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SELECTION OF NON-SYMBIOTIC PHOSPHATE-SOLUBILIZING DIAZOTROPHIC BACTERIA

SELEÇÃO DE BACTÉRIAS DIAZOTRÓFICAS NÃO SIMBIÓTICAS SOLUBILIZADORAS DE FOSFATO

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Abstract

The objective of this study was to isolate and select rhizobacteria non-symbiotic diazotrophs capable of solubilizing phosphates and evaluating their efficiency in establishing interaction with corn seedlings (*Zea mays* L.) in vitro. 57 isolates grew in NFb and JMV culture media, of which 11

showed formations of a transparent halo around the colony cultivated in solid medium added to the NBRIP. The ANOVA detected significance for the parameters: plant height (AP), root length (CR), stem diameter (DC), leaf area (AF), number of leaves (NF) and total dry matter weight (PMST). Regarding FA, all isolates promoted a significant increase compared to the control, with the best results verified for DBSF58, DBSF40 and DBSF20. In the production of PMS mass by seedlings, all isolates differed statistically (p≤0.5) from the control, the highest mean was verified for DBSF40. All isolates selected as diazotrophs showed characteristics for promoting plant growth.
Keywords: growth-promoting bacteria; diazotrophs; biological N fixation.

Resumo

O objetivo deste estudo foi isolar e selecionar rizobactérias diazotróficas não simbióticas capazes de solubilizar fosfatos e avaliar sua eficiência em estabelecer interação com mudas de milho (Zea mays L.) in vitro. 57 isolados cresceram em meios de cultura NFb e JMV, dos quais 11 apresentaram formação de halo transparente ao redor da colônia cultivada em meio sólido adicionado ao NBRIP. A ANAVA detectou significância para os parâmetros: altura da planta (AP), comprimento da raiz (CR), diâmetro do caule (DC), área foliar (AF), número de folhas (NF) e peso de matéria seca total (PMST). Em relação à FA, todos os isolados promoveram aumento significativo em relação ao controle, sendo os melhores resultados verificados para DBSF58, DBSF40 e DBSF20. Na produção de massa de PMS por mudas, todos os isolados diferiram estatisticamente (p≤0,5) da testemunha, sendo a maior média verificada para DBSF40. Todos os isolados selecionados como diazotróficos apresentaram características para promover o crescimento das plantas.
Palvras-chave: bactérias promotoras de crescimento; diazotróficas; fixação biológica de N.

1. Introdução

Nitrogen is an essential element present in many biomolecules needed to sustain life. This element is present in the atmosphere in the form of dinitrogen (N_2), comprising about 78% of the atmosphere. Despite this abundance, organisms are not able to metabolize N_2 directly. One of the main sources of N in the soil is the process of biological fixation of atmospheric N_2, as well as nitrogen. Phosphorus plays an indispensable role in the biosphere, as it is part of nucleic acids, which store the genetic code. Many of the intermediate substances of photosynthesis and cellular respiration are combined with phosphorus, and this is

part of the energy currency of life.

Phosphorus is an essential element for plant growth and continuous applications are necessary, as a small percentage of the applied phosphorus is available to the plants. However, the most used phosphate fertilizers, such as superphosphates or ammonium phosphates, are extracted from phosphate rocks in mines, causing considerable energy costs from non-renewable sources for processing, transport, and distribution. To prevent the deficiency of this nutrient, it is necessary to apply a large amount of phosphorus due to the fixation of this element to the soil, making it poorly soluble and not readily available to plants (Amaral, 2014).

One of the great challenges of modern agriculture is to develop production systems that improve yields in a sustainable way, seeking input efficiency, pest and disease control through biological tools and resistance to environmental stresses Rosenblueth et al., 2018).

