

Accepted Manuscript

Title: Colonization and beneficial effects on annual ryegrass by mixed inoculation with plant growth promoting bacteria

Authors: Nádia L. Castanheira, Ana Catarina Dourado, Isabel Pais, José Semedo, Paula Scotti-Campos, Nuno Borges, Gilda Carvalho, Maria Teresa Barreto Crespo, Paula Fareleira



PII: S0944-5013(16)30560-2
DOI: <http://dx.doi.org/doi:10.1016/j.micres.2017.01.009>
Reference: MICRES 25992

To appear in:

Received date: 12-8-2016
Revised date: 22-12-2016
Accepted date: 23-1-2017

Please cite this article as: {<http://dx.doi.org/>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Colonization and beneficial effects on annual ryegrass by mixed inoculation with plant growth promoting bacteria

Nádia L. Castanheira^{a,b}, Ana Catarina Dourado^c, Isabel Pais^a, José Semedo^a, Paula Scotti-Campos^a, Nuno Borges^b, Gilda Carvalho^d, Maria Teresa Barreto Crespo^{b,c}, Paula Fareleira^{a,*}

^a Instituto Nacional de Investigação Agrária e Veterinária, I. P., Av. da República, 2780-159 Oeiras, Portugal

^b Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal

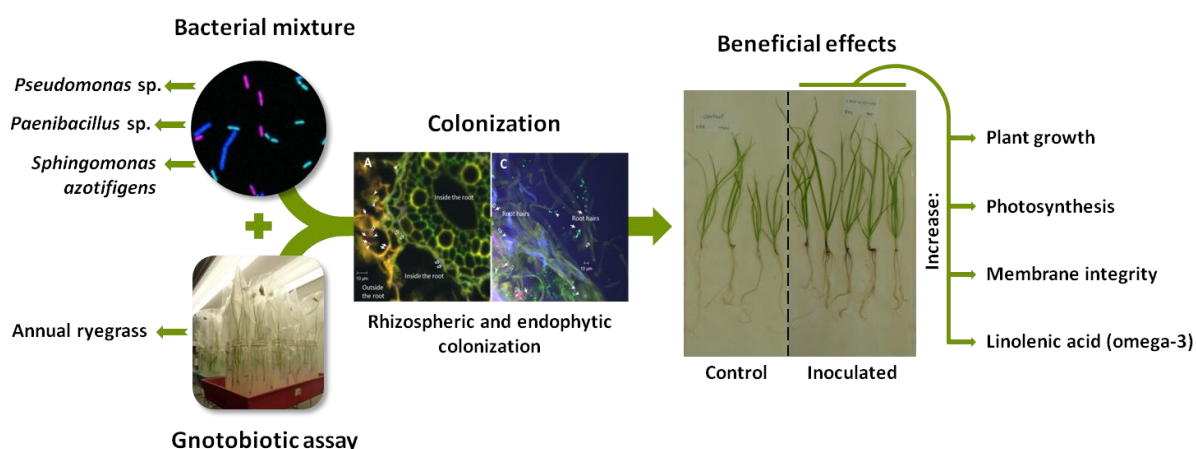
^c iBET - Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2780-901 Oeiras, Portugal

^d UCBIO, REQUIMTE, Chemistry Department, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal

Corresponding author at: Instituto Nacional de Investigação Agrária e Veterinária, I. P., Av. da República, 2780-159 Oeiras, Portugal. Tel.: +351 214463749.

E-mail address: paula.fareleira@iniav.pt (Paula Fareleira).

Graphical abstract



Abstract

Multi-strain inoculants have increased potential to accomplish a diversity of plant needs, mainly attributed to its multi-functionality. This work evaluated the ability of a mixture of three bacteria to colonize and induce a beneficial response on the pasture crop annual ryegrass. *Pseudomonas* G1Dc10 and *Paenibacillus* G3Ac9 were previously isolated from annual ryegrass and were selected for their ability to perform multiple functions related to plant growth promotion. *Sphingomonas azotifigens* DSMZ 18530^T was included due to nitrogen fixing ability. The effects of the bacterial mixture were assessed in gnotobiotic plant inoculation assays and compared with single and dual inoculation treatments. Triple inoculation with 3×10^8 bacteria significantly increased plant dry weight and leaf pigments, indicating improved photosynthetic performance. Plant lipid biosynthesis was enhanced by 65 %, mainly due to the rise of linolenic acid, an omega-3 fatty acid with high dietary value. Electrolyte leakage, an indicator of plant membrane stability under stress, was decreased pointing to a beneficial effect by inoculation. Plants physiological condition was more favoured by triple inoculation than by single, although benefits on biomass were only evident relative to non-inoculated plants. The colonization behaviour and coexistence in plant tissues were assessed using FISH and GFP-labelling, combined with confocal microscopy and a cultivation-based approach for quantification. The three strains occupied the same sites, localizing preferentially along root hairs and in stem epidermis. Endophytic colonization was observed as bacteria entered root and stem inner tissues. This study reveals the potential of this mixture of strains for biofertilization, contributing to improve crop productivity and nutritional value.

Keywords: Plant growth promotion Annual ryegrass colonization *Pseudomonas* *Paenibacillus* *Sphingomonas* Fluorescence *in situ* hybridization

1. Introduction

Healthy plants are naturally associated with a remarkable diversity of microbes, known as the plant microbiota (Bulgarelli et al., 2012; Knief et al., 2012; Lundberg et al., 2012). Chemical compounds exuded by the roots function as carbon and energy sources for microbes colonizing the surface of the roots or the rhizospheric soil (Bowen and Rovira, 1999; Dakora and Phillips, 2002; Nguyen, 2003). Root exudates include a variety of molecules, such as sugars, polysaccharides, organic acids, inorganic ions, amino acids, vitamins, flavonoids, phytosiderophores, peptides, proteins and fatty acids. It is estimated that rhizodeposits account for approximately 11% of net photosynthetically fixed carbon and 10–16% of total plant nitrogen, even though these values can vary greatly depending on plant species and age (Jones et al., 2009). Compounds released by plant roots may also trigger a migratory response in some of the microbes present in the rhizosphere (Kamilova et al., 2006; van Oberbeek and van Elsas, 1995), which enter plant tissues and spread further to the aerial parts of the host plant, adopting an endophytic lifestyle (Raaijmakers et al., 1995). Endophytic colonization may have advantages over root-surface associations, since microbes can establish in a sheltered environment (Reinhold-Hurek and Hurek, 2011; Ryan et al., 2008).

Some plant-associated microbes have been recognized to exert beneficial effects on their hosts by playing important roles in key processes related to nutrient availability and cycling, plant health and growth, enhanced stress tolerance, disease resistance or biological control of plant pathogens (Berg, 2009; Morrissey et al., 2004; Pérez-Montaña et al., 2014). These microbes are known as the functional group of plant growth promoters (PGP). The enhancement of plant nutrition can be achieved by associative nitrogen fixation, the solubilisation of soil-immobilized mineral phosphates (Castagno et al., 2011; Richardson et al., 2009), or through the production of phytohormones that change the root morphology and increase the uptake of water and nutrients from the soil (Bulgarelli et al., 2013; Dobbelaere et al., 2003; Sharma et al., 2013).

