FEMS Microbiology Ecology, 92, 2016, fiw092

doi: 10.1093/femsec/fiw092
Advance Access Publication Date: 28 April 2016
Research Article

RESEARCH ARTICLE

Pseudomonas fluorescens PICF7 displays an endophytic lifestyle in cultivated cereals and enhances yield in barley

Jesús Mercado-Blanco[1], Enriqueta Alós[2,†], María Dolores Rey[2,‡] and Pilar Prieto[2,*]

[1]Departments of Crop Protection, Institute for Sustainable Agriculture, Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), Avda Menéndez Pidal s/n, Campus Alameda del Obispo s/n, E-14004 Córdoba, Spain and [2]Plant Breeding, Institute for Sustainable Agriculture, Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), Avda Menéndez Pidal s/n, Campus Alameda del Obispo s/n, E-14004 Córdoba, Spain

*Corresponding author: Institute for Sustainable Agriculture, Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), Avda Menéndez Pidal s/n, Campus Alameda del Obispo, E-14004 Córdoba, Spain. Tel: +34-957-499-293; Fax: +34-957-499-252; E-mail: pilar.prieto@ias.csic.es
†Present address: Division of Crop Biotechnics, Katholieke Universiteit Leuven, 3001 Leuven, Belgium.
‡Present address: John Innes Centre, Crop Genetics Department, Norwich NR4 7UH, UK.
One sentence summary: Colonization and plant growth promotion abilities of the endophytic bacterium *Pseudomonas fluorescens* PICF7 in barley and wheat.
Editor: Birgit Mitter

ABSTRACT

Pseudomonas fluorescens PICF7, an indigenous inhabitant of olive roots, displays an endophytic lifestyle in this woody crop and exerts biocontrol against the fungal phytopathogen *Verticillium dahliae*. Here we report microscopy evidence that the strain PICF7 is also able to colonize and persist on or in wheat and barley root tissues. Root colonization of both cereal species followed a similar pattern to that previously reported in olive, including inner colonization of the root hairs. This demonstrates that strain PICF7 can colonize root systems of distant botanical species. Barley plants germinated from PICF7-treated seeds showed enhanced vegetative growth. Moreover, significant increases in the number of grains (up to 19.5%) and grain weight (up to 20.5%) per plant were scored in this species. In contrast, growth and yield were not significantly affected in wheat plants by the presence of PICF7. Proteomics analysis of the root systems revealed that different proteins were exclusively found depending on the presence or absence of PICF7 and only one protein with hydrogen ion transmembrane transporter activity was exclusively found in both PICF7-inoculated barley and wheat plants but not in the controls.

Keywords: confocal laser scanning microscopy; olive; plant-growth promotion; proteomics; root colonization; wheat

INTRODUCTION

A fair number of soil-inhabiting bacteria can promote plant growth either directly (e.g. providing nutrients, microelements or bacterially produced plant growth hormones) and/or indirectly (e.g. controlling plant diseases and pests or easing deleterious effects of abiotic stress) (see, for instance, Haas and Défago 2005; Lugtenberg and Kamilova 2009; Borriss 2011; Pliego,

Received: 26 February 2016; **Accepted:** 25 April 2016

Kamilova and Lugtenberg 2011). The beneficial effects exerted by any given plant growth promoting bacterium (PGPB) (Bashan and Holguin 1998) rely first on the successful colonization and persistence of a sufficient population level of the PGPB on or in target niches (Raaijmakers *et al*. 1995; Djavaheri *et al*. 2012). Efficient colonization of plant roots by beneficial bacteria depends on physical–chemical soil characteristics, the host plant genotype, the composition of root exudates, the plant-associated microbiome, as well as specific bacterial phenotypes (Lugtenberg, Dekkers and Bloemberg 2001; Berg *et al*. 2006; Mercado-Blanco and Bakker 2007; Raaijmakers *et al*. 2009). The rhizosphere is the primary site where many plant–microbe and microbe–microbe interactions take place, as well as key multitrophic signalling events influencing them. The consequences of this complex interplay occurring at the below-ground level are of extraordinary significance for the whole plant, determining its development, fitness and productivity (Haas and Défago 2005; Perry *et al*. 2007; Berendsen, Pieterse and Bakker 2012; Coleman-Derr and Tringe 2014; Kowalski *et al*. 2015).

Pseudomonas spp. are essential components of root-associated microbiomes. Because of their ability to promote plant health and productivity many *Pseudomonas* strains pose an interest for exploitation in agricultural biotechnology (Stockwell and Stack 2007; Weller 2007; Höfte and Altier 2010). It is worth mentioning that some beneficial, plant-associated *Pseudomonas* spp. can even establish themselves as endophytes (Mercado-Blanco and Bakker 2007, and references therein). Diverse studies have proved that the presence of endophytic *Pseudomonas* spp. provides benefits for the host plant. Indeed, direct plant growth promotion and/or suppression of phytopathogen-caused deleterious effects are attributed to the presence of pseudomonad strains, either alone or as part of bacterial consortia (Brooks *et al*. 1994; Chen *et al*. 1995; Chanway *et al*. 2000; Nejad and Johnson 2000; Adhikari *et al*. 2001; Kuklinsky-Sobral *et al*. 2004; Grosch *et al*. 2005; Wang *et al*. 2005; Malfanova *et al*. 2013).

Pseudomonas fluorescens PICF7 (Martínez-García *et al*. 2015) is a native olive (*Olea europaea* L.) root inhabitant showing an endophytic lifestyle in this organ (Prieto and Mercado-Blanco 2008) and effective biocontrol activity (Prieto *et al*. 2009; Maldonado-González *et al*. 2015b) against Verticillium wilt of olive (VWO), a devastating disease caused by the soil-borne fungal pathogen *Verticillium dahliae* Kleb. (López-Escudero and Mercado-Blanco 2011). Strain PICF7 is an excellent candidate to be used as a model bacterium in plant–pathogen–endophyte interaction studies. While biocontrol exerted by the strain PICF7 has been demonstrated under different experimental conditions, and both early surface and inner colonization of olive roots are needed for the successful suppression of VWO (Prieto *et al*. 2009), direct plant growth promotion of olive plants is more difficult to assess. Nevertheless, evidence of growth promotion has been obtained (Mercado-Blanco *et al*. 2004).

Currently available '-omics' approaches are steadily enlightening our understanding of the mechanisms underlying plant–beneficial microbe interactions at the soil/root level and how they determine above-ground responses (Massart *et al*. 2015, and references therein). For instance, functional genomics analysis has revealed that colonization of olive roots by *P. fluorescens* PICF7 induces, among others, a broad array of defence responses at both local and systemic level (Schilirò *et al*. 2012; Gómez-Lama Cabanás *et al*. 2014).

Proteomic approaches applied to the study of plant–beneficial microorganism interactions, including endophytic bacteria, have been carried out in the past using gel-based techniques (Cheng *et al*. 2009; Jiang *et al*. 2013; Li *et al*. 2013; Alberton *et al*. 2013; Cordeiro *et al*. 2013). Several studies have reported changes in the host plant proteome as a consequence of the application of beneficial microorganisms, although most referred to the use of *Trichoderma* spp. interacting with herbaceous crops (Segarra *et al*. 2007; Brotman *et al*. 2008; Shoresh and Harman 2008). Some of these studies enabled the identification of potential elicitors of plant defence mechanisms and plant growth stimulation (Hermosa *et al*. 2010). In contrast, information on the influence of PGPBs in plant growth promotion by mean of proteomic approaches is far less frequent (Kandasamy *et al*. 2009; Faleiro *et al*. 2015). Nowadays, high throughput analyses of proteomes by gel-free approaches (based on mass spectrometry) are available and allow the identification of thousands of proteins (Massart *et al*. 2015). Hence, it is expected that the application of these techniques could provide a deeper knowledge of the effects caused by PGPBs in the root proteome and could be useful to identify low abundance proteins such as receptors or signal transduction elements.

More than two-thirds of global cropland features annual grain crops, roughly 70% of humanity's food energy needs and typically grown in monoculture. Annual grain production, at its current scale, is fundamentally unsustainable, but humanity must be fed. The growing human population demands greater crops, more productive and better adapted to specific agro-climatic conditions (Godfray *et al*. 2010). Wheat grains provide a fifth of the calories and the protein for the world's population (Shiferaw *et al*. 2013). The genetic improvement of wheat has been one of the main objectives of crop breeding programmes. Despite major accomplishments, numerous features of wheat grain and plants remain to be improved, such as yield and seed nutritional content among others (Reynolds *et al*. 2010). Similarly, barley is cultivated worldwide and is of considerable economic importance for animal feed and alcohol production. The overall importance of barley as a human food is minor but there is much potential for new uses exploiting the health benefits of whole grain. Although germplasm resources for barley are considerable, new approaches to improve desirable traits such as yield could be also explored.

Within this context, the objectives of this work were to assess whether *P. fluorescens* PICF7 is able to colonize and persist on the surface and within inner tissues of roots of barley and wheat, which are distantly related species to that from what this beneficial bacterium was originally isolated; and to test whether effective colonization of the roots of these two economically relevant cereal crops influences their growth and yield under non-gnotobiotic conditions. Potential changes taking place in the root proteomes of these cultivated Poaceae members upon root colonization by the strain PICF7 were also assessed.

