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

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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.

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 Overbeek 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

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and growth, enhanced stress tolerance, disease resistance or biological control of plant pathogens (Berg, 2009; Morrissey et al., 2004; Pérez-Montaño 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 (Schlaeppi 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 in tryptone-yeast (TY) medium (Beringer, 1974) for 16 h at 30 °C with vigorous shaking. The cultures were centrifuged at 10,000*g* for 10 min. 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, 10^8 cells of a single strain per plant), or combined to yield the inoculation mixtures for dual (treatments 4–6, 0.5×10^8 cells of each strain to a total number of 10^8 bacteria per plant) and triple inoculations (treatment 7, 0.33×10^8 cells of each strain to a total number of 10^8 bacteria per plant; and treatment 8, 10^8 cells of each strain to a total number of 3×10^8 bacteria per plant). Information regarding strains and inoculation treatments used in this study is summarized in Table 1.

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

Bacteria			
	Code	Isolation source	Reference
Pseudomonas sp. G1Dc10	Ps	Stems of annual ryegrass	Castanheira et al. (2014)
Paenibacillus sp. G3Ac9	Pa	Rhizosphere of annual ryegrass	Castanheira et al. (2014)
Sphingomonas azotifigens DSMZ 18530	Sp	Roots of rice	Oyaizu-Masuchi and Komagata (1988); Xie and Yokota (2006)

Inocula			
	Type	Composition[a]	Size
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.

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 et al., 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 1–6) 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 weight 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