Plant growth promoting bacteria (PGPBs) consist of a wide group of microorganisms, inhabiting any part of the plant (phyllosphere and internal tissues) and rhizosphere without causing damage to its host, developing some direct and/or indirect mechanism that promotes improvements in plant growth and development. In recent years, the increased interest in sustainable and ecological agriculture has led producers to reduce the use of chemical fertilizers and to increase the use of natural compounds and practices such as inoculation of crops with PGPBs (Garcia et al., 2017), since the indiscriminate use of fertilizers can cause environmental problems, such as eutrophication of surface and groundwater, soil pollution and greenhouse gas emissions (Xia et al., 2020).

Inoculation of cultures with bacterial strains capable of increasing plant growth, promoting biological N fixation and phosphate solubilization, emerges as a promising technology for sustainable agriculture. Inoculation of seeds with selected strains of plant growth-promoting bacteria is reported to increase plant growth and crop yields. Thus, the objective was to isolate and select rhizobacteria non-symbiotic diazotrophs capable of solubilizing phosphates and evaluating their efficiency in establishing interaction with corn seedlings (*Zea mays* L.) *in vitro*.

2. Material and methods

The study was carried out in the Laboratory of Agricultural Microbiology of the Engineering and Agricultural Sciences Center from the Federal University of Alagoas. Bacterial isolates capable of growing in medium with reduced water activity, previously obtained (Silva et al., 2019) from soil samples from the caatinga in the semi-arid region of the state of Alagoas, were used.

2.1 Isolation and characterization of non-symbiotic diazotrophic bacteria

Test tubes with 10 mL of NFb and JMV culture media were inoculated in triplicate with 100 µL of bacterial inoculum (108 CFU.mL^{-1} (OD550=0.1)) the positive result was characterized by the presence of a halo of growth inside the culture medium, or by the change in color of the medium to blue, which indicates nitrogen fixation, after seven days of incubation at 28 °C. Ten days after incubation, the films were removed with a platinum loop, and transferred to a new semi-solid medium until a new film was formed. After this period, the films were transferred to Petri dishes with a specific solid medium for each diazotroph, plus 20 mg of yeast extract per liter. For final purification, the films were transferred to Petri dishes containing potato media.

2.2 Qualitative evaluation of the solubilization of inorganic phosphates ($Ca_3(PO_4)_2$)

The isolates were evaluated in solid NBRIP culture medium before autoclaving and pH adjusted to 7.0. The inoculation consisted of a spike in the center of the plate, the diameter of the colony halo was measured every five days, for a period of 15 days. The solubilization index (SI) was calculated through the ratio between the diameter of the halo and the diameter of the colony, solubilization can be classified as low solubilization (IS < 2), medium solubilization (2< IS < 3) and high solubilization (IS > 3). The isolates that obtained an index greater than or equal to three, on the 15th day after inoculation, were selected. According to the onset of solubilization, the isolates were further classified as "early", whose onset of

solubilization occurred from the third day onwards, "late" with onset of solubilization from the sixth day, and "non-solubilizing" those that did not showed visible solubilization until the fifteenth day of evaluation. The test was carried out in a completely randomized experimental design in a factorial design with 4 repetitions.

2.3 Colonization of corn radicle *in vitro*

Corn seeds were disinfected with sodium hypochlorite solution at 2.5% for one minute and then washed with distilled and sterilized water. Then, the seeds were placed to germinate in a black Germbox, on moistened filter paper and incubated at a temperature between 28-30 ºC. After seven days, the first count of the germinated seeds was made, and these were placed in bacterial suspensions, corresponding to the inoculation treatments, for 10 minutes, and then placed in test tubes containing 0.6% water-agar medium.

Observation of root colonization was performed visually 15 days after sowing. Considering that the presence of a turbid, whitish colony along and around the root demonstrates the formation of bacterial colonies. For recording these results, it was considered as positive, the growth in four or five of the test tubes, and as negative the absence of growth in four or five of the tubes.