MATERIALS AND METHODS

Production of bacterial inocula

Fresh cultures of wild-type *P. fluorescens* PICF7 (Mercado-Blanco *et al*. 2004; Martínez-García *et al*. 2015) and of its enhanced green fluorescent protein (EGFP)-labelled derivative harbouring plasmid pMP4655 (Bloemberg *et al*. 2000; Prieto and Mercado-Blanco 2008), the latter used for microscopy studies, were obtained by incubation at 25°C on Luria–Bertani (LB) (Miller 1972) agar plates. To propagate strain PICF7(pMP4655) tetracycline (Tc, Sigma-Aldrich, St Louis, MO, USA) was added at 20 mg mL^{-1} when required. Inocula of both strains were prepared from the bacterial biomass grown on LB agar plates at 25°C for 48 h, scraped from the medium with a sterile glass rod, and suspended in

10 mM $MgSO_4 \cdot 7H_2O$. Bacterial cell densities used in different experiments (root colonization and plant growth promotion, see below) were adjusted spectrophotometrically (A_{600} nm) by building up standard curves and culturing viable cells from serial dilution series onto LB plates (to count PICF7 wild type colonies), or LB plates supplemented with Tc (to count EGFP-labelled PICF7 colonies). The density of PICF7(pMP4655) cells present in the inoculum used to monitor wheat and barley root colonization by confocal laser scanning microscopy (CLSM; see below) was adjusted to 5×10^8 colony forming units (CFU)·mL^{-1}. Cells suspensions of wild type PICF7 prepared to bacterize seeds used in plant growth promotion bioassays ranged from 1.3×10^{10} to 2×10^{10} CFU mL^{-1}.

Plant material

Seeds of bread wheat (*Triticum aestivum* L., $2n = 6x = 42$) cv. *Chinese Spring* (CS) and barley (*Hordeum vulgare* L., $2n = 14$) cv. *Betzes* were used for root colonization and plant growth promotion experiments.

Barley and wheat root colonization assay

Thirty seeds per cereal species were placed in 50 mL sterile screw cap prolypropylene tubes and 35 mL of a PICF7(pMP4655) cells suspension, prepared as indicated above, was poured in. Tubes were covered with aluminium foil to avoid direct light exposure and seeds were incubated with gentle shaking using a Duomax 1030 Rocking Shaker (Heidolph®, Schwabach, Germany) during 6 h at room temperature (RT). Then, seeds were grown under three different growing conditions: (i) over sterile water-soaked filter paper placed in Petri dishes (five seeds per plate); (ii) in tubes containing sterile water-agar (one seed per tube) (these two systems were therefore axenic conditions), and (iii) pots containing autoclaved (121°C, 1 h, twice on consecutive days) sandy substrate prepared *ad hoc* (Prieto and Mercado-Blanco 2008). All seeds in their respective containers were randomly distributed and incubated in an I-36LL Controlled Environmental Chamber (Percival Scientific, Perry, IA, USA) at 25°C, 60–80% relative humidity, and 16 h light (115 μmol m^{-2} s^{-1}) for 40 days. Seeds growing in Petri dishes were only possible to maintain until day 16. Plants in pots were watered as needed. Finally, a group of seeds manipulated and grown under the same conditions were used as control treatment [i.e. no PICF7(pMP4655)] to check tissue autofluorescence and/or the presence of native fluorescent bacteria. In this case, pre-germinated seeds were immersed and incubated in a sterile 10 mM $MgSO_4 \cdot 7H_2O$ solution. This experiment (including the three different seed growing conditions) was repeated once.

Assessment of *Pseudomonas fluorescens* PICF7 root colonization by confocal laser scanning microscopy

Barley and wheat seedlings were carefully sampled from the above-mentioned growing conditions (i.e. plates, tubes and pots) at different sampling times from bacterization. Seedlings grown in pots were carefully uprooted to avoid root damage and the root systems were washed by dipping them in tap water to remove attached sand particles. Roots from each seedling were thoroughly analysed by confocal laser scanning microscopy (CLSM) as previously described (Prieto and Mercado-Blanco 2008; Prieto *et al.* 2011). Several root segments (4–5 cm long) representative of the whole root system of each sampled wheat/barley seedling were analysed on the confocal microscope over 40 days after bacterial inoculation (DAB) for seeds growing in tubes and pots and 16 DAB for seeds growing in Petri dishes to monitor *P. fluorescens* PICF7(pMP4655) colonization. Inoculated seedlings were sequentially taken: first, two plants per day from 1 to 10 DAB, and after that two plants every 5 days, from each of the growing conditions used (i.e. plates, tubes and pots). Two non-bacterized, control seedlings were also sampled at the first and last DAB to check for plant tissue autofluorescence, possible fluorescent native bacteria or cross contamination.

Bacterial colonization of barley and wheat root seedlings was analysed from three-dimensional (3D) confocal optical stacks collected using an Axioskop 2 MOT microscope (Carl Zeiss Inc., Jena GmbH, Germany) equipped with an argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss Inc.). EGFP-tagged bacterial cells were excited with the 488 nm argon laser line and were detected in the 500–520 nm window. CLSM microscope data were recorded and then transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss Inc.). Projections from adjacent confocal optical sections were made for building up images finally selected to be presented in this study. Figures were processed with Photoshop 4.0 software (Adobe Systems Inc., San Jose, CA, USA).

Barley– and wheat–*Pseudomonas fluorescens* PICF7 plant growth promotion assessment bioassays

Two independent experiments were conducted in consecutive seasons to evaluate the ability of *P. fluorescens* PICF7 to promote plant growth and to increase yield in barley and wheat. For each bioassay, 120 seeds of both cereal species were prepared, pre-germinated and bacterized as described above. Then, seeds were individually placed on pots filled in with standard commercial organic substrate (Floragard, Oldenburg, Germany).

Barley–*P. fluorescens* PICF7 experiments were carried out in completely randomized designs in the greenhouse at the Institute for Sustainable Agriculture (IAS; Córdoba, Spain) under controlled conditions. During the first bioassay, conditions scored within the greenhouse were as follows: average minimal temperature (T_{min}) = 15.5°C (absolute T_{min} = 10.8°C); average maximal temperature (T_{max}) = 24.9°C (absolute T_{max} = 28.9°C); average temperature (T) = 19°C; average minimal relative humidity (RH_{min}) = 67% (absolute RH_{min} = 53%); average maximal relative humidity (RH_{max}) = 90.1% (absolute RH_{max} = 96%); average HR during the whole period = 82.5%. During the second experiment conditions scored within the greenhouse were as follows: average T_{min} = 14.4°C (absolute T_{min} = 8.8°C); average T_{max} = 26.5°C (absolute T_{max} = 31.3°C); average T = 19.2°C; average minimal relative humidity (RH_{min}) = 61.6% (absolute RH_{min} = 51%); average maximal relative humidity (RH_{max}) = 88.6% (absolute RH_{max} = 95%); average HR during the whole period was 77.6%. In all experiments, plants were watered as needed.

Wheat–*P. fluorescens* PICF7 experiments were carried out in completely randomized designs in an experimental field protected with metallic fencing in the Institute for Sustainable Agriculture (IAS; Córdoba, Spain, coordinates 37°53′0″ N, 4°46′0″W, altitude 120 m.a.s.l.) under open field conditions. Bioassays were conducted over 4 months (January to May) in two consecutive years (2013 and 2014) until harvesting. Wheat plants were kept in the greenhouse during the first month at the same conditions of temperature and RH as barley plants. After 1 month, plants were moved to the experimental field where more favourable conditions for their growth were met. Temperature conditions during the experiment performed in 2013 ranged from an average T_{min} = 8.2°C (absolute T_{min} = −0.5°C) to an average

T_{max} = 19.1°C (absolute T_{max} = 32.8°C); average T during the whole period = 12.9°C. Temperature conditions during the bioassay conducted in 2014 ranged from an average T_{min} = 8.6°C (absolute T_{min} = 0.2°C) to an average T_{max} = 21°C (absolute T_{max} = 34.7°C); average T during the whole period was 14.3°C. Finally, a third wheat–*P. fluorescens* PICF7 bioassay was totally carried out in the greenhouse. In this case, conditions were similar to those described for the barley–*P. fluorescens* PICF7 experiment performed in 2013.

Plant growth parameters such as stem length and above-ground biomass dry weight (early growth parameters at first sampling time; t = 30 DAB; n = 20 plants), dry weight of the aerial biomass and number of stems (intermediate growth parameters at second sampling time; t = 60 DAB, n = 20 plants) and number of spikes, number of grains per plant and grain weight per plant (yield production at harvesting time; t = 120 DAB; n = 20 plants) were scored for each experiment and cereal species. Above-ground biomass dry weight was scored after drying samples in an oven at 65°C for a duration of 72 h. Plants for proteomic analysis (2) were sampled at 15 DAB (see below).

