Biofertilizer potential of diazotrophic bacteria isolated from samples of rhizospheric soil

Laura Y. Moreno y F. Galvis
Grupo de Investigación Majumba, Universidad Francisco de Paula Santander, Cúcuta, Norte de Santander, Colombia
E-mail: lauramoreno_08@hotmail.com

ABSTRACT

Plant growth promoting bacteria are capable of adapting, colonizing and persisting in the plant rhizosphere, which favors plant growth and development. In this work 32 native strains of diazotrophic bacteria were isolated, from rhizospheric soil samples of different crops, identified through traditional soil cultures and the BBL CRYSTAL system. The biochemical identification was made through polymerase chain reaction (PCR). Nine isolates were determined as *Stenotrophomonas maltophilia* and one as *Azotobacter vinelandii*, of which three coincided in the biochemical and molecular identification. A greenhouse trial was conducted to evaluate the fertilizer effect on corn plants, according to a randomized block design and nine treatments, in triplicate: treatment 1: *A. vinelandii* ATCC 9046; treatments from 2 to 7 for the six isolates; treatment 8 for chemical fertilization; and treatment 9 for soil without fertilization (control). The isolates M8-10, M10-1 and M11-3, identified as *S. maltophilia* by PCR, had better results in plant emergence, stem diameter and leaf and stem length. Finally, M10-1 was determined to show plant emergence and growth values higher than the mean and upper limit, as compared with the other isolates and controls, which makes it a potential biofertilizer.

Key words: bacterium, nitrogen fixation

INTRODUCTION

The biological activity of soils has a preponderant role in the achievement of high-yield crops. Microorganisms in association with crops are important as inputs for production improvement and environmental control, in addition to allowing the maintenance of biodiversity and ecosystem sustainability (Carvajal and Mera, 2010).

The free living or symbiotic bacteria which inhabit the rhizosphere may stimulate plant growth through different processes, such as the synthesis of plant growth regulators, nitrogen fixation, nutrient solubilization, production of siderophores and control of soil phytopathogens (Torriente, 2010). The most widely studied microorganisms belong to the genera *Azotobacter*, *Stenotrophomonas*, *Azospirillum*, *Klebsiella*, *Beijerinckia*, *Pseudomonas* and *Bacillus*, among others; they are called plant growth promoting rhizobacteria (PGPR). Some PGPR transform atmospheric nitrogen into ammonium (process known as biological nitrogen fixation), for it to be incorporated to the biosphere, which represents an economic benefit and reduces the negative impact on the environment, due to the exaggerated use of chemical inputs in agricultural production (Bruinsma, 2003).

The isolation and identification of native microorganisms with properties that promote plant growth and show higher permanence in the field, such as *Azotobacter vinelandii*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* is required, to evaluate the biofertilizer potential in crops of agronomic interest. For the identification of the diazotrophic bacteria conventional techniques or techniques based on their biochemical properties are used, such as: description of their cell morphology, Gram coloring, analysis of their capacity to grow under aerobic or anaerobic conditions, or the requirement analysis of special substrata for their cultivation; these techniques allow their identification to genus level. For the characterization of species, subspecies, serovars or strains, more sensitive and specific techniques are necessary, such as polymerase chain reaction (PCR), which identifies a unique sequence in the DNA.

The objective of the work was to identify, through BBL Crystal™ Enteric/Nonfermenter ID Kit and PCR, diazotrophic microorganisms isolated from samples of rhizospheric soil in crops of agronomic interest, as well to test their biofertilizer effect on corn plants.

MATERIALS AND METHODS

Collection and characterization of soil samples. The samples were taken from rhizospheric soils of 16 different sites, with pasture, coffee, sugarcane, corn and fruit crops; they are located in the Chinácota municipality, North of Santander, Colombia. For the collection, transportation and conservation of the samples, the methodologies proposed by Cline (1944) and Sosa (2002) were used. A phytochemical analysis in the laboratory of agricultural soils of the University Francisco de Paula, in Santander –Cúcuta, Colombia- (Olarte *et al*., 1979), and a dry matter analysis were conducted; pH, phosphorus, potassium, calcium and magnesium were determined.