Registration as partial colonization was done when there was growth in two or three tubes. The two forms of mist presence – in the entire root system or just in the collar region – were recorded separately. To verify whether the colonization of the collar region would already indicate the possibility of interaction between the bacteria and the plant or whether, for any interaction to occur, the presence of the bacteria was necessary throughout the root system of the seedling. Treatments were arranged in a fully randomized design, with five replications.

2.4 Efficiency of the interaction between the selected isolates and corn in its early stages of development

The selected bacteria were inoculated separately in Erlenmeyers with TSB culture medium (Tripcase Soy Broth) and subjected to stirring at 28 °C for 24 hours. After the incubation time, bacterial concentrations were adjusted to 108 CFU mL^{-1} (OD550=0.1) with sterilized 0.85% saline solution. The disinfested seeds were immersed for two hours in the bacterial suspensions. The seeds of the control treatment, without bacteria, were immersed for the same period in sterilized saline solution (0.85%). Then, the seeds were placed to germinate in Petri dishes with cotton and filter paper moistened with sterilized distilled water. The plates were wrapped with aluminum foil so that germination occurred in the dark.

After 24 h, the germinated seeds were placed in test tubes (one seed per tube) containing Hoagland & Arnon solution (Hoagland; Arnon, 1950) without N and $Ca_3(PO_4)_2$) as an inorganic source of phosphate and 0.6% of agar. The test was conducted under artificial lighting conditions, for a period of 15 days, the observation of root colonization was done visually, considering that the presence of a turbid, whitish colony along and around the root demonstrates the formation of bacteria colony. The trial was arranged in a completely randomized design, with five replications. The morphometric evaluation was carried out after 15 days of cultivation, where the following were evaluated: a) plant height (PH); b) leaf area (LA), expressed in cm^2, estimated by measuring the width (L) of each leaf and the length (L) of the main vein; c) number of leaves (NL); d) root length (RL); e) stem diameter (SD), expressed in mm, using a caliper with a precision of 0.01mm; f) total dry matter (DM) weight expressed in grams, determined in a forced circulation oven at 60 ºC until constant weight.

3. Results and discussion

3.1 Isolation Characterization of Non-Symbiotic Diazotrophic Bacteria

By the tests, 57 isolates were obtained, which were stored in the incubation room of the Laboratory of Agricultural Microbiology at the Center for Agricultural Sciences/UFAL. The ability of microorganisms to grow in nitrogen-free media is an easy method to prove their ability to fix nitrogen (Figure 1). Nitrogen is necessary for

the synthesis of chlorophyll, amino acids, nucleic acids, and ATP that are necessary for plant growth and survival. Although it is the most abundant element on the planet, plants cannot use N_2 directly, which needs to be reduced to the form of NH_3.

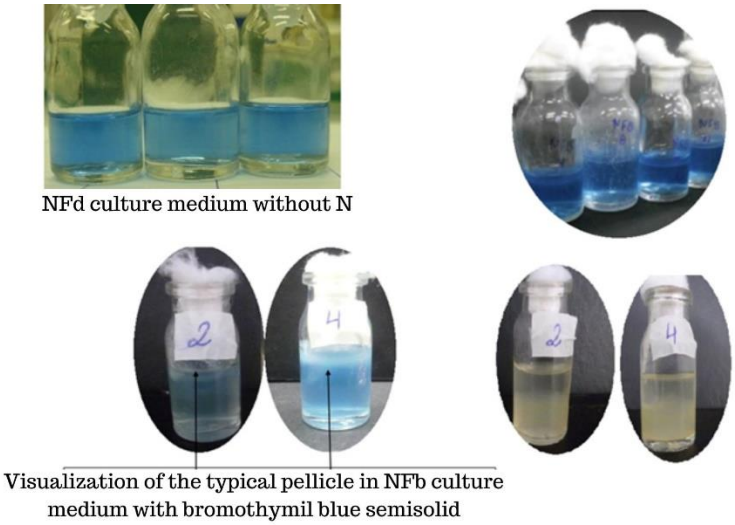


Figure 1. Growth of diazotrophic bacteria isolates in NFB and JMV culture medium. Source: Authors (2024).

