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RESEARCH ARTICLE

Plant growth-promoting bacteria and nitrogen fertilization in initial maize development

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The objective of the present research was to evaluate the initial development of maize (*Zea mays*) inoculated with plant growth-promoting diazotrophic bacteria *Azospirillum brasilense*, *Burkholderia ambifaria* and *Herbaspirillum seropedicae* in vitro and in protected culture. The experiment in protected culture consisted in the comparison of four methods of nitrogen fertilizations in maize crop (absence, broadcast, placement, broadcast and placement) associated or not to inoculation with growth-promoting bacteria. Morphological parameters, foliar nitrogen content and bacterial population were evaluated at 30 and 45 days after sowing. The in vitro essay consisted in the cultivation of inoculated plants in culture medium, with and without nitrogen and then, at seven days after seeding, evaluated morphological parameters, foliar nitrogen content and bacterial population. *A. brasilense* was more responsive to placement fertilization, while *H. seropedicae* for the association between placement and broadcast fertilization, or else the absence of fertilization. Yet, *B. ambifaria* was responsible to the absence of fertilization. Bacterial populations in protected culture varied according to the fertilization used and the period of collection. In vitro, the bacterial population was higher epiphytically.

Highlighted Conclusions

1) *A. brasilense* is more responsive to placement fertilization, and *B. ambifaria* to the absence of fertilization. *H. seropedicae* is more responsive the association between placement and broadcast fertilization, or else the absence of fertilization.
2) Bacterial populations vary according to the fertilization and the period of collection in protected culture, and they are higher epiphytically *in vitro*.

Research conducted in the current agricultural context seeks environmental and economical methods for the establishment of crops of interest, ensuring profitability and preservation of the available natural resources. The use of growth-promoting bacteria stands out as a sustainable alternative to cause an increase in productivity, coupled with environmental preservation.

Maize receives complementary nitrogen fertilizations in broadcast fertilization to, together with the amount from the soil, obtain the ideal quantity to satisfy crop needs, seeking high yields. Different experiments carried out in Brazil, under different climatic conditions, soils and crop systems, resulted in maize responses to nitrogen fertilization (Morais et al. 2017, Mumbach et al. 2017, Mortate et al. 2018).

The mineral needs of a plant have a considerable influence on its development. Biological nitrogen fixation is the most important way of fixing atmospheric nitrogen (Taiz et al. 2017). Endophytic diazotrophic bacteria have been isolated from gramineous and it was found a great diversity of them colonizing maize plants (Guimarães et al. 2017), which demonstrated better use of nutrients and better development when compared to the control, factors that can be highlighted due to better development of aerial part and root system (Santoyo et al. 2016).

Bacteria may promote cellular development of diverse forms, such as producing phytohormones, fixing atmospheric nitrogen, producing siderophores, solubilizing phosphate, among others (Glick 2015).

However, this interaction with grasses does not manifest in the same way as in legumes, with formation of fixation nodules.

There are diverse ways of interaction between plants and endophytic bacteria, representing a major source of study yet to be explored. Many researchers seek to clarify the plant-microorganisms interaction and to demonstrate their application in the agriculture and the environment, as pest and disease control, growth-promotion, biological fixation of atmospheric nitrogen, drought tolerance, phytohormones promotion, among others (Mendes et al. 2007, Assumpção et al. 2009, Babalola 2010, Glick 2012).

Thus, tests about the potential of growth-promoting bacteria with different nitrogen doses are necessary, so that a low-cost and, mainly, low-impact agriculture is promoted, seeking to increase the use of natural resources by the plant.

The objective was to evaluate the initial development of maize plants (*Zea mays*) when inoculated with diazotrophic plant growth-promoting bacteria *Azospirillum brasilense*, *Burkholderia ambifaria* and *Herbaspirillum seropedicae* in vitro and in protected culture.

MATERIAL AND METHODS

Place of experiment. The experiment was carried out in the biotechnology laboratory and in the protected culture environment of the Pontifical Catholic University of Paraná, Campus Toledo.

