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Diversity Of Endophytic Growth-Promoting Bacteria Associated With Medicinal And Aromatic Species

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ABSTRACT

The aim of this study was to isolate endophytic bacteria from medicinal and aromatic plant tissues in the department of Sucre, Colombia and to evaluate plant growth promoting activity. Strains of endophytic bacteria were isolated and their plant growth promoting activity (nitrogen fixation, phosphate solubilization, siderophore and ACC-deaminase production) was evaluated in vitro. The results found the endophyte species Bacillus cereus and Burkholderia contamins of Lippia origanoides, Lippia alba and Melia azederach. These bacteria showed positive activity for nitrogen fixation, phosphate solubilization, siderophore production and ACC-deaminase. Endophyte species with plant growth-promoting capacity are associated with the medicinal plant species of L. origanoides, L. alba and M. azederach.

Key words. *Endophytic bacteria, tissues, plants, medicinal plants, growth promotion.*

INTRODUCTION

In the search for alternatives for the integrated management of plant pathogens in the field, in recent years the concept of endophytic bacteria has gained momentum, given that these microorganisms reside within plant tissues where they live at least part of their lives without causing any apparent disease symptoms in the host (Zinniel et al, 2002; Mano and Morisaki,2008; Stępniewska and Kuźniar, 2013) , creating a symbiotic type association between them (Nair and Padmavathy, 2014), where information is transmitted from the host plant to them and vice versa (Perez et al., 2010).

Several study results reveal that endophytic microorganisms increase the ability to grow and absorb nutrients from the soil for plant development, help fix atmospheric nitrogen (Gupta et al., 2012), solubilize phosphate (Dawwam et al., 2013; Zaidi et al., 2017), remove pollutants (Ma et al., 2011; Stępniewska and Kuźniar, 2013), they can also produce a range of different

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metabolites that can be antibiotic, anti-pathogenic, immunopressor and other biologically active substances (Brader et al., 2014) making them potential candidates for biological control.

According to Gurib-Fakim, 2006), medicinal and aromatic plants have provided the basis for sophisticated traditional medical systems, producing several important medicines that are still used today. The quality and quantity of therapeutic compounds derived from medicinal plants are influenced significantly by such elements as the plant's genetic composition and ecological habitats (Namdar et al., 2019). Ironically, the noteworthy contribution of particular bacteria–host interaction on the crude drugs are reported (Zhai et al., 2018). Therefore, an in-depth understanding of the relationship between endophytic bacteria and medicinal plants is necessary to enhance bioactive molecules. Ideally, an alternative method can be developed to directly manufacture desired bioactive compounds by using endophytic bacteria as an elicitor

Taking what is referred to by J. Yu et al., (2023), the question is whether it is only plants or microorganisms that produce phytochemicals. Are there interactions between plants and endophytic bacteria in the production of bioactive compounds? Can endophytes be considered triggers? For highlighting the roles of the relationship between endophytic bacteria and plants on bioactive compound production, many studies reported the production capacity of bioactive compounds by endogenous bacteria in medicinal plants. Moreover, these studies also provided new and useful insights needed to inhibit pathogens with a view to human health and other potential medical applications (Abla et al., 2015).

For all of the above reasons, the strategy of this research was to inventory the diversity of endophytic bacteria associated with medicinal and aromatic plant species in the department of Sucre with the capacity to promote plant growth.

MATERIALS AND METHODS

Sampling. Plant samples of different plant tissues of medicinal and aromatic plants were collected at different times of the day 6:00 am, 12:00 pm and 18:00 pm. The material collected in the field was labelled, stored and refrigerated for processing in the microbiological research laboratory of the University of Sucre. The plant species were used for the isolation of endophytic bacteria and the other part of the plant material was placed in exsiccate and stored in the laboratory of Biological Conservation of the University of Sucre, for their respective taxonomic confirmation.