The exploitation of PGP to enhance plant productivity in agricultural systems has been acquiring increasing interest (Schlaeppli and Bulgarelli, 2015). Over the past century, crop yields were greatly increased in order to supply the needs of the growing human population. Such productivity increases

have been related with the massive application of chemical fertilizers and pesticides, creating health and environmental problems including soil degradation, contamination of groundwater supplies and loss of biodiversity (Aktar et al., 2009; Tandon, 1996). Also, the production of agrochemicals is energetically expensive and dependent on fossil fuels that are non-renewable resources, which make it no longer sustainable. An increasing number of farmers are choosing biofertilizers (Chatzipavlidis et al., 2013) since they are gentler on the soil and can help reduce the negative impact of global warming. Biofertilizers can make available a wide range of nutrients to plants, particularly micronutrients, and contribute to increase soil organic matter, in addition to being effective in small amounts and able to self-multiply (Berg, 2009; Chatzipavlidis et al., 2013). Some disadvantages are related to technical problems in mass production and upscaling, storage time (because they contain living microorganisms) and loss of efficiency due to high soil temperatures, moisture scarcity, excessive acidity or alkalinity and low nutrient levels (Berg, 2009; Chatzipavlidis et al., 2013). Even though, biofertilizers are promising when considering the rising cost and declining availability of fossil fuels worldwide, as well as the pollution problems induced by agriculture. Taking this into account, studies on plant-associated bacteria with competence to function as biofertilizers are of major importance. A recent survey of annual ryegrass-associated bacteria in Portuguese soils resulted in the report of novel strains that were able to increase the biomass of annual-ryegrass plants in gnotobiotic conditions (Castanheira et al., 2014). The aim of the present work was to evaluate the effects of a mixture of such bacteria on the growth and physiological status of inoculated plants. The selected strains were the phosphate solubilizer and siderophore producer *Pseudomonas* sp. G1Dc10 (class *Gammaproteobacteria*), and the cellulose/pectin hydrolizer *Paenibacillus* sp. G3Ac9 (class *Bacilli*) (Castanheira et al., 2014). *Sphingomonas azotifigens* DSMZ 18530^T (class *Alphaproteobacteria*) was also included in the study due to its nitrogen fixing activity and the ability to stimulate the growth of annual ryegrass when nitrogen is absent in the cultivation medium (our unpublished results). For comparison purposes, single and dual inoculations were also investigated. The colonization patterns and the ability of the bacteria to coexist in plant tissues were addressed using fluorescence *in situ* hybridization (FISH) and GFP-labelling combined with confocal laser scanning microscopy, as well as a cultivation-based approach for quantification purposes.

2. Materials and methods

2.1. Preparation of bacterial suspensions and plant inoculation assays

Bacterial suspensions for plant inoculation were prepared by growing *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *S. azotifigens* DSMZ 18530^T in tryptone-yeast (TY) medium (Beringer, 1974) for 16 hours at 30 °C with vigorous shaking. The cultures were centrifuged at 10,000×g for 10 minutes. Cells were washed with 0.85 % NaCl and suspended in the same solution. Cell density was evaluated by OD measurement at 600 nm and normalized as appropriate. The resulting cell suspensions were either used directly for single inoculation (treatments 1-3, 10⁸ cells of a single strain per plant), or combined to yield the inoculation mixtures for dual (treatments 4-6, 0.5×10⁸ cells of each strain to a total number of 10⁸ bacteria per plant) and triple inoculations (treatment 7, 0.33×10⁸ cells of each strain to a total number of 10⁸ bacteria per plant; and treatment 8, 10⁸ cells of each strain to a total number of 3×10⁸ bacteria per plant). Information regarding strains and inoculation treatments used in this study is summarized in Table 1.

Inoculation of annual ryegrass was performed as described by Castanheira et al. (2014). Briefly, surface sterilized and pre-germinated annual ryegrass seeds were sowed in flasks containing 50 ml of modified Evans medium (Evans, 1970) supplemented with 8 % agar (one seedling per flask), using the procedures described by Vincent (1970). The nitrogen source was included in the medium as potassium nitrate at 50 mg l⁻¹ N. Four-day-old seedlings were inoculated by pouring 0.5 ml of bacterial suspension onto the base of the coleoptile. Plants were grown in a controlled-environment chamber with a 16 h light/8 h dark cycle at 23 °C (day)/18 °C (night), 800 μmol m⁻² s⁻¹ light intensity, and 80 % relative humidity. Plants were harvested at four or seven weeks after inoculation, and respectively assayed for plant growth (dry biomass) or physiological parameters. In seven week assays the plants were watered twice (30 and 45 days after inoculation) with 2 ml 0.25× Evans medium. Non-inoculated plants were used as negative controls. The number of plants per treatment varied between 7 and 9

(treatments 1 to 6) or 14 and 24 (treatments 7 and 8). Some variability in plants size was observed between assays due to the use of different seed lots. In different assays, the average dry weights of non-inoculated plants ranged between 23.4 ± 2.3 mg and 34.7 ± 1.6 mg in the shoots, and 6.9 ± 1.5 mg and 13.7 ± 0.7 mg in the roots.

2.2. *Evaluation of plant growth and physiological parameters*

For evaluation of dry biomass, plants were sectioned into root and shoot portions and dried until constant weight. Total leaf chlorophylls and carotenoids were extracted and quantified as described by Scotti-Campos et al. (2015). Electrolyte leakage was evaluated in leaves according to previous procedures (Scotti-Campos et al., 2014). Total lipid extraction from leaves and fatty acids analysis was performed as described by Scotti-Campos et al. (2014). The value of total fatty acids (TFA) corresponds to the sum of individual fatty acids. The unsaturation degree of TFA was obtained through the double bond index (DBI), calculated according to the formula: $DBI = [(\% \text{ monoenes} + 2 \times \% \text{ dienes} + 3 \times \% \text{ trienes}) / \% \text{ saturated fatty acids}]$ (Mazliak, 1983). All data were analyzed by one-way analysis of variance (ANOVA) with STATISTICA 8, using the Tukey's honestly significance difference (HSD) test at $P \leq 0.05$.

2.3. *Enumeration of plant-colonizing bacteria*

Four week grown plants inoculated with a mixture of the three strains (treatment 8, total number of 3×10^8 bacteria per plant) and incubated in gnotobiotic conditions as described above, were used for enumeration of viable bacteria present in the rhizoplane and within surface disinfected plant tissues (roots, stems and leaves), using the dilution-plating method as described by Castanheira et al. (2016). Ten-fold dilutions of each sample were plated on TY agar and incubated for three-four days at 30 °C. Quantification was achieved by counting the colony forming units (CFU) of each strain (differentiated by the colony morphology) and the bacterial numbers were related to the fresh weight of the

corresponding plant fraction. Between three and four replicate samples (collected from different plants) were analysed for each fraction. Statistical analysis was performed as described above.

2.4. GFP-labelling

S. azotifigens DSMZ 18530^T mutants expressing the *gfp* gene were constructed by transposon mutagenesis. The suicide plasmid pFAJ1819 (Xi et al., 1999), which carries a mini-Tn5 derivative containing a bifunctional *gfp-gusA* cassette (mTn5*gusA-pgfp21*), was mobilized into the recipient strain *S. azotifigens* DSMZ 18530 by triparental mating, using *E. coli* S17 (λ pyr) as donor strain and *E. coli* HB101 (pRK2013) as conjugation helper (Soto et al., 2000). *S. azotifigens* transconjugants were selected based on *E. coli* inability to use sucrose for growth and using kanamycin resistance encoded by Tn5. Colonies expressing the *gfp* gene on minimal medium containing 10% sucrose and 50 $\mu\text{g l}^{-1}$ kanamycin were detected under UV light. One strain exhibiting strong fluorescence (*S. azotifigens*-GFP) was selected for plant inoculation experiments. The stability of the *gfp* marker was evaluated fluorimetrically (Chovanec et al., 2008), by subculturing on TY medium in the absence of kanamycin. After 48 generations, the intensity of GFP fluorescence remained unchanged (data not shown). The growth curve of the *gfp*-transformed strain was identical to the parental strain.