Isolation through selective methods. The isolation of diazotrophic bacteria was made through the Winogradsky and Agar Ashby media. Afterwards, the macroscopic and microscopic characteristics of the pure isolates were determined. For isolate classification the following nomenclature was used: isolate M3-1, where: M corresponds to the soil sample; 3 to the sample number; and 1 to the number of pure strains obtained.

Biochemical characterization by means of BBL CRYSTAL. The biochemical identification was made through BBL Crystal™ Enteric/Nonfermenter ID Kit. The identification system includes tests for fermentation, oxidation, degradation and hydrolysis of different substrata.

Molecular identification. For the isolation of the DNA of the isolates and the control *A. vinelandii* ATCC 9046, the UltraClean® Microbial DNA Isolation Kit of MoBio was used. In the molecular identification the specific primers nifH-g1-for GGTTGTGACCCGAAAGCTGA and nifH-g1-rev GCGTACATGGCCATCATCTC were used, for *A. vinelandii* (Bürgmann, Wilmer, Sigler and Zeyer, 2003; Levy-Booth and Winder, 2010); SM1-for CAGCCTGCGAAAAGTA and SM4-rev TTAAGCTTGCCACGAACAG, for *S. maltophilia* (Whitby *et al.,* 2000; Kisková *et al*., 2012); and G1c-for GCCATGGATACTCCAAAAGGA and G2c-rev TCGGAATCCTGCTGAGAGGC, para *B. cepacia* (Whitby *et al.,* 2000; Rashamuse, Burton and Cowan, 2007). The DNA samples were amplified in a final volume of 50 µl, which contained 1X buffer for PCR; 2,5 mM $MgCl_2$, 200 mM dNTP 1 U of Taq polymerase (Bioline), 1 µM of each primer and 2 µl of DNA. The amplification conditions consisted in an initial denaturalization at 95ºC for five minutes, followed by 30 cycles of 95ºC for one minute, 60ºC for 30 seconds and 72ºC for one minute; and a final extension to 72ºC for 7 minutes. The PCR products were visualized in agarose gels at 1 % and dyed with ethidium bromide.

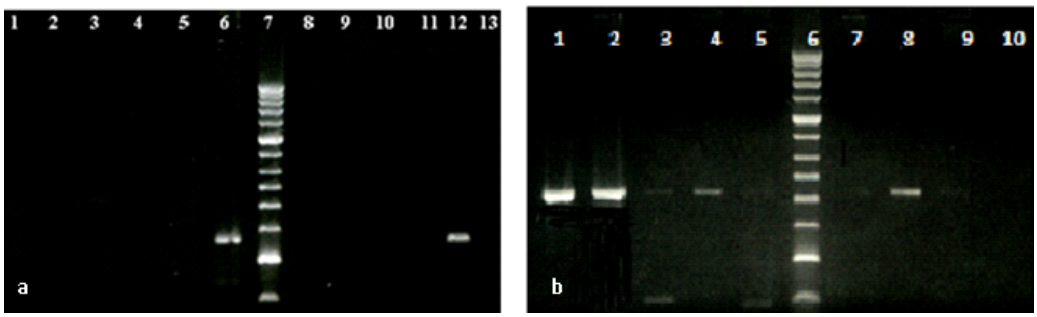
Biofertilizer effect. A trial was conducted in greenhouse with corn plants to evaluate their emergence percentage, stem diameter and leaf and stem length; the effect of six isolates identified by PCR (M-1, M8-4, M8-9, M8-10, M10-1 and M11-3) was compared with that of chemical fertilization and a control. The treatments were inoculated in 80 mL of LB liquid medium (10 g of tryptone, 10 g of NaCl and 5 g of yeast extract/L), with constant agitation at room temperature for 24 hours, until reaching a minimum concentration of 10^8 cells/mL (Macfarland scale). Seed inoculation was made according to the suggestion by Utria-Borges *et al*. (2008). In the essay a randomized block design and nine treatments were used, in triplicate: treatment 1: *A. vinelandii* ATCC 9046; treatments from 2 to 7 for the six isolates; treatment 8 for chemical fertilization; and treatment 9 for soil without fertilization (control). The mean comparisons among means were made according to Duncan (1955) and an ANOVA statistics with the program SPSS 15.0 was used.

