roots of lettuce and carrots, respectively, as well as to increase the uptake of N and P in the edible parts of both plant species. These data confirmed the suitability of *Rhizobium* as biofertilizer for nonlegumes.

**Key words:** PGPR / lettuce / carrot / GFP / rhizobia / endophyte

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1 Introduction

After the Green Revolution, agriculture has been based on the extensive use of chemical fertilizers and pesticides. Throughout the time, the effects of these practices have become hazardous for animal and human health and water ecosystems have been impaired by eutrophication. Moreover, soil quality and rhizosphere diversity have been compromised. For all of these reasons, the reduction in the use of chemical fertilizers is now regarded as an important necessity due to its expensiveness in production and its impact on the environment. This reduction may help to minimize greenhouse-gas emissions and to avoid contamination of ecosystems (Snyder et al., 2009). However, conventional agriculture is unable to provide a solution to this problem without increasing costs or decreasing crop production (Singh et al., 2011). Therefore, the use of biofertilizers may be an efficient and low-cost alternative, able to face the problems described above. Plant growth–promoting rhizobacteria, commonly called PGPR, exhibit several mechanisms influencing the availability of plant nutrients and enhancing plant resistance to stress and pathogen invasion and infection (Berg, 2009; Tikhonovich and Provorov, 2011). Among their benefits, biological nitrogen fixation (BNF), both free-living or in symbiosis, phosphate solubilization, siderophore production, and phytohormone synthesis are some of the most valuable features of these bacteria (Bhattacharyya and Jha, 2011).

Plant–microorganism interactions are efficient due to the direct effect of PGPR in the plant, specifically colonizing and occupying intercellular spaces in leaves, roots, and stems, as endophytes (Hardoim et al., 2008; Bhattacharyya and Jha, 2011). These relationships are based on a complex and well-regulated molecular dialogue between plant and microorganisms. Concurrently, an essential requirement for biofertilizer design is the use of innocuous bacteria for human and animal health, given that some human pathogenic bacteria, such as *Klebsiella pneumoniae*, *Burkholderia cepacia*, *Pseudomonas aeruginosa*, or *Acinetobacter* show plant growth–promoting features but cannot be used due to their pathogenicity (García-Fraile et al., 2012). On the other hand, symbiotic rhizobia are safe microorganisms with a well-known ability to establish nitrogen-fixing endosymbiosis with legumes and present *in vitro* mechanisms of plant growth promotion and have some interesting features, such as siderophore production, phosphate solubilization, and phytohormone production such as indole acetic acid, gibberellins, and cytokinins (Mehboob et al., 2009; García-Fraile et al., 2012). Although the effects of rhizobia in legumes are the best studied, these microorganisms also colonize the roots of nonlegumes such as rice, maize, lettuce, pepper, and tomato and therefore they are good candidates for biofertilization of these plants (Chabot et al., 1996a; Peña and Reyes, 2007; Baset and Shamsuddin, 2010; García-Fraile et al., 2012).

Nevertheless, there are few studies about the ability of *Rhizobium* to promote the growth of vegetables with high agronomic interest and to allow the substitution of chemical fertili-
zers by biofertilizers. Lettuce (Lactuca sativa) is widely consumed in the world and constitutes one of the major fresh vegetables produced in Spain, which is also the major producer of carrots (Dacus carota) in Europe. In both cases the edible parts, shoots and roots, respectively, can be consumed raw and carrot is one of the main components in purees for children. Therefore, it is essential that they are free of hazardous compounds as well as pathogenic. The objective of this study was to investigate whether Rhizobium is able to promote the growth of lettuce and carrot and whether it can be considered as a reliable biofertilizer for these two horticultural crops.

2 Material and methods

2.1 Bacterial strains

Rhizobium leguminosarum L. strain PEPV16 was isolated from an effective nodule of Phaseolus vulgaris growing in a soil from Salamanca (Spain) using the standard method of Vincent (1970) on YMA plates at 28°C (Flores-Félix et al., 2011). The ability of this strain to nodulate P. vulgaris was checked according to Mulas et al. (2011). For GFP-tagged derivative Rhizobium was obtained by biparental mating with E. coli S17.1, carrying plasmid pHc60 (Cheng and Walker, 1998) as described in García-Fraile et al. (2012).