2.5. Fluorescence in situ hybridization (FISH)

2.5.1. Plant tissue fixation and cryosectioning

Plants from treatment 8 were harvested four weeks after inoculation, and roots, stems and leaves were detached and rinsed with phosphate buffer saline (PBS). Plant tissues were cut into small pieces (approximately 1cm length) and fixed with 4 % paraformaldehyde using vacuum infiltration for one hour, followed by overnight fixation at 4 °C. Samples were washed three times with PBS at 4 °C and infiltrated with 30 % sucrose in PBS at 4 °C. Tissues were stored at -20 °C in 50 % ethanol as described by Amann (1995). Samples tissues for cryosectioning were embedded in the tissue-freezing medium O.C.T.TM (Tissue-Tek®; Sakura Finetek, Torrance, CA, USA) in cryomolds (7×7×5 mm,

VWR International) and stored at -20 °C. Transversal sections of roots, stems and leaves (12 µm average thickness) were obtained with a cryostat CM 30505 (Leica, Wetzlar, Germany) at -20 °C and collected onto diagnostic slides with 6 mm wells and adhesion microscope slides (Superfrost® plus, VWR International). Tissue sections showing better cell integrity and anatomy were selected for FISH assays. Samples were collected from approximately 15 plants. Between three and four replicate samples were analysed for each fraction.

2.5.2. FISH procedures

Plant tissues were assayed for the presence of bacterial cells using FISH as detailed by Amann (1995), using fluorescently labeled oligonucleotide probes and respective oligonucleotide competitors (Eurofins MWG Operon, Germany) (Supplementary Table S1).

A general probe (EUBmix, a combination of EUB338, 338I and 338II) was used to target all bacteria. Specific probes for *Gammaproteobacteria* (Gam42a) and *Alphaproteobacteria* (ALF969) were used to target *Pseudomonas* sp. strain G1Dc10 and *Sphingomonas azotifigens* DSMZ 18530^T, respectively. *Paenibacillus* sp. G3Ac9 was detected by exclusion, since it hybridizes with EUBmix, but not with the specific probes Gam42a and ALF969. Probe hybridization was firstly validated by performing FISH in individual suspensions of each bacterium, as well as in mixed cell suspensions.

FISH was carried out in transverse sections of roots, stems and leaves, and in entire root fragments. Hybridization was conducted for 1-2 hours on the slides after dehydration in an ethanol series (50, 80 and 98 %). Dako® pen (Glostrup, Denmark) was used to create a hydrophobic barrier when performing FISH on the superfrost slides. Washing of the probes was performed as detailed by Amann (1995) by rinsing the samples with pre-warmed washing buffer (at 48 °C) and immersing the slides in this washing solution for 10 minutes. Slides were air-flow dried and mounted using the anti-fading medium Vectashield® (Vector Laboratories Inc., Burlingame, CA, USA), covered with 5×2.4 cm coverslip (0.1 mm thickness) and sealed with nail polish.

2.5.3. Confocal laser-scanning microscopy

Hybridized samples were observed with a Zeiss LSM 510 Meta confocal laser-scanning

microscope (CLSM) (Zeiss, Oberkochen, Germany) equipped with two helium neon lasers for excitation of fluorophores Cy3 and Cy5 at wavelengths of 543 nm and 633 nm, respectively, and an argon laser for excitation of FITC and GFP at 488 nm. Bacterial cells were localized with a 63×/1.4NA oil immersion objective. Spectral detection was adjusted for the emission of the Cy3, Cy5 and FITC fluorophores, and GFP. Images were acquired using the standard software package provided by Zeiss. The software *Zeiss LSM Image Browser* version 4.2 was used for image processing.

3. Results

3.1. Influence of inoculation on the growth of annual ryegrass

The effects of inoculation with strains *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *S. azotifigens* DSMZ 18530 on the growth of annual ryegrass were assessed in gnotobiotic plant inoculation assays using a defined cultivation medium. The results obtained with different combinations of these strains (single, dual and triple inoculations, treatments 1 to 8) are presented in Table 2 and Fig. 1.

All treatments induced significant dry weight increases when compared to non-inoculated controls. Treatment 5 (dual inoculation with equal amounts of *Pseudomonas* sp. G1Dc10 and *S. azotifigens* DSMZ 18530, to a total number of 10^8 bacteria per plant) promoted the highest increase in shoots (53.8%). The highest effect in roots was recorded in plants from treatment 2 (single inoculation with *Paenibacillus* sp. G3Ac9, 10^8 cells per plant), presenting 100 % dry weight increase. The smallest effects were observed in treatment 8 (triple inoculation, total number of 3×10^8 bacteria per plant), with 19 % and 37 % increases in shoot and root dry weights, respectively, relative to non-inoculated plants (Fig. 1). However, the differences between inoculated treatments were not statistically significant, except for shoots in treatments 5 and 8.

3.2. Effects of inoculation on plant physiological parameters

Plant physiological parameters, namely leaf pigments, fatty acids content and electrolyte leakage, were evaluated in annual ryegrass plants from treatments 1 to 3 (single inoculations) and 8 (triple inoculation with a total number of 3×10^8 bacteria per plant), as well as in non-inoculated controls (Table 3).

Single inoculation with either *Pseudomonas* sp. G1Dc10 (treatment 1) or *S. azotifigens* DSMZ 18530 (treatment 3) stimulated the leaf chlorophyll and carotenoid contents, and resulted in smaller conductivity values in the electrolyte leakage test. The same parameters were negatively affected by single inoculation with *Paenibacillus* sp. G3Ac9 (treatment 2), which caused the lowest pigments content and the highest electrolyte leakage value. However, the combination of this strain with *Pseudomonas* sp. G1Dc10 and *S. azotifigens* DSMZ 18530 (treatment 8) produced the highest increases in the contents of chlorophylls *a+b* (69 %) and carotenoids (48 %), and the lowest value in electrolyte leakage, corresponding to a 45 % decrease relative to the control.

The total fatty acids (TFA) content was significantly increased in all inoculation treatments, relative to non-inoculated controls (Table 3). The highest TFA value (40.1 mg g⁻¹ dry weight) was found in triple inoculation (treatment 8), corresponding to a 65 % increase relative to control. Such increase was mainly due to higher amounts of the three major fatty acids, C16:0 (palmitic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid), which were respectively increased by 76 %, 55 % and 66 %.

Significant increases were also observed in C16:1 (hexadecenoic acid) and C18:1 (oleic acid).

Distinct proportions of individual fatty acids were obtained in the single inoculation treatments. For example, the amounts of C18:1 and C18:2 in treatment 2 (*Paenibacillus* sp. G3Ac9) were significantly higher than in treatments 1 (*Pseudomonas* sp. G1Dc10) and 3 (*S. azotifigens* DSMZ 18530), but similar to treatment 8 (triple inoculation). On the other hand, all single inoculation treatments originated similar contents of C16:0 (around 4.7 mg g⁻¹ dry weight), yet significantly lower than the obtained upon triple inoculation (6.5 mg g⁻¹ dry weight). Significant increases relative to non-inoculated control were observed in the amounts of polyunsaturated C18:3 in single inoculation treatments, similarly to what was observed in the triple inoculation assay. Despite the higher percentages of C18:3, the double bond index (DBI) remained unchanged in all inoculation treatments, mainly due to the concomitant increase of saturated C16:0.

3.3. Re-isolation of bacteria from inoculated plants

The dilution-plating method was used for quantification of bacteria colonizing annual ryegrass plants four weeks after inoculation with a mixture containing equal amounts of the three bacterial strains (treatment 8) and incubation in gnotobiotic conditions. Bacteria were recovered from the rhizoplane and surface disinfected roots, stems and leaves, indicating both external and internal colonization (Table 4). In most cases, CFU counts revealed identical population densities of the three strains. Statistical differences were only found between *Pseudomonas* sp. G1Dc10 and *Paenibacillus* sp. G3Ac9 in the rhizoplane. The highest colonization density was observed in the rhizoplane (approximately $9 \log_{10}$ CFU g⁻¹ root fresh weight) and the lowest was found in the leaves (approximately $4 \log_{10}$ CFU g⁻¹ leaf fresh weight). Overall, bacterial colonization was more abundant in the roots external environment (rhizoplane) than in plant tissues.

