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Azotobacter vinelandii and Bacillus licheniformis in Phaseolus vulgaris Growth at 50% NH_4NO_3 With Multi-Wall Carbon Nanotubes to Reduce N_2O Release

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ABSTRACT

In *Phaseolus vulgaris* production it is important to regulate the application of NH_4NO_3, by inoculation with *Azotobacter vinelandii* and *Bacillus licheniformis* plus multi-wall carbon nanotubes (MWCN), to decrease the release N_2O from NH_4NO_3 a greenhouse gas, according to soil physical and chemical properties under *P. vulgaris* cultivation. The objective of this work was to analyze the response of *P. vulgaris* with *A. vinelandii/B. licheniformis* with NH_4NO_3 at 50% plus MWCN. For this, *P. vulgaris* seeds were treated with a mixture of *A. vinelandii/B. licheniformis* and 50% NH_4NO_3 plus 10 or 20 ppm of MWCN, by the response variables: germination percentage, phenology and seedling biomass, the experiment was carried out under a random block: *P. vulgaris* fed with 100% NH_4NO_3 uninoculated, non-MWCN or relative control; *P. vulgaris* non NH_4NO_3, uninoculated, irrigated only water or absolute control; the treatments: *P. vulgaris* with *A. vinelandii/B. licheniformis* 50% NH_4NO_3 plus 10 or 20 ppm of MWCN, individually and in mixture. The experimental data were analyzed by ANOVA and Tukey ($P<0.05$). The results showed that *P. vulgaris* with *A. vinelandii/B. licheniformis* with 50% NH_4NO_3 plus MWCN, germinated better and faster, positive response of *P. vulgaris* was also observed on the phenology and biomass of *P. vulgaris*, compared to *P. vulgaris* plus 100% NH_4NO_3, uninoculated. The results support that the positive effect of *A. vinelandii /B. licheniformis*, inside the root system of *P. vulgaris* due to transforming organic compounds, from root metabolisms into phytohormones, to enhance 50% NH_4NO_3 uptake. This root activity of *P. vulgaris* was improved by combination of *A. vinelandii /B. licheniformis* by the MWCN, that accelerated and optimized 50% NH_4NO_3 uptake, consequently there was not NH_4NO_3 remaining, in that sense, to reduce greenhouse gas generation compared to 100% NH_4NO_3 uptake in uninoculated *P. vulgaris*, when non-uptake of NH_4NO_3 caused release of NO_3- with subsequent generation of N_2O, reason is why to decrease NH_4NO_3 with *A. vinelandii /B. licheniformis* plus MWCN are, important to mitigate the impact of greenhouse gases on *P. vulgaris* production, also to preserve soil fertility and prevent surface and groundwater pollution by overfertilization.

Keywords: Soil; Overfertilization; Loss of Fertility; Beneficial Plant Bacteria; Carbon Nanoparticles; NH_4NO_3 Optimization; Greenhouse Effect Mitigation

Introduction

Currently, the generation of greenhouse gases in the production of *Phaseolus vulgaris* causes global warming, due to over nitrogen fertilization with NH_4NO_3, that caused N_2O from NO_3 under common environmental conditions during *P. vulgaris* cropping [1], reason is why strategies are proposals, that combine the reduction of the NH_4NO_3 dose, without risk of compromising healthy plant growth, as well as to inoculate *P. vulgaris* seed with *Azotobacter vinelandii* with *B. licheniformis*, genera of endophytic plant growth bacteria [2,3], that optimize nitrogen fertilizer to the maximum, through the synthesis of phytohormones, to induce maximum activity of the root system that uptakes NH_4NO_3 action [4,5], that can be improved by applying multiwall carbon nanotubes or MWCN [6], non-toxic for plants, animals or human beings [7], that accelerate the action of bacterial phytohormones [8], so that NH_4NO_3 is consumed in such a way, that there is no remaining NH_4NO_3 [9], that generates N_2O from NO_3 greenhouse gas that causes global warming [1]. There is evidence that the combination of seed with genus of plant growth promoting bacteria genera, mixing with MWCN can prevent the loss of soil fertility, due to excess NH_4NO_3 non uptake by the root vegetal system [9-15]. Therefore, the objective of this work was to analyze the response of *P. vulgaris* with *A. vinelandii*/B. *licheniformis*, NH_4NO_3 at 50% plus MWCN to reduce N

