Original Research Article

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Effect of *Azotobacter beijerinckii* from *Zea mays* var *mexicana* and *Gluconacetobacter diazotrophicus* from *Stenocereus queretaroensis* on *Phaseolus vulgaris* at 50% NH_4NO_3 Prevents the Release of N_2O in the Soil

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Abstract: For the healthy growth of *Phaseolus vulgaris* (common bean), nitrogen fertilizers such as NH_4NO_3 are required. Excess nitrogen fertilizer, that is not readily uptaken by plant roots, induces the mineralization of soil organic matter, consequently leading to a loss of biodiversity and decreased soil fertility. Furthermore, it contaminates surface and groundwater with the emission of N_2O (nitrous oxide), a greenhouse gas that contributes to global warming. An ecological option to avoid the excess NH_4NO_3 that causes these negative effects is the use of plant growth-promoting bacteria genera and species: *Azotobacter beijerinckii and Gluconacetobacter diazotrophicus*. Both induce maximum NH_4NO_3 uptake at 50% from the seed stage through phytohormonal action. The objective of this research was to analyze the response of *P. vulgaris* to *A. beijerinckii* and *G. diazotrophicus* at 50% NH_4NO_3. For this purpose, the endophytic *A. beijerinckii* was isolated from the roots of *Zea mays* var. mexicana (teosinte), and *G. diazotrophicus* was isolated from the cactus *Stenocereus queretaroensis*. Seeds of *P. vulgaris* were inoculated individually and with the mixture of both endophytic genera and species using 50% NH_4NO_3 in a randomized block design. The response variables were: germination percentage, phenology, plant height

and root length, and biomass: fresh and dry weight of the aerial and radical, compared to uninoculated *P. vulgaris* fed with 100% NH_4NO_3, that was used as a relative control. The experimental data were analyzed using ANOVA/Tukey HSD ($P < 0.05$). The results showed that regardless of the origin of *A. beijerinckii* and *A. diazotrophicus*, the response of *P. vulgaris* was positive at both germination and seedling stages. This supports two points: first, that plant metabolism in higher plants is similar, since *A. beijerinckii* induced actions involving phytohormones at both germination and seedling stages; and second, that *A. diazotrophicus* was able to colonize plants that are not rich in sugars without problems, thus expanding the usefulness of both genera to just domestic gramines At the same time, by maximizing NH_4NO_3, it prevents soil degradation, environmental pollution, and N_2O generation global warming. For an environmental health conditions for all living things,

Keywords: Soil; Plant metabolism; Nitrogen fertilizer; Beneficial bacterial endophytic genera; Climate change

1. Introduction

In Mexico, *Phaseolus vulgaris* is a legume consumed by farmers. *P. vulgaris* requires NH_4NO_3 for healthy growth. Excess NH_4NO_3 causes rapid mineralization of soil organic matter, leading to a loss of fertility. It is also responsible for the release of greenhouse gases such as nitrous oxide (N_2O). One possible solution to avoid excess NH_4NO_3 in *P. vulgaris* is inoculation with *Azotobacter beijerinckii*, an endophyte from *Zea mays* var. *mexicana* (teosinte), an gramine of modern *Z. mays*, and *Gluconacetobacter diazotrophicus*, an endophyte from *Stenocereus queretaroensis*, a common cactus plant in desert areas. Both plants have different photosynthetic systems. Through evolution, endophytes have adapted to all types of vegetation, that explains the success of endophytes isolated from wild plants and applied to domestic ones (1-5). A wide range of genera and species of bacterial endophytes can help reduce and optimize NH_4NO_3 use by up to 50% (6,7). The literature indicates that genera of endophytic plant growth-promoting bacteria can utilize organic compounds generated during the germination of legume seeds such as *P. vulgaris*, as well as those released during root growth, converting them into phytohormones: auxins, cytokinins, gibberellins, etc (8-10). This accelerates germination and promotes the production of abundant root hairs to optimize NH_4NO_3 uptake, minimize soil fertility loss, water and soil pollution, and N_2O generation, thus mitigating global warming, a global problem (11-13). Based on the above, the objective of this research was to analyze the response of *P. vulgaris* to *A. beijerinckii* and *G. diazotrophicus* with 50% NH_4NO_3.