2.2 Phylogenetic analysis of 16S rRNA gene

The amplification and sequencing of rrs gene was carried out according to Rivas et al. (2007). The sequences obtained were compared with those from EzTaxon-e server (Kim et al., 2012). Sequences were aligned using the Clustal X software (Thompson et al., 1997). The distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). A phylogenetic tree was inferred using the neighbor-joining analysis (Saitou and Nei, 1987). MEGA5 software (Tamura et al., 2011) was used for all analyses.

2.3 Analysis of in vitro PGPR mechanisms

The ability to solubilize phosphate by the strain PEPV16 was tested in YED-P media according to Peix et al. (2004). Side- rophore production was evaluated using M9-CAS-AGAR media (Schwyn and Neilands, 1987) and modified by Alexander and Zuberer (1991). Indole acetic acid production was measured in JMM media (O’Hara et al., 1989) supplemented with tryptophan (0.17g L⁻¹) as described Khalid et al. (2004).

2.4 Colonization assays

Lettuce (Lactuca sativa L. var. romain) and carrot (Daucus carota L. var. nantes) seeds were surface-sterilized with 70% ethanol during 30 s and 5% sodium hypochlorite for 5 min. Several washes were performed and seeds were spread on agar plates. Three days after germination, seedlings were inoculated with 250 μL plant⁻¹ of a bacterial suspension with a turbidity of 5 in McFarland standards (1.5 × 10⁹ CFU mL⁻¹) and incubated in a growth chamber. Uninoculated controls were also included in the study.

Seedlings were viewed under a laser scanning confocal microscope (Leica SP2) 15 d after inoculation. Also, a NIKON Eclipse 80i epifluorescence microscope was used to monitor lettuce and carrot colonization 6 and 7 d after inoculation, respectively, using excitation at 472 nm in an argon laser for green fluorescence and 510 nm for root cells which were stained with 10 μM of propidium iodide (Sigma). Images were processed using Leica confocal software.

2.5 Plant assays

Axenic seedlings were transferred to pots containing a mix of sterilized soil and vermiculite (3 : 1). Eighteen plants per treatment were inoculated with 1 mL of R. leguminosarum PEPV16 suspension with a turbidity of 5 in McFarland standard (1.5 × 10⁹ CFU mL⁻¹) and watered when needed. An uninoculated group was left under the same conditions. Plants were harvested 45 d after inoculation to determine dry weight and nutrient concentrations. The analyses of nitrogen, phosphorous, potassium, magnesium, calcium, sulfur, iron, manganese, zinc, and copper was performed using ICP-OES ICP6500 DUO spectrometer (Thermo Scientific) in the Ionomic Service from CEBAS-CSIC (Spain).

2.6 Statistics

Data were analyzed with one-way ANOVA using Statview 5.0 software (SAS Institute Inc.), with a post-hoc test using Fisher’s protected least significant difference (P ≥ 5%).

3 Results

3.1 Identification of strain PEPV16 and analysis of in vitro PGPR mechanisms

The strain PEPV16 was classified as genus Rhizobium on the basis of its rrs gene sequence showing 99.9% identity with respect to Rhizobium leguminosarum USDA 2370T. Figure 1 shows the phylogenetic position of this strain in the group formed by R. leguminosarum and R. indigoferae, two species that are probably synonyms because they have nearly identical rrs gene as was already pointed out by Ferreira et al. (2011). The strain PEPV16 was able to nodulate P. vulgaris in agreement with other strains from the same species isolated in North Spain where common beans are commonly nodulated by strains of the species R. leguminosarum (Mulas et al., 2011).

