JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2015. Vol. **9**(3), p. 2017-2024

Potential of Rhizobium Strains Isolated from *Agave americana* L. as Plant Growth Promoting Rhizobacteria

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(Received: 05 May 2015; accepted: 06 July 2015)

The use of indigenous Plant Growth-Promoting Rhizobacteria (PGPR) as a biofertilizer is an appealing alternative to enhance the growth and increase the yield of crops grown in nutrient-limited soils. The objective of this study was to determine the efficiency of four rhizobia strains isolated from the rhizosphere of *Agave americana* L. as PGPR. The characteristics of the isolated strains ACO-R05, ACO-R19, ACO-R35, and ACO-R62 were determined using several morphological, physiological, and biochemical tests. The taxonomic status of the strains was done by means of a phylogenetic study based on 16S rDNA chromosomal genes. The efficiency of rhizobia strains to solubilize P, auxins (IAA), and gibberellins (GA), as well as to biosynthesize and fix N_2 was determined. The PGPR potential of the strains was determined through inoculation tests using *Agave americana* as a plant trap. According to the phenotypic characteristics and the phylogenetics based on 16S rDNA sequences, these strains were grouped within the genus *Rhizobium*. All evaluated *Rhizobium* strains were able to solubilize phosphate, synthesize indole-3-acetic acid (IAA) and gibberellins (GA). The inoculation using the rhizobacterias strains had a significant effect ($P<0.05$) on the growth of *A. americana*. Biofertilization using native PGPR-*Rhizobium* proved to be a practical, simple, and efficient alternative to promote growth in this agave species.

Key words: Agave, Biofertilization, PGPR, *Rhizobium*.

Plants establish a relationship with a variety of soil microorganisms in order to obtain essential nutrients (Hayat *et al*., 2010). Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium and Azorhizobium* are known for their capacity to fix atmospheric nitrogen in a symbiotic connection with the roots of leguminous plants. However, Rhizobia also have the ability to form non-specific associative interactions with roots of other plants without forming nodules. It has been suggested that rhizobial strains may be able to produce plant growth regulators, and some of them have already been considered as plant growth promoting rhizobacteria (PGPR) (Galleguillos *et al.*, 2000). Numerous rhizobial species have been isolated from various plants and environmental samples. For example, *Rhizobium cellulosilyticum* was isolated from the sawdust of *Populus alba* L. (García-Fraile *et al*., 2007). *Rhizobium oryzae* was obtained from surface-sterilized roots of wild rice *Oryza alta* L. (Peng *et al.*, 2008) and *R. daejeonense* was isolated from a cyanide treatment bioreactor (Zhe-Xue *et al.*, 2005). These bacteria can establish colonies in plant roots and cause beneficial effects on plant

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development, and are referred to as plant-growth-promoting rhizobacterias (PGPR). Some of these PGPR are diazotrophic bacteria and have the ability to develop root associations with different plants (such as grasses), an ability which may therefore be significant for their use as biofertilizers (Santi *et al*., 2013).

The agave genus includes several species of economical, social, and cultural importance for people around the world. Various Agave species have even been introduced to different international regions such as Australia, Brazil, Tanzania, Kenya, Madagascar, Mexico, China, and across the Caribbean and Mediterranean regions, where it is grown to obtain various products of great importance in the food and pharmaceutical industries (García-Mendoza, 2007; Ahumada-Santos *et al*., 2013, Yang *et al.*, 2015).