Experimental design. In vitro, a completely randomized experimental design was used, in which maize plants, cultivated in culture medium in the presence of nitrogen, were inoculated with three bacteria strains and ten replicates. In greenhouse, was used a complete randomized block design, with a 3x4 factorial scheme, where the first factor represents three bacterial strains and the second factor four methods of nitrogen fertilization, with three replicates.

Material obtainment. The bacterial colonies *A. brasilense*, *H. seropedicae* and *B. ambifaria* were obtained at Federal University of Paraná, located in Palotina-PR, and the maize seeds hybrid PIONNER® 30F53 YH were sold by Pionner, located in Toledo-PR.

Greenhouse essay. Polyethylene plant pots of 25 dm^3 were filled with sieved soil from the experimental area, classified as Oxisol. Soil samples were collected and sent to laboratory analysis and, these samples were used to determine the fertilization correction, which was made by applying and homogenizing 58,33 g pot^{-1} of calcitic lime ($CaCO_3$), 0,80 g pot^{-1} of potassium chloride (KCl), 6,0 g pot^{-1} of super simple (P_2O_5) and 0,375 g pot^{-1} of zinc sulphate ($ZnSO_4$), according to soil analysis (Table 1).

Table 1. Oxisol nutritional content used in the experiment.

SB $cmol_c\,dm^{-3}$	T $cmol_c\,dm^{-3}$	V_1 %	Al^{3+} %	Ca^{2+} %	Mg^{2+} %	K^+ %	Ca^{2+}/Mg^{2+}	Ca^{2+}/K^+	Mg^{2+}/K^+	$(Ca^{2+}+Mg^{2+})/K^+$	$K^+/\sqrt{(Ca^{2+}+Mg^{2+})}$
3.63	7.91	45.89	18.79	24.65	18.84	2.4	1.31	10.26	7.84	18.11	0.1

For nitrogen, each pot received 2,3 g of super N (45% of nitrogen), this amount was applied differently in each pot, according to the respective treatment.

We used for inoculation the *A. brasilense*, *H. seropedicae* and *B. ambifaria* isolates from previous experiment. The bacteria were maintained by successive replications in specific solid medium and incubated in greenhouses at 28 ºC. The inoculum was prepared according to Brito et al. (2017). Table 2 shows the treatments carried out in this experiment.

Seeds were inoculated by putting them inside a beaker containing the corresponding bacteria inoculum, for two hours before sowing. For the implementation of the experiment, six seeds were sowed at 0,05 m deep, being the different sowing and fertilization methods carried in the same period. The pots were thinned after seven days, leaving three plants per pot. Plants were watered when necessary and weeds were manually removed. Plants were sprayed with pesticide DECIS® 25C, after 20 days of implementation due to the presence of defoliation caterpillar in the experiment.

Evaluated parameters. One plant per pot was collected at 30 and 40 days after sowing and then carried the evaluations of aerial fresh mass, aerial length, root epiphytic and endophytic population. Aerial dry mass and foliar nitrogen content were determined after drying the plants in a forced circulation oven at 65 ºC for 72 hours.

The microdrop counting method (Romeiro 2001) for determination of epiphytic population consists in, after previously washing the roots, putting them in Eppendorf with 1000 µL of saline solution. This Eppendorf goes to an ultrasound bath for 1 minute, resulting in the dilution of 10^{-1}, being this dilution sequenced by removing 100 µL of the solution and then resuspending it in a new Eppendorf filled with 900 µL of saline solution forming the 10^{-2}, this process is repeated until 10^{-8}. The endophytic counting method is the same as the previous one, however, after washing the roots, they are macerated in crucibles filled with 1000 µL of saline solution, and then performed the dilution.

Table 2. Relation of tested treatments.