2. Materials and Methods

This research was conducted in the Environmental Microbiology Laboratory Biological Chemical Research Institute at UMSNH, Morelia, Michoacán, México. The studies were carried out in a greenhouse under the following environmental conditions: a temperature of 23°C, a light intensity of 450 µmol m^2/s, and a relative humidity of 67%.

Origin and preparation of available soil

The soil used in this trial came from an agricultural field at the site known as "La Cajita", belonging to Tenencia Zapata, in the municipality of Morelia, Michoacán, México, at km 5 of the Morelia-Pátzcuaro highway. A sodic lateritic soil with a silty loam texture was used, with a poor total nitrogen concentration of 0.10%, poor phosphorus availability of 0.07%, a slightly acidic pH of 6.64, poor organic matter content of 1.20%, poor cation exchange capacity of 10.5, and a field capacity of 30.08 (14). The soil was sieved and solarized for 3 days to minimize the risk of pests and diseases. Subsequently, 1.0 kg of soil was weighed and placed in the upper half of the Leonard jar, shown in **Figure 1**: the mineral solution containing 50% NH_4NO_3 was placed in the lower half of the Leonard jar containing *P. vulgaris* with *A. beijerinckii* and *A. diazotrophicus*, and *P. vulgaris* with water and mineral solution containing 100 % NH_4NO_3. Both portions were connected with a 25 cm long cotton strip for capillary movement of the mineral solution, according to the experimental design in **Table 2**.

Table 1. Physicochemical properties of the soil for trial at greenhouse conditions

Parameter	Value /interpretation
Total nitrogen (%)	0.10 poor
Available Phosphorus (%)	0.07 poor
pH	6.64 light acid
Organic material (%)	1.20 poor
Cation exchange capacity (Cmol(+) Kg^{-1})	10.5 poor
Texture (%)	20.56 (clay)-37.8 (silt)-0.76(sand)
Field capacity (%)	30.08

sand, Si: silt, Cl: clay. Physicochemical parameters for agricultural soils according to literature (14)

2.1. Isolation of endophytic *A. beijerinckii* from roots of *Zea mays* var *mexicana* (teosinte)

Z. mays var *mexicana* (teosinte) was collected from agricultural soil near Morelia, Michoacán, México. The root system was taken and the soil was removed with potable water; a 5 cm cut was made from the main root with scissors disinfected with 3% (v/v) sodium hypochlorite for 10 min, then 10 min with 70% (v/v) alcohol; afterwards it was washed six times with sterile potable water, again with 70% (v/v) alcohol for 5 min and washed six times with sterile water; then 2 cm of the root was placed in a sterile mortar, 5 mL of 0.85% (w/v) NaCl saline solution and 1.0 mL of 0.1% commercial detergent were added: then the root was crushed, and with a sterile pipette 1.0 mL was taken and inoculated into Burk broth (g/L): glucose 10.0; K_2HPO_4 2.0; KH_2PO_4 2.0; $MgSO_4$ 3.0; with a trace element solution 1 mL/L of culture medium, with the following composition (g/L): H_3BO_3 2.86; $ZnSO_4$ $7H_2O$ 0.22; $MnCl_2$ $7H_2O$ 1.81; K_2MnO_4 0.09 at pH 6.4-6.7; Bromothymol blue 1% (w/v) 10 mL/L of culture medium was added, the pH was adjusted to 6.7 and fungal growth was inhibited with the antifungal Tecto 60® (Syngenta) 2% (w/v) 1.0 mL/L of culture medium. After incubation for 48 h at 30°C, 18 g/L of agar was added to Burk broth to prepare Petri dishes. The growth observed in the Burk broth was streaked onto the Petri dishes and incubated for 48 h at 30°C to obtain axenic cultures of *A. beijerinckii*, which were then inoculated onto *P. vulgaris* seeds and biochemically identified (15-17).