Colonies of strain PEPV16 in M9-CAS-AGAR medium were surrounded by a yellow-orange halo (2 mm periphery around colonies) indicative of siderophore production. In YED-P medium, solubilization of insoluble phosphate was detected since strain PEPV16 showed a transparent halo of about 2 cm radius around its colonies. The strain also produced indole-3-acetic acid (IAA) at final concentration 77 mg L⁻¹.
Figure 1: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences of strain PEPV16. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.
3.2 Colonization assays

GFP-tagged bacteria allow confocal and fluorescence microscopy assays. With this technology, the interaction between L. sativa seedlings and PEPV16 strain L. sativa seedlings was tested. This strain is able to colonize the root surface, occupying intercellular spaces in cortical cells. PEPV16 was distributed ubiquitously on the root, forming a smooth layer (Fig. 2A). When checked under confocal microscope, PEPV16 strain was located at root surface and clearly shows invasion of root epidermis intercellular spaces. These results confirm its behavior as an endophyte in this plant (Fig. 2B).

Daucus carota seedlings inoculated with GFP-tagged PEPV16 strain showed uniform colonization along root cell depressions (Fig. 2C), as observed for L. sativa seedlings. Also D. carota seedlings showed similar bacterial distribution (Fig. 2D). Therefore, PEPV16 strain shows the ability to colonize and adhere to root hairs in lettuce and carrots with a similar distribution and to penetrate intercellular spaces during early stages of development in lettuce. The capacity to become an endophyte is expected to be the same as in lettuce and carrot because the colonization of the root surface followed a similar pattern.

3.3 Plant assays

We have analyzed the ability of strain PEPV16 to promote the plant growth of L. sativa and D. carota plants under microcosmos conditions, evaluating effects on edible parts in both cases (Tab. 1).

Concerning L. sativa, the dry weight of shoots was significantly increased for plants inoculated with PEPV16, compared to uninoculated ones. Also the concentrations of N, P, and Ca were significantly higher in inoculated plants indicating that they had higher potential for nutrient uptake than control plants (Tab. 1). The remaining macronutrients were not significantly different between inoculated and uninoculated plants except in the case of K and S whose percentages were significantly lower in inoculated plants. In the case of micronutrients, only the percentage of Fe was significantly higher in the inoculated plants while the rest of micronutrients showed a nonsignificant increase.

The results obtained for D. carota confirmed those for lettuce, since the root dry weight of inoculated plants was significantly higher (40%) than that of the control plants (Tab. 1). As in the case of lettuce, the concentrations of N, P, and Ca were significantly higher in the inoculated plants. In addition, the concentrations of K and Mg were also higher in inoculated plants. The percentage of S was slightly but not significantly higher in the inoculated plants. As in the case of lettuce, the Fe concentration was significantly higher in inoculated plants but no significant differences were found in the remaining micronutrients.

4 Discussion

In the current worldwide scenario of increasing food prices and constant environment impoverishment, the fertilizer application should be optimized by the use of low-cost and
less harmful technologies including the use of microorgan-
isms (Dawson and Hilton, 2011; Van Vuuren et al., 2010). Some PGPR bacteria may act as endophytes, but some of them could be harmful for human and animal health (Rosen-
blueth and Martínez-Romero, 2006) and their use must be avoided in biofertilization schemes (García-Fraile et al., 2012). The genus Rhizobium is an important PGPR whose safety for humans, animals, and plants has been widely shown after decades of inoculation of legumes (Bhattachar-
jee et al., 2012; Glick, 2012). Moreover, the ability of Rhizo-
bium strains to promote the growth of some nonlegumes has been reported as was pointed out in the introduction of this paper. In a recent study, we analyzed the ability of a strain of Rhizobium leguminosarum isolated from P. vulgaris nodules to produce siderophores and IAA and to promote plant growth increasing the fruit production of tomato and pepper (García-Fraile et al., 2012). Nevertheless, there are many species and varieties of vegetables in which the effect of Rhi-
zobium inoculation remains unexplored as occurs in the case of carrots and the variety of lettuce “Romana” used in this study. Moreover, the strain PEVP16, in addition to the pro-
duction of siderophores and indole acetic acid, was able to solubilize phosphate (not shown).