RESULTS AND DISCUSSION

Identification with the use of selective media and biochemical tests. The analysis of rhizospheric soil samples showed between moderately acid (5,1) and moderately alkaline (7,8) pH; averages of: organic matter 7,12 %; phosphorus 262,5 ppm; potassium 0,58 meq/100 g; calcium 17,6 meq/100 g and magnesium 2,1 meq/100 g; and loamy and loamy-sandy texture. These results are within the ranges that favor the permanence and activity of diazotrophic bacteria. No correlation was observed between the soil physical-chemical characteristics and the quantity of isolates obtained from each sample.

In selective media 32 pure isolates were obtained, which were in correspondence with nitrogen fixing bacteria. The analysis with the BBL CRYSTAL system allowed to identify *Klebsiella pneumoniae* subsp. *ozaenae*, *Klebsiella oxytoca* or *Serratia rubidea*, *K. oxytoca* or *K. pneumoniae*, *K. oxytoca*, *S. maltophilia*, *Aeromonas hydrophyla* or *Enterobacter sazakii*, *E. cloacae* or *E. sakazakii*, *E. cloacae*, *B. cepacia*, *B. cepacia* or *Pseudomonas fluorescens* and *Serratia fonticula*.

Molecular identification. In the molecular analysis of *A. vinelandii* through PCR, using primers nifH-g1-for and niH-g1-rev (fig. 1a), a band of 370 pb was observed in isolate M8-4, which is an adequate



a. Primers nifH-g1-forB and nifH-g1-rev for the identification of *A. vinelandii*. Lines 1-6: M6-6, M7, M8-1, M8-2, M8-3, M8-4. Lines: 8-11: M8-5, M8-6, M8-7, M8-8. Line 12: control *A. vinelandii* ATCC9046. Line 13: negative control. Line 7**:** Molecular weight marker *Hyperladder II* of Bioline. In lines 6 and 12 a band of approximately 370 pb was observed. **b.** Primers SM1-SM4, for the identification of *S. maltophilia*. Lines 1-7: M8-9, M8-10, M9-1, M10-1, M10-2. Lines 7-9: M11-2, M11-3, M12, M8-8. Line 10: negative control. Line 6**:** Molecular weight marker *Hyperladder II* of Bioline. A band of approximately 531 pb is observed in lines 1, 2, 4 and 8

Figure 1. Identification, through PCR, of *A. vinelandii* and *S. maltophilia.*

amplification according to Bürgmann *et al*. (2003) and Levy-Booth and Winder (2010). In the identification of *S. maltophilia*, primers SM1-for and SM4-rev recognized the isolations M1, M2, M4-1, M6-3, M8-3, M8-9, M8-10, M10-1 and M11-3 (fig. 1b), and amplified a band of 531 pb, which is in correspondence with the report by Whitby *et al*. (2000) and Kisková *et al*. (2012) (fig. 1b). The amplification using primers G1c-for and G2c-rev for identifying *B. cepacia* did not show results, which was possibly due to the fact that none of the molecularly analyzed isolates corresponded to it. The analysis of primers G1c-for and G2c-rev –conducted in BLAST- showed high specificity for the genus *Burkholderia* because it recognized the species *B. cepacia*, *B. cenocepacia* and *B. ambifaria*.

When comparing the PCR results with those of BBL CRYSTAL, isolate M8-4 was found to correspond to *A. vinelandii* by means of PCR; while with biochemistry, to *S. maltophilia*. Isolates M2, M8-9 and M8-10 were identified through PCR and BBL CRYSTAL as *S. maltophilia*.

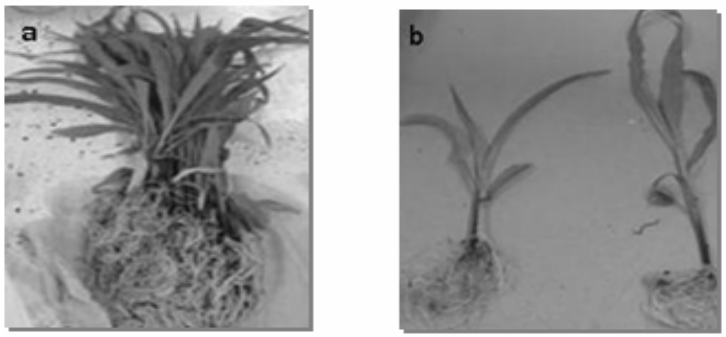
Through biochemistry isolates M4-1, M6-3 and M11-3 were identified as *B. cepacia*; M10-1 as *Klebsiella* sp.; M-1 as *K. pneumoniae* and M8-3 as *E. cloacae*; and by PCR they were identified as *S. maltophilia*. Only three isolates coincided in the molecular identification and biochemistry, probably because the latter is not a sufficiently specific tool for this purpose; while the molecular techniques allow the characterization of microorganisms to achieve their determination at interspecific or intraspecific level (Bou *et al*., 2011). The individuals of the same species not necessarily have the same genetic, biochemical and toxicological attributes. On the other hand, different species may show high genetic, biochemical and toxicological similarity. The classification through selective media and biochemical tests facilitates the identification to genus and, sometimes, to species level. However, the molecular techniques allow to characterize subspecies, serovars and strains in a simpler, fast and reproducible way in a large variety of microorganisms (Godoy *et al*., 2008).