Isolation of endophytic bacteria. The root, stem and leaves of each plant species were washed with sterile distilled water and cut into segments of approximately 1 cm, then subjected to surface disinfection as described by Pérez et al. (2010). The process starts with two separate washes of each tissue in sterile distilled water, followed by shaking for 15 min in potassium phosphate buffer solution 0.05 mol.L-1, pH 7. 0; immersed for 1 min in 70% alcohol; shaken for 5 min in 5% sodium hypochlorite solution and dipped in Tween 80; again immersed for 1 min in 70% alcohol followed by shaking for 15 min in potassium phosphate buffer 0.05 mol.L^{-1}, pH 7.0; finally, washed four times in sterile distilled water. The process was repeated twice. To confirm sterilization of the root surface, the aliquot of the last wash was spread on a plate containing R2A agar medium and incubated at 28 °C for 72 hours. The absence of bacterial growth on R2A medium confirmed that the surface disinfection procedure was effective in removing bacteria from the surface (Pérez et al., 2010).

After the disinfection process, each tissue was placed in a porcelain dish and macerated in liquid nitrogen until a homogeneous mixture was obtained. Serial dilutions were prepared from each homogenate and seeded by diffusion technique on R2A agar surface and incubated at 28 ºC for 72 hours. The population density of endophytic bacteria per tissue (CFU/g tissue) was

estimated by direct colony counting on plates. Bacteria were observed and colonies were selected for shape, surface appearance, color and size. The selected strains were purified and maintained on R2A agar for in vitro evaluation.

In vitro evaluation of the plant growth promotion capacity of endophytic bacteria. The growth-promoting activity was performed in vitro using the capacity (nitrogen fixation, phosphate solubilization, siderophore and ACC Deaminase production) of each strain of endophytic bacteria isolated from medicinal and aromatic plant species.

- **Biological nitrogen fixation**. The fixation capacity of endophytic bacteria was tested in nitrogen-depleted Burk medium following the protocol proposed by Dobereiner et al. (1995). The strains were seeded by compound streak on the surface and incubated at 28°C for 72 hours, after which time those bacteria able to grow on the medium were selected.

- **Phosphate solubilization.** Phosphate solubilization capacity was determined by inoculation of each isolate on NBrip and SRS medium, the strains were incubated for 2 days at 28°C. The appearance of clear halos around the colony on NBrip medium and purple to yellow shift on SRS were used as indicators of phosphate solubilization. In both media the source of insoluble phosphate was calcium phosphate (Ca3 (PO4)) (Franco - Correa, et al., 2010).

- **Siderophore production.** Qualitative assessment of siderophore production was carried out by direct seeding of each strain on the surface of the chromium azurol-S (CAS) medium proposed by Schwyn and Neilands (1987). They were incubated for 7 days at 30°C. The ability of the bacteria to produce siderophores was evidenced by halo formation.

- **ACC Deaminase**. Stretch seeding was performed in Dworkin and Foster (DF) minimal medium (Belimov, et al., 2001, El- tarabily, 2008), supplemented with 0.3 g/L 1-aminocyclopropane carboxylic acid (ACC) 3mm as the sole source of nitrogen. Incubation was carried out for 5 days at 30°C. Boxes that recorded bacterial growth were considered as indicators of the ability of the microorganisms to use ACC as a nitrogen source mediated by ACC-deaminase production (Sgroy et al., 2009).

Identification of endophytic bacteria with growth-promoting activity. Endophytic bacteria with plant growth-promoting activity were selected and identified based on morphological characteristics and by Gram staining. DNA extraction was performed according to the protocol described by (Green and Sambrook, 2012; Oliveira et al., 2013). To verify the presence of DNA, agarose gel electrophoresis was performed in TBE (Tris-borate-edta) buffer. Three µl of pure DNA was taken and mixed with 4 µl of orange dye for visualization with a run time of 2 hours at 70 volts using a Power PacTM Basic 300V/400mA/75W BIO-RAD power supply.

Amplification of the rDNA fragments was carried out with the use of specific oligonucleotides for eubacterial groups. The amplification products obtained were quantified and sent for sequencing, using the service provided by Macrogen (Seoul, South Korea). The consensus sequences obtained were compared with those stored in the NCBI (National Centre for Biotechnology Information) database. The alignment of the bases was performed in the Clustal W program and the analysis and correction with the MEGA 7.0 program. Phylogenetic inferences were obtained by the distance method and maximum parsimony of Neighbor-joining with bootstrap test.

RESULTS AND DICUSSION

Figure 1 shows the growth-promoting activity of endophytic bacterial strains isolated from different medicinal and aromatic plant species.