Protein extraction and quantification

Proteins were extracted following a phenol-based protocol described in Collado-Romero, Alós and Prieto (2014) with slight modifications. Briefly, the complete root systems from two plants of each species were sampled at 15 DAB and independently ground into a fine powder using sterile mortar and pestle and liquid nitrogen. The ground tissue was resuspended in phenol extraction buffer (0.9 M sucrose, 0.5 M Tris–HCl, 50 mM EDTA, 0.1 M KCl, Milli-Q water and freshly added 1% Triton X-100, 2% β-mercaptoethanol and 1% protease inhibitor cocktail set VI (Calbiochem, Madrid, Spain, pH 8) and homogenized on ice using micropestles. Samples were subsequently mixed with one volume of phenol solution equilibrated with 10 mM Tris–HCl pH 8, 1 mM EDTA (Sigma-Aldrich, Madrid, Spain), shaken for 1 min, incubated for 20 min in a tube rotator at 4°C and centrifuged at 18 000 × g for 10 min at 4°C. The upper phenolic phase was collected and proteins were precipitated by adding five volumes of ice cold 0.1 M ammonium acetate and 13 mM DTT in methanol at −80°C for 2 h. A pellet of proteins was obtained by centrifugation at 20 000 × g for 20 min at 4°C. Then, the pellet was washed once with ice cold 0.1 M ammonium acetate, 13 mM DTT in methanol and twice with 80% ice cold acetone. Finally, the pellet was air dried, dissolved in denaturing buffer (6 M urea, 50 mM ammonium bicarbonate pH 8) and stored at −80°C. Protein concentration was determined with the Pierce BCA Protein Assay Kit (Cultek, Madrid, Spain) according to manufacturer's instructions for the microplate procedure. Protein quality was checked by 1D-SDS-PAGE using Mini-Protean cell (Bio-Rad Laboratories, Madrid, Spain) and 12% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad, Madrid, Spain) stained with Coomassie Blue G250 (Supplementary Fig. S1).

Reversed phase-liquid chromatography–tandem mass spectrometry analysis

Protein extracts in 6 M urea and 50 mM ammonium bicarbonate pH 8 were reduced and alkylated. Disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 37°C, and then thiol groups were alkylated with 50 mM iodoacetamide for 1 h at room temperature in the dark. Samples were diluted to reduce urea concentrations below 1.4 M and digested using sequencing grade trypsin (Promega, Madison, WI, USA) overnight at 37°C in a trypsin/protein ratio of 1:5 (w/w). Digestion was stopped by the addition of 1% trifluoroacetic acid (TFA). Then, the supernatants were dried down and desalted onto ZipTip C18 Pipette tips (EMD Millipore Corp., Billerica, MA, USA) until mass spectrometric analysis.

Desalted digested proteins were dried out, resuspended in 0.1% formic acid and analysed by reversed phase-liquid chromatography–tandem mass spectrometry (RP-LC-MS/MS) in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The peptides were concentrated (on-line) by reversed phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Acclaim PepMap100 nanoViper, Dionex, Barcelona, Spain), and then separated using a 0.075 mm × 100 mm C18 RP column (Acclaim PepMap100 nanoViper, Dionex, Barcelona, Spain) operating at 0.3 μl/min.

Peptides from a 5 μg aliquot of the protein extract were eluted in a 180-min gradient of 5 to 40% solvent B (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). Electrospray ionization was carried out using a nano-bore emitter stainless steel ID 30 μm (Proxeon, Odense, Denmark) interface. The Orbitrap resolution was set at 30.000. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by 20 data-dependent MS/MS scans (Top 20), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion mode applied during 30 s periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific, Barcelona, Spain). Database search was performed against Uniprot_Viridiplantae and the *Pseudomonas* database (www.pseudomonas.com). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions. Searches were performed allowing optional Met oxidation and Cys carbamido methylation. Search against decoy database (integrated decoy approach) was performed using false discovery rate (FDR) < 0.01. Protein identification by nLC-MS/MS was carried out at the CBMSO protein chemistry facility that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001.

Bioinformatics and functional analysis of identified proteins

MIAPE guidelines were followed in this work (Taylor *et al.* 2007). The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE (Vizcaíno *et al.* 2016) partner repository with the dataset identifier PXD003782. The output accessions obtained with the Proteome Discoverer software (Thermo Scientific, USA) were exported to Microsoft Excel for data analysis. First, a table containing information for all the proteins identified in the samples analysed, two biological replicates for each treatment and each species, was generated (Supplementary Table S1). Moreover, four tables containing the proteins exclusively identified in the control and treated plants were generated to illustrate the differential protein composition found in roots (see Results and Discussion section).To this end, only the proteins that were present in the two replicates of control or treated plants were considered for the comparison.

Statistical analysis

Statistical analyses of plant growth and yield parameters were performed using STATISTIX 9.0 software (Analytical Software,

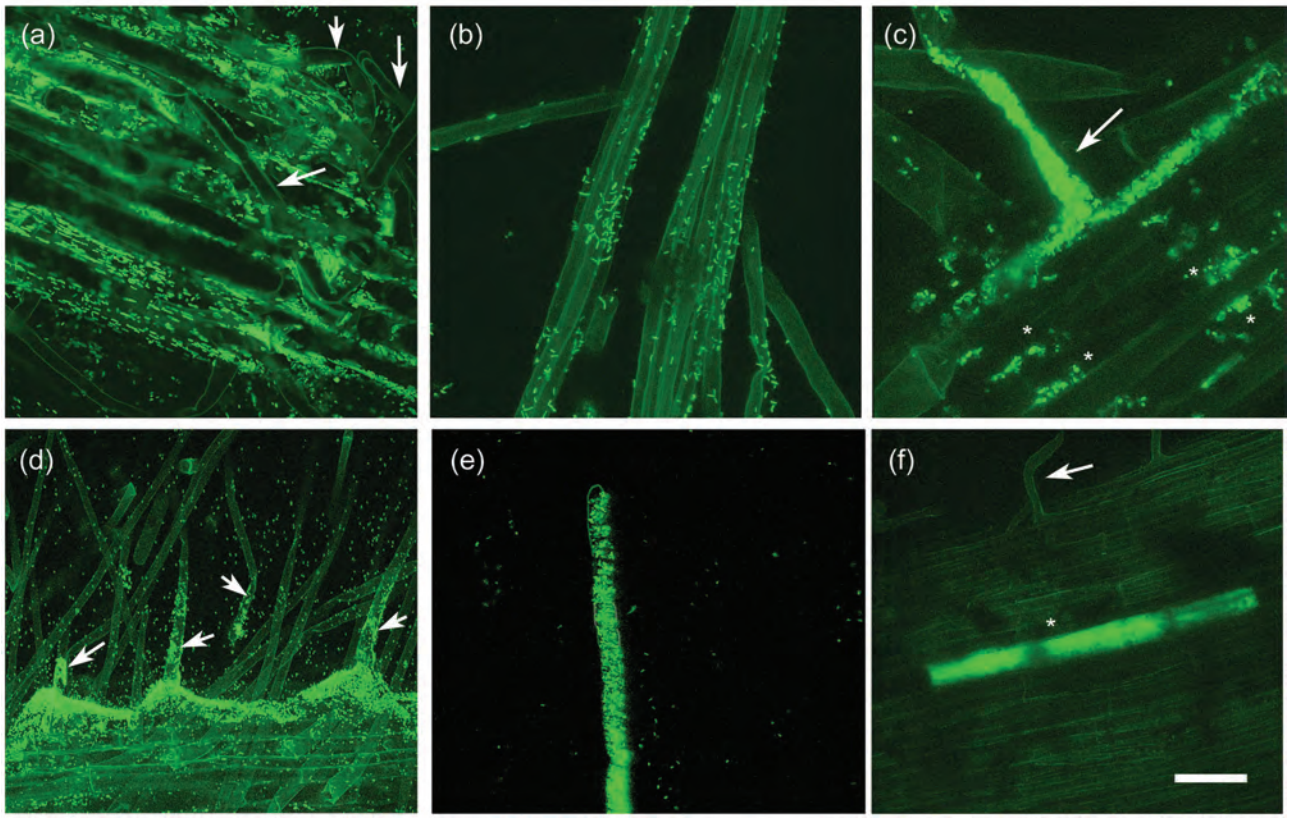

Figure 1. CLSM images of the time course of colonization processes of barley **(a–c)** and wheat **(d–f)** roots by EGFP-tagged *Pseudomonas fluorescens* PICF7 (in green). Confocal analysis was performed on 3–4 cm long root to show surface and inner PICF7 colonization. Single confocal optical sections are shown in (a) and (d). Images in (b) and (e) are projections of 10 adjacent confocal optical sections. Images in (c) and (f) are projections of 25 adjacent confocal optical sections. Focal step between confocal optical sections was 0.5 μm. (a) Surface EGFP-tagged PICF7 colonization of barley roots, 2 DAB. Non-colonized root hairs are shown by arrows. (b) Details of surface EGFP-tagged PICF7 colonization of several barley root hairs, 2 DAB. (c) Confocal optical sections of an internally EGFP-tagged PICF7 colonized root hair cell (shown by arrows) and intercellular colonization of the cortical tissue (*), 20 DAB. Several non-colonized root hairs are also shown. (d) Surface EGFP-tagged PICF7 colonization of wheat roots, 2 DAB. Roots hairs externally colonized by EGFP-tagged PICF7 are shown by arrows. (e) Detail of an entirely EGFP-tagged PICF7 colonized wheat root hair, 35 DAB. (f) Intercellular colonization of the cortical tissue (*) of the wheat root by EGFP-tagged PICF7 cells, 35 DAB. A non-colonized root hair is shown by arrows. Scale bar represents 20 μm in all panels except (c) where it represents 15 μm.

