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Response of *Phaseolus vulgaris* to the leaf endophyte *Stenotrophomonas rhizophila* at reduced dose of nitrogen fertilizer

Abstract

The healthy growth of *Phaseolus vulgaris* demands NH_4NO_3, applied in excess it causes loss of soil productivity and environmental pollution. An alternative solution is to reduce NH_4NO_3 to 50% and inoculate the seed with an endophytic bacterial genus and species: *Stenotrophomonas rhizophila.* The objective of this research was to analyze the effect of *S. rhizophila* on the growth of *P. vulgaris* and NH_4NO_3 at 50%. There isolated *S. rhizophila* from *P. vulgaris* leaves and then was inoculated into *P. vulgaris* seeds with NH_4NO_3 at 50%. Under a randomized block experimental design: 2 controls: *P. vulgaris* with 100% NH_4NO_3 uninoculated or relative control (RC), NH4NO3 irrigated only with water or absolute control, *P. vulgaris* with *S. rhizophila* and NH_4NO_3 at 50%. Through the response variables: germination percentage; Seedling phenology: plant height and root length, biomass: fresh and dry aerial weight, fresh and dry radical weight. The numerical values of the results were analyzed by Tukey, $p < 0.05$. The results showed a positive effect of *S. rhizophila* on the germination of *P. vulgaris* compared to *P. vulgaris* uninoculated and NH_4NO_3 at 100%. A *S. rhizophila* seedling with NH_4NO_3 at 50% had a beneficial effect on *P. vulgaris* in plant height and root length, as well as fresh and dry aerial and radical weight. This data supports that *S. rhizophila* colonized the interior of the roots of *P. vulgaris* by converting organic compounds from the root metabolism into phytohormones and optimizing NH_4NO_3 at 50% Compared to the growth of *P. vulgaris* uninoculated with at 100%. The above indicates that there is a biological option to conserve soil fertility preventing greenhouse gas, without compromising the healthy growth of *P. vulgaris*. At the same time avoid any kind of soil pollution due to NO_3^- excess did not uptake by the root system of the plant. For cropping *P. vulgaris* under sustainable agriculture.

Keywords: soil, legume roots, nitrogen fertilizer, endophytic beneficial bacteria, sustainable agriculture

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Introduction

Healthy growth of *Phaseolus vulgaris* requires NH_4NO_3, but when applied to the soil, in excess, causes loss of fertility, because the nitrogen in NH_4NO_3 is not uptaken by the *P. vulgaris* root system, causing the disappearance of soil organic matter, as well as the NO_3^- being converted into (N_2O) nitrogen oxide, a greenhouse gas that contributes to global warming.[1,2] An alternative ecological solution is to inoculate *P. vulgaris* seeds with *Stenotrophomonas rhizophila*, a beneficial endophyte of legume leave that exerts a positive action on uptake foliar or root NH_4NO_3 and can solubilize PO_4^{-3} (phosphates) to optimize them.[3–5] Although it is believed that when *P. vulgaris* seed is inoculated with *S. rhizophila* and NH_4NO_3 is applied to the soil,[5–7] it could be better, due to the environmental conditions of that environment,[6–8] that are more conducive to that action, compared to what happens in the soil, however, information is limited on the inoculation of *P. vulgaris* seed with *S. rhizophila* compared to foliar *S. rhizophila* inoculation and subsequent application of NH_4NO_3 to the same leaf.[9–11] Therefore, the objectives of this work were to analyze i) the isolation of *S. rhizophila* from *P. vulgaris* leaves, ii) the effect of *S.rhizophila* on the growth of *P. vulgaris* and 50% NH_4NO_3. iii) the identification of *S. rhizophila* isolated from leaves of *P. vulgaris*

Materials and methods

This research was conducted in the Laboratory and Greenhouse of Environmental Microbiology, Institute of Chemical Biological Research, Universidad Michoacana of San Nicolas de Hidalgo, Morelia, Mich, México.