3.4. In situ detection of bacteria in annual ryegrass plants

Plants inoculated with a mixture containing equal amounts of the three strains (treatment 8) were used for *in situ* detection of colonizing bacteria. FISH was carried out using three oligonucleotide probes: a Cy3 (red) fluorescently labelled GAM42a to detect *Pseudomonas* sp., a FITC (green) fluorescently labelled ALF969 to detect *Sphingomonas* sp., and a Cy5 (blue) fluorescently labelled EUBmix that targets all members of Bacteria, thus enabling the detection of *Paenibacillus* sp. by exclusion. The combination of these FISH probes in the co-localization images obtained by CLSM should result in the visualization of *Pseudomonas* sp. as magenta, *Paenibacillus* sp. as blue and *Sphingomonas* sp. as cyan.

Preliminary FISH/CLSM assays in plant tissues confirmed that *Pseudomonas* sp. G1Dc10 and *Paenibacillus* sp. G3Ac9 were visualized as expected. However, *S. azotifigens* was often visualized in either green or cyan, meaning that the hybridization signals of the probes *in planta* were weak and/or unsuccessful. The same occurred in culture, although better hybridization signals were obtained in this

case. To overcome this problem, a modified strain expressing the green fluorescent protein (*S. azotifigens*-GFP) was used in the mixed inoculum. Similar colonization patterns were observed by CLSM in the parental and GFP-labelled *S. azotifigens* strains (data not shown). When FISH was performed in the GFP-labelled strain with the application of the probes ALF969-FITC (green) and EUBmix-Cy5 (blue), the outcome was a reinforcement of the signal and improved visualization of *S. azotifigens* cells.

The combined FISH/CLSM analysis enabled the visualization and localization of the three inoculated strains in different parts of annual ryegrass plants (Fig. 2). As a general trend, the three strains were consistently identified colonizing the same sites. Although the FISH approach used in this work could not be used for quantification purposes, no predominance of any of the strains was detected. The results generally confirmed the dilution-plating counts (Table 4), suggesting similar colonization densities for the three strains. Clusters of bacteria were observed on the surface of the roots, particularly along root hairs (Fig. 2 B-C). Many bacterial cells were visualized near the root tips and surrounding root cap cells (not shown), probably taking advantage of increased amounts of root exudates in this region. The three strains were also observed in the cortical parenchyma and endodermis of the roots (Fig. 2 A). Bacteria were abundant in the epidermis of stems (Fig. 2 D) and some cells were visualized in the stem cortex (not shown). Fewer bacteria were found in leaves, mostly located in the sheaths of the leaves (not shown).

4. Discussion

Multi-strain inoculants are perceived as the current frontier in plant biofertilization (Bashan et al., 2014), mainly because of their potential for higher resilience and enlarged scope of action. However, improvements on the plant performance will depend on the interaction and compatibility between the inoculant strains, as well as their interaction with the host plant. This study aimed to evaluate the ability of a mixture of three strains of the genera *Pseudomonas*, *Paenibacillus* and *Sphingomonas*, to colonize and to stimulate the growth of the pasture crop annual ryegrass. The three genera are

commonly associated with plants and harbour a diversity of plant growth promoting species (Buddrus-Schiemann et al., 2010; Gamalero et al., 2004; Zamioudis et al., 2013; Kim et al., 1998; Ulrich et al., 2008). Besides acting as plant growth stimulators, the strains used in this study combine several other features related to the promotion of plant growth, e.g. phosphate solubilisation, siderophore production, cellulose/pectin hydrolysis and nitrogen fixation, which may be advantageous for use in different field situations.

Our previous work had shown that the total dry weight of annual ryegrass plants was enhanced by more than 50% upon single inoculation with *Pseudomonas* sp. G1Dc10 and *Paenibacillus* sp. G3Ac9, probably due to the production of bacterial phytohormones (Castanheira et al., 2014). Here we evaluated the effects on the shoot and root fractions after inoculation with different combinations of those two strains and *S. azotifigens* DSMZ 18530. Triple inoculation with 3×10^8 bacteria per plant (high-density triple inoculation, treatment 8) significantly increased the growth of annual ryegrass, showing the highest effect in the roots. However, such increases were smaller than the observed in single, dual and triple inoculation treatments containing one-third of the total number of bacteria (10^8 bacteria per plant). Although the differences between treatments had no statistical significance, the observed tendency suggested a saturation effect by the high-density triple inoculum. However, this possibility was not confirmed by physiological evaluations, which revealed a physiological improvement (i.e. lipid synthesis, chlorophyll and carotenoid contents, electrolyte leakage) in plants inoculated with the high-density triple inoculum, relative to either non-inoculated controls or single inoculation treatments. The most relevant result was the 65 % increase in the total fatty acids content, suggesting a dynamic metabolic response and stimulation of membrane biosynthesis (Campos et al., 2003; Partelli et al., 2011). Lipids have higher energy content when compared with carbohydrates and proteins, reinforcing the importance of using lipid-enriched forage grasses for ruminant nutrition (Hegarty et al., 2013). Enhanced lipid synthesis was also observed in single inoculation treatments relative to non-inoculated plants, but at a lower extent. The increase in the total fatty acids content was a consequence of alterations in the lipids profile, mainly reflecting the preferential synthesis of saturated palmitic acid (C16:0) and highly unsaturated linoleic (C18:2) and linolenic (C18:3) acids. The increase of unsaturated membrane lipids can be an important asset for livestock nutrition.

Linolenic and linoleic acids belong to a group of essential fatty acids (respectively omega-3 and omega-6) that cannot be synthesized by humans but must be mandatorily present in their diet. Such obligatory dietary requirement for fatty acids of plant origin may be attained through plants or through organisms which are plant consumers. In mammals these fatty acids are precursors for important regulatory molecules, which help prevent a number of diseases and physiological dysfunctions (Leshem, 1992). It was demonstrated that an increased supply of omega-3 fatty acids in the diet of ruminants can result in increased accumulation of these compounds in animal products, improving their quality and nutraceutical value (Scollan et al. 2001). Therefore, inoculation of annual ryegrass with the three-strain mixture proposed in this work may improve the nutritional value of this pasture crop. The results obtained in this study with the high-density triple inoculum suggest a putative synergistic effect by the three-strains in stimulating lipid biosynthesis and favouring the plants contents of unsaturated fatty acids. It remains unclear whether reducing cell density in the triple inoculum could provide more and/or stronger beneficial effects on plants lipid composition. Clarification of this issue would be important to evaluate a possible saturation effect by the high-density inoculum.

Besides increasing total fatty acids, the high-density triple inoculum also increased leaf chlorophyll and carotenoid contents, indicating an improvement of the photosynthetic apparatus and anti-oxidative capacity (Scotti-Campos et al., 2015). Additionally, membrane permeability was reduced relative to control, as inferred from lower electrolyte leakage values; this parameter has been commonly used as an indicator of membrane stability in a wide range of plant species under different stress conditions (Campos et al., 2003; Scotti-Campos et al., 2013; Scotti-Campos et al., 2014; Scotti-Campos et al., 2015). The pigments content and electrolyte leakage were also improved by single inoculation with *Pseudomonas* sp. G1Dc10 or *S. azotifigens* DSMZ 18530, but not with *Paenibacillus* sp. G3Ac9, indicating a negative impact by this strain in the plant photosynthetic apparatus and membrane stability. There is no obvious explanation for this result, especially in view of the increases in plants biomass provided by this strain. *Paenibacillus* sp. G3Ac9 is an active producer of polysaccharide degrading enzymes, such as cellulases and pectinases (Castanheira et al., 2014), and the possibility that such hydrolytic activities could affect plant physiological parameters should not be ruled out.