Tallahassee, FL, USA). The analysis of variance (ANOVA) was based on a completely randomized design. Means were separate using the least significant difference (LSD) test with a probability level of 0.05.

RESULTS AND DISCUSSION

Pseudomonas fluorescens PICF7 efficiently colonizes barley and wheat roots, even at the endophytic level

Efficient root colonization by a given PGPB is a prerequisite to exert effective growth promotion of the host plant, either by direct (e.g. phytostimulation) or indirect (e.g. disease suppression) means (Lugtenberg and Kamilova 2009). *Pseudomonas fluorescens* PICF7 has been previously characterized as a good colonizer of olive roots, even endophytically, a lifestyle observed under variable experimental conditions and in different olive cultivars (Prieto and Mercado-Blanco 2008; Prieto *et al.* 2011; Maldonado-González *et al.* 2015b). Strain PICF7 is also able to colonize and persist on roots of sunflower (*Helianthus annuus* L.) (Maldonado-González *et al.* 2012) and *Arabidopsis thaliana* (Maldonado-González *et al.* 2015a), although evidence of endophytic colonization in these two species has not been found. Here, we provide microscopy evidence showing that strain PICF7 efficiently colonized the root systems of both barley and wheat (Fig. 1). Moreover, PICF7 was able to develop an endophytic lifestyle in root tissues of these two cereal crops. The combined use of CLSM and an EGFP-tagged PICF7 derivative has thus enabled the *in situ* localization of bacterial cells on or in roots of both species without any tissue manipulation.

CLSM imagery showed that wheat and barley roots were rapidly colonized by PICF7(pMP4655) cells soon after seed bacterization (1 DAB), irrespective on how seedlings were grown (i.e. over sterile filter paper within Petri dishes, in tubes filled with water agar, or in sterile sandy soil pots). Moreover, fluorescently tagged bacteria persisted on/in roots of both cereal species throughout the duration of the bioassay (40 days), independently of how seedlings were grown. Nevertheless, bacterial colonization of wheat and barley root surfaces was not uniform. Thus, some root regions showed an abundance of PICF7 single cells or microcolonies (Fig. 1a, barley) or even areas covered by a bacterial biofilm (Fig. 1d, wheat), while others were completely devoid of fluorescent bacteria. Overall, PICF7 cells were more frequently found near root hairs (Fig. 1a and d), an observation previously reported for olive roots (Prieto and Mercado-Blanco 2008; Prieto *et al.* 2011). This is a common observation in root–bacteria interaction studies, and could be explained as due to an abundance of nutrients and/or bacterial chemo-attractants, to

the presence of preferred attachment sites in these specialized root cells, or simply as a consequence of being a more protective microenvironment for the bacteria. It must be emphasized that studies dealing with root hair colonization by bacteria mostly refer to attachment and/or colonization of the root hair surface (Hansen *et al.* 1997; Rediers *et al.* 2003; Rincón *et al.* 2005; Rothballer, Schmid and Hartmann 2003). For instance, colonization of root hairs in barley by *Pseudomonas* spp. strains was observed, although inner localization of bacteria within these specialized root epidermal cells was not found (Buddrus-Schiemann *et al.* 2010). In fact, internal colonization of root hairs has been rarely reported, although recent studies depict a different scenario that has likely been overlooked. Our previous studies have demonstrated that root hairs play an important role during the endophytic colonization of olive roots by strain PICF7 (Prieto *et al.* 2011). CLSM analysis performed in the present study demonstrates that root hairs of barley and wheat are also internally colonized by PICF7 (Fig. 1c and e). Evidence of internal colonization of root hairs has been reported elsewhere as well (Paungfoo-Lonhienne *et al.* 2010; Upreti and Thomas 2015). It has thus been proposed that endophytic colonization via root hairs by soilborne PGPBs other than Rhizobiaceae members, which display a well-known root hair infection process (Kijne 1992), could be a common phenomenon (Mercado-Blanco and Prieto 2012).

Overall, the colonization process of wheat and barley roots by strain PICF7 showed a similar pattern to that previously observed for olive root colonization regardless of whether gnotobiotic or non-gnotobiotic experimental conditions were used (Prieto and Mercado-Blanco 2008; Prieto *et al.* 2011; Maldonado-González *et al.* 2015b).

The colonization pattern of PICF7 was similar in the two species for the three growth conditions (paper in Petri dishes, tubes or pots). Briefly, from 1 to 20 DAB PICF7-tagged populations were observed predominantly in both species on the root surface (Fig. 1a and d). Nevertheless internal colonization of wheat and barley root tissues by PICF7(pMP4655) cells occurred differently in time for each species. In fact, PICF7(pMP4655) cells were internally detected in barley earlier (20 DAB) than in wheat (35 DAB, Fig. 1). From this time point on, no significant changes in sampled plants were observed until the end of the bioassay (40 DAB). *Pseudomonas fluorescens* PICF7 was not found in the vascular tissue at any time. Finally, wheat and barley roots from non-bacterized, control plants did not show fluorescent bacteria at any time during the bioassay (data not shown).

Many studies on the surface and endophytic colonization pattern by diverse PGPBs of barley and wheat tissues, starting from seed inoculation, are available (Weller 1983; Kragelund and Nybroe 1996; Hansen *et al.* 1997; Schloter and Hartmann 1998; Tombolini *et al.* 1999). In this study we have provided evidence that a beneficial bacterium isolated from a distant plant host (olive) showed a similar root colonization pattern in at least one dicot and two monocots. Moreover, strain PICF7's ability to develop an endophytic lifestyle in some species (olive, barley and wheat) (Prieto and Mercado-Blanco 2008; this study) but not in others (*A. thaliana*; Maldonado-González *et al.* 2015a), offers a good scenario to disentangle underlying mechanisms leading to root endophytism displayed by this bacterium.

Pseudomonas fluorescens PICF7 enhances yield in barley but not in wheat

Once effective colonization of wheat and barley roots by *P. fluorescens* PICF7 was demonstrated, we aimed to assess whether this bacterium was able to promote plant growth and yield in both cereals when grown under non-gnotobiotic conditions and after seed treatment. We pursued an objective with practical implications; that is, to demonstrate growth promotion in annual, fast-growing species, thereby overcoming limitations encountered when working with olive, and to explore potential benefits in crops taxonomically distant from the natural host. Microbe-assisted crop production is an active research area and reports on the isolation, identification and characterization of PGPBs are abundant. Thus, the search for new PGPBs able to enhance growth promotion of cereal crops offers continuous advances. For instance, it is of particular relevance to identify novel PGPBs, including those showing an endophytic lifestyle, originating from plants and/or geographical and environmental conditions to which they are adapted and will be eventually deployed (Majeed *et al.* 2015; Pang *et al.* 2016). Alternatively, engineering well-characterized rhizobacteria to enhance their capabilities as PGPBs in alternative plant hosts is an interesting approach already providing promising results (Setten *et al.* 2013).

Among PGPBs interacting with cereal crops, *Pseudomonas* spp. strains able to increase their growth and health upon seed treatment have been studied for decades (Weller and Cook 1986). *Pseudomonas* spp.-assisted growth promotion of cereals has been demonstrated, even under field conditions (de Freitas and Germida 1992). For instance, strain *Pseudomonas* sp. DSMZ 13134 promoted enhanced growth and yield in barley plants under nutrient deprivation and greenhouse conditions. Moreover, under field conditions 20% yield increase was obtained supporting the successful agronomical application of this strain (Fröhlich *et al.* 2012).

Results obtained from independent experiments performed in two consecutive seasons after *P. fluorescens* PICF7 treatment of wheat and barley seeds differed depending on the cereal species. While seed bacterization led to enhanced growth of barley plants and eventually to a significant ($P < 0.05$) increase in yield (Table 1), wheat plants were not affected by PICF7 treatment at the end of the experiments (Table 2). The increase in barley biomass was constant over time. Thus, above-ground biomass dry weight and the number of stems per plant were significantly ($P < 0.05$) higher in PICF7-treated plants than in control, non-bacterized plants already at 60 DAB in both experiments (Table 1). Eventually, at harvesting time (i.e. end of the bioassays, 120 DAB), the number of grains per plant increased by 19.5% (experiment I) or 16.6% (experiment II) in PICF7-treated plants compared with control plants (Table 1). Likewise, grain weight per plant increased by 20.5% (experiment I) or 19% (experiment II) in plants developed from bacterized seeds (Table 1). These yield increases in barley plants were very similar to values reported by Fröhlich *et al.* (2012) when using strain *Pseudomonas* sp. DSMZ 13134 under field conditions. In contrast, wheat plants did not show significant ($P > 0.05$) yield increases in any experiment (Table 2) although above-ground biomass showed significant ($P < 0.05$), variable differences at first and second sampling times. For instance, above-ground biomass dry weight and number of stems were significantly ($P < 0.05$) higher in PICF7-treated plants at 60 DAB in experiment I. To the contrary, these growth parameters were significantly ($P < 0.05$) lower in plants from bacterized seeds at the same sampling point in experiment II.