Origin of *Stenotrophomonas rhizophila.*

This genus and endophyte species were isolated from *P. vulgaris* leaves. For this, 4 healthy leaves of *P. vulgaris* were washed with soap and water to remove excess soil or then cut into 1.0 cm pieces, placed in a 50 ml beaker, disinfected with 15.0 ml of 0.5% hypochlorite/1 min, then the leaves were rinsed with sterile tap water 5 times, then disinfected in 15.0 ml of 70% ethanol/1 min, rinsed 5 times with sterile tap water. The pieces of *P. vulgaris* leaves were placed in a sterile 50 ml mortar with 9.0 ml of sterile saline solution (NaCl 0.85%) and 0.1% commercial detergent powder (Roma) after crushing the leaves was inoculated 0.1 ml on selective agar for *S. rhizophila* with the following chemical composition: (g / L): xylose 10, casein peptone 5, yeast extract 3, NaCl 5, adjusted pH of 6.7 according to the differences in growth observed during isolation from the leaves were classified as C1 and C2 after incubation at 30°C for 72 h. Axenic colonies were observed under a microscope stained by Gram, were observed.[3–5,7–9]

For conservation, both isolated were suspended in a 18 x 150 tube with sterile saline solution to have an approximate concentration of 1.0 x 10^8UFC/ml on the McFarland scale and then placed in sterilized agricultural soil (1.30h at 121°C). Tubes were incubated for 48 h and then the purity and viability were checked in the selective medium for *S. rhizophila*. The soil with the *S. rhizophila* isolates was kept in a refrigerator at 5°C until use. To activate, 0.1 g of soil with *S.*

rhizophila was taken and sown in 2 tubes of selective broth for *S. rhizophila* and incubated at 30°C/72h. Purity was ensured by Gram staining before inoculating *P. vulgaris*.[2,6,7]

To inoculate *P. vulgaris*, the seeds were disinfected with 5% sodium hypochlorite/5 minutes, rinsed with sterile tap water 6 times, then disinfected with 70% alcohol/5 minutes and rinsed 6 times with sterile water. The suspension of each *S. rhizophila* C1 and C2 was adjusted to 1 x 10^8CFU/ml to inoculate 10 seeds of *P. vulgaris,* in plastic bags seeds were left in contact for 30 minutes and then 5 seeds were planted per Leonard jar, according to the experimental design in Table 1 as well as Figure 1 shown the isolation and inoculation of *S.rhizophila* in *P. vulgaris* and 50% NH_4NO_3.[9–11]

Table 1 Experimental design of the response of *Phaseolus vulgaris* to *Stenotrophomonas rhizophila* and 50% NH_4NO_3

Phaseolus vulgaris*	**Isolated *S. rhizophila C1***	**Isolated *S. rhizophila C2***	**NH_4NO_3**
Absolute control (AC)	–	–	Water
Relative control (RC)	–	–	100%
Treatment 1 (T1)	+	–	50%
Treatment 2 (T2)	--	+	50%
Treatment 3 (T3)	+	+	50%

*n= 6 repetitions, (+) = applied, (–) =no applied.

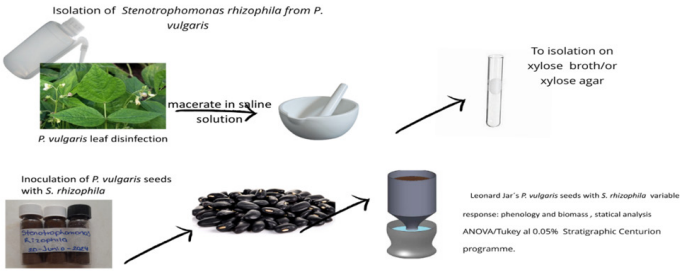

Figure 1 Isolation and inoculation of *Stenotrophomonas rhizophila* in *Phaseolus vulgaris* at 50% NH_4NO_3.

Sowing *Phaseolus vulgaris* with *Stenotrophomonas rhizophila*

Seeds inoculated with *S. rhizophila* were sown in 1 kg of agricultural soil in Leonard jars according to the experimental design shown in Table 1. This soil was collected from a site located at 19° 41 '23.5 " north latitude 101° 15' 00.5" west longitude, with an altitude of 1920 over the sea, from the facilities of the Center for the Research of Resources Institute of Morelia, Michoacán, México, this soil was sifted with a No. 20 mesh and solarized for 48 h to reduce pests and diseases. This soil was classified as loam according to NOM-021. RECNAT-2000.Soil is added to Leonard's jars are pots where 5 seeds were sown per jar, and of these, 6 replicates were used per treatment. The number of plants per treatment was 30, sufficient for statistical analysis. *P. vulgaris* seeds were inoculated with *S. rhizophila* isolates, which were fed with 40 mL/Kg of soil with the 50% mineral solution, with a field capacity of 80%. The response variables of the effects of *S. rhizophila on P. vulgaris* were germination percentage; during germination, the seeds that emerge are observed; these seeds are never touched until they reach the seedling stage, its added water to the jars to separate the roots before measuring and weighing them. The damage is minimal, this procedure is similar for all treatments, while phenology: seedling height (PH) and root length (RL); as well as biomass: aerial and root fresh and dry weight (AFW/RFW) and (ADW/RDW) at the seedling level.[5,6] The numerical data of the results were statistically analyzed by ANOVA/Tukey at 0.05% with the Stratigraphic Centurion program.[7,10]