Further work will be required to evaluate the effects of strain *Paenibacillus* sp. G3Ac9 on plants exposed to different environments and stress situations. Interestingly, the combination of this strain with *Pseudomonas* sp. G1Dc10 and *S. azotifigens* DSMZ 18530 in the triple inoculation treatment eliminated the negative effects on the pigments content and electrolyte leakage. Single inoculation with *Paenibacillus* sp. G3Ac9 provided the highest increases in monounsaturated oleic acid and polyunsaturated linoleic acid, probably contributing to the corresponding increases in the triple inoculation treatment. This effect, together with the large increase in plants biomass, justified the inclusion of *Paenibacillus* sp. G3Ac9 in the inoculation mixture. The genus *Paenibacillus* is referred in the literature as harbouring several strains that are plant-growth promoters and prominent members of the endophytic population in healthy tissues of medicinal plants, functioning as prolific producers of bioactive compounds, such as polymyxin and fusaricidin antibiotics, or hydrolytic enzymes that may facilitate the colonization process of inner plant tissues (Reinhold-Hurek et al., 1993; Choi et al., 2009; Li and Jensen, 2008; Ulrich et al., 2008). The ability to hydrolyse cellulose may also represent an advantage for the biocontrol of soil-borne phytopathogens containing cellulose in the cell wall composition, such as the oomycete *Phytophthora cinnamomi*.

The colonization of annual ryegrass by the high-density triple inoculum was evaluated using GFP-labelling and FISH/CLSM, combined with the conventional dilution-plating technique for quantification purposes. The results firmly established that the three strains are rhizospheric, since they colonize the surface of the roots, and endophytic, as they also enter plant tissues. It has been recognized that rhizosphere and/or rhizoplane colonization is the first step of plant colonization and is essential for the success of inoculation (Andreote et al., 2009). On the other hand, it is commonly accepted that the endosphere provides a protected environment for those bacteria that colonize and establish *in planta*, increasing their chances of survival (Ryan et al., 2008). The strains used in this study colonize both the rhizosphere and the endosphere of annual ryegrass, and such versatility regarding plant colonization may represent an asset for their use as field inoculants.

FISH/CLSM visualizations made four weeks after inoculation showed that the three bacterial strains were predominantly located around root cap cells and surrounding root hairs, as well as in stem epidermis. In addition, bacteria were found up to cortical cell layers, but not in the vascular system.

This may indicate that the spreading of bacteria inside annual ryegrass plants did not involve their transport through the xylem vessels, contrarily to what was suggested for other host/endophyte systems (Compant et al., 2005; Hurek et al., 1994; James et al., 2002). Migration along intercellular spaces (Compant et al., 2010) would therefore be the most plausible route for the distribution of bacteria within annual ryegrass plants.

In this study, we observed that bacterial cells in annual ryegrass leaves were mostly located in the sheaths. Interestingly, the quantification of bacteria re-isolated from inoculated plants showed stronger colonization of the stems as compared with the root interior and leaves. Many reports describe the presence of endophytic bacteria in various plant compartments, but their distribution is often uneven (Compant et al., 2010). Most plants have higher numbers of endophytes in roots than in above ground tissues (Rosenblueth and Martínez-Romero, 2006). Studies involving the inoculation of tomato and grapevine with endophytic plant growth promoting bacteria showed greater accumulation of endophytes in the leaves than in stems (Compant et al., 2005; Yan et al., 2003). The occurrence of higher numbers of endophytes in certain plant tissues may be related to increased availability of nutrients or activating compounds (Afzal et al., 2011). More studies would be needed to confirm if this is the case of stems in annual ryegrass.

All strains used in this study showed identical colonization behaviour on annual ryegrass, and similar results were described for the plant growth promoting strain *Burkholderia graminis* G2Bd5 on the same host (Castanheira et al., 2016), suggesting that there is a common pattern for annual ryegrass colonization by different bacteria. This seems to reinforce the idea that the host plant plays an active role in controlling the colonization process (Rosenblueth and Martínez-Romero, 2006). Further studies on the interaction of these strains with other host plants could help clarify this issue. The observation of the three strains coexisting in the various plant compartments indicates that there is no antagonism between them.

In conclusion, gnotobiotic inoculation of the pasture crop annual ryegrass with a mixture of three plant growth promoting bacteria significantly increased plant growth and physiological status, enhancing leaf photosynthetic pigments, lipid biosynthesis and the content in linolenic acid, an omega-3 fatty acid with high dietary value. Overall, biomass evaluations suggested an inverse trend between plants

dry weight and the inoculum size/number of strains. Nevertheless, triple inoculation revealed advantages over one-strain inocula in terms of plants physiological condition. Due to the taxonomic and functional diversity of these strains, their combined use in a triple inoculation mixture may represent a valuable asset for plant inoculation in a diversity of field conditions, providing a more versatile inoculum. Additionally, their competence for plant colonization and absence of antagonistic effects reinforce the use of these strains in a mixture. Annual ryegrass is a fodder crop that is often used in mixed legume-grass pastures in Mediterranean ecosystems. In such systems, inoculation of annual ryegrass with the bacterial mixture proposed in this study could also benefit the legumes present in the pasture, for instance by increasing phosphorus bioavailability or providing protection against soil-borne phytopathogens. However, eventual interferences on the establishment of other beneficial plant microbe associations (e.g. nitrogen-fixing symbiosis between legumes and rhizobia, and mycorrhizae) would have to be addressed. Undoubtedly, these are important issues deserving additional investigation. Knowledge on the capacity of these strains to thrive and interact with the soil and plant natural microbiomes will be essential to determine their full potential for biofertilization.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors are grateful to Jan Michiels (University of Leuven, Belgium) for providing *E. coli* S17 (λ pyr) and plasmid pFAJ1819. Seeds of *Lolium multiflorum* were kindly supplied by Fertiprado (Vaia Monte, Portugal). The authors also acknowledge Anabela Amado for laboratory assistance and Isabel Videira e Castro for scientific advising. Financial support was provided by: Project PTDC/AGRAAM/100577/2008 (Fundação para a Ciência e a Tecnologia, Portugal); Project 54971 (ProDer – Programa de Desenvolvimento Rural, Portugal); and iNOVA4Health - UID/Multi/04462/2013 program financially supported by Fundação para a Ciência e a Tecnologia, through national funds and co-funded by FEDER under the PT2020 Partnership Agreement. N.

Castanheira was supported by a fellowship from Fundação para a Ciência e a Tecnologia (SFRH/BD/69185/ 2010).

References

- Afzal, M., Yousaf, S., Reichenauer, T.G., Kuffner, M., Sessitsch, A., 2011. Soil type affects plant colonization, activity and catabolic gene expression of inoculated bacterial strains during phytoremediation of diesel. *J Hazard Mater* 186, 1568-1575.
- Aktar, M.W., Sengupta, D., Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. *Interdiscip Toxicol* 2, 1-12.
- Amann, R.L., 1995. *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes, in: Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), *Molecular Microbial Ecology Manual*, Vol. 3.3.6. Kluwer Academic Publications, Dordrecht, pp. 1-15.
- Andreote, F.D., Araújo, W.L., Azevedo, J.L., van Elsas, J.D., Rocha, U.N., van Overbeek, L.S., 2009. Endophytic colonization of potato (*Solanum tuberosum* L.) by a novel competent bacterial endophyte, *Pseudomonas putida* strain P9, and its effect on associated bacterial communities. *App. Environ Microbiol* 11, 3396-3406.
- Bashan, Y., de-Bashan, L.E., Prabhu, S.R., Hernandez, J.P., 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant Soil* 378, 1-33.
- Berg, G., 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84, 11-18.
- Beringer, J.E., 1974. R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* 84, 188-198.
- Bowen, G.D., Rovira, A.D., 1999. The rhizosphere and its management to improve plant growth. *Adv Agron* 66, 1-102.
- Buddrus-Schiemann, K., Schimid, M., Schreiner, K., Welzl, G., Hartmann, A., 2010. Root colonization by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. *Microb Ecol* 60, 381-393.

- Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P., 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95.
- Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E.V.L., Schulze-Lefert, P., 2013. Structure and functions of the bacterial microbiota of plants. *Annu Rev Plant Biol* 64, 807-838.
- Campos P.S., Quartin V., Ramalho J.C., Nunes M.A., 2003. Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *J Plant Physiol* 160, 283-292.
- Castagno, L.N., Estrella, M.J., Sannazzaro, A.I., Grassano, A.E., Ruiz, O.A., 2011. Phosphate-solubilization mechanism and *in vitro* plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina). *J Appl Microbiol* 110, 1151-1165.
- Castanheira, N., Dourado, A.C., Alves, P.I., Cortés-Pallero, A.M., Delgado-Rodríguez, A.I., Prazeres, Â., Borges, N., Sánchez, C., Barreto Crespo, M.T., Fareleira, P., 2014. Annual ryegrass-associated bacteria with potential for plant growth promotion. *Microbiol Res* 169, 768-779.
- Castanheira, N., Dourado, A., Kruz, S., Alves, P.I., Delgado-Rodríguez, A.I., Pais, I., Semedo, J., Scotti-Campos, P., Sanchez, C.S., Borges, N., Carvalho, G., Barreto Crespo, M.T., Fareleira, P., 2016. Plant growth-promoting *Burkholderia* species isolated from annual ryegrass in Portuguese soils. *J Appl Microbiol* 120, 724-739.
- Chatzipavlidis, I., Kefalogianni, I., Venieraki, A., Holzapfel, W., 2013. Status and trends of the conservation and sustainable use of microorganisms in agroindustrial processes. FAO Background Study Paper No. 64. Commission on Genetic Resources for Food and Agriculture.
- Choi, S.K., Park, S.Y., Kim, R., Kim, S.B., Lee, C.H., Kim, J.F., Park, S.H., 2009. Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J Bacteriol* 191, 3350-3358.
- Chovanec, P., Hovorka, O., Novák, K., 2008. Visualization of symbiotic tissue in intact root nodules of *Vicia tetrasperma* using GFP-marked *Rhizobium leguminosarum* bv. *viciae*. *Folia Microbiol* 53, 139-146.

- Compant, S., Clément, C., Sessitsch, A., 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* 42, 669-678.
- Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C., Ait Barka, E., 2005. Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. *Appl Environ Microbiol* 71, 1685-1693.
- Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low nutrient environments. *Plant Soil* 245, 35–47.
- Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Crit Rev Plant Sci* 22, 107-149.
- Evans, C.G.T., Herbert, D., Tempest, D.W., 1970. The continuous cultivation of microorganisms. 2. Construction of a chemostat. *Meth Microbiol* 2, 277-327.
- Gamalero, E., Lingua, G., Giusy Capri, F., Fusconi, A., Berta, G., Lemanceau, P., 2004. Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution, plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. *FEMS Microbiol Ecol* 48, 79-87.
- Hegarty, M., Yadav, R., Lee, M., Armstead, I., Sanderson, R., Scollan, N., Powell, W., Skøt, L., 2013. Genotyping by RAD sequencing enables mapping of fatty acid composition traits in perennial ryegrass (*Lolium perenne* (L.)). *Plant Biotechnol J* 11, 572-581.
- Hurek, T., Reinhold-Hurek, B., Van Montagu, M., Kellenberger, E., 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *J Bacteriol* 176, 1913-1923.
- James, E.K., Gyaneshwar, P., Mathan, N., Barraquio, W.L., Reddy, P.M., Iannetta, P.P.M., Olivares, F.L., Ladha, J.K., 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. *Mol Plant Microbe Interact* 15, 894-906.
- Jones, D.L., Nguyen, C., Finlay, R.D., 2009. Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant Soil* 321, 5–33.

- Kamilova, F., Kravchenko, L.V., Shaposhnikov, A.I., Azarova, T., Makarova, N., Lugtenberg, B., 2006. Organic acids, sugars, and L-tryptophan in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Mol Plant Microbe Interact* 19, 250-256.
- Kim, H., Nishiyama, M., Kunito, T., Senoo, K., Kawahara, K., Murakami, K., Oyaizu, H., 1998. High population of *Sphingomonas* species on plant surface. *J App Microbiol* 85, 731-736.
- Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., von Mering, C., Vorholt, J.A., 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J* 6, 1378–1390.
- Leshem, Y., 1992. Plant membranes: a biophysical approach to structure, development and senescence. Kluwer Academic Publishers, Dordrecht.
- Li, J., Jensen, S.E., 2008. Nonribosomal biosynthesis of fusaricidins by *Paenibacillus polymyxa* PKB1 involves direct activation of a D-amino acid. *Chem Biol* 15, 118-127.
- Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., del Rio, T.G., Edgar, R.C., Eickhorst, T., Ley, R.E., Hugenholtz, P., Tringe, S.G., Dangl, J.L., 2012. Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90.
- Mazliak, P., 1983. Plant membrane lipids: changes and alterations during aging and senescence, in: Lieberman M (Ed.), Post-harvest physiology and crop preservation, Nato Advanced Study Institutes Series vol. 46, Plenum Press, New York, pp. 123-140.
- Morrissey, J.P., Dow, J.M., Mark, L.G., O’Gara, F., 2004. Are microbes at the root of a solution to world food production? *EMBO Rep* 5, 922-926.
- Nguyen, C., 2003. Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie* 23, 375-396.
- Oyaizu-Masuchi, Y., Komagata, K., 1988. Isolation of free-living nitrogen-fixing bacteria from the rhizosphere of rice. *J Gen Microbiol* 34, 127-164.
- Partelli, F.L., Batista-Santos, P., Scotti-Campos, P., Pais, I.P., Quartin, V.L., Vieira, H.D., Ramalho, J.C., 2011. Characterization of the main lipid components of chloroplast membranes and cold induced changes in *Coffea* spp. *Environm Exp Bot* 74, 194-204.

- Pérez-Montaña, F., Alías-Villegas, C., Bellogín, R.A., del Cerro, P., Espuny, M.R., Jiménez-Guerrero, I., López-Baena, F.J., Ollero, F.J., Cubo, T., 2014. Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. *Microbiol Res* 169, 325-336.
- Raaijmakers, J.M., van der Sluis, I., van den Hout, M., Bakker, A.P.H.M., Schippers, B., 1995. Dispersal of wild-type and genetically-modified *Pseudomonas* spp from treated seeds or soil to aerial parts of radish plants. *Soil Biol Biochem* 27, 1473-1478.
- Reinhold-Hurek, B., Hurek, T., 2011. Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* 14, 435-443.
- Reinhold-Hurek, B., Hurek, T., Claeyssens, M., van Montagu, M., 1993. Cloning, expression in *Escherichia coli*, and characterization of cellulolytic enzymes of *Azoarcus* sp., a root-invading diazotroph. *J Bacteriol* 17, 7056-7065.
- Richardson, A.E., Barea, J.M., McNeill, A.M., Prigent-Combaret, C., 2009. Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 111–117.
- Rosenblueth, M., Martínez-Romero, E., 2006. Bacterial endophytes and their interaction with hosts. *Mol Plant Microbe Interact* 19, 827-837.
- Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., 2008. Bacterial endophytes: recent developments and applications. *FEMS Microbiol Lett* 278, 1-9.
- Schlaeppli, K., Bulgarelli, D., 2015. The plant microbiome at work. *Mol Plant Microbe Interact* 28, 212-217.
- Scollan, N.D., Dhanoa, M.S., Choi, N.J., Maeng, W.J., Enser, M., Wood, J.D., 2001. Biohydrogenation and digestion of long chain fatty acids in steers fed on different sources of lipid. *J Agric Sci* 136, 345-355.
- Scotti-Campos, P., Pham-Thi, A.T., Semedo, J.N., Pais, I.P., Ramalho, J.C., Matos, M.C., 2013. Physiological responses and membrane integrity in three *Vigna* genotypes with contrasting drought tolerance. *Emir J Food Agric* 25, 1002-1013.