To explain why growth and yield promotion was effective in barley but not in wheat further investigation would be needed. Several factors (i.e. genetic, environmental, physiological, etc.), alone or in combination, could be responsible for this differential outcome. Without excluding other possibilities, a plausible explanation for final yield promotion in barley would be the different temperature at which barley and wheat experiments

Table 1. Enhanced yield obtained upon bacterization of barley seeds with *Pseudomonas fluorescens* PICF7. Values are given as mean ± standard error of the mean (SE). Letters a and b indicate if there are differences among treatments (presence or absence of PIC7) at $P < 0.05$. Twenty control and twenty PICF7 bacterized plants were scored at each sampling time. Stem dry weight refers to complete above-ground biomass.

	First sampling time (30 days)		Second sampling time (60 days)		Third sampling time (120 days)		
	Stem length (cm)	Stem dry weight (g)	Stem dry weight (g)	Number of stems	Number of spikes	Number of grains per plant	Grain weight per plant (g)
Experiment I							
Control	38.68 ± 3.70[a]	0.17 ± 0.04[b]	2.55 ± 0.35[b]	4.615 ± 0.27[b]	9.78 ± 2.44[a]	121.38 ± 27.05[b]	4.63 ± 0.95[b]
PICF7	40.15 ± 6.00[a]	0.21 ± 0.06[a]	3.20 ± 1.03[a]	6.000 ± 1.12[a]	10.30 ± 2.06[a]	145.00 ± 19.62[a]	5.58 ± 0.69[a]
P-value	0.3554	0.0113	0.0001	0.0015	0.6191	0.0473	0.0302
Experiment II							
Control	43.83 ± 3.12[a]	0.28 ± 0.03[a]	3.30 ± 0.29[a]	8.20 ± 0.42[b]	7.20 ± 2.46[a]	130.35 ± 26.54[b]	5.35 ± 1.34[b]
PICF7	43.09 ± 3.86[a]	0.28 ± 0.04[a]	2.92 ± 0.30[b]	8.70 ± 0.48[a]	7.75 ± 1.73[a]	151.96 ± 35.46[a]	6.35 ± 1.75[a]
P-value	0.5093	0.6297	0.0103	0.0239	0.39	0.0298	0.0419

Table 2. Effect on growth and yield parameters observed in wheat plants upon bacterization of seeds with *Pseudomonas fluorescens* PICF7. Values are given as mean ± standard error of the mean (SE). Letters a and b indicate if there are differences among treatments (presence or absence of PIC7) at $P < 0.05$. Twenty control and twenty PICF7 bacterized plants were scored at each sampling time. Stem dry weight refers to complete above-ground biomass.

	First sampling time (30 days)		Second sampling time (60 days)		Third sampling time (120 days)		
	Stem length (cm)	Stem dry weight (g)	Stem dry weight (g)	Number of stems	Number of spikes	Number of grains per plant	Grain weight per plant (g)
Experiment I							
Control	46.20 ± 2.84[a]	0.13 ± 0.03[a]	2.44 ± 0.16[b]	5.70 ± 0.82[b]	4.53 ± 0.74[a]	207.67 ± 47.76[a]	6.33 ± 1.53[a]
PICF7	41.02 ± 9.34[b]	0.14 ± 0.03[a]	2.73 ± 0.37[a]	7.00 ± 0.94[a]	4.53 ± 1.06[a]	201.07 ± 35.15[a]	5.96 ± 1.31[a]
P-value	0.0267	0.7972	0.0372	0.0041	1.000	0.6698	0.4803
Experiment II							
Control	46.84 ± 2.36[a]	0.21 ± 0.03[a]	2.64 ± 0.31[a]	7.70 ± 0.48[a]	5.10 ± 0.81[a]	234.47 ± 29.089[a]	5.30 ± 0.63[a]
PICF7	43.09 ± 3.86[b]	0.18 ± 0.04[b]	2.32 ± 0.28[b]	6.10 ± 0.57[b]	5.55 ± 1.05[a]	216.45 ± 39.351[a]	4.82 ± 1.48[a]
P-value	0.0007	0.0279	0.0294	0	0.1485	0.1138	0.2031

were performed at the reproductive stages of the plant life cycle. Barley experiments were entirely carried out in a greenhouse where average temperature values were $T_{min} = 15.5°C$, $T_{max} = 24.9°C$, (average $T = 19°C$) for experiment I, and $T_{min} = 14.4°C$, $T_{max} = 26.5°C$ (average $T = 19.2°C$) during experiment II. These *T* values are optimal for plant development and not inhibitory for PICF7, although they are below the optimal *T* (28°C) for the growth of this bacterium (Martínez-García *et al.* 2015). In contrast, wheat plants were grown in pots under open-air (field) conditions after the first sampling time, resembling cropping conditions for winter wheat in southern Spain. Therefore, average *T* during these experiments was 5–7°C lower for wheat than for barley, and during a prolonged period of time. Suboptimal *T* values could have greatly influenced strain PICF7 growth and its metabolism, thereby limiting its effectiveness as a PGPB in this host even though this bacterium is still able to show slight growth at 5°C (Martínez-García *et al.* 2015). Although temperature values were different for wheat and barley experiments after the first sampling time (30 DAB), a third wheat experiment was entirely performed in the greenhouse, simultaneously with the first barley experiment. No plant growth promotion was observed in wheat in these conditions either (data not shown). In addition, the first sampling time was always when all wheat plants were still growing in the greenhouse, even in the experiments in which wheat plants were transferred into open field conditions, and no effect due to the presence of PICF7 was observed in any case (Table 2). How prolonged low temperatures could affect PICF7 performance needs further experimental evidence, but results suggested that continued temperatures below the optimal *T* for PICF7 could limit its potential as a PGPB under specific agronomical scenarios.

Effective colonization of both the surface and the interior of root tissues by beneficial soil-borne microorganisms can lead to important benefits to above-ground organs. Positive effects on plant growth mediated by endophytic colonization of beneficial microorganisms have been demonstrated in several studies (Triplett 1996; Nejad and Johnson 2000; Adhikari *et al.* 2001; Maciá-Vicente *et al.* 2009a,b). We have proved that seed bacterization with strain PICF7 significantly increased yield in barley. Although evidence of growth promotion exerted by this bacterium in its natural host was earlier reported (Mercado-Blanco *et al.* 2004), plant growth promotion in herbaceous, fast-growing hosts with economical relevance has been easier to demonstrate. This outcome has both practical and fundamental implications. On the one hand, the potential of this root endophyte as a bioformulation (seed treatment) aimed to enhance yield in cereal crops with high economic relevance can be explored. On the other hand, since barley and wheat showed a different response upon PICF7 treatment, an excellent study system is available to help unravel bacterial traits involved in plant growth

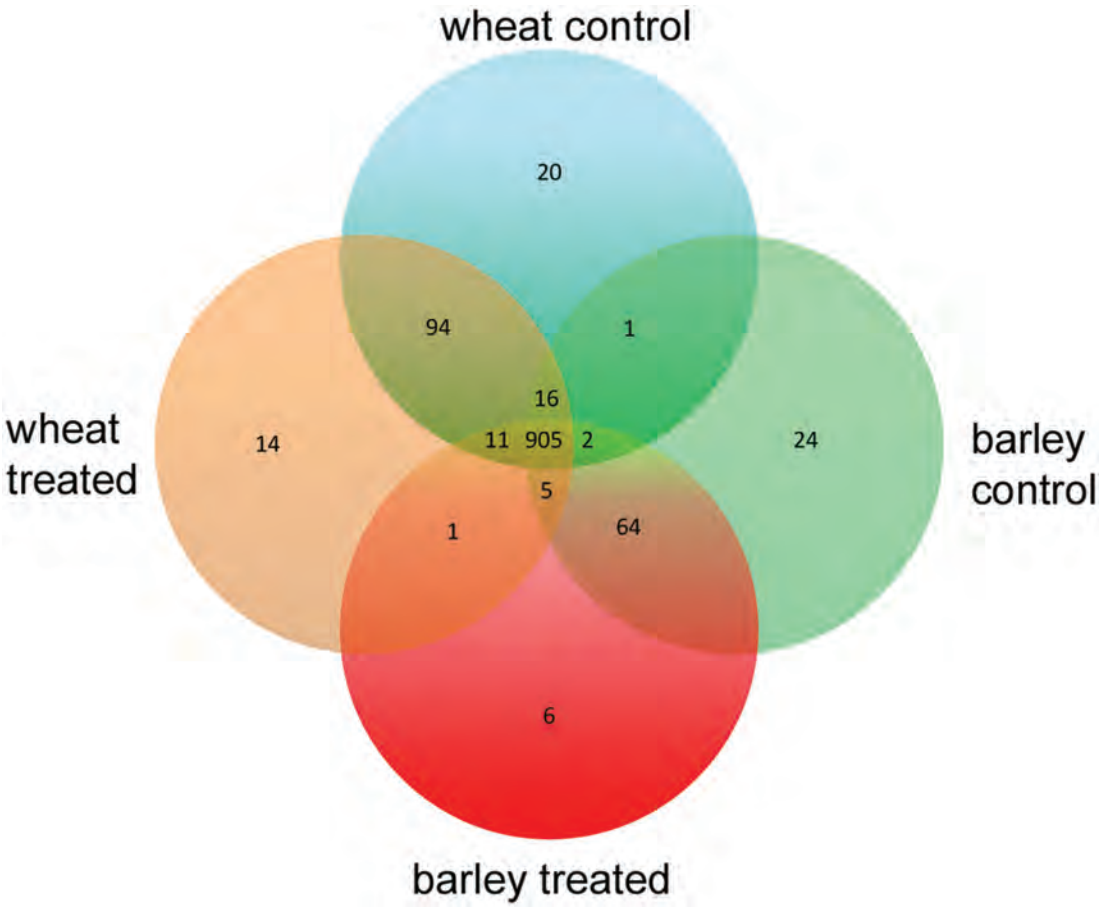

Figure 2. Venn diagram representing the proteins identified in wheat and barley roots in the presence or in the absence of *Pseudomonas fluorescens* PICF7. Only peptides with 5–30 amino acids and a minimum of two peptides per protein were used for positive identification, and peptide FDR < 0.01.

promotion operative at specific environmental conditions but not at others.