For instance, *Agave salmiana* is grown in the Eastern Cape province of South Africa for the production of fibers and fructans (Smith and Figueiredo, 2012). Agave plants are of course of great relevance to Mexico, since this country is considered to be the point of origin for the evolution and diversification of the genus (García-Mendoza, 2002). Approximately 163 species occur in Mexico, 123 of these being endemic to this country (Delgado-Lemus *et al*., 2014). One of these, *Agave americana* L. (Agavaceae), is an agave species found in the highlands of Chiapas, Mexico, where it is an important source of natural fiber, medicine, and fructans, as well as being used for the production of alcoholic beverages (García-Mendoza, 2002). Local farmers have established plantations of *A. americana* to obtain sufficient raw materials for agro-industrial use. However, low water availability and nutrient content (especially phosphorus) in the soil limits growth, and consequently, plants only become mature after 5 to 7 years. An alternative already being used in other crops is to apply plant growth-promoting rhizobacteria (PGPR); however, it is necessary to assess the possible effects in *A. americana* specifically, in order to improve chances for the survival and growth of plantlets. PGPR are the rhizosphere bacteria which have the capacity to enhance plant growth by means of a wide variety of mechanisms, such as phosphate solubilization, siderophore production, biological nitrogen fixation, phytohormone production, antifungal activity, induction of systemic resistance, promotion of beneficial plant-microbe symbioses, and more (Saharan and Nehra, 2011; Bhattacharyya and Jha, 2012).

As part of a project to promote sustainable agriculture and organic farming in Chiapas, the objective of this study was to determine the phenotypic and genotypic characteristics of four *Rhizobium* strains isolated from *A. americana*, and to evaluate their symbiotic potential as plant-growth-promoting rhizobacterias (PGPR)

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains ACO-R05, ACO-R19, ACO-R35, and ACO-R62 were selected from 235 strains, which were isolated previously from the rhizosphere of *Agave americana*. They were pre-selected for their potential as biofertilizers and were provided by the '*Instituto Tecnologico de Tuxtla Gutiérrez*'. All strains were grown in yeast extract-mannitol (YEM) medium at 28°C and then conserved at 4°C until used (Vincent, 1970).

Phenotypic Characteristics of Strains

The cell morphology of the strains was analyzed using an optic microscope (Zeiss® PS7, Germany). The Gram reaction was done using a stain kit (Merck®, Germany) according to the manufacturer's procedure and colony morphology was determined using cells grown on YEM agar plates at 28ºC for 5 days (Toledo *et al.*, 2003).

Bacterial growth was determined in a YEM medium with 0.5, 1.0, 2.0, 3.0 or 5.0% (*w*/*v*) NaCl and at pH 4.0, 5.0, 9.0 and 11.0 at 28ºC. Tolerance to high temperatures was also tested at 37ºC and 44ºC. The acid/alkaline reaction was verified by spreading the inoculum on YEM plates (pH 7.0) containing 25 mg mL^{-1} bromothymol blue (Vincent, 1970). The resistance to antibiotics was tested on YEM agar plates as recommended by Martínez-Romero *et al*. (1991). Following the same procedures, tolerance to Al^{3+} and Cu^{2+} was tested on a solid YEM medium. $AlCl_3.6H_2O$, 500 µg mL^{-1} and $CuCl_2.2H_2O$, 100 µg mL^{-1} solutions were filtered, sterilized, and added to the sterilized YEM medium. The ACO-R05, ACO-R19, ACO-R35, and ACO-R62 strains were then added to the medium and incubated at 28ºC for 5 days.

16S rDNA Gene Sequencing and Phylogenetic Analysis

The strains were grown in 2.0 ml YEM medium overnight. Total genomic DNA was extracted using the DNA isolation kit for cells and tissues (Roche®, Basel**,** Switzerland), according to the manufacturer specifications. PCR was done with the universal bacterial 16S rRNA primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'), which amplify products of approximately 1500 bases. Procedures were done as described by Weisburg *et al*. (1991). The PCR products were purified using the PCR product purification system kit of Roche® (Basel**,** Switzerland) and sequenced at Macrogen® (Seoul, Korea). Partial sequences obtained were matched against nucleotide sequences present in GenBank using the BLASTn program. The phylogenetic tree with the 16S rDNA gene sequences from the type strains was constructed by neighbour-joining (Saitou and Nei, 1987) and through a bootstrap analysis with 1000 replicates with the Tamura-Nei model (Tamura and Nei, 1993).

Measurement of Rhizobium Strains Efficiency

Phosphate solubilizing capacity

The P solubilizing capacity of the strains isolated from the *Agave americana* and of the reference strains was tested by plate assay using Pikovskaya medium (PVK) supplemented with 1.5% agar (Nautiyal, 1999). The strains were stabbed in triplicate using sterile toothpicks. The halo and colony diameters were measured after incubating the plates at 28°C for 5 days.