Biofertilizer effect. The treatments which showed the best results in plant emergence, stem diameter, and leaf and stem length were M8-10, M10-1 and M11-3, identified as *S. maltophilia* by PCR (table 1). They had significant differences with regards to the control and the chemical fertilization; besides, higher efficiency was observed in corn plant emergence and growth. The treatment with M10-1 showed higher efficiency in plant development, which promoted fast emergence and higher stem diameter and root and stem length, as well as higher dry weight (fig. 2). According to the statistical analysis of plant emergence and growth, M10-1 showed values over the mean and upper limit, as compared with the other isolates and controls (table 1). It is important to emphasize that the other isolates had adequate results, without significant differences with regards to M10-1, for which they become potential biofertilizers.

The beneficial effect of rhizobacteria lies on different mechanisms, such as: production of growth promoting substances, siderophores and antibiotics; as well as resistance induction in the plant and nitrogen fixation (Torriente, 2010). For such reason the isolation of diazotrophic bacteria, their identification through

Table 1. Biofertilizer effect of diazotrophic isolates on corn plants.

Treatment	Emergence (%)			Stem diameter (cm)	Leaf length (cm)	Stem length (cm)	Root length (cm)	Dry weight (g)
	4 days	6 days	9 days					
Azotobacter	70	97	100	0,32	0,31	9,70	18,20	14,50
M1	72	93	100	0,35	0,34	10,40	19,60	12,20
M8-4	69	93	97	0,31	0,36	10,10	19,00	12,00
M8-9	77	96	100	0,33	0,37	9,80	18,40	15,00
M8-10	83	100	100	0,38	0,39	10,20	19,20	16,90
M10-1	88	100	100	0,50	0,42	12,70	24,00	24,00
M11-3	83	97	100	0,40	0,38	11,20	21,00	15,30
Chemical fertilization	23	60	79	0,40	0,34	8,50	16,00	13,90
Control	57	78	90	0,23	0,28	7,50	14,10	11,00
Statistical analysis								
Mean	69,11	90,44	96,22	0,36	0,35	10,01	18,83	14,98
Deviation	19,64	13,20	7,25	0,08	0,04	1,48	2,81	3,86
Lower limit	54,01	80,29	90,64	0,30	0,32	8,87	16,67	12,01
Upper limit	84,21	100,59	101,80	0,41	0,39	11,15	20,99	17,94



a. Isolate M10-1, evaluation of root growth at 20 days. **b.** Comparison of stem size with the treatment without inoculation and with the inoculated treatment with isolate M10-1, at 20 days.

Figure 2. Biofertilizer effect on corn plants.

reliable methods and the evaluation of their capacity as plant growth promoters, in addition to being an option in research processes with agricultural purposes, are a good alternative to improve crop nutrition and quality, which contributes to the improvement of the plant-soil-microorganism system.

It is concluded that the results obtained through conventional techniques are usually little reliable and cause errors at the moment of defining genus and species of a soil microorganism. In addition, the essay applied for evaluating the capacity of some isolates with regards to the promotion of plant growth allowed to prove their potential as biofertilizers; this will be tested in a study at greenhouse level, with a non-sterile soil, to compare it with native microorganisms and controlled conditions, with possibilities of being applied in field on crops of agronomic interest.

BIBLIOGRAPHIC REFERENCES

Bou, G. *et al*. 2011. Métodos de identificación bacteriana en el laboratorio de microbiología. *Enferm. Infecc. Microbiol. Clin*. 29 (8):601.