- Scotti-Campos, P., Semedo, J.N., Pais, I., Oliveira, M., Passarinho, J., Ramalho, J.C., 2014. Heat tolerance of Portuguese old bread wheat varieties. *Emir J Food Agric* 26, 170-179.
- Scotti-Campos, P., Semedo, J.N., Pais, I.P., Oliveira, M., Passarinho, J., Santos, M., Almeida, A.S., Costa, A.R., Pinheiro, N., Bagorro, C., Coco, J., Costa, A., Coutinho, J., Maças, B., 2015. Physiological responses to drought in four developed *Triticum aestivum* groups. *Emir J Food Agric* 27, 178-185.
- Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A., 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* 2, 587. doi: 10.1186/2193-1801-2-587.
- Soto, M.J., Jiménez-Zurdo, J.I., van Dillewijn, P., Toro, N., 2000. *Sinorhizobium meliloti putA* gene regulation: a new model within the family *Rhizobiaceae*. *J Bacteriol* 182, 1935-1941.
- Tandon H.L.S., 1996. Nitrogen research and crop production, Fertiliser, Development and Consultation Organization, New Delhi.
- Ulrich, K., Stauber, T., Ewald, D., 2008. *Paenibacillus* - a predominant endophytic bacterium colonising tissue cultures of woody plants. *Plant Cell Tiss Org Cult* 93, 347-351.
- van Overbeek, L.S., van Elsas, J.D., 1995. Root exudates-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. *Appl Environ Microbiol* 61, 890-898.
- Vincent, J.M., 1970. A Manual for Practical Study of Root Nodule Bacteria, IBP Handbook No. 15, Blackwell Scientific Publishers, Oxford.
- Xi, C., Lambrecht, M., Vanderleyden, J., Michiels, J., 1999. Bi-functional *gfp*- and *gusA*-containing mini-Tn5 transposon derivatives for combined gene expression and bacterial localization studies. *J Microbiol Meth* 35, 85-92.
- Xie, C.H., Yokota, A., 2006. *Sphingomonas azotifigens* sp. nov., a nitrogen-fixing bacterium isolated from the roots of *Oryza sativa*. *Int J System Evol Microbiol* 56, 889-893.
- Yan, Z., Reddy, M.S., Kloepper, J.W., 2003. Survival and colonization of rhizobacteria in a tomato transplant system. *Can J Microbiol* 49, 383-389.

Zamioudis, C., Mastranesti, P., Dhonukshe, P., Blilou, I., Pieterse, C.M., 2013. Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. bacteria. *Plant Physiol* 162, 304-318.

Figure captions



Fig. 1. Annual ryegrass plants grown in Evans medium for 4 weeks in a controlled environment growth chamber. (A) Non-inoculated (control); (B) Inoculated with a bacterial mixture (treatment 8) containing equal amounts of *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *Sphingomonas azotifigens* DSMZ 18530^T (total number of 3×10^8 bacteria per plant).

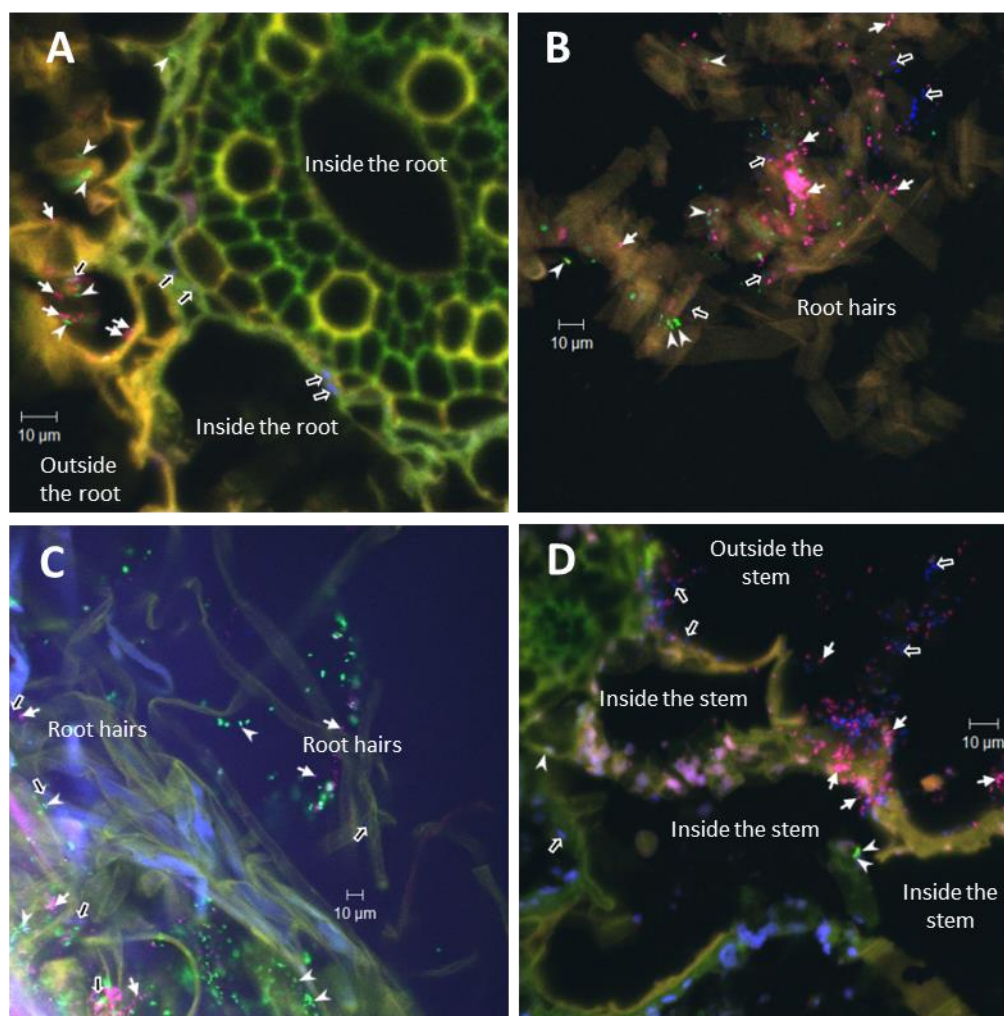


Fig. 2. Confocal laser scanning microscopic images of fluorescence *in situ* hybridization (FISH) of annual ryegrass plants inoculated with a mixed inoculum containing *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and GFP-labelled *Sphingomonas azotifigens* DSMZ 18530T. FISH analysis was performed to visualize each bacterium: GAM42a(Cy3)+EUBmix(Cy5)-targeted *Pseudomonas* (➤) appeared magenta, ALF969(FITC)+EUBmix(Cy5)-targeted *S. azotifigens*-GFP (➤) appeared cyan/green, and EUBmix(Cy5)-targeted *Paenibacillus* (⇨) appeared blue. Micrographs of root transverse sections (A-B) and entire root fragment (C), showing the presence of the three bacteria in the root cortical parenchyma and endodermis (A), and in root hairs (B-C). Cross section of a stem (D) showing endophytic colonization by inoculated bacteria.

Tables

Table 1 Bacterial strains and plant inoculation treatments used in this study.

Bacteria			
	Code	Isolation source	Reference
<i>Pseudomonas</i> sp. G1Dc10	Ps	Stems of annual ryegrass	Castanheira et al. (2014)
<i>Paenibacillus</i> sp. G3Ac9	Pa	Rhizosphere of annual ryegrass	Castanheira et al. (2014)
<i>Sphingomonas azotifigens</i> DSMZ 18530	Sp	Roots of rice	Oyaizu-Masuchi and Komagata (1988); Xie and Yokota (2006)
Inocula			
	Type	Composition ^a	Size (total number of cells per plant)
Treatment 1	Single	Ps	10 ⁸
Treatment 2	Single	Pa	10 ⁸
Treatment 3	Single	Sp	10 ⁸
Treatment 4	Dual	Ps + Pa	10 ⁸
Treatment 5	Dual	Ps + Sp	10 ⁸
Treatment 6	Dual	Sp + Pa	10 ⁸
Treatment 7	Triple	Ps + Pa + Sp	10 ⁸
Treatment 8	Triple	Ps + Pa + Sp	3×10 ⁸

^a Multiple inocula (treatments 4-8) contained equal amounts of each strain.

Table 2 Influence of inoculation with different combinations of strains *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *Sphingomonas azotifigens* DSMZ 18530 on the growth of annual ryegrass after incubation for 4 weeks in gnotobiotic conditions.

