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Rhizobium as plant probiotic for strawberry production under microcosm conditions

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Abstract There is increasing interest in the use of plant growth-promoting rhizobacteria (PGPR) as environmental-friendly and healthy biofertilizers. Strawberries (*Fragraria x ananassa*) are mainly consumed fresh and hence any PGPRs used for biofertilization must be safe for humans, which is the case for members of the genus *Rhizobium*. In this study, the effects of inoculation of strawberry plants with *Rhizobium* sp. strain PEPV16, which belongs to the phylogenetic group of *R. leguminosarum*, and whose plant growth promotion ability has been reported previously for lettuce (*Lactuca sativa*) and carrots (*Daucus carota*), was examined. The results demonstrated that PEPV16 promotes strawberry growth through significant increases in the number of stolons, flowers and fruits as compared with uninoculated controls. Compared to uninoculated controls, the fruits of the inoculated plants had higher concentrations of Fe, Zn, Mn and Mo, and they also had higher concentrations of organic acids, such as citric and malic acid, and lower amounts of ascorbic acid than fruits. Although decreases in ascorbic acid have previously been described after the inoculation of strawberry with strains from different PGPR genera, this is the first study to report increases in organic acids after PGPR inoculation.

Keywords: Strawberry · *Rhizobium* · Colonization · Organic acids · PGPR

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1 Introduction

Biofertilizers or plant probiotics (Berlec 2012) include rhizospheric and endophytic microorganisms with the ability to promote and directly and/or indirectly regulate plant growth (Compant et al. 2010 and Glick 2012). Direct plant growth mechanisms involve those that facilitate the acquisition of nutrients through biological nitrogen fixation (BNF), phosphate solubilization, iron sequestration and the modulation of phytohormone levels. Indirect mechanisms involve the induction of systemic resistance and the production of antibiotics, enzymes (lytic enzymes and ACC deaminase) and siderophores (Glick 2012). There are many bacteria that possess one or more of these beneficial mechanisms. Many of them are human pathogens and, therefore, cannot be used as biofertilizers (García-Fraile et al. 2012). Strains from different species of *Rhizobium* are now well known as plant growth-promoters capable of fixing atmospheric nitrogen within legume root nodules (Peix et al. 2015), and they have also been reported to act as root colonizers and plant growth-promoters in some vegetables, such as pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*) and carrot (*Daucus carota*) (García-Fraile et al. 2012; Flores-

Félix et al. 2013). Recently, we have shown that *Phyllobacterium*, related phylogenetically to the *Rhizobiaceae* family, promotes the growth of strawberries (Flores-Félix et al. 2015b), which are one of the most important soft fruits consumed in European countries, including Spain (López-Aranda et al. 2011). They contain nutritive compounds comprising sugars, vitamins and minerals (Giampieri et al. 2015), together with bioactive compounds such as vitamin C, flavonoids, anthocyanins and phenolic acids (Basu et al. 2014; Giampieri et al. 2015). Their high antioxidant potential is mainly due to their vitamin C content (Giampieri et al. 2015) and several studies have focused on the effect of bacterial inoculation on the vitamin C content and the contents of various minerals in the fruits (Pirlak and Köse 2009; Erturk et al. 2012; Bona et al. 2015; Ipek et al. 2014; Flores-Félix et al. 2015b). Interestingly, however, strawberry is peculiar regarding its ability to propagate both sexually, via seeds and vegetatively via stolons (Heide et al. 2013). Accordingly, stolon production is as important as that of fruits, and the production of both can be enhanced by biofertilization with some PGPRs, as recently shown for *Phyllobacterium* strains (Flores-Félix et al. 2015b).