A specialized group of bacteria called diazotrophs can convert N_2 to NH_3 using the nitrogenase enzyme complex (Hoffman; Dean; Seefeldt, 2009). This ammonia can be further oxidized to nitrate by the action of nitrifying bacteria. As plants lack the enzyme nitrogenase, they are unable to synthesize ammonia from aerial nitrogen, thus they use ammonia fixed by microorganisms to produce the nitrogenous biomolecules for their growth and survival. The isolation and screening of possible diazotrophic bacteria are important steps towards the discovery of isolates efficient in nitrogen fixation.

In grasses, BNF (biological N fixation) is carried out by nitrogen-fixing bacteria (NFB), significant advances have occurred in research on the process, with the elaboration of the semi-solid medium NFb. With no nitrogen source, the semi-solid condition creates an environment with a low level of oxygen, like what happens in the soil or in the plant, where diazotrophic bacteria are located microaerophilic bacteria associated with plant roots (Kuss et al., 2007). The formulation of this medium facilitated the isolation of bacteria of the genus *Azospirillum* and, after this

discovery, many other species of diazotrophic bacteria were isolated in Brazil, belonging to the genus *Gluconacetobacter*, *Herbaspirillum* and *Burkholderia*. These bacteria have been detected in high numbers between 104 and 107 cells g^{-1} fresh weight, and many studies have been conducted to evaluate the supply of N supplied to host plants.

3.2 Qualitative evaluation of the solubilization of inorganic phosphates ($Ca_3(PO_4)_2$)

Of the 57 isolates, 11 (19%) showed the formation of a transparent halo around the colony cultivated in solid medium, indicating the ability to solubilize calcium phosphate ($Ca_3(PO_4)_2$) added to NBRIP. Most isolates did not present solubilization with sufficient diameter to exceed the colony growth limit, these received zero value for SI. Of the eleven isolates, three showed low solubilization with IS ranging from 1.140 to 1.417; one obtained a medium index (SI 2.735), and 7 obtained a high solubilization index (SI ranging from 3.102 to 12.750).

The F test detected significant differences ($p \leq 0.01$) between bacterial isolates, incubation time and for interaction, indicating dependence between the two factors. The means obtained are shown in Table 1. The highest solubilization index was found for isolate DBSF30 (SI 12.750) followed by isolate DBSF20 (IS 11.450) at 15 days. DBSF30, DBSF20, DBSF40, DBSF37 and DBSF,58 at fifteen days showed indices that differed significantly from the other incubation times. For DBSF21, DBSF33, DBSF41, DBSF42, DBSF47 and DBSF64 no significant increases were detected at 5, 10 and 15 days.

The DBSF41, DBSF47 and DBSF42 isolates solubilized phosphate, however, not capable of producing a halo greater than colony growth, indicating low solubilization capacity with IS less than 2.00. It was also observed that all isolates presented the solubilization halo at five days, therefore being classified as early.

Table 1 Efficiency of phosphate solubilization in solid NBRIP medium indicated by the solubilization index (SI) calculated by the ratio between the mean diameter of the halos and the mean diameter of the colonies of each isolate after 15 days of incubation.

Isolated	**Time in incubation (days)**		
	5 days	10 days	15 days
DBSF30	4.105BC*	11.620aB	12,750aA
DBSF20	3.007bB	3.204cB	11,450bA
DBSF40	2,140cB	3.107cB	8,388cA
DBSF37	2.834bB	7,600bA	8,090cA
DBSF33	3.592aA	3.592aA	3,592dA
DBSF58	1.383cB	2.003dB	3.109dA
DBSF47	2.852bA	2,852cA	3.102dA
DBSF21	1,828cA	2,000dA	2,733dA
DBSF41	1,100dA	1.287eA	1.417eA
DBSF64	1,008dA	1.080eA	1.177eA
DBSF42	0.300dA	0.300eA	1.140eA

*SI < 2 low solubilization; IS between 2 and 3 middle solubilization; IS >3 high solubilization). Means followed by the same letter do not differ statistically, Scott- Knott test (p≤0.05). Columns - lowercase letters; lines - capital letters. Source: Authors (2024).