2.2. Isolation of endophytic *G. diazotrophicus* from roots of *Stenocereus queratoerensis*.

Sections of 4 cm from the root of *S. queretaroensis*, rinsed with sterile tap water, disinfected with alcohol at 70%/2.5 min, then with NaClO at 10%/2.5 min; root was macerated in a mortar with commercial detergent at 0.01% mixing with saline solution or NaCl at 0.85% then 1.0 mL and sow i in LGI broth with this chemical composition (g/L): sucrose 100.0; K_2HPO_4 2. 0; KH_2PO_4 2.0; NaCl 1.0; $MgSO_4$ 3.0; yeast extract 1.0; 10 mL 2.0% (w/v) bromothymol blue and 10 mL/L of trace element solution with chemical composition (g/L^{-1}): H_3BO_4 2.86; $ZnSO_4$ 0.22; $MnCl_2$ 1.81; K_2MnO_4 0.09; pH was adjusted to 5.7. The cultivation media were incubated at 30°C/72 h; when was growth of *G. diazotrophicus* was reseeded on LGI agar to observe the microscopic and macroscopic morphological characteristics of these genera and species of endophytic bacteria on LGI agar, round and shiny colonies with a yellow intracellular pigment similar to that of *G. diazotrophicus* were observed [14]. Seeds were then disinfected in 70% (v/v) alcohol for 5 minutes and rinsed six times with deionized water. For every 10 *P. vulgaris* seeds, 1 ml of *A. beijerinckii* and *G. diazotrophicus* in a 1:1 ratio, suspended in a 0.85% saline solution and 0.01% detergent, were inoculated. Next, the inoculated seeds were agitated at 200 rpm for 30 minutes at 30°C, then sown in Leonard Jar, where 5 seeds per treatment and control were sown in 100 g of soil, soil that had been exposed to sunlight for 40h, and fed with a mineral solution with the recommended dose of nitrogen fertilizer as NH_4NO_3 for *P. vulgaris* in the following chemical composition (g/L): NH_4NO_3 10; K_2HPO_4, 2.5; KH_2PO_4, 2.0; $MgSO_4$ 0.5; NaCl, 0.1; $CaCl_2$, 0.1; $FeSO_4$, 1.0. The mineral solution was used to feed *P. vulgaris* with a volume of 10 ml to ensure field capacity at 80%. The inoculated *P. vulgaris* seeds were then sown in Leonard Jar until the seedling physiological stage. At the end of this period, phenology was measured: plant height (PH), root

length (RL), and leaves length (LH), while biomass was measured: aerial and root fresh and dry weight (AFW/RFW)/(ADW/RDW) of *P. vulgaris* (18,19). The experimental data were subjected to an analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$).

2.3. Recovery of *A. beijerinckii* from the spermosphere and rhizosphere of *P. vulgaris*

Isolation of *A. beijerinckii* from the interior of *P. vulgaris* roots: roots were washed and disinfected with 96% alcohol for 5 min, then washed seven times with sterile water. Pieces 5.0 cm in length were ground in a mortar with saline solution (0.85% NaCl) with 0.01% commercial detergent 1.0 mL was then used to dilute 10^{-2} and 10^{-3} and 0.2 mL was inoculated onto nutrient agar (g/L): Only antibiotic-free nutrient agar was used to recover *A. beijerinckii*, as the experiment was conducted in sterilized soil 121°C/60 min (12,13). All tests were performed in triplicate.

Table 2. Experimental design to analyze the effect of *A. beijerinckii* and *G. diazotrophicus* on *P. vulgaris* at 50% NH_4NO_3.

*P vulgaris	*A. beijerinckii*	*Gluconacetobacter diazotrophicus*	Water	NH_4NO_3
Absolute control (AC)	-	-	+	-
Relative control (RC)	-	-	-	100%
Treatment 1	+	-	-	50%
Treatment 2	-	+	-	50%
Treatment 3	+	+	-	50%

*Number of replications (n) = 6; applied (+); not applied (-).

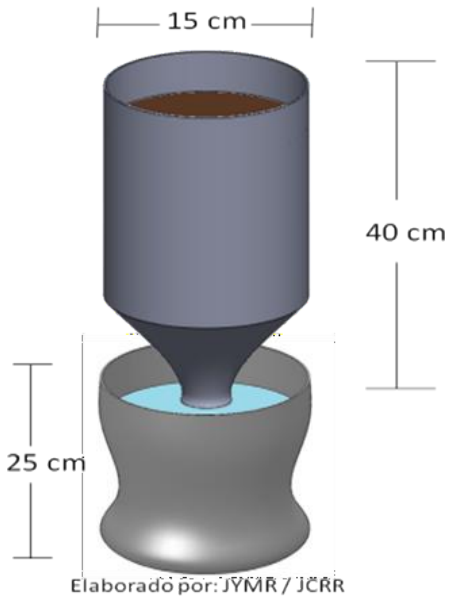

Figure 1. Diagram of the Leonard jar.