Although there are several reports addressing the ability of *Rhizobium* strains to enhance the growth of the aerial parts of non-legumes (García-Fraile et al. 2012; Glick 2012), there are no studies about the effect of *Rhizobium* strains on strawberry plants. We have recently reported a strain of *Rhizobium*, PEPV16, which nodulates *Phaseolus vulgaris* and has several *in vitro* plant promotion mechanisms, that was able to colonize the roots of lettuce, an important step required for obtaining beneficial effects on plant growth (Lugtenberg et al. 2001; Compant et al. 2010); the strain had a positive effect on the growth of lettuce leaves (Flores-Félix et al. 2013). This strain was identified in that previous study as *R. leguminosarum* on the basis of its 16S rRNA gene sequence, but since 2013 several species of the genus *Rhizobium* with identical 16S rRNA genes have been published, such as *R. laguerreae* (Saïdi et al. 2014), *R. sophorae* (Jiao et al. 2015), *R. anhuiense* (Zhang et al. 2015), and *Rhizobium acidisoli* (Román-Ponce et al. 2015). Moreover, there are two accession numbers in Genbank for the 16S rRNA gene of the *R. indigoferae* CCBAU71042^T type strain, one of them identical to that of the above sequences. Nevertheless, these species are distinguishable on the basis of the results of different housekeeping gene analyses, such as *recA* and *atpD*, which are available for all type strains of these species (Peix et al. 2015).

The aims of this study were to analyse the *recA* and *atpD* genes of strain PEPV16 in order to establish more exactly its relationship with species in the *R. leguminosarum* group, and to explore the effect of inoculation with this strain on the growth of strawberry plants under greenhouse conditions, as well as on the content of minerals and organic acids, including ascorbic acid (vitamin C), of the fruits.

2 Material and methods

2.1 Housekeeping gene analysis

The *atpD* and *recA* genes of strain PEPV16 were amplified and sequenced as described by Gaunt et al. (2001). The sequences obtained were compared with those held in GenBank using the BLASTN program (Altschul et al. 1990). They were aligned using Clustal W software (Thompson et al. 1997). Distances calculated according to Kimura's two-parameter model (Kimura 1980) were used to infer phylogenetic trees with the neighbour-joining method (Saitou and Nei 1987), using MEGA5 software (Tamura et al. 2011). Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets.

2.2 Biofilm production and plant colonization assays

The ability of strain PEPV16 to form biofilms on abiotic surfaces at the macro and micro-scale and to produce cellulose was analysed as described by Flores-Félix et al. (2015b). The microstructure of the biofilm was detected using 25 ml of TY medium placed in a 50 ml glass tube containing a sterile microscope slide. The medium was inoculated with a 2 day old culture of strain PEPV16 (measured at 600 nm) using 100 μl of a 0.5 OD suspension. The inoculated slides were examined after 7 days of incubation. The slides were then removed and placed in water for 5 s three times to remove the cells that had not adhered to the slide surface, after which they were placed in a solution of 40 mg l^{-1} acridine orange in phosphate buffer (pH 7.2) for 30 s. Microscopic examination was carried out using an appropriate filter with a NIKON Eclipse 80i fluorescence microscope. Cellulose production was checked on plates containing YMA medium supplemented with 0.25 % Congo Red, inoculated with strain PEPV16 and incubated at 28 °C for 2 days (Robledo et al. 2012). In order to confirm cellulose production, the strain was inoculated in 30 ml of YMB medium, which was shaken at 180 rpm and 28 °C for 5 days, followed by static growth for 2 days. Five ml were taken from the bottom of the flask and centrifuged at 1500 × g for 5 min. The flocs were then washed with 5 ml of 100 mM phosphate citrate buffer (PCA) pH 5, recovered by centrifugation, and resuspended in 5 ml of the same buffer. This suspension was placed in 5 cm diameter Petri plates and treated with 10 U ml^{-1} cellulase produced by *Trichoderma viride* (Sigma Co., USA) for 2 h at 37 °C in an orbital shaker at 180 rpm. Controls without cellulase were incubated under the same conditions.

