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Characterization of rhizobia isolates obtained from nodules of wild genotypes of common bean

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ABSTRACT

This study aimed to evaluate the tolerance to salinity and temperature, the genetic diversity and the symbiotic efficiency of rhizobia isolates obtained from wild genotypes of common bean cultivated in soil samples from the States of Goiás, Minas Gerais and Paraná. The isolates were subjected to different NaCl concentrations (0%, 1%, 2%, 4% and 6%) at different temperatures (28 °C, 33 °C, 38 °C, 43 °C and 48 °C). Genotypic characterization was performed based on BOX-PCR, REP-PCR markers and 16S rRNA sequencing. An evaluation of symbiotic efficiency was carried out under greenhouse conditions in autoclaved Leonard jars. Among 98 isolates about 45% of them and *Rhizobium freirei* PRF81 showed a high tolerance to temperature, while 24 isolates and *Rhizobium tropici* CIAT899 were able to use all of the carbon sources studied. Clustering analysis based on the ability to use carbon sources and on the tolerance to salinity and temperature grouped 49 isolates, *R. tropici* CIAT899 and *R. tropici* H12 with a similarity level of 76%. Based on genotypic characterization, 65% of the isolates showed an approximately 66% similarity with *R. tropici* CIAT899 and *R. tropici* H12. About 20% of the isolates showed symbiotic efficiency similar to or better than the best *Rhizobium* reference strain (*R. tropici* CIAT899). Phylogenetic analysis of the 16S rRNA revealed that two efficient isolates (ALSG5A1 and JPrG6A8) belong to the group of strains used as commercial inoculant for common bean in Brazil and must be assayed in field experiments.

Introduction

The common bean (*Phaseolus vulgaris* L.) is a leguminous plant of worldwide social and economic importance, providing most of the daily requirements of protein and carbohydrates for the poorest populations of South and Central America, Africa and India.[1] With respect to international agriculture, Brazil is the world's third largest producer of common bean, accounting for 12.7% of worldwide production.[2] In Brazil, the common bean is cultivated on a total area of 3.1 million hectares with a total grain production of approximately 2.8 million tons,[3] for which high amounts of nitrogen (N) are required.

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Despite its abundance in the atmosphere, N is scarce in tropical soils due to the fast mineralization of organic matter in tropical conditions. Although the decomposition of organic matter is an important source of N for crops, the adequate supply of N to crops depends largely on the use of nitrogen fertilizers.[4] However, biological nitrogen fixation (BNF) is considered a more sustainable approach for supplying N to the production system.

BNF is a key process for the conversion of nitrogen gas (N_2) into ammonia (NH_3) performed by bacteria belonging to the group of rhizobia. The reduction reaction of N_2 to NH_3 is carried out by N-fixing bacteria or diazotrophic microorganisms containing the enzymatic complex in which nitrogenase takes part.[5] Among N-fixing bacteria of the rhizobia group, a variety of *Rhizobium* and *Ensifer* species is able to colonize and establish a symbiotic partnership with common bean.[6,7]

To improve BNF efficiency, more efficient rhizobia strains are needed. Many isolating works have been performed using soil from different sites; however, as trap plant usually is used a commercial variety of common bean. The strategy used in our work was to collect soil in different sites and to use wild genotypes of common bean as trap plant looking for a better exploration of the rhizobial community, since wild genotypes show a broader genetic base. This work aimed to characterize and determine the symbiotic efficiency of rhizobia isolates obtained from the root nodules of wild genotypes of common bean.

Materials and methods

Bacterial strains and rhizobia isolates

The isolates evaluated in this work were obtained by Sampaio FB[8] and are available at the Collection of Microorganisms and Multifunctional Fungi of Embrapa Rice and Beans. Strains of *Rhizobium tropici* (CIAT899 and H12), *Rhizobium freirei* (PRF81) were used as reference strains in all analyses and, *Rhizobium etli* bv. *phaseoli* (CFN42) used in the BOX- and REP-PCR analyses.

Carbon source use (CSU) and tolerance to salinity and temperature (TST) assays

CSU was assayed for 98 isolates and for the *R. tropici* reference strains. Bacteria were kept for growth on modified YMA (Yeast Mannitol Agar) culture medium, without mannitol, added with individual carbon sources sucrose, glucose, malic acid, maleic acid, nicotinic acid, inositol, sorbitol, arabinose, fructose and glycerol. After incubation at 28 °C, bacterial growth was verified from 48 to 96 h at each 24 h.