T1	Control (Absence of nitrogen and inoculation)
T2	Control and broadcast nitrogen fertilization
T3	Control and placement of nitrogen fertilization
T4	Control with placement and broadcast nitrogen fertilization
T5	*A. brasilense* only
T6	*A. brasilense* and broadcast nitrogen fertilization
T7	*A. brasilense* and placement of nitrogen fertilization
T8	*A. brasilense* with placement and broadcast nitrogen fertilization
T9	*H. seropedicae* only
T10	*H. seropedicae* and broadcast nitrogen fertilization
T11	*H. seropedicae* and placement of nitrogen fertilization
T12	*H. seropedicae* with placement and broadcast nitrogen fertilization
T13	*B. ambifaria* only
T14	*B. ambifaria* and broadcast nitrogen fertilization
T15	*B. ambifaria* and placement of nitrogen fertilization
T16	*B. ambifaria* with placement and broadcast nitrogen fertilization

The Kjeldahl method was used to determine the nitrogen content, which is based in the decomposition of organic matter through the digestion of the sample at 400 ºC with concentrated Sulphur acid with copper sulphate as a catalyst, speeding up the organic matter oxidation. The nitrogen present in the resulting acid solution is determined by steam distillation followed by titration with diluted acid (H_3BO_3) (Nogueira and Souza 2005).

In vitro essay. The root production in the in vitro essay was performed according to the methodology of Brito et al. (2017). Then, the seeds were inoculated just as in the previous stage of the experiment. After 2 hours, the inoculated seeds were conditioned in test tubes with 20 mL of MS culture medium (Murashige and Skoog 1962) with nitrogen and 5 cm of polypropylene spheres to keep the seeds over the medium.

Plants were removed from the test tubes seven days after implementation and then carried out the following evaluations: aerial and root fresh mass, root epiphytic and endophytic population count, root morphology and aerial and root fresh mass. Aerial and root dry mass and nitrogen content were determined after drying the material in a vertical forced circulation oven at 65 ºC for 72 hours.

Three plants of each treatment were chosen to determine root morphology, these were colored with bromothymol blue and observed in a 10x electronic microscope for visualization of the bacterial colony on the roots.

Statistical Analysis. The data were submitted to variance analysis and, when significant, the means were compared by the Tukey test at 5% probability, by means of statistical program SISVAR (Ferreira 2014).

RESULTS AND DISCUSSION

For the results collected at 30 days of development (Table 3), about the parameters aerial fresh and dry mass, the treatment without nitrogen fertilization and absence of microbial population presented superior results to the others. For the treatment where the seeds were inoculated with *A. brasilense* and with placement nitrogen fertilization, results of aerial fresh mass were statistically lower to the other treatments.

The bacterial action, according to each fertilization method used, showed that only when the placement of fertilization was used, the action of the *B. ambifaria* influenced in aerial dry mass, fresh mass and length which were superior to the control, not differing statistically from the other bacteria. Plants inoculated with *A. brasilense* in the absence of fertilization showed superior results to the other treatments for the aerial length parameter.

Plants inoculated with *H. seropedicae*, that received placement of fertilization, showed statistically superior levels of foliar nitrogen content than the other fertilization methods. *A. brasilense* with placement and broadcast nitrogen fertilization resulted in a superior foliar nitrogen content to the control but not statistically differing among themselves from the other tested bacteria.

At 45 days of development (Table 4), the aerial fresh mass parameter presented a statistically significant difference only when plants inoculated with *A. brasilense* were cultivated with broadcast fertilization, that presented superior results to the control and did not differ statistically from the other bacteria, the same occurred for the aerial dry mass parameter.

Table 3. Effect of inoculation with plant growth-promoting bacteria associated with different nitrogen fertilizations in maize plants at 30 days after sowing in protected culture.

Bacteria	Nitrogen							
	Absence		Broadcast		Placement		Broadcast + Placement	
	Aerial fresh mass							
Control	96.42	aA	31.77	bA	29.82	bB	10.79	bA
A. brasilense	115.20	aA	86.84	abA	50.68	bAB	57.95	abA
B. ambifaria	61.69	aA	85.73	aA	85.29	aA	74.55	aA
H. seropedicae	82.03	aA	88.85	aA	64.54	aAB	69.13	aA
	Aerial dry mass							
Control	11.55	aA	4.22	abA	1.52	bB	4.14	abA
A. brasilense	14.15	aA	9.44	aA	6.57	aAB	6.45	aA
B. ambifaria	7.40	aA	10.72	aA	11.81	aA	6.65	aA
H. seropedicae	9.86	aA	10.05	aA	6.37	aAB	7.65	aA
	Aerial length							
Control	105.00	aAB	72.00	aA	62.00	aB	73.30	aA
A. brasilense	146.00	aA	86.00	bA	95.33	bAB	99.67	bA
B. ambifaria	109.83	aAB	91.00	aA	109.00	aA	78.33	aA
H. seropedicae	85.67	aB	92.67	aA	82.33	aAB	106.67	aA
	Nitrogen content							
Control	4.14	aA	2.68	aB	4.17	aA	2.55	aB
A. brasilense	5.45	aA	5.64	aA	6.35	aA	5.68	aA
B. ambifaria	3.90	aA	4.09	aAB	4.26	aA	4.45	aAB
H. seropedicae	3.67	bA	3.88	bAB	6.86	aA	2.85	bAB