Indole-3-acetic acid (IAA) production by the *Rhizobium* strains

The colorimetric Salkowski's method was used to determine the amounts of IAA produced by each *Rhizobium* strain (Ehmann, 1977). Each strain ($3´10^9$ CFU mL^{-1}) was grown in triplicate in 100 mL conical flasks containing 10 mL YEM broth, supplemented with L-tryptophan (100 mg mL^{-1}) at 28±2 °C in the dark, and shaken for 24 h. The culture medium after growth was centrifuged and the cell-free supernatant was used for IAA extractions. The extracted IAA was estimated colorimetrically at 530 nm using a standard curve with IAA obtained from Sigma Chemical (St. Louis, MO, USA).

Determination of gibberellins produced by the *Rhizobium* Strains

The method described by Holbrook *et al.* (1961) was used with minor modifications in order to determine gibberellins. All strains ($3´10^9$ CFU mL^{-1}) were grown in 25 mL of nutrient broth in triplicate at 28°C in the dark, and shaken for 24 h. Culture supernatants were recovered after centrifugation. 2 mL of zinc-acetate reagent was added to 15 mL supernatant. After 2 min, 2 mL 10.6% potassium ferrocyanide was added and the solution was centrifuged at 2000 rpm for 15 min. A 5 mL aliquot of the supernatant was added to 30% HCl, mixed, and then incubated in a dark room at 20°C for 75 min. 5 ml 5% HCl served as control and absorbance was read at 254 nm. Concentration of GA was calculated by preparing a standard curve using Gibberellic acid (GA_3, Hi-Media) as standard (100-1000 µg mL^{-1}). The experiment was done in triplicates.

Biofertilizer Test

The efficiency of PGPR strains ACO-R05, ACO-R19, ACO-R35, and ACO-R62 isolated from *A. americana* rhizosphere was evaluated in a biofertilization test. *A. americana* plantlets, previously obtained through micropropagation, were planted in polystyrene trays which contained peat-moss as substrate, and covered with a polyethylene sheet to maintain humidity and prevent dehydration. The plantlets were placed in the growth chamber at 25°C to achieve acclimatization. After two months (60 days after transplantation, DAT), the plants were transferred to pots containing peat-moss with the Fahraeus medium as nutrient (Fahraeus, 1957) and placed in the greenhouse. The plants were inoculated with each of the PGPR strains, with 2 mL of the bacterial suspension at a concentration of $1x10^6$cel mL^{-1}. Plants without inoculum, served as control. Four replicas were used per treatment, and these were arranged in a completely randomized design. The plants were grown under greenhouse conditions for 90 days. Measurements of the plants´ fresh weight, the diameter of the stem, the number of leaves, and the length of the roots were carried out on the plants during the transplantation phase (m_1) and after 90 days (m_2) at the greenhouse. The data used for statistical analysis were the difference between m_2-m_1 measurements.

Statistical Analysis

The effect of the four *Rhizobium* strains on inoculation, P-solubilizing, GA, and IAA production was determined by analysis of variance

(ANOVA) and comparison of means with the Tukey test (P<0.05) (SAS Institute, 1989).

RESULTS AND DISCUSSION

Characteristics of the Strains

The morphological and physiological characteristics of the bacterial strains isolated from the *Agave americana* rhizosphere with the potential to be use as biofertilizers are shown in Table 1. The strains ACO-R05, ACO-R19, ACO-R35, and ACO-R62 present the typical phenotypic characteristics of the *Rhizobium* genus. Cells are motile and rod-shaped, gram-negative, strictly aerobic, and of rapid growth (24-48 h). The optimum growth temperature was of 28±2º C, and they presented an acidic reaction. In the YEM medium, the strains ACO-R05 and ACO-R35 formed circular colonies, a cream-white in colour, between 1.5 and 3.0 mm in diameter and of a glossy appearance. The strains ACO-R19 and ACO-R62 formed circular colonies of a cream-white colour, with regular borders and between approximately 1.0 and 1.5 mm in diameter and of a mucoid appearance.