The same isolates and reference strains were assessed for TST on YMA culture medium on a factorial (5 × 5) arrangement (concentrations of NaCl – 0%, 1%, 2%, 4%, 6% and temperature – 28 °C, 33 °C, 38 °C, 43 °C, 48 °C) incubated for a period of 48 h.

Genotypic characterization based on molecular markers

Based on CSU and TST, 55 isolates were selected for genotypic characterization. Genomic DNA was extracted according to Ausubel et al.[9] DNA quantity was estimated by spectrophotometry (NanoDrop®, Thermo scientific, Wilmington, USA), and DNA concentration was adjusted to 50 ng μL^{-1} for all samples. BOX-PCR was performed using the primer BOX A1R (5′-CTACGGCAAGGCGACG-3′), while REP-PCR was performed using the primers REP-1 (5′-IIIICGICGICATCIGGC-3′) and REP-2 (5′-ICGICTTATCIGGCCTAC-3′) according to Versalovic et al.[10]

The BOX-PCR reaction was performed in a final volume of 15 μL, containing 2.9 μL of milli-Q water, 7.5 μL of 2× QIAGEN Multiplex PCR Master Mix (3 mM Mg^{2+}), 1.6 μL of primer BOX A1R (50 pmol μL^{-1}) and 3 μL of DNA template (50 ng μL^{-1}). The REP-PCR reaction was performed in a final volume of 15 μL, containing 2.0 μL of milli-Q water, 7.5 μL of 2× QIAGEN Multiplex PCR Master Mix (3 mM Mg^{2+}), 1.25 μL of each primer REP 1 and REP 2 (10 pmol μL^{-1}) and 3 μL of DNA template (10 ng μL^{-1}).

The amplification program was designed according to Kaschuk et al.[11] PCR amplification consisted of an initial denaturing step (95 °C; 7 min); followed by 35 cycles of denaturation (94 °C; 1 min), annealing (55 °C; 1 min for BOX-PCR and 40 °C; 1 min for REP-PCR) and extension (65 °C; 8 min); followed by a final extension cycle (65 °C; 15 min). The PCR program was performed in a thermocycler Biocycler® (Applied Biosystems).

PCR products were subjected to electrophoresis on an agarose gel 1% (50 V; 7 h) in TAE buffer 0.75×[12] using 1 kb DNA Ladder® (Norgen) as a DNA band position marker. The agarose gel was stained with SYBR® green (Life Technologies) and visualized with a MultiDoc-it® system.

Symbiotic efficiency under greenhouse conditions

Based on genotypic characterization, 30 isolates were selected to evaluate their symbiotic efficiency. In addition to the isolates, the treatments were composed of two *R. tropici* strains (CIAT899 and H12), one *R. freirei* strain (PRF81), two nitrogen fertilized treatments (NT1 = 60 and NT2 = 120 kg ha^{-1} of N) and one control treatment (CT – without inoculation and without N).

Seeds of common bean cv. Pérola were sown in autoclaved Leonard jars in a random block design with three replicates. At five days after emergence (DAE), plantlets were inoculated with a cell suspension containing 1×10^9 cell mL^{-1} of each isolate and reference strain. Once a week, 200 mL of nutritive solution without N were added.[13] To the nitrogen fertilized treatments NT1 and NT2, 1 and 2 mL, respectively, of a solution containing 106.68 mg mL^{-1} of urea were added.

Plants were harvested at 35 DAE. Roots were carefully washed, dried in a paper towel, and the nodules were detached and counted to determine the number of nodules (NN). The leaves were detached from shoots to determine the leaf area (LA) using a leaf area meter LI-COR model 3100. Shoots and nodules were dried (65 °C; 72 h) to determine the shoot dry mass (SDM) and nodule dry mass (NDM). Subsequently, to determine SDM, shoot of plants were milled to determine the total N (N-Total) using the Kjedahl method, as described by Silva and Queiroz.[14]

16S rRNA sequencing analysis

Based on the symbiotic efficiency five isolates (ALSG5A1, JPrG1A1, JPrG6A8, JPrG8A7 and PCG4A2) were selected for

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