Material and Methods

The genera of plant growth-promoting endophytic bacteria: *A. vinelandii*/B. *licheniformis* are part of the bacterial collection of the Environmental Microbiology Laboratory of the Research Institute, and were isolated from the interior of roots of *Zea mays var mexicana* (teocintle); *A. vinelandii* was grown in Burk broth while *B. licheniformis* was grown in nutrient broth to inoculate a dose of 1.0 x 10^6 CFU/ml/0.5 g of *P. vulgaris* seeds individually and in a mixture of 0.5 ml of each to have the dose 1.0 x 10^6/ml/0.5 g of *P. vulgaris* seed [15]. The seeds of *P. vulgaris* var canario were obtained from the Secretariat of Livestock and Hydraulic Resources and Environment, Mexican government delegation, Michoacán, México. The MWCN were acquired from Sigma-Adrich; were suspended in saline solution (0.85% NaCl and 0.01% commercial detergent, sterile by autoclave), applied to the *P. vulgaris* seed at doses of 10 and 20 ppm [16]. The response variables used to evaluate the response of *P. vulgaris* with 50% NH_4NO_3 with *A. vinelandii* and/or *B. licheniformis*, plus *carbon nanotubes* were: germination percentage, seedling phenology: plant height (PH), root length (RL), fresh and dry aerial and root weight (AFW/RFW/ADW/RDW). The experiment was carried out under a random block design with *P. vulgaris* fed with 100% NH_4NO_3 uninoculated or relative control, *P. vulgaris* non NH_4NO_3 irrigated only with uninoculated water or absolute control and the treatments: *P. vulgaris* with *A. vinelandii*/B. *licheniformis* 50% NH_4NO_3 with 10 and 20 ppm of MWCN, individually and in mixture with and without MWCN, *P. vulgaris* with 50% NH_4NO_3 uninoculated with 10 ppm of MWCN. The experimental data were analyzed by ANOVA and Tukey [17]. On Figure1 show scheme of the experimental design to evaluate the response of *P. vulgaris* with 50% NH_4NO_3 with *A. vinelandii* and/or *B. licheniformis* plus MWCN.

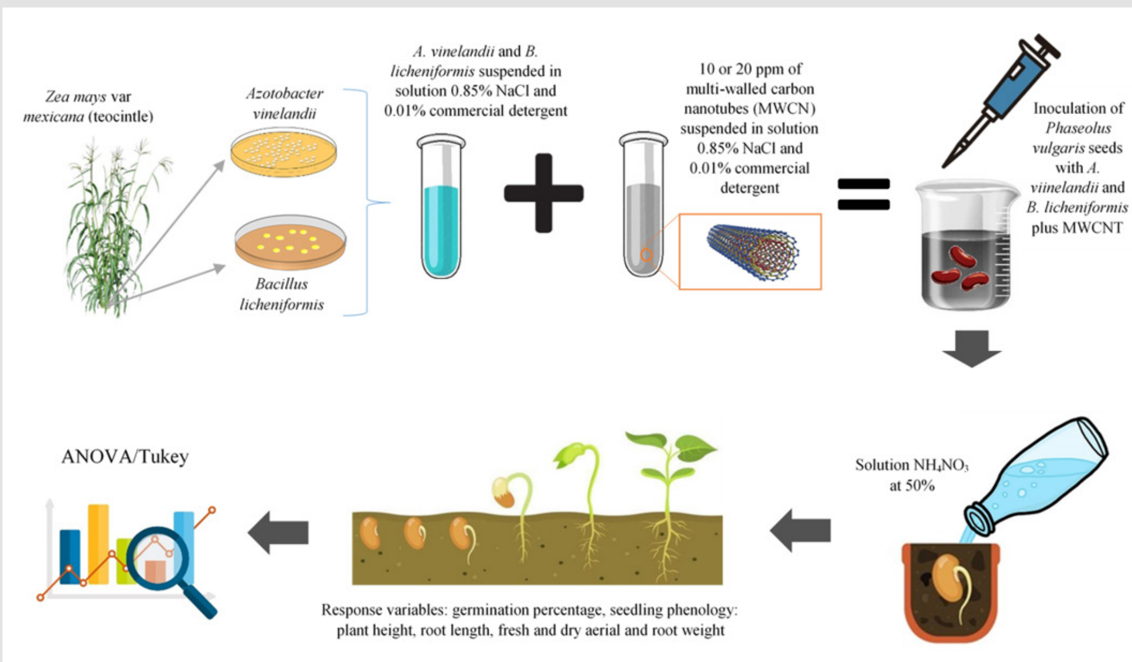

Figure 1: Scheme of the experimental design to evaluate the response of *Phaseolus vulgaris to A. vinelandii*/B. l*icheniformis* plus 50% NH_4NO_3 with 10 or 20 ppm of MWCN.