3. Results and Discussion

Table 3 shows the germination percentage of *P. vulgaris* with *A. beijerinckii* and *G. diazotrophicus* using 50% NH_4NO_3 in treatment 1, with 93.33% germination. This indicates that by inducing root formation in *P. vulgaris*, this genus and species invaded the interior of these young roots to utilize them. In contrast, in treatment 2, *P. vulgaris* with *G. diazotrophicus* plus 50% NH_4NO_3, germination was 83.33%, and in treatment 3, *A. beijerinckii* and *G. diazotrophicus* on *P. vulgaris* with 86.66% NH_4NO_3: these values were statistically different compared to the 76.66% germination of *P. vulgaris* with 100% *A. beijerinckii* and *G. diazotrophicus* uninoculated. This fact made clear that in the case of *A. beijerinckii*, it was able to invade the interior of young roots of *P. vulgaris* to utilize the organic compounds derived first from root metabolism and then from the initiation of photosynthesis into phytohormones (18-21). Meanwhile, it was demonstrated that *G. diazotrophicus*

can invade and reproduce inside *P. vulgaris*, a plant in that sucrose circulates in the phloem to facilitate the induction of young root growth (12,16). This, in turn, enhance better germination of *P. vulgaris* seeds compared to germination with 100% NH_4NO_3 doses. This makes it clear that the lack of these endophytic plant genera and species reduces the capacity of *P. vulgaris* to fully utilize the nutrients that promote maximum seed germination (21,22).

Table 3. Germination percentage of *P. vulgaris* with a *A. beijerinckii* and *G. diazotrophicus* and NH_4NO_3 at 50%.

*P. vulgaris**	Germination percentage
Absolute control irrigated with water	73.33[d]**
Relative control fed with NH_4NO_3 at 100% uninoculated	76.66[c]
Treatment 1 *G. diazotrophicus* + NH_4NO_3 at 50%	93.33[a]
Treatment 2 *A. beijerinckii* +NH_4NO_3 at 50%	83.33[b]
Treatment 3 *G. diazotrophicus* + *A. beijerinckii* + NH_4NO_3 at 50%	86.66[a]

*n = 6; **different letters indicate statistical difference according to ANOVA/Tukey (0.05).

Table 4. Colonization of the spermosphere/rhizosphere of **P. vulgaris* by *A. beijerinckii* and NH_4NO_3 at 50%.

Days after sowing	Log CFU x 10^6/g of seed or spermo-rhizophere
0*	4.7[b]**
3	5.1[a]
6	5.0[a]
10	4.3[b]
15	4.1[b]

*n = all values are average of 3 repetitions. **Different letters indicate statistical difference according to ANOVA/Tukey (0.05)

Table 4 shows the colonization dynamics of *A. beijerinckii* in the seed and NH_4NO_3 at 50%. It illustrates the invasion of this endophyte into the spermosphere-rhizosphere of *P. vulgaris*, where it was evident that the applied inoculum density of 4.7 x 10^6 CFU/g in the *A. beijerinckii* seed facilitated colonization of both the interior and exterior of young *P. vulgaris* roots. The transformation of root metabolites by *A. beijerinckii* generated growth phytohormones that increased the number of root hairs, optimizing and NH_4NO_3 at 50% uptake for a healthy increase in *P. vulgaris* (6,23). This ensures that there is no longer free and NH_4NO_3 at 50% that causes loss of fertility, contamination of surface water or aquifers, and prevents the generation of N_2O, thus mitigating the climate change problem caused by this gas during *P. vulgaris* cultivation (15-17) compared to what was observed in, uninoculated *P. vulgaris* with *A. beijerinckii* fed with 100% NH_4NO_3, it is evident that the *P. vulgaris* roots cannot uptake a sufficient amount of NH_4NO_3 to avoid the environmental damage caused by over-fertilization (11,21). Meanwhile, seed colonization by *G. diazotrophicus* after the spermosphere-rhizosphere of *P. vulgaris* with 50% NH_4NO_3 (data not shown) indicates that both endophytic genera and species can be useful for effectively regulating the reduced dose of NH_4NO_3 to prevent environmental problems (23,24). Therefore, both could be considered an ecological alternative for the sustainable production of *P. vulgaris* (25-27)