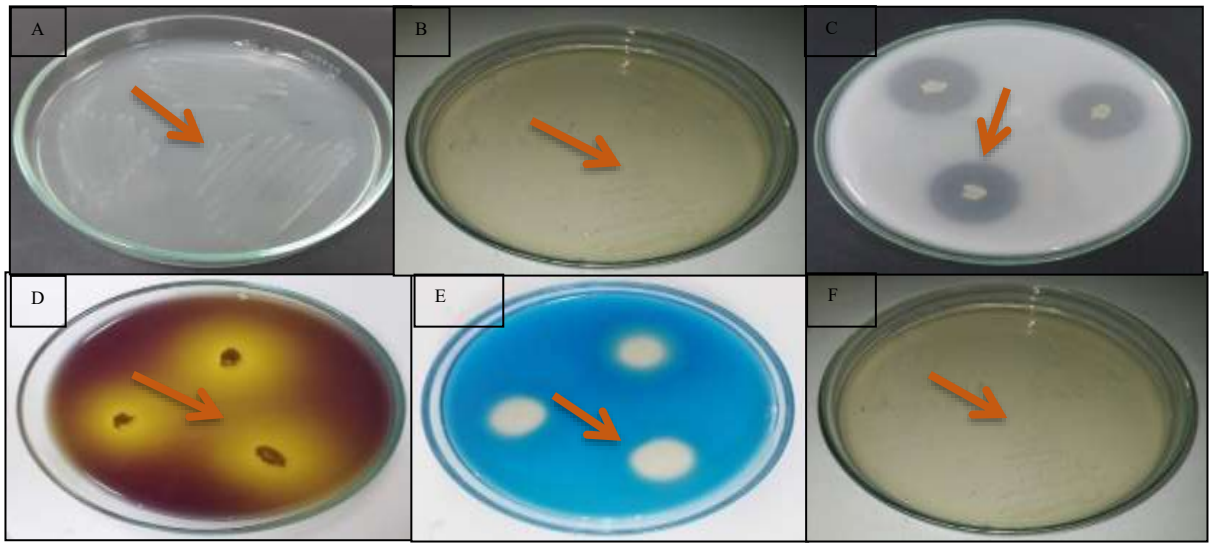

Figure 1. In vitro growth-promoting activity. A-B. Biological nitrogen fixation of RLO4 and TLO5 isolates, respectively. C-D. Phosphate solubilization. D. Siderophore production. D. ACC deaminase production of the isolates evaluated. Source: Lina Chamorro Anaya, 2021.

Bacteria that are able to grow in nitrogen-deficient media may be because the microorganism is using atmospheric nitrogen as a nutrient and in the absence of this element the nifHDK genes are expressed (Rubio and Luden, 2005), allowing the transcription of genes encoding the nitrogenase enzyme complex, which reduces the triple covalent bond of the nitrogen gas molecule (Dos Santos et al., 2012), being able to combine it with oxygen or hydrogen to form ammonium (NH4+) and nitrate (NO3+), these chemical forms can be used by plants and by the microorganisms themselves to partially or totally supply the requirements for the synthesis of proteins and other organic compounds (Dawe, 2000).

Endophytic bacteria are characterized by their ability to increase phosphate mobilization through mechanisms that allow them to capture insoluble phosphorus and convert it to the soluble forms monobasic ion ($H_2PO_4^-$) and dibasic ion ($H_2PO_4^{-2}$), which are available to both plants and microorganisms (Behera et al., 2014).

The mechanism of action of iron sequestration by these microorganisms in conditions of iron scarcity is the excretion of siderophores. Once iron is sequestered from the medium, the siderophore-iron complex is recognized by specific membrane receptors and once inside the cell, it is deposited in a specific site by a process involving ligand exchange that may or may not be preceded by iron reduction or hydrolysis of the siderophore (Ardon et al., 1998). Microorganisms that produce this type of compound have the advantage of not making it available to pathogenic microbial communities, whose growth is highly dependent on this element (considered a biocontrol mechanism), arousing great interest in its potential and for playing an important role in the promotion of plant growth, as it allows the increase of iron available to plants in environments lacking this element (Radzki et al., 2013).