* Lowercase letters represent difference between columns. Capital letters represent the difference between lines.

Table 4. Effect of inoculation with plant growth-promoting bacteria associated with different nitrogen fertilizations in maize plants at 45 days after sowing in protected culture.

Bacteria	Nitrogen							
	Absence		Broadcast		Placement		Broadcast + Placement	
	Aerial fresh mass							
Control	156.86	aA	39.35	aB	37.24	aA	70.93	aA
A. brasilense	219.05	aA	168.87	aA	111.39	aA	175.24	aA
B. ambifaria	155.02	aA	143.86	aAB	144.16	aA	176.99	aA
H. seropedicae	208.45	aA	133.05	aAB	145.03	aA	178.19	aA
	Aerial dry mass							
Control	24.45	aA	6.36	abB	6.00	bA	10.75	abA
A. brasilense	31.60	aA	27.12	aA	17.37	aA	28.50	aA
B. ambifaria	22.29	aA	21.63	aAB	23.43	aA	23.96	aA
H. seropedicae	27.55	aA	20.45	aAB	19.77	aA	22.64	aA
	Aerial length							
Control	141.00	aA	87.00	bB	85.00	bB	120.00	abA
A. brasilense	164.33	aA	139.33	abA	117.67	bAB	148.00	abA
B. ambifaria	131.00	aA	130.00	aA	128.00	aA	139.00	aA
H. seropedicae	153.67	aA	134.33	aA	128.33	aA	148.00	aA
	Nitrogen content							
Control	1.57	aA	1.61	aA	2.81	aB	1.85	aA
A. brasilense	2.27	bA	2.20	bA	5.09	aA	2.26	bA
B. ambifaria	1.53	aA	1.95	aA	2.64	aB	2.37	aA
H. seropedicae	1.47	bA	1.57	bA	4.34	aAB	3.17	abA

* Lowercase letters represent difference between columns. Capital letters represent the difference between lines.

The aerial length, in the absence of inoculation and fertilization, was statistically superior to plants cultivated with placement or broadcast fertilization, on the other hand no statistical difference was observed between broadcast and placement of fertilization for this parameter. Plants inoculated with *A. brasilense* and in the absence of nitrogen fertilization were statistically superior to the ones inoculated and cultivated with nitrogen in the base.

Still, at 45 days, for nitrogen foliar content, plants inoculated with *A. brasilense* cultivated with placement of fertilization were statistically superior to the other fertilization methods. Also, *H. seropedicae* with placement of nitrogen fertilization was statistically superior to the plants inoculated with the same bacteria cultivated in the absence of fertilization or with broadcast fertilization.

Figure 1 shows the different populations of plant growth promoting bacteria in the roots, according to the treatments. To evaluate this parameter a culture medium that identify all the bacteria with the ability to act as plant growth promoters was used.