Biochemical, physiological and genetic identification besides the microscopic and macroscopic morphology Gram reaction as well as colonial growth was observed at 4 °C, 30°C and 37 °C; tolerance to 1, 2, and 3% NaCl. While the biochemical profile: catalase, gelatinase, and amylase, etc. according to serval works reported in literature.[5,7,9] All isolates were able to growth on selective agar for *S. rhizophila* with the following chemical composition: (g / L): xylose 10, casein peptone 5, yeast extract 3, NaCl 5, adjusted pH of 6.7. While genetic tests of the isolates were sequential analysis of the 16S rRNA gene, PCR to purify and sequence rRNA products by Macrogen Inc. (South Korea). The rRNA low subunit gene was amplified from DNA by extraction with 16S rRNA gene-specific oligonucleotide primers 8F (5'-3': AGAGTTT- GATCCTGGCTCAG) and 1492R - (5'-3': GGTTACCTTGTTACGGACTT), based on nucleotide sequences of the isolates compared with homologous sequences from the GenBank database, the BLAST program for phylogenetically close species.[3,4,7,9]

Results and discussion

Table 2 shows the positive effect of S. *rhizophila* C1 and C2, individually or in mixture, on the germination of *P. vulgaris* seeds with NH_4NO_3 at 50%, with 90%, this value was statistically different compared to the 60% germination of the non-inoculated *P. vulgaris* seed used as RC. This result shows that *S. rhizophila* invades the seed to induce greater and faster germination by synthesizing phytohormones that accelerate the rapid formation of what will be the aerial part of *P. vulgaris* and at the same time the growth of the root,[12–15] that uses the conduction system of the legume to reach the entire plant, at the seedling level disinfection was performed, grinding of stems and leaves to detect the existence of *S. rhizophila*[15,16] (data not shown), that shows that it can be inoculated into the seed without problem to reach the foliar part which could help the defense against foliar phytopathogens,[17–19] by colonizing this tissue an additional benefit to the capacity for the solubilization of PO_4^{-3} as well as optimization of NH_4NO_3, reduced to 50% an advantage over *Rhizobium etli,*[20–22] that although it contributed to the healthy growth of *P. vulgaris* it does not have protective activity against phytopathogens, a property of *S. rhizophila* interesting to exploit in order not to not only reduce and optimize NH_4NO_3, but also prevent the attack of root and foliar phytopathogens.[23,24–26]

Table 2 Effect of *Stenotrophomonas rhizophila* on germinating *Phaseolus vulgaris* with 50% NH_4NO_3

Phaseolus vulgaris	**Germination percentage**
CA: P. vulgaris + water	50.0 ^{c*}
RC: P. vulgaris + NH_4NO_3 100%	60.0 ^{b}
T1: P. vulgaris + *S. rhizophila* C1 + NH_4NO_3 50%	90 ^{a}
T2: P. vulgaris + *S. rhizophila* C2 + NH_4NO_3 50%	90.0 ^{a}
T3: P. vulgaris + *S. rhizophila* C1 + *S. rhizophila* C2 NH_4NO_3 50%	90.0 ^{a}

*n=6** Different letters indicate statistical difference by ANOVA/ Tukey HSD P<0.05%.

In Figure 2, the large and more abundant roots of *P. vuglaris* with *S. rhizophila* are observed, supports that *S. rhizophila,* when invading the root system, synthesized sufficient phytohormones for the optimization of NH_4NO_3 reduced at 50%,[10–14] that ensured healthy growth without risk of excess NH_4NO_3 that would cause loss of organic matter,[13] release of greenhouse gases, and/or contamination of surface water or aquifers,[10,14–16] compared to the response observed

in *P. vulgaris* not inoculated with 100% NH_4NO_3, where it was evident that the stem and leaves were smaller and less intense in color despite having the maximum dose of NH_4NO_3 and PO_4^{-3}, that demonstrated that *P. vulgaris* was unable to sufficiently uptake nitrogen fertilizer, consequently that fertilizer causes loss of organic matter, release of greenhouse gases, and contamination of surface water or aquifers.[27–30]