With regard to ACC deaminase production B. cereus was able to grow on medium containing ACC as the sole nitrogen source. Endophytic bacteria have been reported to produce the microbially derived enzyme 1-amino cyclopropane-carboxylate deaminase (ACC), a key enzyme in the metabolism of α-ketobutyrate and ammonia, thereby decreasing high ethylene levels in host plants (Sessitsch et al., 2005; Sun et al., 2009), providing resistance to various stresses (Chaudhary et al., 2012).

From the sequences of the amplified products and the homologous sequences obtained at NCBI, analyzed by Clustal W and Mega 7, a phylogenetic identification was obtained by the distance and maximum parsimony method of Neighbor-joining with bootstrap test.

Sequencing results for each strain, with the respective activated growth promoter and plant species are listed in table 1.

Table 1. Endophytic bacterial species with growth-promoting activity and plant species isolated.

Species of endophytic bacteria identified	**Plant growth promotion activity**				**Isolated medicinal and aromatic plant species**
	Biological nitrogen fixation	Phosphate solubilization	Siderophore production	ACC Deaminase	
Bacillus cereus	+	+	+	+	Lippia origanoides
Burkholderia contamins	+		+		Melia azedarach
Bacillus cereus	+	+			Melia azedarach
Bacillus cereus		+	+		Lippia alba

Studies by Beneduzi et al. (2008) report Bacillus sp strains as nitrogen fixers, producing indole compounds: indole-3-acetic-acid (IIA) and indole-pyruvic acid (IPyA). There are reports that bacteria belonging to the genus Bacillus, such as Bacillus aryabhattai, B. megaterium and B. subtilis have shown the ability to synthesize siderophores, regulating the concentration of iron in the medium through its chelation (Fe3+- siderophore) (Scharf et al., 2014), controlling iron-dependent plant pathogen diseases.
The production of this enzyme is widely distributed among bacteria of the genus Bacillus sp, Enterobacter spp, Pseudomonas spp, Streptomyces spp, Burkholderia spp and Rhizobium spp, the most studied (Lugtenberg and Kamilova, 2009, Bhattacharyya and Jha, 2012).

Studies by Andrade et al. (2014) report that endophytic bacteria isolated from banana roots correspond to the genus Bacillus sp and are highly efficient P solubilisers. Likewise, Matos et al. (2017), report that the strains evaluated to solubilise calcium phosphate were from the genus Bacillus, exhibiting acid phosphatase activity and inducing a significant reduction in the pH of the culture medium.
Burkholderia contaminans are Gram-negative, aerobic, non-sporulating bacilli. Most strains are pigmented, greenish-yellow and hemolytic. Setu et al., (2018), suggest that the inhibition of pathogens by B. contaminans strain may be attributed to its ability to produce antifungal metabolites, toxin inhibitory metabolites, proteolytic enzymes, siderophore competition and nutrients.
On the other hand, studies by Da Silva et al. (2012) show that two bacterial strains, the first Burkholderia contaminans and one Burkholderia lata isolated from Phaseolus vulgaris L. and Pithecellobium sp. respectively, were able to fix nitrogen in free life, which represents a

novelty, since for the first time species of the B. cepacia diazotrophic complex other than Burkholderia vietnamiensis were reported (Da Silva et al., 2012).
On the other hand, endophytic bacteria, which live inside plants without showing evidence of physiological symptoms of pathogen attack and nutritional deficiencies, have the ability to provide multiple benefits in terms of plant growth and survival (Kim et al., 2011). They proceed to act through two mechanisms, a direct mechanism is the production of some phytohormones necessary for the plant such as auxins, gibberellins, ethylene and cytokinins, production of organic acids and as growth promoters (Wall, 2001), which was also evaluated in this research, biological nitrogen fixation, production of siderophores for iron assimilation and phosphate solubilization figure 11, showing good results for the endophytic bacteria isolated from Lippia alba and Lippia origanoides.

CONCLUSION

The plant species Lippia origanoides, Lippia alba and Melia azedarach, have associated species of endophytic bacteria with the capacity to fix nitrogen, solubilize phosphate, produce siderophore and ACC deaminase, activities that help in the nutrition and protection of plants against phytopathogens of commercial interest.

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DECLARATIONS

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Conflict of interest
The authors declare that they have no competing interests.