Table 5 shows the effect of *P. vulgaris* with *A. beijerinckii* and *G. diazotrophicus* on biomass, where a plant height (PH) of 35.8 cm, an aerial fresh weight (AFW) of 0.3618 g, radical fresh weight (RFW) of 0.370 g, aerial dry weight (ADW) of 0.1457 g, and radical dry weight (RDW) of 0.1457 g were registered. This is because both genera of endophytic bacteria, upon initially invading the seed and then the first roots, used organic compounds derived from seed metabolism, such as organic acids and amino acids (28,29). During leaf and root formation, they used other organic compounds derived from photosynthesis, which allowed the mobility of sucrose through the phloem (17,30). This enabled them to use sucrose as a source of carbon and energy in the synthesis of

other phytohormones that are transported by phloem from the roots to the foliage of *P. vulgaris* (1,2,31). Both *A. beijerinckii* and *A. diazotrophicus*, these phytohormones, enhance healthy growth of both the aerial and root parts, increasing both the fresh and dry weight of the foliage and roots with the 50% NH_4NO_3 dose (31-33). These values were statistically different AFW of 0.509g, RFW of 0.1231g, ADW of 0.509g, and RDW of 0.0542g in *P. vulgaris* fed with 100% NH_4NO_3 or uninoculated used as a RC. This demonstrates that colonization of *P. vulgaris* by endophyte genera is crucial for maximizing NH_4NO_3 uptake, preventing over-fertilization (34,35), that is responsible for soil fertility loss and contamination of surface and groundwater by NO_3 not uptaken by the *P. vulgaris* root system (9,35). In the soil, this remaining NO_3^- can be anaerobically converted to N_2O, a greenhouse gas that causes global warming (4,7). This makes the use of *A. beijerinckii* and *A. diazotrophicus* even more important for optimizing the 50% dose of NH_4NO_3, an additional biological alternative to the use of plant growth-promoting bacteria in sustainable agricultural production (8,10,17,34).

Table 5. Response of *P. vulgaris* to *A. beijerinckii* and *G. diazotrophicus* to 50% NH_4NO_3 at seedlings

*P. vulgaris**	Plant height (cm)	Length root (cm)	Aerial fresh weight (g)	Radical fresh weight (g)	Aerial dry weight (g)	Radical dry weight (g)
Absolute control: irrigated water uninoculated	25.16[b]**	10.85[d]	0.380[c]	0.280[c]	0.110[d]	0.030[c]
Relative control (RC) NH_4NO_3 at 100% uninoculated	22.74[b]	11.1[c]	0.409[a]	0.509[a]	0.123[c]	0.054[a]
Treatment 1 *G. diazotrophicus* + NH_4NO_3 at 50%	25.82[b]	11.04[c]	0.383[b]	0.384[b]	0.140[b]	0.038[b]
Treatment 2 *A. beijerinckii* + NH_4NO_3 at 50%	24.06[b]	12.48[b]	0.235[d]	0.235[d]	0.147[a]	0.039[b]
Treatment 3 *G. diazotrophicus* + *A. beijerinckii* + NH_4NO_3 at 50%	35.8[a]	21[a]	0.361[b]	0.370[b]	0.145[a]	0.034[b]

*n = 6; **different letters indicate statistical difference according to ANOVA/Tukey (0.05)

4. Conclusion

It was evident that *A. beijerinckii*, an endophyte of *Z. mays* var. *mexicana* (teosinte), the ancestor of modern *Z. mays* and commonly considered to induce healthy grass growth, as well as *G. diazotrophicus*, isolated from the cactus *S. queretaroensis* and recommended for sucrose-rich plants with sugarcane (*Saccharum officinarum*), were also effective in colonizing the roots of *P. vulgaris*. This confirms that the sugar content of higher plants, according to the type of photosynthesis that distinguishes gramines from legumes, was not a factor preventing both *A. beijerinckii* and *A. diazotrophicus* from having a positive interaction with *P. vulgaris* to colonize the root system. This optimized NH_4NO_3 to 50%, thus preventing the decrease in soil fertility, the contamination of surface water or aquifers, and the generation of N_2O, thereby mitigating climate change.

Acknowledgments

To the Coordinación de Investigación Científica de la UMSNH "Aislamiento y selección de microorganismos endófitos promotores de crecimiento vegetal para la agricultura y biorecuperación de suelos" from the Research Project (2026), Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México. To Phytonutrimentos de México and BIONUTRA S, A de CV, Maravatío, Michoacán, México for the *P. vulgaris* seeds and verification of greenhouse tests.

Conflicts of Interest

The authors declare no conflicts of interest.

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