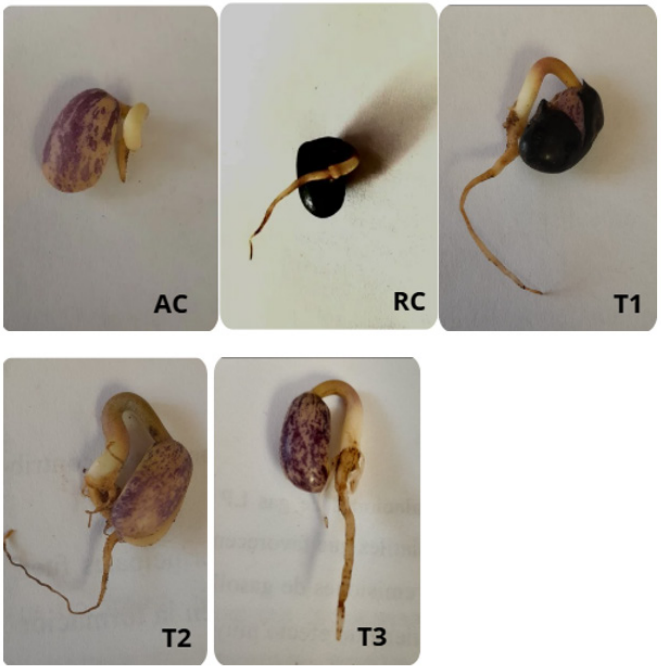

Figure 2 Effect of *Stenotrophomonas rhizophila* inoculation on germinating *Phaseolus vulgaris* with 50% NH_4NO_3.

AC=*P. vulgaris* + water,

RC= *P. vulgaris* + NH_4NO_3 at 100%,

T1= *P. vulgaris* + *S. rhizophila C1* + NH_4NO_3 at 50%,

T2= *P. vulgaris* + *S. rhizophila C2* + NH_4NO_3 at 50%

T3=*P.vulgaris* + *S. rhizophila C1* + S. *rhizophila C2* + NH_4NO_3 at 50%.

Table 3 shows the positive effect of *S. rhizophila* C1 and C2 on *P. vulgaris* at the seedling level with 50% NH_4NO_3, that registered 24.9 cm in PH, 12.45 cm in RL, 1.26 g AFW, 0.100 g RFW; as well as the positive effect of *S. rhizophila C2 on P. vulgaris* with NH_4NO_3, with 3.32 g in ADW and 0.80 g in RDW. Compared to the data of the relative control that corresponds to uninoculated *P. vulgaris* fed at 100% with NH_4NO_3, with PH= 21.65 cm, RL=11.61 cm, AFW= 2.08 g RFW, =0.71 g, ADW=0.22 g and RDW=0.047 g, that were statistically different. This effect is attributed to the stimulation of root growth, *S. rhizophila* inside vegetal tissue, can promote root development and branching, that improves water and NH_4NO_3 uptake;[31–33] in the synthesis of phytohormones, *S. rhizophila* can move from root system to aerial tissues[21–24] to influence positively to plant health growth;[24-28] improved of NH_4NO_3 availability, *S. rhizophila* can solubilize PO_4^{-3}, that are not available to root system.[29–32] The above demonstrated that *S. rhizophila* can be inoculated into *P. vulgaris* seeds, whereby invading the interior of the root system by utilizing organic compounds derived from the root metabolite,[33–35] *S. rhizophila* transformed them into phytohormones that optimize NH_4NO_3, reduced to 50%, as well as the solubilization of PO_4^{-3}, for healthy plant growth.[36–39] In addition to preventing the possible release of N_2O from free NO_3^- from NH_4NO_3, due to the physicochemical conditions of the soil, that contributes to global warming.[40]

Figure 3 shows the effect of S. rhizophila on the phenology of *P. vulgaris* with 50% NH_4NO_3 at the seedling level, where it was evident that in the aerial part there were more larger leaves when inoculated with isolate C1 or T1 and C2 or T2 individually, while it was even better when both C1 and C2 or T3 were mixed, similarly a denser root system was observed in *P. vulgaris* induced by C1 or T1 and C2 or T2 and even more evident when C1 and C2 were mixed, because both isolates colonized the interior of the root system from where both isolates of *S. rhizophila* C1 and C2 converted organic compounds of the root metabolism into phytohormones that optimized the NH_4NO_3 reduced to 50%.[20, 21,31,32] Then through the conduction system they reached the leaf part where both isolates were detected (data not shown), there it was observed as a result of the maximum uptake of NH_4NO_3.[27,34,37] These plants reached a plant height, root length, and number of leaves higher than *P. vulgaris* fed with the 100% dose of NH_4NO_3. The above demonstrates that *S. rhizophila* is an option that can be excellent for the healthy growth of *P. vulgaris* comparable to that reported with *R. etli*.[28,29,36] In this sense, inoculating P. vulgaris with S. rhizophila allows to reduce the dose of NH_4NO_3, to avoid excess fertilizer that contributes to the loss of soil fertility,[38–40] the contamination of surface water such as aquifers as well as the generation of N_2O from NH_4NO_3, consequently mitigating climate change that negatively affects plant life on Earth.[17,27,28,33]