REFERENCES

Abla Elhartiti, Elhabchi Souad, Hichar Abdelhadi, Omar Bazdi, Ounine Khadija. Antagonistic activity of endophytic bacteria isolated from Mentha rotundifolia L International Journal of Scientific & Technology Research 4(12): 36-39.

Andrade, L. F., de Souza, G. L. O. D., Nietsche, S., Xavier, A. A., Costa, M. R., Cardoso, A. M. S., Pereira, D. F. G. S. 2014. Analysis of the abilities of endophytic bacteria associated with banana tree roots to promote plant growth. Journal of Microbiology. 52(1), 27-34.
Ardon, O., Nudelman, R., Caris, C., Libman, J., Shanzer, A., Chen, Y., Hadar, Y. 1998. Iron uptake in Ustilago maydis: tracking the iron path. Journal of bacteriology. 180(8), 2021-2026.
Bhattacharyya, PN., Jha, DK. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol. 28(4), 1327-50.

Behera, B. C., Singdevsachan, S. K., Mishra, R. R., Dutta, S. K., Thatoi, H. N. 2014. Diversity, mechanism and biotechnology of phosphate solubilising microorganism in mangrove—a review. Biocatalysis and Agricultural Biotechnology. 3(2), 97-110.

Belimov, A. A., Safronova, V. I., Sergeyeva, T. A., Egorova, T. N., Matveyeva, V. A., Tsyganov, V. E., Dietz, K. J. 2001. Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. Canadian Journal of Microbiology. 47(7), 642-652.

Beneduzi, A., Peres, D., da Costa, P. B., Zanettini, M. H. B., Passaglia, L. M. P. 2008. Genetic and phenotypic diversity of plant-growth-promoting bacilli isolated from wheat fields in southern Brazil. Research in microbiology. 159(4), 244-250.

Brader, G., Compant, S., Mitter, B., Trognitz, F., Sessitsch, A. 2014. Metabolic potential of endophytic bacteria. Current Opinion in Biotechnology. 27, 30-37.

Chaudhary, H. J., Peng, G., Hu, M., He, Y., Yang, L., Luo, Y., Tan, Z. 2012. Genetic diversity of endophytic diazotrophs of the wild rice, Oryza alta and identification of the new diazotroph, Acinetobacter oryzae sp. nov. Microbial ecology. 63(4), 813-821.

Dawe, D. 2000. The potential role of biological nitrogen fixation in meeting future demand for rice and fertilizer. The quest for nitrogen fixation in rice (1-9). Philippines: International Rice Research Institute. Dawwam, G., Elbeltagy, A., Emara, H., Abbas, I., Hassan, M. 2013. Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. Annals of Agricultural Sciences. 58(2), 195-201.

Dos Santos, P. C., Fang, Z., Mason, S. W., Setubal, J. C., Dixon, R. 2012. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial

Da Silva, K., Cassetari, A.E.S., Lima, A.S., De Brandt, E., Pinnock, E., Vandamme, P. & Moreira F.M. (2012). Diazotrophic Burkholderia species isolated from the Amazon region exhibit phenotypical, functional and genetic diversity. Syst Appl Microbiol, 35:253-62.

Döbereiner, J., Baldani, VLD, Baldani, JI 1995. Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas . Embrapa SPI.

El-Tarabily, K. A. 2008. Promotion of tomato (Lycopersicon esculentum Mill.) plant growth by rhizosphere competent 1-aminocyclopropane-1-carboxylic acid deaminase-producing streptomycete actinomycetes. Plant and Soil. 308(1-2), 161-174.

Franco-Correa, M., Quintana, A., Duque, C., Suarez, C., Rodríguez, M. X., Barea, J. M. 2010. Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. Applied Soil Ecology.45(3),209-217.

Gupta, G., Panwar, J., Akhtar, M. S., Jha, P. N. 2012. Endophytic nitrogen-fixing bacteria as biofertilizer Sustainable agriculture reviews (pp. 183-221): Springer.

J. Yu, Y. Zheng, C. Song et al., New insights into the roles of fungi and bacteria in the development of medicinal plant, Journal of Advanced Research, https://doi.org/10.1016/j.jare.2023.12.007

Kim, Y. C., Leveau, J., Gardener, B. B. M., Pierson, E. A., Pierson, L. S., & Ryu, C. M. (2011). The multifactorial basis for plant health promotion by plant-associated bacteria. Applied and Environmental Microbiology, 77(5), 1548-1555.