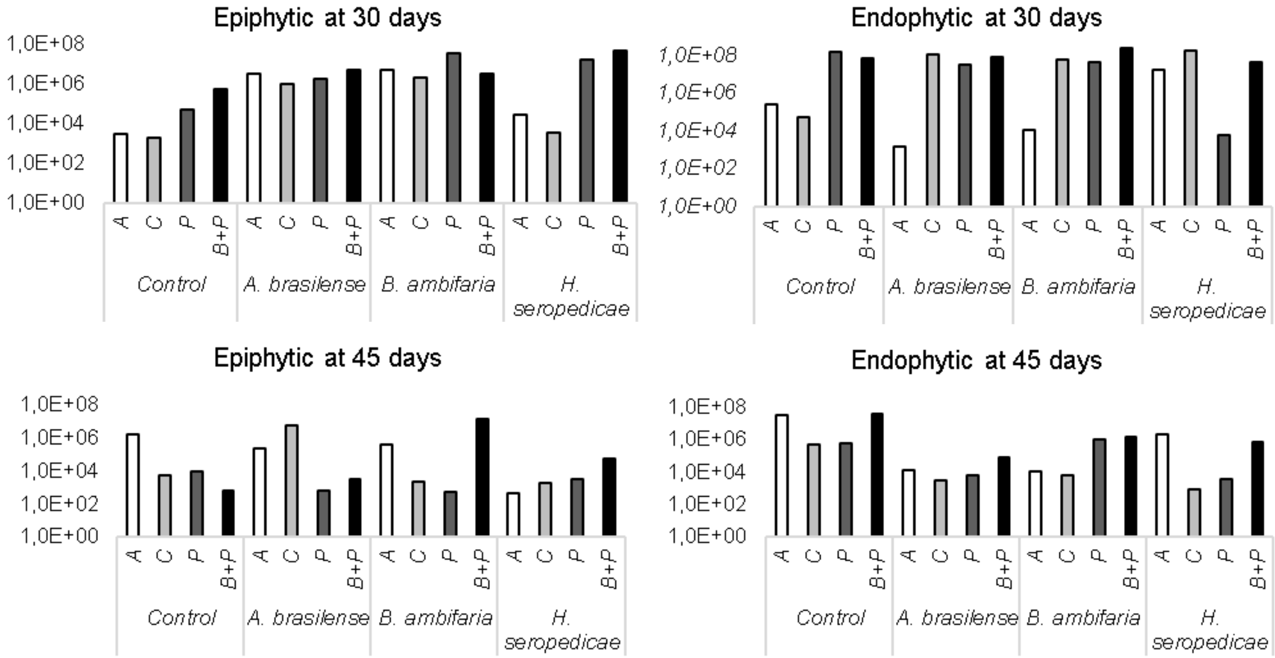

Figure 1. Epiphytic and endophytic population in maize plants inoculated with different growth promoting bacteria in greenhouse at 30 and 45 days. A – Absence of fertilization, B – Broadcast fertilization, P – Placement of fertilization, B + P – Broadcast and Placement of fertilization.

Epiphytically, at 30 days, split and broadcast fertilization resulted in larger bacterial populations for treatments control and inoculation with *A. brasilense* and *H. seropedicae*. The bacteria *B. ambifaria* was favored by placement of fertilization.

When in the absence of fertilization or in broadcast fertilization, plants inoculated with *A. brasilense* or *B. ambifaria* had larger microbial populations. On the other hand, placement of fertilization stimulated the bacterial population of plants inoculated with *B. ambifaria.* Plants inoculated with *H. seropedicae*, under the effect of split of broadcast and placement of fertilization, showed higher bacterial population on roots.

The endophytic population from control treatment was superior in plants cultivated with placement of fertilization, while *A. brasilense* and *H. seropedicae* with broadcast fertilization and *B. ambifaria* with association between broadcast and placement of fertilization.

At 45 days, roots collected from the control treatment in the absence of nitrogen fertilization, presented greater bacterial endophytic population while for the treatment of inoculation with *A. brasilense* these results appeared for the broadcast fertilization and, for the treatment with *B. ambifaria* and *H. seropedicae,* for the association between placement and broadcast fertilization.

In broadcast fertilization, *A. brasilense* is the largest epiphytic populations, while for *B. ambifaria* the large values were in the association between broadcast and placement of fertilization. For the endophytic population in the control treatments, the inoculation with *A. brasilense* and *B. ambifaria* showed higher bacterial populations when associated with broadcast and placement fertilization and in the absence of fertilization for *H. seropedicae.*

The absence of fertilization, broadcast fertilization and association between broadcast and placement of fertilization, resulted in larger populations of bacteria with potential for nitrogen fixation in plants belonging to the control treatment, while the placement of fertilization for plants inoculated with *B. ambifaria*.