Barley and wheat root proteomes showed specific and distinct patterns upon *Pseudomonas fluorescens* PICF7 colonization

Proteomics constitutes a powerful approach to explore in detail mechanisms underlying plant–beneficial bacteria interactions. Even though it has not been broadly implemented in this specific area of research, a few examples using proteome analysis in PGPB–cereal interactions are available. For instance, to understand the molecular basis of plant growth promotion exerted by a *P. fluorescens* strain, Kandasamy *et al.* (2009) analysed the differential expression of diverse proteins involved in defence, primary and energy metabolism in rice seedlings. In another study, Cangahuala-Inocente *et al.* (2013) reported the identification of six proteins differentially expressed in roots of maize plants inoculated with the PGPB *Azospirillum brasilense* strain FP2. More recently, Faleiro *et al.* (2015) have shown that a number of proteins (46) were differentially accumulated in maize roots inoculated with strain FP2, identifying three of them (two up-regulated and one down-regulated) by MS. These authors correlated the observed changes in the maize root proteome profile with early steps (7 DAB) in the maize–FP2 interaction leading to several differences in plant growth parameters.

By the comparison of the proteins analysed by RP-LC-MS/MS, we have identified changes in the proteomic profile (15 DAB) of wheat and barley roots as a consequence of *P. fluorescens* PICF7 seed colonization (Fig. 2). The vast majority of the proteins were present in both treated and control roots of wheat and barley at 15 DAB. Thus, 905 proteins were found in all four root systems, namely wheat and barley, bacterized or not. A hundred and twenty-eight proteins were exclusively found in wheat roots, while 94 proteins were specific for barley roots (Fig. 2). As for wheat roots, only 14 proteins were solely found in PICF7-treated plants (Table 3, Fig. 2) while untreated plants displayed 20 proteins that were not detected in bacterized plants (Table 4, Fig. 2). Ninety-four proteins exclusively present in the wheat root proteome were found in both bacterized and not bacterized plants (Fig. 2). Regarding barley seedlings, only six proteins were exclusively present in the root proteome of PICF7-treated plants (Table 5, Fig. 2), whilst 24 proteins were specific to control untreated root plants (Table 6, Fig. 2). Sixty-four proteins of the barley root proteome were found in both bacterized and non-bacterized plants.

Whereas the information obtained by using this approach was abundant and more than a thousand proteins were analysed, it is interesting to remark that most of the proteins found in our study corresponded to uncharacterized, predicted or putative proteins (see Tables 3–6). This situation is frequently reported in proteomic studies dealing with plant–beneficial microbe interactions (Palmieri *et al.* 2012; Kwasiborski *et al.* 2014;

Table 3. Proteins exclusively found in roots of wheat seedlings originated from seeds treated with *Pseudomonas fluorescens* PICF7. The proteins were identified in the two replicates of the bacterized wheat roots and not in any other protein extracts. The Uniprot identification number (ID; http://www.uniprot.org/) and the protein name of the best match of the identified peptides are included.

Uniprot ID	Protein name
M8BCQ7	Nodal modulator 3 (*Aegilops tauschii*)
Q8S916	Phosphoenolpyruvate carboxylase (*Nicotiana sylvestris*)
F2CWC9	Predicted protein (*Hordeum vulgare*)
F2DEN3	Predicted protein (*Hordeum vulgare*)
S5A8C3	S-Formylglutathione hydrolase-like protein (*Triticum monococcum* subsp. monococcum)
W5AC36	Uncharacterized protein (*Triticum aestivum*)
W5GIY6	Uncharacterized protein (*Triticum aestivum*)
M8CVP3	Uncharacterized protein (*Aegilops tauschii*)
W5B1M0	Uncharacterized protein (*Triticum aestivum*)
W5FLX0	Uncharacterized protein (*Triticum aestivum*)
W5G3R0	Uncharacterized protein (*Triticum aestivum*)
W5GST5	Uncharacterized protein (*Triticum aestivum*)
M7ZLF7	Uncharacterized protein (*Triticum urartu*)
M7ZE29	Uncharacterized protein (*Triticum urartu*)

Table 4. Proteins exclusively found in roots of wheat seedling originated from untreated control seeds. The proteins were identified in the two replicates of the control wheat roots and not in any other protein extracts. The Uniprot identification number (ID; http://www.uniprot.org/) and the protein name of the best match of the identified peptides are included.

Uniprot ID	Protein name
W5DZ64	Acyl carrier protein (*Triticum aestivum*)
P08215	ATP synthase subunit alpha, chloroplastic (*Pisum sativum*)
B9RNI5	Importin subunit alpha (*Ricinus communis*)
F2CVI7	Predicted protein (*Hordeum vulgare* var. distichum)
B8B4A1	Putative uncharacterized protein (*Oryza sativa* subsp. indica)
A2ZM75	Putative uncharacterized protein (*Oryza sativa* subsp. indica)
I1HQ21	Pyruvate kinase (*Brachypodium distachyon*)
B4FYH2	Pyruvate kinase (*Zea mays*)
P00869	Ribulose bisphosphate carboxylase small chain (*Pisum sativum*)
Q9ZP07	Ribulose bisphosphate carboxylase small chain (*Cicer arietinum*)
W5GEJ9	Uncharacterized protein (*Triticum aestivum*)
W5BZ40	Uncharacterized protein (*Triticum aestivum*)
M8BZN7	Uncharacterized protein (*Aegilops tauschii*)
M0WRU5	Uncharacterized protein (*Hordeum vulgare* var. distichum)
W5E0N3	Uncharacterized protein (*Triticum aestivum*)
W5HB43	Uncharacterized protein (*Triticum aestivum*)
W5FES4	Uncharacterized protein (*Triticum aestivum*)
W5FZ36	Uncharacterized protein (*Triticum aestivum*)
W5G255	Uncharacterized protein (*Triticum aestivum*)
M7Z0P7	Uncharacterized protein (*Triticum urartu*)

Sharma *et al.* 2014), which highlights the urgent need for further investigation in this area. For instance, in PICF7-treated wheat plants only 3 out of the 14 proteins showed relevant identities: a putative Nodal modulator 3, a phosphoenolpyruvate carboxylase and an S-formylglutathione hydrolase-like protein (Table 3). In PICF7-treated barley plants the six proteins exclusively found in their roots were categorized as uncharacterized or predicted (Table 5). Regarding non-bacterized plants, only 7 out of 20 proteins in wheat (i.e. an acyl carrier protein, a chloroplastic ATP synthase subunit alpha), and 2 out of 24 proteins in barley (ATP synthase subunit beta and a eukaryotic initiation factor) showed relevant identities (Tables 4 and 6). The similarity of the unknown proteins with the *Pseudomonas* database (www.pseudomonas.com) was assessed, but no matches were found between the peptides and the proteins from the database.

Besides differences found in the proteomic profiles of each cereal species under two treatments (bacterized or not), which would need future in-depth insight to unravel the involvement and roles of the specific proteins accumulated in each condition, only two distinctive commonalities related to the presence or absence of PICF7 were found. On the one hand, a putative hydrogen ion transmembrane transporter (Q35784; http://www.uniprot.org/uniprot/Q35784) was identified in roots from both wheat and barley PICF7-treated plants but not in untreated plants. On the other hand, one uncharacterized protein ([M0S5K3], http://www.uniprot.org/uniprot/M0S1U3) was the only one found in both wheat and barley untreated plants. The role of these two proteins in cereal-PICF7 interaction is unknown.

Analysis of the root proteome of barley and wheat showed subtle differences in protein patterns depending on the

Table 5. Proteins exclusively found in roots of barley seedlings originated from seeds treated with *Pseudomonas fluorescens* PICF7. The proteins were identified in the two replicates of the bacterized barley roots and not in any other protein extracts. The Uniprot identification number (ID; http://www.uniprot.org/) and the protein name of the best matches of the identified peptides are included.