Microorganisms directly affect the ability of plants to acquire P from the soil through several mechanisms. These mechanisms include: increasing root surface area by extending the root system or by promoting the growth of lateral roots and root hairs (growth promotion via phytohormones); shifting the adsorption equilibrium, which results in a transfer of phosphate ions to the soil solution or increasing the mobility of organic forms of P and stimulating metabolic processes that are effective in solubilizing and mineralizing P from poorly available forms of phosphorus inorganic and organic (Rampim et al., 2020).

Solubilizing large amounts in a short time in the NBRIP culture medium, in addition to maintaining constant solubilization capacity over the evaluated period, is an important feature for selection of isolates. Some isolates may be able to

solubilize phosphate, however, they often do not do so in a solid medium (Nautiyal, 1999). The physical state of the culture medium and agitation may be factors that influence these results.

Isolates with little solubilization capacity in solid medium, may have a better ability to solubilize phosphorus when inoculated in liquid medium. The diffusion coefficient in solid medium of acids produced by bacteria is different according to the acid produced and the culture medium (Delvasto et al, 2006).

3.3 *In vitro* colonization of corn seedlings

Eight isolates completely colonized the root system and the lap region, one colonized partially (DBSF47) and two colonized only the neck region (DBSF40 and DBSF58). Assessment of *in vitro* colonization allows rapid assessment of large numbers of isolates. Colonization can occur both in the roots and in the collar region, depending on the bacterial isolates. Competitive colonization of the rhizosphere is crucial for beneficial bacteria to exert their many mechanisms of action. The process comprises a series of steps: migration towards the roots, attack, distribution along the roots, population growth and establishment. Colonization is the initial and fundamental step in the beneficial interaction. After the initial contact, comes the crucial phase, which is maintenance or persistence, where the bacterium uses the exudates released by the plant (Melo, 1998) and, in exchange, produces and supplies a series of bioactive substances to benefit plant growth directly or indirectly. indirectly.

These recognition mechanisms are mediated by specific plant exudates. They attract bacteria to the host's rhizosphere, and these exudates can serve as a source of carbon and act as signaling molecules Albareda et al., 2006). Thus, growth-promoting bacteria must have the ability to adhere to plant seeds or roots for subsequent colonization and a competitive process that is affected by the genotypic characteristics of the RPCP and the host plant variety (Muñoz-Rojas; Caballhero-Mellado, 2004).

Colonization of the colon in culture medium may be sufficient to consider the bacteria capable of associating with the plant. Partial colonization of the root system may be an indicator of the ability of bacteria to colonize the colon. Bacterial establishment in the rhizosphere is fundamental for the microorganism to interact with the plant. The concentration of bacteria in the region close to the collar of the plant, but without any colonization along the roots, may indicate a bacterial isolate that, due to its greater O_2 needs, moves to the surface in search of O_2, resulting in failure in general root colonization.

4.4 Efficiency of the interaction between the selected isolates and corn in its early stages of development

The analysis of variance detected significance (F Test p≤0.05) for the parameters: plant height (PH), root length (RL) stem diameter (SD) leaf area (LA) number of leaves (NL) and total dry matter weight (DM).

Table 2 Means of plant height (AP), root length (CR) stem diameter (DC) leaf area (AF) number of leaves (NF) and total dry matter weight (PMST), total dry matter (DM) of corn seedlings.