Table 5 shows the data from the *in vitro* experiment and, when cultivated in culture medium in the absence of nitrogen, plants inoculated with *A. brasilense* were superior to the control treatment for the parameters aerial fresh mass and aerial length. For the parameters root length and foliar nitrogen content, plants inoculated with *A. brasilense* were statistically superior to the other tested bacteria, not without differing from the control. Plants inoculated with *B. ambifaria* showed statistically superior parameters to the control for root fresh and dry mass.

Table 5. Effect of inoculation with growth promoting bacteria in culture medium with presence of nitrogen in the laboratory at 7 days (Aerial fresh mass – AFM, Aerial dry mass – ADM, Root fresh mass – RFM, Root dry mass – RDM, Aerial length – AL, Root length – RL, Nitrogen content – N%).

Bacteria	AFM	ADM	RFM	RDM	AL	RL	N%
Control	248.0 b	26.7 b	926.0 b	242.0 a	9.3 b	13.1 a	13.0 a
A. brasilense	351.0 a	31.0 ab	863.0 b	232.0 a	12.0 a	15.6 a	11.9 a
B. ambifaria	320.0 ab	38.8 a	1118.0 a	230.5 a	9.3 b	7.8 b	6.6 b
H. seropedicae	295.0 ab	33.9 ab	891.7 b	233.0 a	8.3 b	5.7 b	8.0 b

* lowercase letters represent the difference between rows.

The conditions obtained *in vitro,* of ideal temperature and humidity, in addition to a specific culture medium and absence of external determinant factors, allow the inoculation to may result in distinct characteristics from the ones obtained in the field. However, this information is essential for the initial characterization of the bacterial activity, thus identifying the microorganisms with potential to be growth promoters in non-legumes.

The bacterial population in vitro (Figure 2), showed no bacteria in the control treatment, guaranteeing the efficiency of the seed sanitization process. Concerning the treatments with bacterial presence, plants inoculated with *A. brasilense* showed a higher epiphytic and endophytic bacterial population in the analyzed roots.

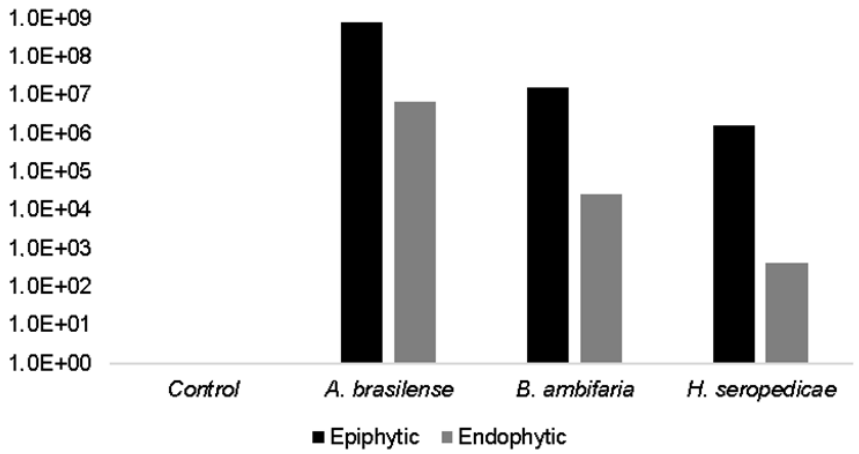

Figure 2. Epiphytic and endophytic population in maize roots inoculated with different plant growth promoting bacteria grown in vitro.

The bacterial population in the roots confirms the previous questioning, where all the epiphytic bacterial populations were larger than the endophytic ones, reinforcing the hypothesis that the bacteria can have a mechanism similar to that occurring in legumes (Glyan'ko et al. 2009), not yet defined.

Plant growth promoting bacteria act in the development of plants through the production of metabolites (Glick 2015). The high nutritional concentration may cause the use of energy for the realization of compound producing processes, as the nitrogen biological fixation, to be useless, causing the organism to stablish itself but not necessarily associate with the roots, not acting in the plant development.

This characteristic is similar to the one that occurs in symbiotic processes, where legumes inoculated with *Rhizobium* spp. in soils with high concentration of nitrogen, recognize the organism as a pathogen, reducing the nodules formation and, consequently, biological nitrogen fixation (Glyan’ko et al. 2009).

