Applied Soil Ecology 55 (2012) 44–52

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

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

Diversity of plant growth-promoting rhizobacteria communities associated with the stages of canola growth

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form
22 December 2011
Accepted 28 December 2011

Keywords:
Canola
PGPR abilities
Bacterial diversity
Inoculants
Plant growth response

ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) stimulate the growth of their host plant and the presence of the plant clearly has a significant effect on rhizospheric bacterial community structure. In this study, cultivable bacteria associated with soil, rhizosphere and the roots of canola (*Brassica napus*) were isolated and identified by amplifying the V6–V8 region on the 16S rDNA sequence, in order to verify if possible changes in the microbial communities were associated with some stages of crop rotation and canola growth. Several PGP activities of all isolates were also evaluated. The bacterial richness associated to the rhizospheric soil of canola at the rosette stage was more than 20% larger than the other stages indicating that the microbial community structure was influenced by seasonal variation. There was also a relationship between bacterial diversity and monthly rainfall. *Agrobacterium*, *Burkholderia*, *Enterobacter*, and *Pseudomonas* were the most abundant among all the bacterial genera identified. Several of those bacteria could produce indolic compounds and siderophores, to solubilize phosphate, and some could also fix nitrogen. Some of the isolates tested for growth-promoting effects of bacterial treatment in canola were able to promote plant growth. The presence of specific PGP traits suggests that these particular organisms can promote plant growth by more than one mechanism and that some of these strains should be tested in further field inoculation experiments.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) stimulate the growth of their host plants (Vessey, 2003). These bacteria can colonize either only the rhizosphere (epiphytic bacteria), or also inside the roots (endophytic bacteria) (Glick, 1995). Endophytic bacteria can be either associative or facultative. Facultative endophytes live in the spaces between the cells of the root cortex (Elmerich and Newton, 2007; Kobayashi et al., 1995), whereas associative bacteria can establish a symbiotic relationship with plant cells, living inside the cells in specialized root structures called nodules (Gray and Smith, 2005).

PGPR stimulate plant growth directly or indirectly. Indirect stimulation occurs when PGPR prevent the deleterious effects of phytopathogenic microorganisms, while direct plant growth stimulation occurs when PGPR synthesize some plant-growth substances or facilitate the uptake of certain nutrients. Direct stimulation involves nitrogen fixation (performed by diazotrophic organisms), solubilization of phosphate, production of phytohormones (such as auxin and cytokines), and production of siderophore that helps the transportation of ferric iron into plant cells (Ghosh et al., 2003).

While the rhizobacteria may benefit the plant, the presence of the plant clearly has a significant effect on rhizosphere bacterial community structure due to differences in root exudation, rhizodeposition in different root zones and the growth stage of the plant (Di Cello et al., 1997; Smalla et al., 2001; Dunfield and Germida, 2003; Houlden et al., 2008). Furthermore, other factors might influence the composition of the microbial community in the rhizosphere, such as cropping practices (like crop rotation) and seasonal change (Di Cello et al., 1997; Lupwayi et al., 1998; Smalla et al., 2001; Dunfield and Germida, 2003).

Canola is an option for ground cover plant for crop rotation and the production of oil and both as food and biodiesel (Schuchardt et al., 1998; Tomm, 2005). Among the available technologies that increase crop productivity and decrease the use of nitrogen fertilizers is the use of diazotrophs because of their abilities to promote

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doi:10.1016/j.apsoil.2011.12.011

plant growth. However, studies on the diversity of such microorganisms associated with canola crops are scarce (Germida et al., 1998; Siciliano et al., 1998; Dunfield and Germida, 2003). Likewise, studies towards the isolation of bacteria from canola crops and the evaluation of their potential to promote plant growth are also rare (Hong et al., 1991; Bertrand et al., 2001; Ghosh et al., 2003).

The purpose of this study was to isolate and identify cultivable bacteria associated with soil, rhizosphere and the roots of canola (*Brassica napus*) to verify if possible changes in the microbial communities are associated with some stages of crop rotation and canola growth. Moreover, we also evaluated the PGP abilities of bacterial isolates and selected some as potential PGPR for canola.

2. Material and methods

2.1. Sample processing

Both field soil and rhizosphere samples from canola (variety Hyola 60) were collected in Vacaria, a canola producing region of the Rio Grande do Sul State, Brazil (28°30′43″S, 50°56′02″W). The soil type was classified as Hapludox (pH 6.0) with an organic matter content of 5.6% and a clay content of 64%. The samples were collected between March 2009 and January 2010 in five different periods in order to cover all the growth stages of canola. Samples were collected in the following periods: (1) March 2009, field soil used for soybean growth; (2) July 2009, field soil prepared for seeding canola (pre-seeding of canola); (3) September 2009, rhizospheric soil and roots of canola at the rosette stage; (4) October 2009, rhizospheric soil and roots of canola at the flowering stage; (5) January 2010, field soil after canola harvest (postharvest fall stubble stage). At growth stages at which plants were present (rosette and flowering stages), five plants with adhering soil at least 2 m away from each other were randomly taken and then mixed. Roots and soil were separated from the rest of the canola plants. At the crop rotation stages at which canola was not present (soil previously used with soybean, pre-seeding of canola and postharvest fall stubble), ten soil samples (0.5 kg) were randomly taken and bulked to obtain a representative composite sample.