For plant colonization assays, 30 achenes of strawberry (*Fragraria x ananassa*) var. Camarosa were surface-sterilized by immersion in 70 % ethanol for 30 s, followed by soaking in an aqueous 5 % sodium hypochlorite solution for 15 min. The achenes were then washed six times with

sterile water and germinated in 1 % water-agar plates overlaid with sterile Whatman number 1 filter paper wetted with sterile water. Five day old strawberry seedlings were inoculated with 1 ml of a suspension (10^8 CFU ml^{-1}) of the GFP-tagged PEPV16 strain obtained in a previous study (Flores-Félix et al. 2013). The plates were placed in the darkness in a growth chamber at 24 °C with mixed incandescent and fluorescent illumination (400 microeinsteins m^{-2} s^{-1}; 400–700 nm) programmed for a 16 h photoperiod and 50–60 % relative humidity (Robledo et al. 2008) until the seedling roots were 1–2 cm in length. In order to remove unbound bacteria, the roots were gently washed three times with sterile distilled water before microscopic examination. Uninoculated strawberry roots were included in the experiment as negative controls. Fluorescence microscopy was carried out using a Nikon Eclipse 80i, and excitation of the green fluorescent protein (GFP) was accomplished using a mercury lamp. The seedlings were examined at 3, 5, 8 and 12 days after inoculation.

To increase the resolution of the biofilm observations, calcofluor white staining was used as described by Flores-Félix et al. (2015a). Seedlings were placed on slides and stained with 50 µl of 50 mg l^{-1} Calcofluor White solution (Calcofluor White stain, Sigma®) and 50 µl of 10 % potassium hydroxide solution to improve resolution, as recommended by the manufacturer. Preparations were covered and incubated for 1 min before examination under an epifluorescence microscope.

2.3 Growth promotion assays *in planta*

The ability of strain PEPV16 to promote the growth of strawberry var. Camarosa plants was evaluated. Fifteen plants were included in each treatment: a control without inoculation and inoculation with strain PEPV16. The seedlings (conserved at −4 °C) were obtained from a commercial distributor of strawberry plants in Canada and were planted on "SEED PRO 6040"/vermiculite (3:1 *V*/*V*) (PROJAR, Spain) non-sterile commercial peat, using black plastic trays containing 6 Kg each. Six days after planting, each tray was inoculated with 100 ml of a suspension of strain PEPV16 containing 10^6 CFU ml^{-1}. To obtain this suspension, the cells of the strain were cultivated on YMA plates for 48 h at 28 °C and then suspended in sterile water.

The plants were irrigated with water from a bottom reservoir every 48 h for 3 months in a lab greenhouse under day/night temperatures of 25–35/15–20 °C, humidity being set at 70 %. During the experiment, stolons, flowers and fruits were counted, the fully ripened fruits from first to third categories were harvested. The fresh and dry weights of 25 fully ripened fruits were measured and their mineral content was analysed. Determination of N, P, K, Ca and Mg was performed at the Ionomic Service of the *Centro de Edafología y Biología Aplicada del Segura* (CEBAS)-CSIC (Spain). Statistical analyses using One-way Analysis of Variance were carried out using the StatView 4.1 program for Macintosh computers (Abacus Concepts, USA) and mean values were compared with Fisher's Protected LSD test (Least Significant Differences (LSD) at a confidence level of 95 %, $P \leq 0.05$).

2.4 Analysis of organic acids

Fifteen fully ripened fruits from each treatment were frozen at −20 °C, lyophilized (Labconco Freezone 4.5 apparatus, USA), and ground to a mean particle size of less than 910 µm. The material obtained was divided into three aliquots, which were analyzed separately. Organic acids were analyzed according to the procedure described by Dopico-García et al. (2007). Each lyophilized powdered sample (ca 0.3 g) was extracted using 0.01 N H_2SO_4 (ca. 50 ml) for 30 min whilst stirring at 300 rpm. The aqueous solution was then passed through a Chromabond C18 NEC column previously conditioned with 30 ml of methanol and 70 mL of water acidified to pH 2 using HCl. The aqueous extract containing the organic acids was evaporated to dryness under reduced pressure at 30 °C and redissolved in 1 ml 0.001 N H_2SO_4 for HPLC-UV analysis, using 20 µl of the suspension.

