Microbiological Research 169 (2014) 768–779

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Annual ryegrass-associated bacteria with potential for plant growth promotion

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ARTICLE INFO

Article history:
Received 16 September 2013
Received in revised form
30 December 2013
Accepted 31 December 2013
Available online 8 January 2014

Keywords:
Plant-associated bacteria
Nitrogen fixation
Plant growth promoting activities
Plant inoculation
Biofertilizers

ABSTRACT

Annual ryegrass is a fast-growing cool-season grass broadly present in the Portuguese "montado", a typically Mediterranean agro-forestry-pastoral ecosystem. A culture-dependent approach was used to investigate natural associations of this crop with potentially beneficial bacteria, aiming to identify strains suitable for biofertilization purposes. Annual ryegrass seedlings were used to trap bacteria from three different soils in laboratory conditions. Using a nitrogen-free microaerophilic medium, 147 isolates were recovered from the rhizosphere, rhizoplane, and surface-sterilized plant tissues, which were assigned to 12 genera in classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacilli* and *Actinobacteria*. All isolates were able to grow in the absence of nitrogen and several of them were able to perform *in vitro* activities related to plant growth promotion. Isolates of the genera *Sphingomonas* and *Achromobacter* were found to be the most effective stimulators of annual ryegrass growth under nitrogen limitation (47–92% biomass increases). Major enhancements were obtained with isolates G3Dc4 (*Achromobacter* sp.) and G2Ac10 (*Sphingomonas* sp.). The latest isolate was also able to increment plant growth in nitrogen-supplemented medium, as well as the phosphate solubilizer and siderophore producer, G1Dc10 (*Pseudomonas* sp.), and the cellulose/pectin hydrolyser, G3Ac9 (*Paenibacillus* sp.). This study represents the first survey of annual ryegrass-associated bacteria in the "montado" ecosystem and unveiled a set of strains with potential for use as inoculants.

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1. Introduction

Many bacteria form beneficial associations with plants in their natural environments. Some of them thrive at the root surface or in the surrounding soil, taking advantage of carbon and energy-sources in root exudates. Other can live endophytically, invading the inner parts of plant hosts without causing symptoms of disease (Cocking 2003; Vessey 2003). In either case, plant-associated bacteria may exert beneficial effects on their hosts by participating in key processes related to nutrient cycling and availability, seedling establishment, or biological control of plant pathogens. The best studied case of such beneficial associations is undoubtedly the symbiotic interaction between nitrogen-fixing rhizobia and legume plants. Because of their contribution to the nitrogen balance of important leguminous crops, these symbioses have attracted increasing attention, mainly since the use of nitrogenous fertilizers in agriculture has resulted in unacceptable levels of environmental pollution.

Biological nitrogen fixation is not limited to symbiotic associations and may also be accomplished by rhizospheric and endophytic bacteria. Free-living diazotrophs of the genera *Azotobacter* and *Azospirillum* have been found associated with a number of important crops (Tilak et al. 2005). Endophytic diazotrophs have been encountered within plant tissues of several gramineae, such as rice (Stoltzfus et al. 1997), maize (Chelius and Triplett 2001), oat (Soares et al. 2006), and sugarcane (Baldani et al. 1997; Boddey and Dobereiner 1995; James 2000). Though, firm evidences on the direct transfer of fixed nitrogen from associative diazotrophs to the host plants were obtained in only a few studies involving ^{15}N incorporation experiments coupled to the use of non-nitrogen fixing (Nif^-) mutants as negative controls. Those studies have addressed, for example, the associations between *Gluconacetobacter diazotrophicus* and sugarcane (Sevilla et al. 2001), *Azoarcus* and kallar grass (Hurek et al. 1998; Hurek et al. 2002), or *Klebsiella pneumonia* and wheat (Iniguez et al. 2004). In other cases, the

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http://dx.doi.org/10.1016/j.micres.2013.12.010

observed plant growth responses have never been unequivocally related to nitrogen fixation by the diazotroph, and considerable evidence suggested that these effects may be primarily derived from other mechanisms, such as the production of plant growth substances (Dobbelaere et al. 2003). For instance, in the associative diazotroph *Azospirillum*, the ability to promote plant growth seems to be mainly related to production of the plant hormone indole acetic acid (Barbieri et al. 1991; Okon and Labandera-Gonzalez 1994). Bacterial synthesized phytohormones may cause changes in the root morphology and physiology, resulting in increased uptake of water and nutrients from the soil, including nitrogen (Yanni et al. 2001). Other mechanisms involved in the stimulation of plant growth by plant-associated bacteria include the increased availability of nutrients (*e.g.* solubilization of mineral phosphate), stimulation of disease-resistance mechanisms (induced systemic resistance) and protection against soil-borne pathogens, for instance through the production of siderophores that solubilize and sequester iron from the rhizospheric environment (Bulgarelli et al. 2013; Dobbelaere et al. 2003). As in the case of nitrogen-fixation, these plant growth promoting activities also have acquired increased importance due to their potential use in sustainable agriculture.

