Tropentag 2009
University of Hamburg, October 6-8, 2009
Conference on International Research on Food Security, Natural Resource Management and Rural Development

Morphological Characterization and Genetic Identification of Rhizobacteria in Cuban Agricultural Soils

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Introduction

In subsistence and low input agricultural systems, crop yields are directly dependent on the inherent soil fertility and on microbial processes that govern the mineralization and mobilization of nutrients required for plant growth. The study of microbial diversity in agricultural soils provides valuable ecological information by defining host preferences and predominance of strains, knowledge of the genetic relationships and structure of bacteria, as well as the dynamics of exchange of genetic material. This is also a necessary source for the selection of efficient strains to be used in inoculation trials in agricultural fields (Smit et al., 2001).

Although a great deal of knowledge has been amassed concerning the diversity and genetics of bean symbionts, the basis of a successful inoculation and efficient N fixation remains elusive, as well as the influence of other rhizobacteria on nodulation and N fixation (Muresul et al., 2008). In our work we aim to identify genetically isolates from soil, plants in intercropping with common bean and beans nodules, as well as the phenotypic characterization of diazotroph isolates from bean nodules. This should contribute to the understanding of microbial community in tropical soils and to obtain efficient strains for inoculants production.

Material and Methods

Sample preparation, culture conditions and isolation of bacterial colonies

Soil dilutions were performed as described by Jensen (1962). Root segments from sorghum and bean bearing nodules were washed under running water, then surface-sterilized by immersion in 90% ethanol for 1 minute, followed by 3% sodium hypochlorite for 3 min, and finally washed with sterile distilled water. Bean nodules were also surface-sterilized by immersion in 0.1% $HgCl_2$ for 2 minutes. Sterile sorghum roots were crushed in 1 ml sterile distilled water. Bean nodules were carefully excised from the roots with a flamed-sterile scalpel. A total of 20 nodules were randomly collected and crushed in 1 ml of sterile distilled water. Both, sorghum roots and bean nodules suspensions were streaked on nutrient agar (NA) medium plates and incubated for 7 days at room temperature. All the colonies obtained from soil, sorghum or nodule samples were purified by repeated streaking.

DNA extraction and PCR amplification of the 16S rRNA gene

DNA from the colonies isolated was extracted by alkaline lysis (Vanparys et al., 2005). The 16S rRNA genes were amplified with the conserved primers: 5'CTGGCTCAGGAC/TGAACGCTG3' (ARI C/T) and 5'AAGGAGGTGATCCAGCCGCA3' (pH), which amplify almost the full length of the gene (1500 bp) corresponding to the 16S rRNA.

The PCR-amplified 16S rRNA gene products were purified using a QIAquick PCR Purification Kit (Qiagen) and analyzed afterwards by electrophoresis. For each sequence reaction 8 sequencing primers were used. Sequence analysis was performed using an Applied Biosystems 3100 DNA Sequencer. Sequence assembly was performed with BioNumerics version 4.5. Phylogenetic analysis was performed using the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium). The sequences of strains with strong resemblance to the consensus sequences of the different isolates were retrieved from the EMBL database and aligned.

Phenotypic characterization under controlled growth condition

The phenotypic characterization of the diazotrophic isolates consisted in the nodulation test. We used the *Rhizobium etli* CNPAF512 strain as a control and the identified *Rhizobium* and *Agrobacterium* isolates. For *Agrobacterium tumefaciens* strains isolated only two of them were analyzed due to the homology with the Genbank database.

Seeds of bean cv. ICA Pijao were surface-sterilized as described previously and pre-germinated during two days on water agar (15 g l^{-1}) in the dark at 30^{o}C. One pre-germinated seedling was planted per square dish (12x12 cm) containing 50 ml of Snoeck medium. The seedlings were inoculated with 100 µl inoculum, containing 10^{7} *Rhizobium* or *Agrobacterium* cells. Bean plants were grown in a Sanyo Gallenkamp Fytotron plant growth room with a 12-h photoperiod (day/night temperature, 22°C/18°C; day/night relative humidity, 65%/75%).

Data were processed using SAS 9.1 Entreprise Guide 4. Analysis of Variance (ANOVA) mixed model was applied with specific settings: Kenward and Roger calculation as degree of freedom method and Tukey HSD ($P<0.05$) as posthoc significance test. Ten replicates for the nodulation parameters were considered as the experimental unit.