Table 3 Effect of *Stenotrophomonas rhizophila* in on the phenology and biomass of *Phaseolus vulgaris* and 50% NH_4NO_3

Phaseolus vulgaris	**Plant heigth(cm)**	**Radical lenght (cm)**	**Fresh aerial weight (g)**	**Radical fresh weight (g)**	**Aerial dry weight (g)**	**Radical dry weight (g)**
AC: P. vulgaris + water	18.46 ^{c**}	8.13^{c}	1.52^{c}	0.38^{d}	0.21^{c}	0.047^{c}
RC: P. vulgaris + NH_4NO_3 at 100%	21.65 ^{b}	11.61^{b}	2.08^{b}	0.71^{c}	0.22^{c}	0.034^{d}
T1: P. vulgaris + *S. rhizophila* C1 + NH_4NO_3 at 50%	23.93^{b}	12.21^{a}	3.11^{a}	0.91^{bc}	0.59^{b}	0.064^{b}
T2: P. vulgaris + *S. rhizophila* C2 + NH_4NO_3 at 50%	23.93^{b}	12.39^{a}	3.32^{a}	1.22^{ab}	0.80^{a}	0.094^{ab}
T3: P. vulgaris + *S. rhizophila* C1 + C2 NH_4NO_3 at 50%	24.9^{a}	12.45.^{a}	2.93^{ab}	1.26^{a}	0.42^{b}	0.100^{a}

*n=6; **Different letters indicate statistical difference by ANOVA/Tukey HSD P<0.05%.

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Figure 3 Effect of *Stenotrophomonas rhizophila* on *Phaseolus vulgaris* with 50% NH_4NO_3 at seedling stage.

AC = *P. vulgaris* + water, RC= *P. vulgaris* + NH_4NO_3 at 100%,

T1= *P. vulgaris* + *S. rhizophila C1* + NH_4NO_3 at 50%,

T2= *P.vulgaris* + *S. rhizophila C2* + NH_4NO_3 at 50%,

T3=*P.vulgaris* + *S. rhizophila C1* + *S. rhizophila C2* + NH_4NO_3 at 50%.

Biochemical and genetic characterization of *S. rhizophila* isolates from *P. vulgaris* leaves.

P. vulgaris leaf isolates were characterized as heterotrophic aerobic growers at temperatures between 5 and 30 °C. The colonies are light brown, convex, smooth, up to 3 mm in diameter, the cells are rod-shaped, Gram-negative bacteria, ranging in size from 1.5—3 × 0.8—1 µm.[7,8, 24] Comparative and phylogenetic analyzes of the aerobic chemoorganotrophic *S. maltophilia* as reference based on the nucleotide sequences of the 16S rRNA gene revealed closely related species The nucleotide sequence of the isolates was similar to a strain *S. rhizophila* with 83.7% homologyin contrast to *S. maltophilia* growth at 4 °C, not 37 °C; uses xylose as the only source of carbon and energy; poor osmolytic tolerance. *S. rhizophila* was isolated from the leaf of *P. vulgaris*. The BLAST phylogenetic analysis supports that Gammaproteobacteria belongs to the genus *Stenotrophomonas,* species *S. rhizophila.*[3,5,7,9,26]

Conclusion

Based on the above results, *S. rhizophila* endophyte of *P. vulgaris* leaves can be used as a biofertilizer in legume production, since it is a biological tool for the reduction and optimization of nitrogen fertilizer that ensures soil fertility, prevents contamination of surface water such as aquifers, and today mitigates global warming by preventing the formation of N_2O. To contribute to sustainable management in favor of a healthy environment, and to ensure the conservation of soil as a natural resource for future generations.

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Conflicts of interest

The authors declare that there is no type of conflict of interest in its planning, execution and writing with the institutions involved, as well as those that financially supported this research.

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