2.2. Bacterial isolation

Isolation of putative diazotrophic bacteria was performed according to Döbereiner et al. (1995). Root-associated bacteria were isolated from canola roots, which were previously surface-sterilized (surface disinfection was done by washing the roots in running tap water, followed by 70% ethanol washing for 1 min, sodium hypochlorite solution (2%, v/v) washing for 2 min and five serial rinses in sterilized distilled water). Ten grams of roots were sliced into small segments and added to 90 ml of saline solution (0.85%), followed by shaking and incubation at 4 °C for 16 h to release the bacteria that were inside the plant material. Soil and epiphytic bacteria were isolated by vigorously shaking 10 g of soil dispersed in 90 ml of saline solution (0.85%), followed by shaking and incubation at 4 °C for 16 h. Aliquots of 0.1 ml of three-fold serial dilutions of all samples were inoculated, in triplicate, into vials containing 5 ml of semi-solid N-free medium, NFb, LGI or LGI-P (Döbereiner et al., 1995), to select bacteria that could grow in N-depleted culture conditions. Five days after incubation at 28 °C, the vials showing a veil-like pellicle near the surface of the medium were considered positive and used to reinoculate other vials containing the semi-solid N-free medium previously utilized. Cultures from the positive vials were subjected to further purification steps by streaking them onto specific agar plate containing 20 mg l^{-1} of yeast extract and incubated at 28 °C for 2 days. One colony from each plate was randomly selected and grown in liquid LB medium (Sambrook and Russel, 2001) at 28 °C under agitation (200 rpm). Strains were individually screened by Gram-staining reaction and conserved in 20% glycerol solution at −20 °C.

2.3. DNA isolation

For the DNA extraction, bacterial cells were rinsed with TES buffer (10 mM Tris pH 8.0, 25 mM EDTA, and 150 mM NaCl) and resuspended in TE buffer (10 mM Tris pH 8.0, 25 mM EDTA). Cell lyses took place in 20 mg ml^{-1} lysozyme at 37 °C and 4% sodium dodecyl sulfate. Extractions with phenol/chloroform and precipitation in ethanol were performed as described (Sambrook and Russel, 2001). DNA quality and integrity was checked by electrophoresis on 0.8% agarose ethidium bromide gel. DNA was quantified by spectrophotometer.

2.4. PCR amplification and partial sequencing of the 16S rRNA gene

Fifty nanogram of bacterial DNA was used as template for PCR procedures. The selected primers U968 (5′-AACGCGAAGAACCTTAC-3′) and L1401 (5′-CGGTGTGTACAAGACCC-3′, Felske et al., 1997) were used to amplify a region of about 450 base pairs between nucleotides 968 and 1401 of the *Escherichia coli* 16S rRNA gene. This fragment includes variable regions V6–V8 (Brosius et al., 1978). The amplification of 16S rRNA gene portions from different bacterial samples was performed in a PCR Express (Thermo Hybaid) thermal cycler in 25 μl reaction volume containing 0.1 mM of each primer, 1 mM $MgCl_2$ (Invitrogen), 20 μM of each dNTP (Amersham Biosciences), and 1 U Taq DNA polymerase (recombinant, Invitrogen®). PCR products were analyzed by electrophoresis in 1% agarose gels in 1× TBE buffer with ethidium bromide and visualized with UV light.

Sequences of partial 16S rRNA genes were determined in both forward and reverse directions in a Megabace 1000 automatic 117 sequencer using the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (GE 118 HealthCare). Sequence analyses were performed with BioEdit version 7.0.9.0 software (Hall, 1999) to verify their qualities and check for possible chimeric origins. DNA sequences were compared with those from the GenBank database using BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/). The nucleotide sequences of the 308 partial 16S rRNA gene segments determined in this study have been deposited in the GenBank database under accession numbers JN699229–JN699532.

2.5. Diversity index

Diversity index (H', Shannon and Weaver, 1949) was estimated based on the total number of individuals and the number of genera identified for each sampled stage. The degree of association between H' and monthly precipitation or temperature variations was analyzed by Pearson's correlation method. Correlations were considered significant at $P < 0.05$.

2.6. Evaluation of plant growth promotion abilities

Analysis of indolic compounds and siderophore production, and phosphate solubilization activities were carried out for all bacterial isolates. *In vitro* biological nitrogen fixation assays were performed for seven selected isolates.

Bacterial indolic compounds production was measured according to Glickmann and Dessaux (1995) and Beneduzi et al. (2008). Production of siderophores was investigated by performing the universal assay (Schwyn and Neilands, 1987; Beneduzi et al., 2008) using King B medium. Isolates able to solubilize phosphates were identified by the method described by Sylvester-Bradley et al.