The separation and quantification of organic acids was carried out in a system consisting of an analytic HPLC–UV unit (Gilson Inc., Middleton, WI) with a Nucleogel® Ion 300 O ion-exclusion column (300 × 7.7 mm; Macherey–Nagel, Düren, Germany), as previously reported by Dopico-García et al. (2007). Elution was performed in isocratic mode with 0.01 N H_2SO_4 at a flow rate of 0.2 ml min^{-1}. Detection of organic acids was achieved using a UV detector set at 214 nm. Organic acid quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards: oxalic, *cis*- and *trans*-aconitic, citric, ascorbic, malic, shikimic and fumaric acids (Sigma–Aldrich, St. Louis, MO). The data thus acquired were analyzed using One-way Analysis of Variance, as reported above (García-Fraile et al. 2013).

3 Results and discussion

3.1 Housekeeping gene analysis

Strain PEPV16 was classified within the phylogenetic group of *R. leguminosarum* because its 16S rRNA gene exhibited 100 % similarity with the type strain of this species (Flores-Félix et al. 2013). Nevertheless, since 2013 several new species of *Rhizobium* with identical 16S rRNA genes have been described, such as *R. laguerreae* (Saïdi et al. 2014), *R. sophorae* (Jiao et al. 2015), *R. anhuiense* (Zhang et al. 2015) and *R. acidisoli* (Román-Ponce et al. 2015). These species are distinguishable by their housekeeping genes, which are

phylogenetically divergent from one another. This does not occur in the case of *R. indigoferae*, an old species (Wei et al. 2002), whose housekeeping genes suggest that it would be synonymous with *R. leguminosarum* and that its taxonomic status should be revised according to the current rules of bacterial taxonomy (Ferreira et al. 2011).

The results for the *recA* and *atpD* genes of strain PEPV16 and the closely related species on the basis of the 16S rRNA gene revealed that this strain is closely related to different species, depending on the gene analysed (Fig. 1). The high relatedness of the *atpD* gene to the type strain of *R. laguerreae* (98.9 % similarity) suggested that strain PEPV16 belonged to this species (Fig. 1a). Nevertheless, the *recA* gene was more closely related to *R. leguminosarum*, with 98.3 % similarity (Fig. 1b). Therefore, strain PEPV16 could be an example of genome recombination, as has been shown previously for *R. leguminosarum* strains (Kumar et al. 2015), and it could be a good candidate for further characterization through complete genome sequencing.

3.2 Biofilm production and colonization of strawberry roots

Strain PEPV16 was able to produce biofilms on abiotic surfaces (Fig. 2a) and cellulose (Fig. 2a and b), as confirmed by treatment with cellulase (data not shown). Cellulose microfibrils constitute part of the biofilm polysaccharides and the production of cellulose is an important mechanism for legume root colonization by *Rhizobium* and *Phyllobacterium* (Robledo et al. 2012; Flores-Félix et al. 2015b). The examination by fluorescence microscopy of GFP-tagged *Rhizobium* sp. PEPV16 revealed the attachment of its cells to the strawberry seedling roots, forming typical microcolonies (Fig. 2c and d). Moreover, contrast staining with Calcofluor White revealed the presence of biofilms around the seedling roots (Fig. 2e) and the presence of globular masses on root hairs (Fig. 2f). These results are in agreement with those previously found in strawberry roots inoculated with the *Phyllobacterium* strain PEPV15, which was also able to promote the growth of strawberries (Flores-Félix et al. 2015b). Therefore, the effect of strain PEPV16, which is also able to colonize strawberry roots and has demonstrated *in vitro* mechanisms of plant growth promotion (Flores-Félix et al. 2013), was analysed.