Annual ryegrass (*Lolium multiflorum* Lam.) is a forage crop that is extensively used in southern and central Portugal, mainly in areas dedicated to the exploitation of cork and holm oaks ("montado"). It is a cool season annual bunchgrass with high palatability and digestibility, which make it highly valued for forage and livestock systems. It is also suited for soil conservation uses, as plants develop extensive, fibrous, and shallow root systems that can contribute to impair soil erosion. Ryegrass is highly responsive to nitrogen fertilization, and recommended rates can reach 100 kg N ha^{-1}. Despite the efforts invested so far on the characterization of plant-growth promoting bacteria associated with many important grasses, little is known about the natural associations of this crop with potentially beneficial bacteria. A better understanding of annual ryegrass-associated bacteria in native environments is an essential tool for the development of an integrated strategy towards the reduction of the needs for chemical fertilizers.

The aims of the present study were the isolation of annual ryegrass-associated bacteria in natural pastures of the Portuguese "montado" and the evaluation of their plant growth promoting activities. The main goal was to identify strains with potential for the improvement of plant growth.

2. Materials and methods

2.1. Soil sampling

Composite soil samples were collected from the top layer (0–7 cm depth) of three natural pasture fields in "montado" ecosystems in the centre of Portugal, with different geological characteristics. The sampling sites were designated as follows: site X, schist soil (Herdade da Zambujeira, Crato); site G, granite soil (Herdade da Zambujeira, Crato); and site A, sandy soil (Companhia das Lezírias, Porto Alto). The samples were homogenized, sieved through a 2 mm mesh screen and stored at 4 °C until use. Soils chemical and granulometric properties were determined using standard procedures (Ramos et al. 2009) (Table 1).

Table 1
Soil properties in sampling sites.

Description and properties	Sampling site		
	G	X	A
Soil type	Granite	Schist	Sand
Location	Crato, Alentejo	Crato, Alentejo	Porto Alto, Ribatejo
Texture	Loamy-sand	Sandy-loam	Sandy
pH (H_2O)	5.56	4.98	5.26
Organic matter (%)	3.1	7.3	1.4
Total N (g kg^{-1})	1.34	3.05	0.28
NO_3-N (mg kg^{-1})	88.0	163.0	8.4
P_2O_5 (mg kg^{-1})	114.0	18.0	19.0
K_2O (mg kg^{-1})	315.0	225.0	29.0
Na (mg kg^{-1})	0.07	0.06	0.02
Ca (mg kg^{-1})	2.98	3.08	0.70
Mg (mg kg^{-1})	0.98	0.68	0.43
K (mg kg^{-1})	0.32	0.21	0.04

2.2. Bacterial enumeration and isolation

Seeds of a commercial variety of annual ryegrass (*L. multiflorum* Lam. cv. Pollanum) were surface disinfected, pre-germinated and transferred to flasks containing 60 g of soil (3 seedlings per flask). After incubation for eight weeks in a controlled-environment growth chamber, three groups of 6–8 plants from each soil were selected and used for enumeration and isolation of diazotrophic bacteria using described procedures (Estrada-de los Santos et al. 2001). The root-adherent soil fraction (rhizosphere) was recovered, as well as rhizoplane-associated bacteria. Roots, stems and leaves were pooled and sectioned, surface sterilized with 3% sodium hypochlorite and homogenized. The rhizosphere soil, rhizoplane bacterial suspensions and plant tissues homogenates were serially diluted (tenfold) in 10 mmol l^{-1} $MgSO_4 \cdot 7H_2O$. Culture tubes containing nitrogen-free semisolid medium (NFb; Döbereiner et al. 1976) supplemented with 20 mg l^{-1} cycloheximide were inoculated with 100 μl aliquots of diluted samples (three replicates for each dilution) and incubated for 7–14 d at 30 °C. Tubes showing a subsurface pellicle were transferred at least two times in the same medium, and the population size was estimated by the Most Probable Number (MPN) technique considering the fraction of positive tubes at each dilution. The MPN counts were subjected to analysis of variance (ANOVA) using the STATISTICA 8 program. The values were compared using the Fisher's least significance difference (LSD) test ($P \leq 0.05$).

Bacterial growth in positive tubes was streaked out on NFb agar plates supplemented with 0.2% NH_4Cl and incubated for 5–7 d at 30 °C. Single colonies were selected on the basis of their origin and morphology, and individually transferred to fresh agar plates. Further purification proceeded on Congo Red (Rodríguez-Cáceres 1982) and tryptone-yeast (TY) (Beringer 1974) agar plates. Once purified, isolates were routinely grown on TY medium at 30 °C, and stock cultures were preserved in 45% glycerol at −20 °C.

2.3. Growth in nitrogen-free media and acetylene reduction assay

Isolates and reference strains were grown in liquid NFb medium supplemented with 0.02% NH_4Cl, at 30 °C with vigorous shaking for 16 h. Cells were centrifuged at 10,000 × *g* for 10 min, washed with 0.85% NaCl and suspended in the same solution at an OD (600 nm) of 0.73 (approximately 10^8 cells ml^{-1}). Aliquots (25 μl) of the washed cell suspensions were inoculated into 3 ml semi-solid NFb medium in 18 ml vials sealed with rubber septa, and incubated statically for 48–96 h at 30 °C. Growth was evaluated by the formation of a subsurface pellicle.

The nitrogenase activity of the isolates was assessed by the acetylene reduction assay (ARA), using a modification of previously reported procedures (Mascarua-Esparza et al. 1988). Briefly, cultures in 5 ml semi-solid NFb medium were prepared as described above but using 10 ml rubber stoppered vials. After incubation for 48 h at 30 °C, 10% (v/v) of the air atmosphere in each vial was replaced with acetylene and incubation proceeded for further 24 h in the same conditions. Gas samples (0.5 ml) were withdraw and

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