Uniprot ID	Protein name
F2DAW8	Predicted protein (*Hordeum vulgare* var. distichum)
F2DHM1	Predicted protein (*Hordeum vulgare* var. distichum)
V4TS58	Uncharacterized protein (*Citrus clementina*)
M0V3M9	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0Z4W3	Uncharacterized protein (*Hordeum vulgare* var. distichum)
K7TFC7	Uncharacterized protein (*Zea mays*)

Table 6. Proteins exclusively found in roots of barley seedlings originated from untreated control seeds. The proteins were identified in the two replicates of the control barley roots and not in any other protein extracts. The Uniprot identification number (ID; http://www.uniprot.org/) and the protein name of the best match of the identified peptides are included.

Uniprot ID	Protein name
F2EFA8	ATP synthase subunit beta (*Hordeum vulgare* var. distichum)
G8CLP1	Eukaryotic initiation factor (*Aegilops speltoides* subsp. speltoides)
F2CXH7	Predicted protein (*Hordeum vulgare* var. distichum)
F2CYB5	Predicted protein (*Hordeum vulgare* var. distichum)
F2DNQ0	Predicted protein (*Hordeum vulgare* var. distichum)
F2CXL5	Predicted protein (*Hordeum vulgare* var. distichum)
F2DA07	Predicted protein (*Hordeum vulgare* var. distichum)
F2DDF2	Predicted protein (*Hordeum vulgare* var. distichum)
F2DFW6	Predicted protein (*Hordeum vulgare* var. distichum)
F2DGN6	Predicted protein (*Hordeum vulgare* var. distichum)
F2DJA8	Predicted protein(*Hordeum vulgare* var. distichum)
F2E1T7	Predicted protein (*Hordeum vulgare* var. distichum)
F2E2M2	Predicted protein (*Hordeum vulgare* var. distichum)
F2E7R5	Predicted protein (*Hordeum vulgare* var. distichum)
U5DCB1	Uncharacterized protein (*Amborella trichopoda*)
I1HF20	Uncharacterized protein (*Brachypodium dystachion*)
M0XLU3	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0W8A5	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0WMC6	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0ZCR3	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0ZD47	Uncharacterized protein (*Hordeum vulgare* var. distichum)
M0S176	Uncharacterized protein (*Musa acuminata* subsp. malaccensis)
W5H6×2	Uncharacterized protein (*Triticum aestivum*)
W5HSU0	Uncharacterized protein (*Triticum aestivum*)

presence/absence of PICF7. These differences were specific for each cereal species, suggesting that the presence of the PGPB altered their root proteomes in distinctive ways. These scarce differences do not seem to be due to different environmental/growing conditions since the sampling time (15 DAB) was performed when barley and wheat plants were still under stable greenhouse conditions (see Materials and Methods). Therefore, protein pattern alterations by PICF7 seemed to be species-specific. Whether these differences conditioned growth and yield differences at harvesting time is still unknown. Since most of the proteins differentially expressed were uncharacterized and/or predicted, no definitive conclusion can be established so far. However, this study provides an exciting starting point to further analyse their true involvement in aspects such as barley and wheat root colonization by PICF7, including endophytism, and/or the differential plant growth and yield increase here reported.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

Thanks are due to Professor Antonio Martín for his support and the use of CLSM and vibratome facilities.

FUNDING

This work was supported by grants AGL2012-33264 from Spanish Ministerio de Economía y Competitividad (MINECO) and P12-AGR-667 (Convocatoria Proyectos de Excelencia from Junta de Andalucía, Spain), both co-funded by the European Regional Development Fund (ERDF) from the European Union (UE).

Conflict of interest. None declared.

REFERENCES

Adhikari TB, Joseph CM, Yang GP *et al.* Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. *Can J Microbiol* 2001;**47**:916–24.

Alberton D, Mueller-Santos M, Campos Brusamarello-Santos LC *et al.* Comparative proteomics analysis of the rice roots colonized by *Herbaspirillum seropedicae* strain SmR1 reveals induction of the methionine recycling in the plant host. *J Proteome Res* 2013;**12**:4757–68.

Bashan Y, Holguin G. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol Biochem* 1998;**30**:1225–8.

Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and plant health. *Trends Plant Sci* 2012;**17**:478–86.

Berg G, Opelt K, Zachow C *et al.* The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol Ecol* 2006;**56**:250–61.

Bloemberg GV, Wijfjes AHM, Lamers GEM *et al.* Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Mol Plant Microbe Interact* 2000;**13**:1170–6.

Borriss R. Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents. In: Maheshwari DK (ed.). *Bacteria in Agrobiology: Plant Growth Responses*. Berlin, Heidelberg: Springer, 2011, 41–76.

Brooks DS, Gonzalez CF, Appel DN *et al.* Evaluation of endophytic bacteria as potential biocontrol agents for oak wilt. *Biol Control* 1994;**4**:373–81.

Brotman Y, Briff E, Viterbo A *et al.* Role of swollenin, an expansin-like protein from *Trichoderma*, in plant root colonization. *Plant Physiol* 2008;**147**:779–89.

Buddrus-Schiemann K, Schmid M, Schreiner K *et al.* Root colonization by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. *Microb Ecol* 2010;**60**:381–93.

Cangahuala-Inocente GC, do Amaral FP, Faleiro AC *et al.* Identification of six differentially accumulated proteins of *Zea mays* seedlings (DKB240 variety) inoculated with *Azospirillum brasilense* strain FP2. *Eur J Soil Biol* 2013;**58**:45–50.

Chanway CP, Shishido M, Nairn J *et al.* Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. *For Ecol Manage* 2000;**133**:81–8.

Chen C, Bauske EM, Musson G *et al.* Biological control of Fusarium wilt on cotton by use of endophytic bacteria. *Biol Control* 1995;**5**:83–91.

Cheng Z, Duan J, Hao Y *et al.* Identification of bacterial proteins mediating the interactions between *Pseudomonas putida* UW4 and *Brassica napus* (Canola). *Mol Plant Microbe Interact* 2009;**22**:686–94.

Coleman-Derr D, Tringe SG. Building the crops of tomorrow: advantages of symbiont-based approaches to improving abiotic stress tolerance. *Front Microbiol* 2014;**5**:283.

Collado-Romero M, Alós E, Prieto P. Unravelling the proteomic profile of rice meiocytes during early meiosis. *Front Plant Sci* 2014;**5**:356.

Cordeiro FA, Tadra-Sfeir MZ, Huergo LF *et al.* Proteomic analysis of *Herbaspirillum seropedicae* cultivated in the presence of sugar cane extract. *J Proteome Res* 2013;**12**:1142–50.

de Freitas J, Germida JJ. Growth promotion of winter wheat by fluorescent pseudomomads under field conditions. *Soil Biol Biochem* 1992;**24**:1137–46.

Djavaheri M, Mercado-Blanco J, Versluis C *et al.* Iron regulated metabolites produced by *Pseudomonas fluorescens* WCS374 are not required for eliciting induced systemic resistance against *Pseudomonas syringae* pv. tomato in Arabidopsis. *Microbiology-open* 2012;**1**:311–25.

Faleiro AC, Neto PAV, de Souza TV *et al.* Microscopic and proteomic analysis of *Zea mays* roots (P30F53 variety) inoculated with *Azospirillum brasilense* strain FP2. *J Crop Sci Biotech* 2015;**18**:63–71.

Fröhlich A, Buddrus-Schiemann K, Durner J *et al.* Response of barley to root colonization by *Pseudomonas* sp. DSMZ 13134 under laboratory, greenhouse, and field conditions. *J Plant Interact* 2012;**7**:1–9.

Godfray HCJ, Beddington JR, Crute IR *et al.* Food security: the challenge of feeding 9 billion people. *Science* 2010;**327**:812.

Gómez-Lama Cabanás C, Schilirò E, Valverde-Corredor A *et al.* The biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. *Front Microbiol* 2014;**5**:427.

Grosch R, Faltin F, Lottman J *et al.* Effectiveness of 3 antagonistic bacterial isolates to control *Rhizoctonia solani* Kuhn on lettuce and potato. *Can J Microbiol* 2005;**51**:345–53.

Haas D, Défago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat Rev Microbiol* 2005;**3**:307–19.

Hansen M, Kragelund L, Nybroe O *et al.* Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. *FEMS Microbiol Ecol* 1997;**23**:353–60.

Hermosa RL, Woo S, Lorito M *et al.* Proteomic approaches to understand *Trichoderma* biocontrol mechanisms and plant interactions. *Curr Proteomics* 2010;**7**:298–305.

Höfte M, Altier N. Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. *Res Microbiol* 2010;**161**:464–71.

Jiang W, Cheng Z, McConkey BJ *et al.* Investigating the role of protein UnkG from the *Pseudomonas putida* UW4 in the ability of the bacterium to facilitate plant growth. *Curr Microbiol* 2013;**66**:331–6.

Kandasamy S, Loganathan K, Muthuraj R *et al.* Understanding the molecular basis of plant growth promotional effect of *Pseudomonas fluorescens* on rice through protein profiling. *Proteome Sci* 2009;**7**:1–8.

Kijne JW. The Rhizobium infection process. In: Stacey G, Burris RH, Evans HJ (eds). *Biological Nitrogen Fixation*. New York: Chapman & Hall, 1992, 349–98.

Kowalski KP, Bacon C, Bickford W *et al.* Advancing the science of microbial symbiosis to support invasive species management: a case study on *Phragmites* in the Great Lakes. *Front Microbiol* 2015;**6**:95.