The ability of Rhizobium to colonize lettuce roots previously reported by Chabot et al. (1996b) has been confirmed in this study using GFP-labeled strains and confocal microscopy. Moreover the strain PEVP16 was able to colonize the inner part of roots showing that this strain is an endophyte in lettuce. In carrots, although the colonization of intercellular spaces was not clear, the colonization of root surfaces was abundant as observed using confocal microscopy. These results confirm the need of performing colonization assays on different plant species, since the ability of different strains to establish effective molecular interactions depends on the host plant and interaction efficiency (Bais et al., 2006).

Both lettuce and carrot seedlings, 6 d after inoculation, showed more higher root-hair density on their roots and an increased root length in comparison to uninoculated seedlings (data not shown). An increase in root-hair number and, consequently, larger absorption surface is closely related to the improved ability for nutrient uptake, compared to uninocu-
lated seedlings. These data match perfectly with the ability of PEVP16 strain to produce indole-3-acetic acid since Methboob et al. (2009) described a relationship between inoculation with indole acetic acid–producing bacteria and an increased development and root-hair number in early stages.

The increase in the ability to take up nutrients was clearly shown since N and P concentrations were significantly in-
creased in shoots (lettuce) and roots (carrot) inoculated with the strain PEVP16. Although Rhizobium strains are unable to fix nitrogen with nonlegumes, an increased level of this ele-
ment in pepper has been also reported after inoculation with a Rhizobium strain isolated from P. vulgaris (García-Fraile et al., 2012). The P uptake was increased by 15% in the let-
tuce shoots, in concordance with the results obtained by Cha-
bot et al. (1996a) when lettuce was inoculated with a R. leguminosarum strain isolated from P. vulgaris. In carrots, the increase of P concentration in roots of inoculated plants compared to the control ones went up by 40%, with the same increase in dry weight. This increase was higher than that found by Antoun et al. (1998) for radish which was inoculated with a R. leguminosarum strain also isolated from P. vulgaris. With regard to the micronutrients, it is remarkable that Fe in-
creased in inoculated plants of both crops. This can be related to the ability of the strain PEVP16 to produce sidero-
phores since it has been reported that plants inoculated with PGPR are able to produce siderophores and are capable of absorbing Fe from these compounds (Glick, 2012). Magnesium also plays an important role as cofactor in many enzymes (Mengel and Kirkby, 2001). The increase of this ele-
ment in carrot root leading to enhanced metabolic activity could be related with the higher dry weight of these plants.

### 5 Conclusion
The results show that Rhizobium leguminosarum PEVP16, a strain that actively colonizes the rhizosphere of L. sativa and D. carota, increased plant growth as well as the content of N

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**Table 1**: Effect of R. leguminosarum strain PEVP16 on dry weights and nutrient concentrations of lettuce shoots and carrot roots.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry weight per edible part</th>
<th>Macronutrients</th>
<th>Micronutrients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>/ g (± SE)</td>
<td>N / % (± SE)</td>
<td>P / % (± SE)</td>
</tr>
<tr>
<td>Lettuce</td>
<td>Control</td>
<td>4.53±0.19</td>
<td>3.44±0.01</td>
</tr>
<tr>
<td></td>
<td>PEVP16</td>
<td>5.05±0.16</td>
<td>3.72±0.03</td>
</tr>
<tr>
<td>Carrot</td>
<td>Control</td>
<td>0.90±0.08</td>
<td>2.87±0.02</td>
</tr>
<tr>
<td></td>
<td>PEVP16</td>
<td>1.53±0.9</td>
<td>3.51±0.06</td>
</tr>
</tbody>
</table>

§ SE = standard error.

Values followed by the same letter in each treatment are not significantly different from each other at P = 5% according to Fisher’s Protected LSD (least significant differences).
and P of the edible parts in both vegetables being a good potential biofertilizer for these non-legume crops.

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