Lugtenberg, B., Kamilova, F. 2009. Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology. 63(1), 541-56.

Ma, Y., Prasad, M., Rajkumar, M., Freitas, H. 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology advances, 29(2), 248-258.

Mano, H., Morisaki, H. 2008. Endophytic bacteria in the rice plant. Microbes and environments. 23(2),109-117.
Matos, A. D., Gomes, I. C., Nietsche, S., Xavier, A. A., Gomes, W. S., Dos Santos Neto, J. A., Pereira, M. C. 2017. Phosphate solubilization by endophytic bacteria isolated from banana trees. Anais da Academia Brasileira de Ciências. 89(4), 2945-2954.

Nair, D. N., Padmavathy, S. 2014. Impact of endophytic microorganisms on plants, environment and humans. The Scientific World Journal.14, 1-11.

Oliveira, M. N., Santos, T. M., Vale, H. M., Delvaux, J. C., Cordero, A. P., Ferreira, A. B.,.Borges, A. C. 2013. Endophytic microbial diversity in coffee cherries of Coffea arabica from southeastern Brazil. Canadian journal of microbiology. 59(4), 221-230.

Pérez Cordero, A. F., Rojas Sierra, J. N., Fuentes Cuello, J. R. 2010. Diversidad de bacterias endófitas asociadas a raíces del pasto colosuana (Bothriochloa pertusa) en tres localidades del departamento de Sucre, Colombia. Acta Biológica Colombian. 15(2), 219-228.

Radzki, W., Gutierrez-Mañero, F., Algar, E., Lucas- Garcia, J., García-Villaraco, A., Ramos- Solano, B. 2013. Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie van Leeuwenhoek.104(3).

Rubio, L. M., Ludden, P. W. 2005. Maturation of nitrogenase: a biochemical puzzle. Journal of Bacteriology. 187(2), 405-414.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Wang-Pruski, G. 2005. Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. International Journal of Systematic and Evolutionary Microbiology. 55(3), 1187-1192.

Setu, B.T., Sang, W.K., Hyun, G.L., Hyun S.K & Youn, S.L. (2018). Effectiveness of multi-trait Burkholderia contaminans KNU17BI1 in growth promotion and management of banded leaf and sheath blight in maize seedling. Microbiological Research. 38(2): 1-34.

Schwyn, B., Neilands, J. B. 1987. Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry. 160(1), 47-56.

Stępniewska, Z., Kuźniar, A. 2013. Endophytic microorganisms—promising applications in bioremediation of greenhouse gases. Applied microbiology and biotechnology. 97(22), 9589-9596.

Sun, Y., Cheng, Z., Glick, B. R. 2009. The presence of a 1-aminocyclopropane-1-carboxylate (ACC) deaminase deletion mutation alters the physiology of the endophytic plant growth-promoting bacterium Burkholderia phytofirmans PsJN. FEMS microbiology letter. 296(1), 131-136.

Thanh-Dung Nguyen, Thi-Tho Nguyen, Minh-Nhut Pham, Huu-Nghia Duong, Thuy-Trang Pham, Thi-Pha Nguyen, Phu-Tho Nguyen, To-Uyen Thi Nguyen, Huu-Hiep Nguyen, Huu-Thanh Nguyen. 2023. Relationships between endophytic bacteria and medicinal plants on bioactive compounds production. Rhizosphere. 27, 100720. https://doi.org/10.1016/j.rhisph.2023.100720.

Wall, L. (2001). Consequences of an overview on PGPR work in Argentina: the Field should be wider. Programa de investigación en interacciones biologicas, Universidad Nacional de Quilmes. Argentina. WEBER, O., CRUZ, L. & DOBEREINER, J.

Zaidi, A., Khan, M., Ahemad, M., Oves, M. 2009. Plant growth promotion by phosphate solubilizing bacteria. Acta microbiologica et immunologica Hungarica. 56(3), 263-284.

Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczmarski, D., Higley, P., ... Vidaver, A. K. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Applied and environmental microbiology. 68(5), 2198-2208.