



Diversity of *Bradyrhizobium* populations associated to soybean–maize rotations in Québec, Eastern Canada, and their potential to improve growth of both plant species

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ABSTRACT

Bradyrhizobial populations associated with soybean–maize rotations have not been extensively studied, especially in Canada. In this work, we estimated the diversity of 255 bradyrhizobial strains isolated from soil, rhizosphere and nodules in farms under soybean or maize grown in rotation in Québec, Eastern Canada, and we characterized them for their ability to promote growth of both plants. The population was distributed into six IGS types. The major types IGS I and III included 98 and 70 strains respectively. About 50% of strains from soils of both plants and from soybean nodules were included in IGS I. Reference strains of *Bradyrhizobium japonicum* and inoculant products were included in IGS III and VI. Diversity indices were similar for both plants but were lower in soil and nodules than in rhizosphere of soybean. REP-PCR analysis was congruent with IGS typing, the major REP-clusters including a majority of strains within a same IGS type.

With soybean, 25 strains allowed higher symbiotic effectiveness (shoot dry yield) than the commercial strain 532c and 5 strains (out of 8 tested) were more competitive for nodule occupancy. With maize, 20 strains showed a PGP effect increasing shoot dry weight by 10–20%. There was no link between the IGS type and symbiotic effectiveness or PGP effect, neither with siderophore or IAA production. Our results indicate that diverse bradyrhizobial strains may compete with inoculant strains, but this diversity can be exploited to select superior strains able to improve growth of both soybean and maize.

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1. Introduction

Rhizobia are well known for their capacity to fix atmospheric nitrogen in root nodules of legumes, supplying most of the plant requirement for nitrogen. The rhizobium–legume symbiosis can be successfully improved through the selection of superior nitrogen-fixing rhizobial strains resulting in increased legume productivity (Slattery and Pearce, 2002). Beyond nitrogen fixation, rhizobia can also colonize roots of non-legume species and promote their growth without forming any nodule-like structure as reported in several studies during the last decades (reviewed by Mehboob et al., 2009; Baset Mia and Shamsuddin, 2010). Therefore, it is possible to select rhizobial strains for growth enhancement of non-legumes species; this has been recently demonstrated in an extensive field study showing increased rice production by inoculation with a *Rhizobium leguminosarum* bv. *trifolii* strain (Yanni and Dazzo, 2010). The beneficial effect of rhizobia with non-legumes is likely due to the same mechanisms of action as other soil bacterial species

known as PGPR (plant growth promoting rhizobacteria) (Kloepper et al., 1980). Most rhizobial species show typical plant growth promoting characteristics such as phytohormones and siderophores production, P solubilization and plant nutrient uptake improvement (Antoun et al., 1998).

Populations of rhizobia are naturally associated with gram-inaceous plants and other cereals, especially when they are cultivated in systems including legumes as it has been reported in rice/berseem clover rotation (Yanni et al., 1997), in maize/bean association (Gutiérrez-Zamora and Martínez-Romero, 2001) or in barley–wheat–canola in rotation with pea (Lupwayi et al., 2004). Since rhizobial diversity is favoured by crop rotations (Depret et al., 2004; Ferreira et al., 2000), rhizobial populations associated with each legume and cereal species cultivated in rotation or intercropping systems could be genetically different. For instance, in the maize and bean intercropping system, diversity of *R. etli* isolates obtained from bean nodules, soil or rhizosphere differed from that of maize stem and root isolates (Rosenblueth and Martínez-Romero, 2004).

In Eastern Canada, soybean (*Glycine max* (L.) Merr.) is one of the most important legume crops with an annual production area of more than 1,572,400 ha (Statistics Canada, 2011). Maize (*Zea mays*

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L.), another major crop in Canada, is often cultivated in rotation with soybean, which favours higher grain yields at lower N application rates (Ennin and Clegg, 2001). Soybean inoculation with *Bradyrhizobium japonicum* is generally recommended in Canada (Hume and Blair, 1992) but, the introduced inoculant strain must compete with indigenous populations for nodulation (Botha et al., 2004).

Diversity of indigenous populations of *Bradyrhizobium* has been mainly investigated on isolates obtained from soybean grown fields in the world's top producers countries such as USA (Noel and Brill, 1980; Schmidt et al., 1986; Sadowsky et al., 1987; Mpepereki and Wollum, 1991) and Brazil (Boddey and Hungria, 1997; Ferreira et al., 2000; Loureiro et al., 2007; Giongo et al., 2008). In soybean fields of the Great Plains of Canada and USA, the different levels of *B. japonicum* diversity between isolates from the two countries is likely due to the different crop rotations and to the longer soybean history in USA (Farooq and Vessey, 2009). In Eastern Canada, a preliminary study using 37 strains of *B. japonicum* isolated from soybean showed that some strains could improve maize yield (Prévost et al., 2000), but very little is still known about *Bradyrhizobium* diversity in soybean/maize rotations.

The objective of the present study was to examine the diversity of 255 indigenous *Bradyrhizobium* strains isolated in farms under soybean/maize rotations in the province of Québec, Canada. Strains were tested for their genetic diversity, their symbiotic effectiveness and competitive ability for nodulation of soybean, and for their PGP (plant growth promoting) effect on maize and *in vitro*. A greater knowledge of indigenous populations of *Bradyrhizobium* in Canadian soils will contribute to select highly competitive strains adapted to their environment and usable for the development of inoculants for soybean and maize.