Results and Discussions

The samples of rhizosphere soil, sorghum roots and bean nodules, were processed to perform the isolation of bacteria in each condition, which turned out a total of 32 isolates with different characteristic of colony growth, color, slimy production, borders, elevation and Gram reaction. All different colonies were isolated to set up the genetic identification using 16S rDNA protocol.

Identification of bacterial isolates by 16S rDNA sequence analysis

Eight different taxa were recovered from the 16S rDNA sequence analysis (figure 1). Most of the sequences showed from 99 to 100% of similarity with sequences in the GenBank database and from the entire lineages, which indicate the confidence of the identification at genus level and often at species level.

Strains from *Sorghum* root samples represent the most microbial diversity and most of them showed 100% similarity with entries in the GenBank database. Two groups of isolates are related with symbiotic bacteria from the genus *Rhizobium* and *Ochrobactum*, however, *Ochrobactrum cytisi* (AM411072) is only observed in soil and not in bean nodules samples.

The *Rhizobium* strains isolated from the bean nodules revealed a close match with the accessions CP000133 and EF054889 representing *Rhizobium etli* (RL-1, RL-5) and *Rhizobium tropici* (RL-2) respectively, giving 100% of similarity with GenBank database sequences. The distribution of rhizobia that nodulate *P. vulgaris* varies among geographical locations (Laguerre et al., 2001), although *R. etli* and *R. tropici* appear to be distributed worldwide.

These results show the diversity of rhizobia in Cuban soils, which could be useful to identify the efficiency of the strains for the nodulation and N fixation in interaction with common bean and

also could be the reason of the lack of response in some field trials due to the promiscuity of the crop to be infected by different *Rhizobium* strains.

The presence of *Agrobacterium* strains in nodule samples was a quite unexpected. This genus is present in bean nodules and *Sorghum* roots, but surprisingly, was not recovered in the soil sample. In bean nodules, Mhamdi et al. (2005) identified along with *Rhizobium*, *Agrobacterium*-like bacteria, and proved that these could invade new nodules upon co-inoculation with rhizobia and affect their nodulation performance. This might possibly be related to the lack of response of legumes to *Rhizobium* inoculation in tropical conditions.

All *Sphingomonas* isolates were identified as *Sphingomonas yanoikuyae* (100% identity with GenBank database) and for group 5 the *Stenotrophomonas* (EF695449, AB294557) were characterized by the specie *maltophilia* (100% and 98.7% sequence similarity, respectively).

From the Gram positive isolates, only one sequence matched 100% (AF260750) with a *Bacillus subtilis* strain in the GenBank database; however; all the other identifications were above the limit (97.5%) of species level identification and represent the presence of *Bacillus*, *Brevibacillus* and *Paenibacillus* strains.

Representatives of the genera *Sphingomonas*, *Stenotrophomonas*, *Bacillus* and *Paenibacillus*, have been reported to establish beneficial interactions with plants.