3.3 Effect on plant growth

Inoculation with the test strain resulted in an increase in the number and length of stolons as compared to the uninoculated control (p-value < 0.05), together with a significantly higher number of flowers and fruits (Table 1). When the fruits produced were classified using the commercial categories determined by the EU (Commission Regulation (EEC) No 899/87), depending on their calibre (first, second and third category), all of them were found to correspond to fresh fruits weighing more than 7 g. Although no significant differences were found in the fresh weight of the fruits from the second and third categories (data not shown), a significant increase was found in the fresh and dry weights of fruits from the first category after inoculation with the test strain (Table 1). This is in accordance with the results of other authors, who found significant increases in fruit weight after inoculation with strains of *Pseudomonas* (Esitken et al. 2010; Bona et al. 2015), *Bacillus megaterium* and *Bacillus* sp. (Erturk et al. 2012), and *Alcaligenes faecalis* and *Agrobacterium rubi* (Ipek et al. 2014). Other authors found no differences after the inoculation of two combined strains of *Bacillus* and *Pseudomonas* (Pırlak and Köse 2009). Variable results, depending on the inoculated strain, have been reported in other studies after the inoculation of strawberris with several strains of *Bacillus megaterium*, *B. simplex*, *Bacillus* sp. and *Paenibacillus polymyxa* (Erturk et al. 2012) or after the inoculation with strains of *Alcaligenes faecalis*, *Staphylococcus arlettae*, *S. simulans*, *Pantoea agglomerans*, *Agrobacterium rubi* and *Bacillus megaterium* (Ipek et al. 2014).

In this study the content of N, P and K increased slightly while the Ca content decreased slightly after inoculation with the test strain, but the differences with respect to the uninoculated plants were not statistically significant (Table 1). Inoculation significantly increased the Na content. However, other researchers have found increases in the contents of several macronutrients when non-rhizobial strains were inoculated onto strawberry plants (Ipek et al. 2014; Flores-Félix et al. 2015b).

With regard to micronutrients, inoculation with strain PEPV16 increased the iron content of the strawberry fruits (Table 1), as also occurred after inoculation with the *Phyllobacterium* strain PEPV15 (Flores-Félix et al. 2015b) and with several strains from different genera and species, such as *Alcaligenes faecalis*, *Staphylococcus arlettae*, *S. simulans*, *Pantoea agglomerans*, *Agrobacterium rubi* and *Bacillus megaterium* (Ipek et al. 2014). Inoculation with strain PEPV16 increased the Mn content in strawberry fruits, in agreement with the results of Ipek et al. (2014). Nevertheless, an increase in the Zn content and a decrease in the B content were also found after inoculation with strain PEPV16, in contrast to the results of Ipek et al. (2014). The Mo content also increased after inoculation with strain PEPV16; this element is not usually analyzed in strawberries despite its importance for fruit development (Kaiser et al. 2005) and also in human health (Mendel and Schwarz 2011).

Taken together with previous studies on the effects of strain PEPV16 on lettuce and carrots, it can be concluded that it is a good plant probiotic that is able to increase the yield and mineral contents of the edible parts of various horticultural plants, such as strawberries, carrots and lettuce.