Dartora et al. (2013) using *A. brasilense* and *H. seropedicae* in maize, isolated and combined, highlight that doses of nitrogen did not influence in the action of the diazotrophic bacteria, however, the combined use of the bacteria resulted in an increase of morphological characteristics of interest as dry mass and final yield. The authors point out that the efficiency of bacterial colonization in roots is associated to biotic and abiotic factors, which directly influence in the final efficiency of inoculum use.

Rice plants inoculated with *H. seropedicae* and *B. ambifaria* cultivated under rainfed conditions, with different doses of nitrogen fertilizer, demonstrated that the use of plant growth promoting bacteria increases aerial dry mass and nitrogen content (Guimarães et al. 2010), as exposed in the present work.

Analyzing the different bacterial strains with broadcast fertilization, it is highlighted that the plant length was statistically higher than the control for all the tested bacteria. The results obtained in this work continue to be in harmony with the work of Dartora et al. (2013) and Guimarães et al. (2010), whose use of plant growth promoting bacteria acts positively in different parameters of plant development.

The test of *H. seropedicae* in maize, allied to different nitrogen doses, resulted in an increase of nitrogen foliar content and better use of the nitrogen applied. The present work did not evaluate final yield of the crop, not even the nitrogen content of distinct parts of the plant, but the use of *H. seropedicae* resulted in higher levels of nitrogen in the leaves, which during the physiological development are translocated to the seeds, as occurred in the work of Araújo et al. (2013).

The absence of fertilization or broadcast fertilization resulted in a greater endophytic population in plants inoculated with *H. seropedicae,* while the treatment that associated broadcast and placement of fertilization had greater populations in plants inoculated with *B. ambifaria.*

The ability to colonize plant tissue directly influences the contribution of the bacteria in the processes that participate in the plant organism, and the exchange between the two organisms happens directly (Baldani et al. 1997). Thus, bacteria with a greater potential for endophytic colonization may result in greater effects on the growth promotion of crops of interest, as is the case of *A. brasilense*, widely cited in the current literature.

In rice plants, evaluating the variety of bacteria belonging to the genus *Herbaspirillum* and the genus *Burkholderia*, Rodrigues et al. (2006) concluded that bacteria from the genus *Burkholderia* are present in a greater quantity in the rice varieties tested, and that the population of plant growth promoting bacteria in the roots varies according to the crop development stage.

The different populations found throughout the development of the culture, point out the results found by Rodrigues et al. (2006). Using different diazotrophic microorganisms in wheat, Sala et al. (2005) reported that the interaction between plant genotype and nitrogen fertilization influences the amount of PGPB present in maize roots.

Evaluating the nitrogen distribution in the plant, together with the colonization by *Herbaspirillum* spp. and *Glucinacetobacter diazotrophicus*, Gomes et al. (2005) concluded that there is a difference in the nitrogen content concentration in different parts of the plant and in different genotypes during its development, however, the population of *Herbaspirillum* spp. is higher in roots regardless of these factors.

The inoculation with *A. brasilense* reflected in the accumulation of fresh and dry mass in the plants analyzed, as well as the work developed in the field of Dartora et al. (2013). The inoculation with *B. ambifaria*, operated mainly on aerial parameters, as occurred in the work of Guimarães et al. (2010). *H. seropedicae* did not stand out in the *in vitro* development, leading to the questioning that, if in ideal conditions, this microorganism chooses not to spend its energy colonizing the roots, as happened with *Rhizobium* spp. in legumes in the work of Glyan'ko et al. (2009).

In conclusion, the use of plant growth promoting bacteria associated to nitrogen fertilization positively affects the parameters aerial fresh and dry mass, aerial length and foliar nitrogen content in the initial development of maize, cv PIONNER® 30F53 YH, under greenhouse conditions. *A. brasilense* is more responsive to placement of fertilization, while *H. seropedicae* for the association between placement and broadcast fertilization, or else the absence of fertilization. Yet, *B. ambifaria* is responsible to the absence of fertilization. Bacterial populations in protected culture varied according to the fertilization used and the period of collection. *In vitro*, the bacterial population was higher epiphytically.

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