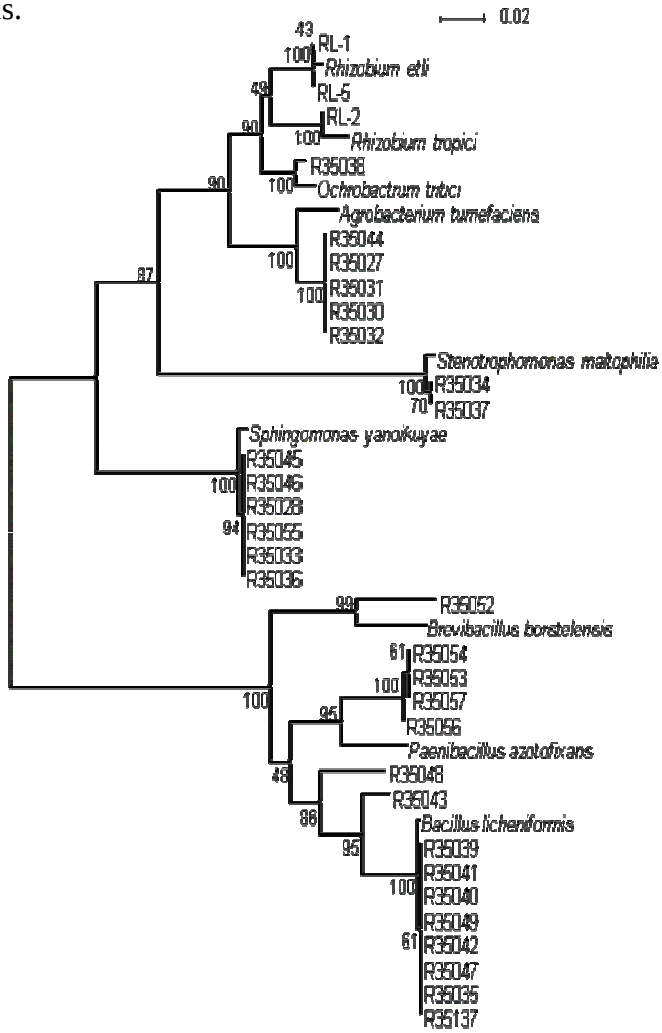

Fig. 1 Dendogram showing the distances among the isolates strains turned out from the 16S rDNA and the reference strains.

In some cases like *Bacillus* sp., *Bacillus subtilis*, *Bacillus licheniformis* and *Paenibacillus* have been reported as plant growth promoting rhizobacteria (PGPR). Furthermore, *Sphingomonas* isolates, though reported previously in legumes, have also been shown to be effective for bioremediation soils contaminated with heavy metals (Cakmakci et al., 2007).

Analysis of nodulation tests

The nodulation of the *Rhizobium* and *Agrobacterium* isolates was compared with *Rhizobium etli* wild-type reference strain CNPAF512. Figure 2 shows the abundant nodule formation by *R. etli* (reference and isolated strains) and *R. tropici* (RL-2, EF054889), while none of *Agrobacterium* isolates did elicit nodules on ICA Pijao roots. Several studies have reported the presence of *Agrobacterium* in several tropical legumes. Muresul et al. (2008) have detected *Agrobacterium* e.g. in nodules of *Hedysarum spinosissimum, Scorpiurus muricatus, Glycine max, Phaseolus vulgaris,* but the plant tests showed that the isolates were unable to nodulate their original host.

N_2-fixing rhizobia resembling agrobacteria were isolated from root nodules of *Acacia* spp. and common bean in Africa, but the isolates were not able to maintain the symbiotic effectiveness. Their presence in nodules, according to Mhamdi et al. (2005) could be explained either by a mixed infection with rhizobia or by the acquisition of a symbiotic plasmid by the *Agrobacterium* which might be highly unstable and lost during the isolation and preservation processes.

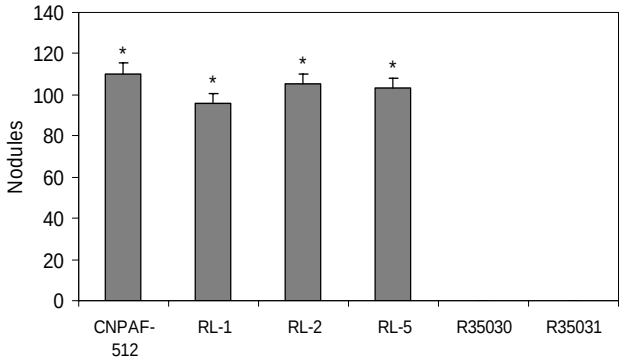

Fig. 2 Nodulation test on *P. vulgaris* c.v. ICA Pijao. Strains analyzed: *Rhizobium etli* (CNPAF512, reference wild-type), *R. etli* (RL-1), *R. tropici* (RL-2), *R. etli* (RL-5), *A. tumefaciens* (Acc: R35030), *A. tumefaciens* (Acc:R35031). *significant diference P<0.05 for Tukey HSD.

Conclusions and perspectives

The identification of bacteria in agricultural soils has a valuable importance to unravel the biodiversity of beneficial and pathogenic interaction that take place in the rhizosphere, which can predict the effect on the crop production. In this study the determination of several PGPRs, like *Bacillus*, *Paenibacillus*, *Stenotrophomonas* and *Sphingomonas* can be used as inoculants for *Sorghum*, while both strains of *Rhizobium tropici* and *Rhizobium etli* can be used for phaseolus beans inoculation. The lack of response for some Cuban bean trials might be due to the co-habitat of several *Rhizobium* strains and also *Agrobacterium* in nodules of common bean. The influence of *Agrobacterium* in the symbiosis, nodule performance and N fixation, and also the phenotypic characterization of *Rhizobium* strains are the source for further studies.

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