2. Materials and methods

2.1. *Bradyrhizobium* strains

255 *Bradyrhizobium* strains were obtained from soils, rhizospheres or nodules collected at middle plant growing stage in soybean–maize rotations systems in farms located in five municipalities of the province of Québec, Canada (Table S1). In each field, soil rhizosphere samples from two soybean or maize plants and five soil samples between rows were mixed to obtain a composite sample. Nodules were collected from roots of the two soybean plants. *Bradyrhizobium* strains were isolated directly from soybean field nodules and from nodules formed *via* the host–plant infection technique, where soybean cv. *Lotus* (early cultivar, CRAAQ, 2003), used as a trap plant, was inoculated with soil or rhizosphere samples. After 28 days growth, nodules were surface sterilized and crushed, and the suspension was streaked on yeast extract mannitol agar (YMA) (Vincent, 1970) to obtain pure cultures. Since these strains were slow-growers and could re-nodulate soybean in symbiotic tests (described below), they were considered to belong to the *Bradyrhizobium* genus. Reference and commercial *B. japonicum* strains used in inoculants in Canada were included in the study. Strains were maintained on YMA slants.

2.2. PCR-RFLP of the IGS region

Bradyrhizobium strains were grown on agar slopes of TY (Tryptone yeast extract) medium and lysed with proteinase K (Laguerre et al., 1997). PCR amplification of the IGS (intergeneric spacer) region was performed on lysed cells using the primers 1490 and 132' as described by Laguerre et al. (1996), except that reactions were carried in a 50 µl volume (MgCl₂ 1.25 mM, dNTP's nucleotide mix 0.2 mM, each primer 0.1 µM, Taq polymerase 1.25 U). PCR products were examined by horizontal electrophoresis in 0.9% agarose

and aliquots were digested with the restriction endonucleases *HaeIII* and *MspI* (Boehringer, Germany) as specified by the manufacturer. These two restriction enzymes showed good polymorphism for *Bradyrhizobium* in our preliminary work and in a study in Senegal (Krasova-Wade et al., 2003). Restricted DNA was analysed by horizontal electrophoresis in 2% Nusieve agarose (Lonza Rockland, USA) and gels were stained with ethidium bromide and photographed under UV illumination. The restriction patterns were determined and strains were assigned within an IGS type defined by the combination of the restriction patterns. The Shannon diversity index (Shannon and Weaver, 1963) was used to determine diversity in IGS types in populations across soil, rhizosphere, nodules and across plant species (soybean and maize). Type richness was used to compare genetic diversity among these populations of different sizes, by determining the expected number of IGS types for a sample size of 22 strains, corresponding to the smallest population (in maize soil), and was calculated by rarefaction method online (<http://www.biology.ualberta.ca/jbrzusto/rarefact.php> (Simberloff, 1972).

2.3. Determination of REP patterns

Bacterial cells were prepared for REP (repetitive extragenic palindromic) analysis as described above for IGS region. The primers used were REP1R-I and REP2-I (de Bruijn, 1992). The cycling programme and reactions were performed according to Versalovic et al. (1994) and Vinuesa et al. (1998), but mixture composition was modified using dNTP 1.25 mM, each primer 2 µM, Taq polymerase 4 U and MgCl₂ buffer 2.5 mM, in a 15 µl reaction volume. Cluster analysis was performed by SAHN UPGMA (unweighted pair group method with averages) method using NTSys PC (v2.02) and a dendrogram was constructed (Sneath and Sokal, 1973).

2.4. Intrinsic antibiotics resistance (IAR)

Strains were tested for their resistance to the following antibiotics: rifampicin (100 µg mL⁻¹), streptomycin (60 µg mL⁻¹), nalidixic acid (20 µg mL⁻¹), tetracycline (55 µg mL⁻¹), chloramphenicol (100 µg mL⁻¹), kanamycin (35 µg mL⁻¹) and carbenicillin (200 µg mL⁻¹). Briefly, 150 µL of a fresh culture grown in yeast extract mannitol broth (YMB) medium at 25 °C for 5–7 days (D.O. 0.9) was used to fill each well of an ELISA plate. A matching replica plater (Sigma, USA) was used to inoculate plates containing YMA supplemented with antibiotics. Each isolate was tested in triplicate (3 wells randomly chosen) on two plates of each antibiotic medium. After 10 days of incubation at 25 °C, isolates were recorded resistant when growth or poor growth occurred and sensitive when no growth occurred.

2.5. *In vitro* PGPR characteristics

2.5.1. Determination of IAA

Production of IAA (Indol Acetic Acid) was determined using a slight modification of the rapid assay described by Bric et al. (1991). A Petri dish containing YMA amended with 5 mM L-tryptophan was overlaid with four pieces (each about 4 cm²) of sterilized nitrocellulose membrane (Pall NT). One nitrocellulose membrane piece was inoculated with a loopful of a fresh culture (grown in YMB) of one *Bradyrhizobium* strain. Each strain was tested in duplicate. After 7 days incubation at 30 °C, membranes showing good growth were removed from the agar plates and placed on a filter paper Whatman No. 2 saturated with Salkowski reagent (Gordon and Weber, 1951) prepared in sulphuric acid (Bric et al., 1991). Strains were identified

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