Inoculation treatments ^a	Dry weight relative to non-inoculated controls (%) ± SE ^{d, e}	
	Shoot	Root
<i>Single inoculation</i> ^b		
Treatment 1 (Ps)	140.6 ± 10.1 * AB	188.9 ± 21.1 * A
Treatment 2 (Pa)	140.1 ± 4.3 * AB	200.4 ± 17.8 * A
Treatment 3 (Sp)	128.9 ± 13.3 * AB	140.2 ± 16.6 * A
<i>Dual inoculation</i> ^b		
Treatment 4 (Ps + Pa)	137.8 ± 14.0 * AB	155.2 ± 21.7 * A
Treatment 5 (Ps + Sp)	153.8 ± 8.2 * A	168.1 ± 6.9 * A
Treatment 6 (Sp + Pa)	140.4 ± 10.7 * AB	166.7 ± 16.7 * A
<i>Triple inoculation</i> ^c		
Treatment 7 (Ps + Pa + Sp, 10 ⁸ cells per plant)	126.1 ± 5.4 * AB	143.9 ± 7.9 * A
Treatment 8 (Ps + Pa + Sp, 3×10 ⁸ cells per plant)	119.1 ± 4.5 * B	136.7 ± 16.7 * A

^a Multiple inocula (treatments 4-7) contained equal amounts of each strain. Ps, *Pseudomonas* sp. G1Dc10; Pa, *Paenibacillus* sp. G3Ac9; Sp, *Sphingomonas azotifigens* DSMZ 18530.

^b Average values of 7-9 replicate samples ± SE.

^c Average values of 14-24 replicate samples ± SE.

^d Values followed by (*) were significantly different from non-inoculated controls according to Tukey's HSD test at $P \leq 0.05$.

^e Different capital letters (A,B) express significant differences between treatments according to Tukey's HSD test at $P \leq 0.05$.

Table 3 Influence of inoculation with different combinations of strains *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *Sphingomonas azotifigens* DSMZ 18530^T on pigments content, fatty acid composition and electrolyte leakage in leaves of annual ryegrass plants grown for 7 weeks in gnotobiotic conditions.

Physiological parameter ^{b, c}	Inoculation treatments ^a					
	Non-inoculated	Single inoculation				Triple inoculation
	control	Treatment 1 (Ps)	Treatment 2 (Pa)	Treatment 3 (Sp)	Treatment 8 (Ps + Pa + Sp)	
<i>Pigments</i> ^d						
Chlorophylls (<i>a+b</i>) (mg g ⁻¹ dry weight)	10.5 ± 0.3	13.9 ± 0.2 * ^A	8.6 ± 0.6 * ^B	14.7 ± 0.4 * ^A	17.7 ± 1.1 * ^A	
Chlorophyll <i>a/b</i>	3.3 ± 0.2	4.0 ± 0.2 ^A	3.3 ± 0.4 ^A	3.3 ± 0.0 ^A	3.6 ± 0.1 ^A	
Carotenoids (mg g ⁻¹ dry weight)	2.5 ± 0.1	3.4 ± 0.1 * ^A	2.0 ± 0.0 ^B	3.5 ± 0.1 * ^A	3.7 ± 0.2 * ^A	
<i>Fatty acids</i> (mg g ⁻¹ dry weight) ^e						
<C16:0	0.3 ± 0.0	0.4 ± 0.0 ^B	0.4 ± 0.0 ^B	0.2 ± 0.0 ^C	0.8 ± 0.0 * ^A	
C16:0 (palmitic acid)	3.7 ± 0.2	4.6 ± 0.2 * ^B	4.7 ± 0.2 * ^B	4.8 ± 0.1 * ^B	6.5 ± 0.4 * ^A	
C16:1 (hexadecenoic acid)	0.7 ± 0.0	1.4 ± 0.1 * ^A	1.1 ± 0.1 * ^{AB}	0.9 ± 0.1 * ^B	1.1 ± 0.1 * ^{AB}	
C18:0 (stearic acid)	0.5 ± 0.1	0.4 ± 0.0 ^B	0.6 ± 0.0 ^A	0.5 ± 0.0 ^{AB}	0.3 ± 0.0 ^C	
C18:1 (oleic acid)	0.6 ± 0.1	0.8 ± 0.1 ^B	1.1 ± 0.0 * ^A	0.6 ± 0.0 ^B	1.1 ± 0.1 * ^A	
C18:2 (linoleic acid)	3.1 ± 0.1	3.8 ± 0.2 * ^{AB}	4.9 ± 0.4 * ^A	3.3 ± 0.1 ^B	4.8 ± 0.5 * ^A	
C18:3 (linolenic acid)	15.4 ± 0.6	21.4 ± 0.9 * ^{BC}	18.6 ± 0.4 * ^C	22.3 ± 0.6 * ^{AB}	25.5 ± 1.4 * ^A	
Total fatty acids (TFA)	24.3 ± 0.5	32.7 ± 1.0 * ^B	31.4 ± 1.0 * ^B	32.6 ± 0.7 * ^B	40.1 ± 1.9 * ^A	
Double Bond Index (DBI)	14.0 ± 0.6	15.1 ± 0.6 ^A	14.1 ± 0.3 ^{AB}	15.4 ± 0.3 ^A	12.9 ± 0.5 ^B	
<i>Electrolyte leakage</i> ^d						
(% of total conductivity)	5.8 ± 0.3	4.4 ± 0.2 * ^B	11.7 ± 0.0 * ^C	4.1 ± 0.2 * ^{AB}	3.2 ± 0.2 * ^A	

^a Inocula contained 10⁸ (single inoculation) or 3×10⁸ (triple inoculation) bacteria per plant. The triple inoculum (treatment 8) contained equal amounts of each strain. Ps, *Pseudomonas* sp. G1Dc10; Pa, *Paenibacillus* sp. G3Ac9; Sp, *S. azotifigens* DSMZ 18530.

^b Values followed by (*) were significantly different from non-inoculated controls according to Tukey's HSD test at $P \leq 0.05$.

^c Different capital letters (^{A, B}) express significant differences between inoculated treatments according to Tukey's HSD test at $P \leq 0.05$.

^d Average values of 3 (single inoculation), 5 (triple inoculation) or 11 (non-inoculated control) replicate samples ± SE.

^e Average values of 6 (single and triple inoculation) or 18 (non-inoculated control) replicate samples ± SE.

Table 4 Enumeration of viable bacteria recovered from the rhizoplane and surface disinfected roots, stems and leaves of annual ryegrass plants using the dilution-plating method. Plants were inoculated with a bacterial mixture (treatment 8) containing equal amounts of *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *Sphingomonas azotifigens* DSMZ 18530^T (total number of 3×10^8 bacteria per plant), and were grown for 4 weeks in gnotobiotic conditions.

	Number of recovered bacteria ^{a, b, c} (\log_{10} CFU g ⁻¹)		
Plant fraction	<i>Pseudomonas</i> sp. G1Dc10	<i>Paenibacillus</i> sp. G3Ac9	<i>Sphingomonas azotifigens</i> DSMZ 18530 ^T
Rhizoplane	9.38 ± 0.05 ^{a A}	8.76 ± 0.30 ^{a B}	9.10 ± 0.04 ^{a AB}
Root	4.84 ± 0.29 ^{c A}	4.32 ± 0.45 ^{c A}	4.71 ± 0.22 ^{c A}
Stem	7.58 ± 0.20 ^{b A}	7.32 ± 0.30 ^{b A}	6.67 ± 0.43 ^{b A}
Leaves	4.32 ± 0.48 ^{c A}	4.58 ± 0.40 ^{c A}	3.94 ± 0.17 ^{c A}

^a Average values of 3-4 replicate samples \pm SE.

^b Different letters express significant differences between \log_{10} CFU counts of each strain in different parts of the plant (lowercase letters) or between \log_{10} CFU counts of the different strains in the same part of the plant (uppercase letters), according to Tukey's HSD test at $P \leq 0.05$.

^c CFU, colony forming units.