The four strains can grow at 37°C but not at 44°C. The strains ACO-R19 and ACO-R62 can grow within a wide pH range between 4.0 and 11.0, in contrast to the strains ACO-R05 and ACO-R35, which only grow between a pH of 5.0 and 9.0. As for the capacity to tolerate NaCl, the strains ACO-R05 and ACO-R35 have the ability to grow within a NaCl range of 0.5 to 3.0, with the exception of the strain ACO-R19 and ACO-R62, which tolerates up to 2.0% salt. The same behavior is observed in the reference strains *Rhizobium radiobacter* NCPPB 2437 (Sawada *et al.,* 1993) and *R. radiobacter* NF20A (Verástegui-Valdés *et al.,* 2014). However, the strain ACO-R05 and ACO-R35 were most resistant to the tested antibiotics in comparison to the other evaluated strains, and at the same time proved to be sensitive to the Amikacin (10 µg ml^{-1}) and Netilmicin (20 µg ml^{-1}). It was also determined that all strains have the capacity to grow in the presence of Al^{+3} (500 µg mL^{-1}) and of Cu^{+2} (100 µg mL^{-1}).

The taxonomic position of the bacterial strains was determined according to the phylogenetic analysis performed with partial sequences of the chromosomal gene 16S rDNA (Figure 1). The 16S rDNA gene sequences of strains ACO-R05, ACO-R19, ACO-R35, and ACO-R62 have been deposited in the GenBank database under accession numbers KR232945, KR232948, KR232946, and KR232947 respectively. The four strains were affiliated with the genus *Rhizobium*. The ACO-R05, ACO-R19, ACO-R35, and ACO-R62 strains showed a 98.6% genetic similarity with *Rhizobium radiobacter* (Sawada *et al.,* 1993). Considering the relationship with *R. radiobacter* (previously considered *Agrobacterium radiobacter*), an additional experiment was performed to determine the pathogenicity of the *Rhizobium* strains on sunflower plants (*Helianthus annus*) and found that these strains are not tumorogenic. Also, the *nif*H gene was amplified for all isolates with primer pair *nif*HF/*nif*H1 and the protocol described by Laguerre *et al.,* 2001. Only, the ACO-R05 and ACO-R35 strain could amplify the gene *nif*H (Table 1). Similar results were reported by Verástegui-Valdés *et al*. (2014),

Efficiency of the *Rhizobium* Strain

The abilities for phosphate solubilization by plant-associated bacterial species have been the object of various research projects (Alikhani *et al*., 2006; Chen *et al*., 2006). Nevertheless, a very limited number of studies have reported strains of *Rhizobium* as P-solubilizing plant-associated bacteria (Sridevi and Mallaiah, 2009). In the current study, results showed that the solubilization of inorganic phosphate by the four *Rhizobium* strains was accompanied by a significant drop in pH after 72 h (Table 2). The maximum P-solubilization was recorded by the strain ACO-R35 (9.2 mm) with a maximum drop in the pH to 4.3, followed by the ACO-R62 (8.0 mm), ACO-R05 (6.3 mm), and ACO-R19 (5.7 mm) with a maximum drop in the pH to 5.3, 5.5, and 5.8, respectively. The P-solubilizing activity is determined by the microbial biochemical ability to produce and release organic acids, which through their carboxylic groups chelate the cations (mainly Ca) bound to phosphate converting them into the soluble forms (Chen *et al.,* 2006).

On the other hand, the biosynthesis of phytohormones by the *Rhizobium* strains used in the current study showed that all differed in their capacity to produce IAA and GA (Table 2). After 72 h of growth, the IAA production was significantly higher (P<0.05) in ACO-R35 (17.6 mg mL^{-1}) as compared with the others strains, which did not show significant changes in IAA

concentrations according to the Tukey test ($P< 0.05$). The production of gibberelins by *Rhizobium* strains showed that ACO-R35 produced a significantly higher amount of GA (4.5 mg L^{-1}), followed by ACO-R05 (3.3 mg L^{-1}), ACO-19 (2.9 mg L^{-1}), and ACO-R62 (2.7 mg L^{-1}). The production of IAA and gibberellins has been confirmed in numerous rhizobia species (Bottini *et al*., 2004; Boiero *et al*., 2007). Therefore, these rhizobial strains can be used as biofertilizer and improve the growth of *A. americana*.