Kragelund L, Nybroe O. Competition between *Pseudomonas fluorescens* Ag1 and *Alcaligenes eutrophus* JMP134(pHP4) during colonization of barley roots. *FEMS Microbiol Ecol* 1996;**20**:41–51.

Kuklinsky-Sobral HL, Araujo WL, Mendes R *et al.* Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environ Microbiol* 2004;**6**:1244–51.

Kwasiborski A, Bajji M, Renaut J *et al.* Identification of metabolic pathways expressed by *Pichia anomala* Kh6 in the presence of the pathogen *Botrytis cinerea* on apple: new

possible targets for biocontrol improvement. *PLoS One* 2014; **9**:e91434.

Li J, McConkey BJ, Cheng Z *et al.* Identification of plant growth-promoting bacteria-responsive proteins in cucumber roots under hypoxic stress using a proteomic approach. *J Proteomics* 2013;**12**:119–31.

López-Escudero FJ, Mercado-Blanco J. Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant Soil* 2011;**344**:1–50.

Lugtenberg BJJ, Dekkers LC, Bloemberg GV. Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* 2001;**39**:461–90.

Lugtenberg BJJ, Kamilova F. Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 2009;**63**:541–56.

Maciá-Vicente JG, Jansson HB, Talbot NJ *et al.* Real-time PCR quantification and live-cell imaging of endophytic colonization of barley (*Hordeum vulgare*) roots by *Fusarium equiseti* and *Pochonia chlamydosporia*. *New Phytologist* 2009a; **182**:213–28.

Maciá-Vicente JG, Rosso LC, Ciancio A *et al.* Colonisation of barley roots by endophytic *Fusarium equiseti* and *Pochonia chlamydosporia*: effects on plant growth and disease. *Ann Appl Biol* 2009b;**155**:391–401.

Majeed A, Abbasi MK, Hameed S *et al.* Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Front Microbiol* 2015;**6**:198.

Maldonado-González MM, Bakker PAHM, Prieto P *et al.* *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. *Front Microbiol* 2015a;**6**:266.

Maldonado-González MM, García-Carneros A, Prieto P *et al.* Evaluation of *Pseudomonas fluorescens* PICF7as potential biocontrol agent against *Plasmopara halstedii* in sunflower. *Poster presented at the 18th International Sunflower Conference*, Asociación Argentina de Girasol, Buenos Aires, Argentina, 2012.

Maldonado-González MM, Schilirò E, Prieto P *et al.* Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. *Environ Microbiol* 2015b;**17**:3139–53.

Malfanova N, Kamilova F, Validov S *et al.* Is L-arabinose important for the endophytic lifestyle of *Pseudomonas* spp.? *Arch Microbiol* 2013;**195**:9–17.

Martínez-García PM, Ruano-Rosa D, Schilirò E *et al.* Complete genome sequence of *Pseudomonas fluorescens* strain PICF7, an indigenous root endophyte from olive (*Olea europaea* L.) and effective biocontrol agent against *Verticillium dahliae*. *Stand Genomic Sci* 2015;**10**:10.

Massart S, Perazzolli M, Höfte M *et al.* Impact of the omic technologies for understanding the modes of action of biological control agents against plant pathogens. *Biocontrol* 2015;**60**:725–46.

Mercado-Blanco J, Bakker PAHM. Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek* 2007;**92**:367–89.

Mercado-Blanco J, Prieto P. Bacterial endophytes and root hairs. *Plant Soil* 2012;**361**:301–6.

Mercado-Blanco J, Rodríguez-Jurado D, Hervás A *et al.* Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biol Control* 2004;**30**:474–86.

Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1972.

Nejad P, Johnson PA. Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. *Biol Control* 2000;**18**:208–15.

Palmieri MC, Perazzolli M, Matafora V *et al.* Proteomic analysis of grapevine resistance induced by *Trichoderma harzianum* T39 reveals specific defence pathways activated against downy mildew. *J Exp Bot* 2012;**63**:6237–51.

Pang F, Wang T, Zhao C *et al.* Novel bacterial endophytes isolated from winter wheat plants as biocontrol agent against stripe rust of wheat. *Biocontrol* 2016;**61**:207–19.

Paungfoo-Lonhienne C, Rentsch D, Robatzek S *et al.* Turning the table: plants consume microbes as a source of nutrients. *PLoS One* 2010;**7**:e11915.

Perry LG, Alford ER, Horiuchi J *et al.* Chemical signals in the rhizosphere: root-root and root-microbe communication. In: Pinton R, Varanini Z, Nannipieri P (eds). *The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface*, 2nd edn. Boca Raton: CRC/Taylor & Francis, 2007, 297–330.

Pliego C, Kamilova F, Lugtenberg BJJ. Plant growth-promoting bacteria: fundamentals and exploitation. In: Maheshwari DK (ed.). *Bacteria in Agrobiology: Crop Ecosystems*. Berlin, Heidelberg: Springer, 2011, 295–343.

Prieto P, Mercado-Blanco J. Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7. *FEMS Microbiol Ecol* 2008;**64**:297–306.

Prieto P, Navarro-Raya C, Valverde-Corredor A *et al.* Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. *Microb Biotechnol* 2009;**2**:499–511.

Prieto P, Schilirò E, Maldonado-González MM *et al.* Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. *Microbial Ecol* 2011;**62**:435–45.

Raaijmakers JM, Leeman M, Van Oorschot MMP *et al.* Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. *Phytopathology* 1995;**85**:1075–81.

Raaijmakers JM, Paulitz TC, Steinberg C *et al.* The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 2009;**321**:341–61.

Rediers H, Bonnecarrére V, Rainey PB *et al.* Development and application of a dapB-based *in vivo* expression technology system to study colonization of rice by the endophytic nitrogen-fixing bacterium *Pseudomonas stutzeri* A15. *Appl Environ Microbiol* 2003;**69**:6864–74.

Reynolds M, Bonnett D, Chapman SC *et al.* Raising yield potential of wheat. I. Overview of a consortium approach and breeding strategies. *J Exp Bot* 2010;**6**:439–52.

Rincón A, Ruíz-Díez B, García-Fraile S *et al.* Colonisation of *Pinues halepensis* roots by *Pseudomonas fluorescens* and interaction with the ectomycorrhizal fungus *Suillus granalatus*. *FEMS Microbiol Ecol* 2005;**51**:303–11.

Rothballer M, Schmid M, Hartmann A. *In situ* localization and PGPR-effect of *Azospirillum brasilense* strains colonizing roots of different wheat varieties. *Symbiosis* 2003;**34**:261–79.

Schilirò E, Ferrara M, Nigro F *et al.* Genetic responses induced in olive roots upon colonization by the biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7. *PLoS One* 2012;**7**:e48646.

Schloter M, Hartmann A. Endophytic and surface colonization of wheat roots (*Triticum aestivum*) by different *Azospirillum brasilense* strains studied with strain-specific monoclonal antibodies. *Symbiosis* 1998;**25**:159–79.

Segarra G, Casanova E, Bellido D *et al*. Proteome, salicylic acid, and jasmonic acid changes in cucumber plants inoculated with *Trichoderma asperellum* strain T34. *Proteomics* 2007;**7**:3943–52.

Setten L, Soto G, Mozzicafreddo M *et al*. Engineering *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. *PLoS One* 2013;**8**:e63666.

Sharma S, Aggarwal R, Yadav A *et al*. Protein mapping of *Chaetomium globosum*, a potential biological control agent through proteomics approach. *J Plant Biochem Biotechnol* 2014;**23**:284–92.

Shiferaw B, Smale M, Braun HJ *et al*. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Sec* 2013;**5**:291–317.

Shoresh M, Harman GE. The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. *Plant Physiol* 2008;**147**:2147–63.

Stockwell VO, Stack JP. Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* 2007;**97**:244–9.

Taylor CF, Paton NW, Lilley KS *et al*. The minimum information about a proteomics experiment (MIAPE). *Nat Biotechnol* 2007;**25**:887–93.

Tombolini R, Van der Gaag DJ, Gerhardson B *et al*. Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA 342 on barley seeds visualized by using green fluorescent protein. *Appl Environ Microbiol* 1999;**65**:3674–80.

Triplett EW. Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. *Plant Soil* 1996; **186**:29–38.

Upreti R, Thomas P. Root-associated bacterial endophytes from *Ralstonia solanacearum* resistant and susceptible tomato cultivars and their pathogen antagonistic effects. *Front Microbiol* 2015;**6**:255.

Vizcaíno JA, Csordas A, del-Toro N *et al*. 2016 update of the PRIDE database and related tools. *Nucleic Acids Res* 2016;**44**: D447–56

Wang YQ, Ohara Y, Nakayashiki H *et al*. Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in Arabidopsis. *Mol Plant Microbe Interact* 2005;**18**:385–96.

Weller DM. Colonization of wheat roots by a fluorescent pseudomonad suppressive to Take-All. *Phytopathology* 1983;**73**:1548–53.

Weller DM. *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 2007;**97**:250–6.

Weller DM, Cook RJ. Increased growth of wheat by seed treatments with fluorescent pseudomonads, and implications of *Pythium* control. *Can J Plant Pathol* 1986;**8**:328–34.