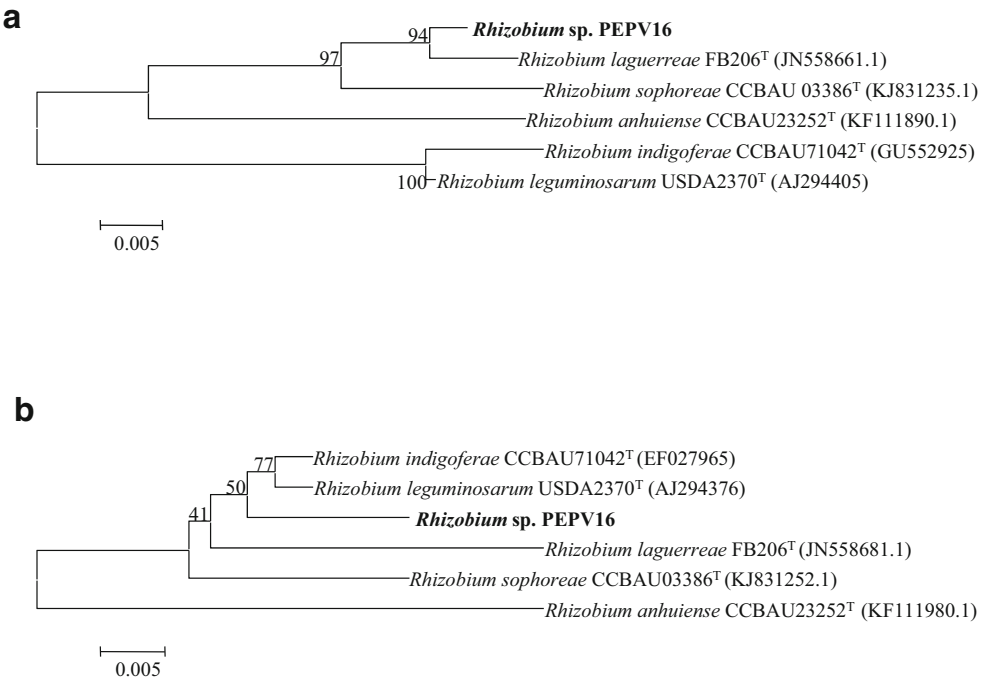

Fig. 1 Neighbour-joining phylogenetic trees of the *atpD* (**a**) and *recA* (**b**) genes showing the position of strain PEPV16 within the phylogenetic group of *Rhizobium leguminosarum*. Bootstrap values (percentages) calculated for 1000 replications are indicated. The Genbank accesion numbers of *atpD* and *recA* genes of strain PEPV16 are KU196776 and KU196777, respectively. Bar, 0.5 nucleotide substitution per 100 nt