Isolated*	PH (cm)	RL (cm)	SD (cm)	LA (cm^2)*	NF	DM (g)
Control	8.157f	9.670c	0.317 B	5.110f	2,000b	0.039f
DBSF20	14,700b	16,500b	0.750a	15.910a	2,000b	0.087b
DBSF21	16,970a	15.867b	0.523b	14.081b	2,000b	0.076d
DBSF30	13.733c	22.367ª	0.280c	13.220b	2,000b	0.099a
DBSF33	16,767a	16.733b	0.727a	11,997c	2,000b	0.063e
DBSF37	15.410b	21,500ª	0.230c	10.987c	2,000b	0.099a
DBSF40	7,670f	22,500ª	0.730a	15,950a	2,000b	0.100a
DBSF41	9,580e	15.430b	0.680a	8,280e	2,000b	0.059e
DBSF42	15,600b	16.610b	0.260c	11.974c	2.333b	0.082c
DBSF47	15.160b	19.767ª	0.263c	11.137c	2,000b	0.056e
DBSF58	13.953b	20.067ª	0.730a	16,763a	3,000a	0.099a

DBSF64	11,890d	15.143b	0.197c	9,430d	2,000b	0.072d

*Means followed by the same letter do not differ statistically, Scott- Knott test (p≤0.05).

For PH, only the isolated DBSF40 did not differ significantly from the control, while the others promoted a significant increase, with emphasis on DBSF21 (16.970cm) and DBSF33 (16.767cm). Regarding RL, significant differences were also found between the control and the isolates, the largest lengths were with the isolates DBSF30, DBSF37, DBSF40, DBSF47 and DBSF58.

The length of primary roots is one of the important parameters to evaluate plant development (Vejan et al., 2016). Roots with greater surface area absorb more water and nutrients from the soils and translocate them to the aerial part of the plants, resulting in greater growth and increased biomass production and grain production. The production of hormones such as cytokinin by diazotrophic bacteria, enables greater development of the root system, initially detected by the increase in density and length of root hairs, which results in the appearance of lateral roots and an increase in the volume of the root surface; more specifically due to the increased differentiation of epidermal cells (Moreira et al., 2010).

For SD, the averages observed for isolates DBSF30, DBSF37, DBSF42, DBSF47 and DBSF64 did not show significant difference in relation to the control. The highest value was found for DBSF20, which did not show significant differences in relation to isolates DBSF33, DBSF40, DBSF41 and DBSF58. Larger stem diameters are important because they have greater resistance to lodging, the increase in stem diameter favors the transport of water, nutrients and elaborated sap between root and leaves, therefore, a plant with greater stem diameters in suitable environments will be favored when expressing its productive potential (Kappes et al., 2011).

As for the number of leaves, only the isolate DBSF58 differed from the control, presenting 3 leaves. About LA, all isolates promoted a significant increase compared to the control, with the best results verified for DBSF58, DBSF40 and DBSF20 (16,763, 15,950 and 15,950 cm^2 respectively). In the production of DM

mass by seedlings, all isolates differed statistically ($p<0.5$) from the control, the highest average was verified for DBSF40 (1.00g).

The leaf area is a parameter that reflects the significant growth of the plant, since the leaves are organs that capture the light energy of the sun and the production of organic matter through the photosynthetic process of absorbing minerals from the soil. Also, it is directly related to the final production of dry mass, because during the development cycle, the plant depends on the leaves, and the growth rate of the plant depends both on the rate of expansion of the leaf area and on the rate of photosynthesis per unit. of leaf area (Terra et al., 2012).

The results observed for all traits show the positive potential of inoculation of the isolates in maize plants. These responses observed in seedlings, occur by hormone production, greater absorption of water and nutrients that resulted in vigorous initial growth. Considering that nitrogen, a necessary element for the composition of the most important biomolecules, such as ATP, NADH, NADPH, chlorophyll, proteins, and numerous enzymes, was not provided, the results show that it was provided by the association with bacteria.

4. Conclusions

In the present study, it was evidenced that all bacterial isolates have characteristics for promoting plant growth.

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