Plant growth of *A. americana* inoculated with *Rhizobium* strains

The inoculation with *Rhizobium* strains had a positive effect on the growth parameters of *A. americana* plants (Table 3). The ACO-R05, ACO-

Table 1. Characteristics that distinguish Rhizobium strain Isolated from *Agave americana L* from Phylogenetically Closely Related Taxa

Characteristics	*Rhizobium* isolated from *A. americana*				Reference Strains	
	1	2	3	4	5	6
Gram stain	-	-	-	-	-	-
Colony aspect on YEM medium:	Cream-white Glossy	Cream-white Mucoid	Cream-white Glossy	Cream-white Mucoid	Cream-white Mucoid	Cream-white Mucoid
Growth on YEM agar at:						
37ºC	+	+	+	+	+	+
44 ºC	-	-	-	-	-	-
Tolerance to NaCl:						
0.5 %	+	+	+	+	+	+
1.0 %	+	+	+	+	+	+
2.0 %	+	+	+	+	+	+
3.0 %	+	-	+	-	-	-
5.0 %	-	-	-	-	-	-
Growth in YEM medium to different pH at 28º C:						
4.0	-	+	-	+	+	+
5.0	+	+	+	+	+	+
7.0	+	+	+	+	+	+
9.0	+	+	+	+	+	+
11.0	-	+	-	+	+	+
Acid production in YEM (25 µg L^{-1} bromothymol blue)	+	+	+	+	+	+
Tolerance to antibiotics (ug mL^{-1}):						
Ampicillin (100)	+	+	+	+	+	+
Amikacin (10)	-	-	-	-	-	-
Carbencillin (20)	+	+	+	-	-	-
Chloramphenicol (100)	+	+	+	+	+	+
Gentamicin (10)	+	-	+	-	-	-
Kanamycin (100)	+	+	+	+	+	+
Netilmicin (20)	-	-	-	-	-	-
Tolerance to heavy metals (ug mL^{-1}):						
Al^{+3} (500)	+	+	+	+	+	+
Cu^{+2} (100)	+	+	+	+	+	+
Pathogenicity test on: sunflower plants (*Helianthus annus*)	-	-	-	-	+	-
nifH[b]	+	-	+	-	-	+

[b]nifH gene was detected by PCR after isolation.

Taxon 1, ACO-R05; 2, ACO-R19; 3, ACO-R35; 4, ACO-R62; 5, *Rhizobium radiobacter* NCPPB 2437 (Sawada *et al*., 1993) and 6, *Rhizobium radiobacter* NF20A (Verástegui-Valdés *et al*., 2014). +, Positive; -, Negative'.

R19, ACO-R35, and ACO-R62 strains had a positive effect on plant fresh weight, stem diameter, number of plant, as compared to the uninoculated control plants. Plants inoculated with ACO-R35 were on average weighted at 2.34 g more than uninoculated plants at 90 days post inoculation. The stem diameter obtained with ACO-R35 differed significantly (P<0.05) from that obtained with the rest of the treatments. Plants treated with *Rhizobium* strains ACO-R35, ACO-R19, and ACO-R62 experiences similar effects as to number of leaves, compared to uninoculated plants according to the Tukey test (P<0.05). The plants inoculated with ACO-R35 showed significantly higher root length as compared to other treatments (P<0.05). As for *Rhizobium´*s potential as a PGPR, an important quantity of rhizobial species isolated from various types of plants and environmental samples have been reported to date. For example, *Rhizobium cellulosilyticum* was isolated from the sawdust of *Populus alba* L. (García-Fraile *et al.*, 2007). *R. oryzae* was obtained from surface-sterilized roots of wild rice *Oryza alta* L. (Peng *et al.*, 2008), and *R. daejeonense* was isolated from a cyanide treatment bioreactor (Zhe-Xue *et al.*, 2005). These *Rhizobium* species were isolated from non-leguminous plants or other natural sources, and have shown high potential for nitrogen fixation, as well as presenting other desirable biological features for their use as biofertilizers. Similar results have been reported by Santos *et al.* (2014), which concern the occurrence and diversity of diazotrophic bacteria in rhizosphere soil and root and leaf tissues of *Agave sisalana* plants, as well as to test for their potential for plant growth promotion. Therefore, the PGPR strains from this study could constitute an alternative as an *A. americana* biofertilizer, in order to improve its growth and development.