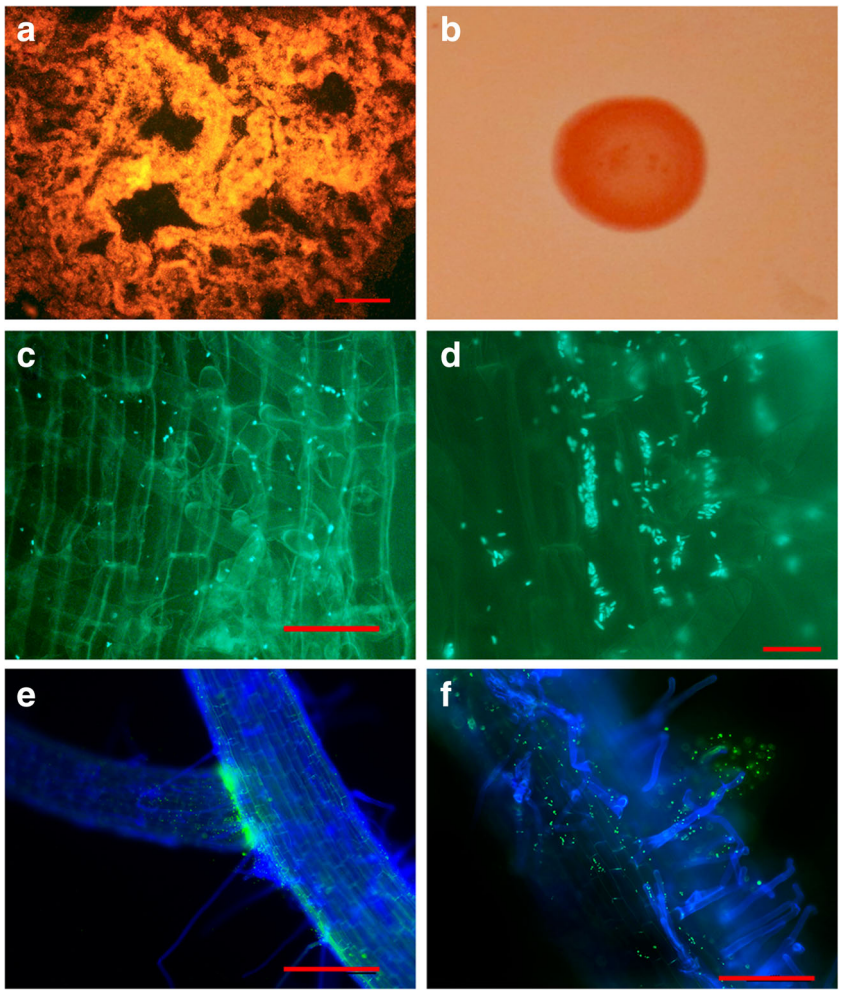

Fig. 2 Biofilm formation by *Rhizobium* strain PEPV16 on abiotic surfaces observed in glass slides with *acridine orange* (**a**, bar 500 μm). Cellulose formed in plates containing *Congo Red* (**b**, *left*). Fluorescence optical micrographs of roots of strawberry seedlings colonized by GFP-tagged cells of PEPV16 (**c**, bar 100 μm, and **d**, bar 10 μm). The micrographs show the ability of strain PEPV16 to colonize the root surfaces of strawberry 3 days post-inoculation (**c**) and the initiation of microcolonies 5 days post-inoculation (**d**). Fluorescence optical micrographs of roots of strawberry seedlings colonized with GFP-tagged cells of PEPV16 contrast stained with calcofluor white revealing the presence of biofilms on the strawberry roots 8 days post-inoculation (**e**, bar 500 μm) and a globular mass of bacteria on root hairs 12 days post-inoculation (**f**, bar 100 μm)

Table 1 Effect of inoculation with *Rhizobium* sp. PEPV16 on the growth and fruit production of strawberry under microcosm conditions

Treatment	Stolons per plant (± S.E.)	Stolons length (cm) (± S.E.)	Flowers per plant (± S.E.)	Fruits per plant (± S.E.)	Fresh weight per fruit from first category (g)[¥] (± S.E.)	Dry weight per fruit from first category (g)[¥] (± S.E.)
Vegetative parameters						
Control	3 (±0.40)[a]	38.14 (±2.19)[a]	8 (±1.14)[a]	3 (±0.37)[a]	16.47 (±0.15)[a]	1.11 (±0.03)[a]
PEPV16	6 (±0.55)[b]	76.74 (±4.01)[b]	11 (±1.18)[b]	5 (±0.69)[b]	18.06 (±0.58)[b]	1.40 (±0.09)[b]
Macroelements[‡]						
	N (%) (± S.E.)	P (%) (± S.E.)	K (%) (± S.E.)	Mg (%) (± S.E.)	Ca (%) (± S.E.)	Na (%) (± S.E.)
Control	0.94 (±0.01)[a]	0.22 (±0.05)[a]	1.47 (±0.03)[a]	0.11 (±0.03)[a]	0.11 (±0.01)[a]	0.07 (±0.01)[a]
PEPV16	1.00 (±0.07)[a]	0.25 (±0.06)[a]	1.61 (±0.04)[a]	0.11 (±0.01)[a]	0.09 (±0.01)[a]	0.10 (±0.01)[b]
Microelements[‡]						
	Fe (mg kg^{-1}) (± S.E.)	Mn (mg kg^{-1}) (± S.E.)	Zn (mg kg^{-1}) (± S.E.)	Cu (mg kg^{-1}) (± S.E.)	B (mg kg^{-1}) (± S.E.)	Mo (mg kg^{-1}) (± S.E.)
Control	18.94 (±0.04)[a]	37.73 (±1.62)[a]	12.10 (±0.51)[a]	2.75 (±1.13)[a]	8.78 (±0.76)[a]	1.47 (±0.06)[a]
PEPV16	24.70 (±0.19)[b]	41.37 (±0.69)[b]	13.57 (±0.37)[b]	2.21 (±0.14)[a]	8.70 (±0.01)[a]	2.99 (±0.17)[b]