Bruinsma, Jelle (Ed.). 2003. World agriculture: Towards 2015/2030 an FAO perspective. FAO, Rome. 432 p .

Bürgmann, H.; Widmer, F.; Sigler, W.V. & Zeyer, J. 2003. mRNA extraction and reverse transcription-PCR Protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in Soil. *Appl. Environm. Microbiol*. 69 (4):1928.

Carvajal, J. & Mera, A.C. 2010. Fertilización biológica: técnicas de vanguardia para el desarrollo agrícola sostenible. *Producción + Limpia*. 5 (2):77.

Cline, M.G. 1944. Principles of soil sampling. *Soil Science*. 58:275.

Godoy, M. *et al*. 2008. Identificación de micobacterias no tuberculosas: comparación de métodos bioquímicos y moleculares. *Revista de la Sociedad Venezolana de Microbiología*. 28:96.

Kisková, J. *et al*. 2012. Bacterial prevalence in the Dunnock (*Prunella modularis*) in sub-alpine habitats of the Western Carpathians, Slovak Republic. *Ornis Fennica*. 89 (1):34.

Levy-Booth, D.J. & Winder, R.S. 2010. Quantification of nitrogen reductase and nitrite reductase Genes in soil of thinned and clear-cut douglas-fir stands by using real-time PCR. *Appl. Environm. Microbiol*. 76 (21):7116.

Olarte, R.L. *et al*. 1979. Métodos analíticos del laboratorio de suelos. 4a. ed. Instituto Geográfico Agustín Codazzi, Bogotá. 613 p .

Rashamuse, K.J.; Burton, S.G. & Cowan, D.A. 2007. A novel recombinant ethyl ferulate esterase from *Burkholderia multivorans*. *J. Appl. Microbiol.* 103:1610.

Sosa, A. 2002. Muestreo de suelos. Instituto Nacional de Tecnología Agropecuaria, Buenos Aires.

Torriente, D. 2010. Aplicación de bacterias promotoras del crecimiento vegetal en el cultivo de la caña de azúcar. Perspectivas de su uso en Cuba. *Cultivos Tropicales*. 31 (1):19.

Utria-Borges, E. *et al*. 2008. Utilización agraria de los biosólidos y su influencia en el crecimiento de plántulas de tomate (*Lycopersicon esculentum* Mill.). *Revista Chapingo. Serie horticultura*. 14 (1):33.

Whitby, P. *et al*. 2000. Identification and detection of *Stenotrophomonas maltophilia* by rRNA- directed PCR. *J. Clin. Microbiol*. 38 (12):4305.

Received: February 10, 2012
Accepted: December 22, 2012

Reseñas de Publicaciones

VOCES, FINCAS, INNOVACIÓN

COLECTIVO DE AUTORES

EDITORIAL FEIJOÓ

UNIVERSIDAD CENTRAL "MARTA ABREU" DE LAS VILLAS

2011

El libro titulado *Voces, Fincas, Innovación* recopila 41 historietas sobre la pasión por el cambio que ha generado el Programa de Innovación Agropecuaria Local (PIAL) en Cuba, como reflejo directo y personal de experiencias vividas por campesinas y campesinos, investigadoras e investigadores u otros técnicos del sector agropecuario y de fuera de él. Describe el entusiasmo y el reto de aquellos que se enrumbaron por senderos nuevos, desconocidos y prometedores.

Este libro le da voz a la gente que le aporta vida y sentido al PIAL, con su capacidad innovadora y de experimentación desde el aprendizaje en la acción, que se realiza a través del intercambio de experiencias y el fortalecimiento de sus conocimientos basados en evidencias prácticas de la realidad cotidiana, como fiel reflejo de la sabiduría de la gente del campo. Asimismo recrea la virtud de quienes, apegados a la madre naturaleza, crecen cada día imponiéndose a las adversidades. Sirve para estimular a otros campesinos a innovar, a despertar al sol cada mañana con la mano amiga del investigador.

Constituye la presente obra científica un material de consulta necesaria para investigadores, docentes y personal técnico, en el propósito de alcanzar un mayor conocimiento en las temáticas que son abordadas por este colectivo de autores.

M.Sc. Yuván Contino Esquijerosa