Table 2. Phosphate Solubilizing, Production IAA and GA (Gibberellic acid) by PGPR- *Rhizobium* Strains Isolated from *Agave americana* L.

Strain	P-solubilizingAgar (halo size (mm))	IAA (mg L^{-1})	GA (mg L^{-1})
ACO-R05	6.3 C[a]	9.1 B	3.3 B
ACO-R19	5.7 C	9.0 B	2.9 B
ACO-R35	9.2 A	17.6 A	4.5 A
ACO-R62	8.0 B	12.5 B	2.7 B
MSD[b] (P<0.05)	1.1810	4.6659	0.8435

[a] Mean values of three replicates. The means followed by the same letter are not significantly different (P<0.05).
[b] MSD: Minimum Significant Difference.

Table 3. Growth Parameters for *Agave americana* Plants Inoculated with Rhizobium Strains

Treatment	Plant fresh weight(g)	Stem diameter(cm)	Number of leaves	Root length (cm)
ACO-R05	1.15 C	0.30 B	1.25 B	10.18 B
ACO-R19	1.38 B	0.27 B	1.75 AB	13.0 AB
ACO-R35	2.85 A	0.77 A	2.25 A	15.62 A
ACO-R62	0.75 D	0.24 B	1.75 AB	10.60 AB
Uninoculated	0.51 E	0.21 B	1.00 B	10.05 B
MSD[¥] (P<0.05)	0.1434	0.2027	0.9799	5.1173

*Mean values of four replicates. Means followed by the same letter do not show a significant difference (P<0.05).
?MSD: Minimum significant difference.

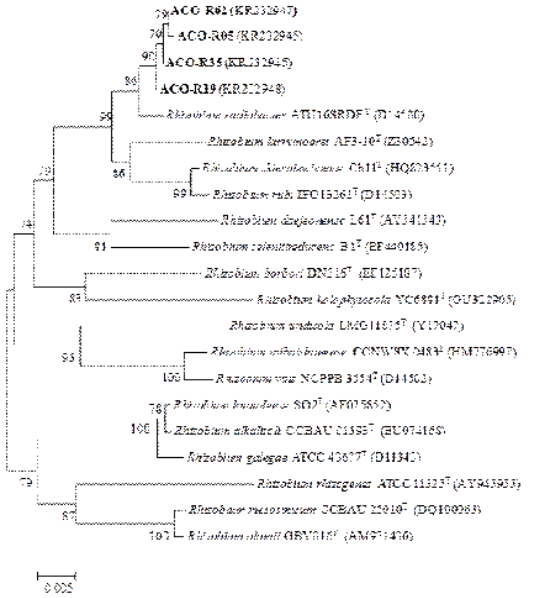

Fig. 1. Neighbour-joining phylogenetic trees based on 16S rDNA gene sequences of the *Rhizobium* strains isolated from *Agave americana*. Only bootstrap values > 50% are shown. Type strains are indicated by superscripts T. The accession numbers for the sequences are indicated within parentheses. Those generated in this study are shown in bold

CONCLUSION

Finally, the four strains evaluated in the present study, which were isolated from the rhizosphere of the *Agave americana*, proved to possess the ability to solubilize phosphate and biosynthesize IAA and GA, which suggests that these strains have a high potential to be used as growth promoters in this agave species

ACKNOWLEDGEMENTS

We thank the 'Coordinación de Posgrado en Ciencias Biologicas-UNAM'. The project was funded by '*Tecnológico Nacional de México*' (TNM, Mexico) and the 'Infraestructura 251805' project from the '*Consejo Nacional de Ciencia y Tecnologia'* (CONACyT, Mexico). We also thank Elisabeth Tremblay for reading this manuscript.

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