Values followed by a different letter in each treatment are significantly different from each other at $p < 0.05$. S.E. = Standard Error

[‡] Results are from 15 fully ripened fruits per treatment

[¥] Results are from 25 fully ripened fruits per treatment

Table 2 Analysis of organic acids of strawberry fruits harvested from control plants and from plants inoculated with *Rhizobium* sp. PEPV16

Treatment	Oxalic (mg/Kg) (± S.E.)	Aconitic (mg/Kg) (± S.E.)	Citric (mg/Kg) (± S.E.)	Ascorbic (mg/Kg) (± S.E.)	Malic (mg/Kg) (± S.E.)	Shikimic (mg/Kg) (± S.E.)	Fumaric (mg/Kg) (± S.E.)	∑ (mg/Kg)
Control	38.4 (±0.7)[a]	29.9 (±0.7)[a]	22,498.2 ± 1771.9[a]	2258.0 ± 79.1[a]	11,009.2 ± 166.9[a]	3.6 ± 0.0[a]	10.2 ± 0.1[a]	35,846.1
PEPV16	75.1 (±10.7)[b]	56.1 (±0.6)[b]	24,928.0 ± 367.4[b]	2074.4 ± 595.3[b]	15,040.5 ± 549.0[b]	4.4 ± 0.0[b]	11.7 ± 0.3[b]	42,190.2

Values followed by different letters in each treatment are significantly different from each other ($p < 0.05$). Results are from 15 fully ripened fruits per treatment

3.4 Effect on fruit organic acid content

Strawberries are particularly appealing for human consumption because of their antioxidant potential, which is connected to the preservation of cardiovascular health (Giampieri et al. 2012) and the enhancement of bodily defences against oxidative challenges (Tulipani et al. 2014). Giampieri et al. (2012) reported that ascorbic acid (vitamin C) was involved in these antioxidant activities. In other studies on strawberries, increases in this vitamin have been found after inoculation with several strains of *Bacillus megaterium*, *B. simplex*, *Bacillus* sp. and *Paenibacillus polymyxa* (Erturk et al. 2012), *Pseudomonas fluorescens* and *Pseudomonas* sp. (Bona et al. 2015) and *Phyllobacterium endophyticum* (Flores-Félix et al. 2015b). However, as occurred after inoculation with strain PEPV16, decreases in ascorbic acid have been reported by other authors after inoculation with *Bacillus* sp. and *Pseudomonas* sp. (Pırlak and Köse 2009), *Bacillus* sp. and *Pseudomonas* sp. (Esitken et al. 2010) and with *Alcaligenes faecalis*, *Staphylococcus arlettae*, *S. simulans*, *Pantoea agglomerans*, *Agrobacterium rubi* and *Bacillus megaterium* (Ipek et al. 2014).

Contrary to what was observed for ascorbic acid, significant increases were noted in oxalic, aconitic, citric, malic, shikimic and fumaric acid contents after inoculation with strain PEPV16 (Table 2), although in all cases they were within the range of levels commonly found in strawberry fruits. To date, no studies have addressed the changes in the content of these organic acids in strawberries after bacterial inoculation, and hence further work is necessary to determine whether this would be a general effect of this practice.

3.5 Concluding remarks

Strain PEPV16 of the genus *Rhizobium* colonized the roots of strawberry, and exerted a positive effect on strawberry yield in both its production modes (seeds and stolons). Inoculation can increase the number of stolons and fruits as well as the weight of first-category fruits, which are those most appreciated by consumers, and also the content of some minerals and organic acids. Our results show that biofertilization with *Rhizobium* can increase both the yield and quality of strawberries, contributing to preserving both human health and the environment.

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