Results and Discussion

Table 1 shows the effect of *A. vinelandii*/B. l*icheniformis* on the germination of *P. vulgaris* seeds, with 50% NH_4NO_3 and 10 or 20 ppm of MWCN. A germination percentage of 71.43% was registered in *P. vulgaris*, irrigated with water or AC alone, a statistically different numerical value compared to 90.47% of *P. vulgaris* with 100% NH_4NO_3, non- MWCN, in contrast to the value of 83.33% germination in *P. vulgaris* with *A. vinelandii*, 50% NH_4NO_3 plus 10 ppm of MWCN: numerical value with statistical difference compared to 76.19% in *P. vulgaris* with 50% NH_4NO_3, uninoculated plus 10 ppm of MWCN; by germination percentage, that was also statistically different compared to the 90.19 % in *P. vulgaris*, with *A. vinelandii/B. licheniformis* and NH_4NO_3 at 50% plus 10 ppm of MWCN, in evident contrast with the germination percentage registered for *P. vulgaris* and *A. vinelandii/B. licheniformis* at 50% NH_4NO_3, plus 20 ppm of MWCN, with a value of 66.66%. According to this results, 3 fundamental facts can be inferred. First, that the germination of *P. vulgaris* with NH_4NO_3 at 50% alone, was equal to germination of *P: vulgaris* at 100% NH_4NO_3, with the combination of *A. vinelandii/B. licheniformis*, plus 10 ppm of MWCN [18]. Second to increase germination that *P. vulgaris* was only possible with *A. vinelandii*/B. l*icheniformis* plus 50% NH_4NO_3 [19]; because individually neither of the two bacterial genera reached a higher percentage of germination, including at seedling stage (data not shown). Third that although MWCN increase and accelerate the production of phytohormones associated with germination [20], only if the concentration is not greater or less than 10 ppm, because as it was not registered when 20 ppm was applied germination was reduced, due to negative effect both on the seed of *P. vuglaris* and on the beneficial phytohormonal activity of *A. vinelandii/B. licheniformis* [21,22]. Table 2 shows the effect of *A. vinelandii*/B. l*icheniformis* on *P. vulgaris* with 50% NH_4NO_3 plus 20 ppm MWCN, on the phenology and biomass of *P. vulgaris* at the seedling level, where a plant height (PH) of 33.08 cm was registered, a statistically different value compared to 28.42 cm HP of *P. vulgaris* uninoculated, with 100% NH_4NO_3 or RC, a numerical value with statistical difference compared, to 35.67 cm HP of *P. vulgaris* with *A. vinelandii/B. licheniformis* at 50% NH_4NO_3, plus 10 ppm MWCN. This value was statistically different compared to 31.83 cm PH of *P. vulgaris* at 50% NH_4NO_3, *A. vinelandii/B. licheniformis* non MWCN, compared to the PH of *P. vulgaris* used as relative control (RC), also with the 32.92 cm of uninoculated *P. vulgaris*, non NH_4NO_3 or neither MWCN. While the root length (RL) of *P. vulgaris* was: 18.33 cm with *A. vinelandii*/B. l*icheniformis* plus 10 ppm of MWCN, a statistically different value compared to the 15 cm RL of *P. vulgaris* with *A. vinelandii/B. licheniformis* 50% NH_4NO_3, with 20 ppm of MWCN and the 14. 66 cm RL of *P. vulgaris* with *A. vinelandii/B. licheniformis*, 50% NH_4NO_3 non- MWCN, a statistically different value compared to the 16.92 cm RL o0f *P. vulgaris* non NH_4NO_3, uninoculated, or neither MWCN, used as absolute control (AC).

Table 1: Effect of *A. vinelandii/B.licheniformis* at 50% NH_4NO_3 plus MWCN+ on the germination of *Phaseolus vulgaris* var Canario.

[*]*Phaseolus vulgaris* seeds	Germination per centage (%)
Irrigated water only or absolute control (AC)	71.43[c**]
Uninoculated fed 100% NH_4NO_3, non-MWC[N]+	90.47[a]
A. vinelandii/B.licheniformis 50% NH_4NO_3 non-MWCN	83.33[b]
50% NH_4NO_3, uninoculated plus10 ppm MWCN	76.19[c]
A. vinelandii/B.licheniformis + 50% NH_4NO_3 plus 10 ppm MWCN	90.19[a]
A. vinelandii/B.licheniformis + 50% NH_4NO_3 20 ppm MWCN	66.66[d]

Note: *n= 50 **Values with different letters had statistical difference (P<0.05) according to ANOVA-Tukey. +MWCN= multi-wall carbon nanotubes.

Table 2: Effect of *Azotobacter vinelandii* and *Bacillus licheniformis* on growth of *Phaseolus vulgaris* at seedling stage at 50% NH_4NO_3 plus multi-wall carbon nanotubes (MWCN)+.

	Phenology			Biomass		
	(cm)			(g)		
Treatment	Plant	Radical	Aerial fresh	Radical	Dry aerial	Dry radical weight
*Phaseolus vulgaris**	height	length	weight	fresh weight	weight	
Irrigated only water Absolute control (AC)	32.92c**	16.83[b]	1.21[c]	0.34[c]	0.11[d]	0.02[d]
Fed 100% NH_4NO_3 Uninoculated Non- MWCN[+] Relative control (RC)	28.42[d]	17.02[b]	1.38[b]	0.52[a]	0.14[b]	0.04[b]
Fed 50% NH_4NO_3 *A. vinelandii/B. licheniformis* Non- MWCN	31.83[c]	17.08[b]	1.39[b]	0.53[b]	0.14[b]	0.04[b]
Fed 50% NH_4NO_3 uninoculated 10 ppm MWCN	32.00[c]	14.66[c]	1.34[b]	0.50[b]	0.13[c]	0.03[c]
A. vinelandii/B. licheniformis 50% NH_4NO_3, 10 ppm MWCN	35.67[a]	18.33[a]	1.59[a]	0.57[a]	0.17[a]	0.05[a]
A. vinelandii/B. licheniformis 50% NH_4NO_3, 20 ppm c MWCN	33.08[b]	15.00[c]	1.40[b]	0.52[b]	0.14[b]	0.04[b]

Note: *n = 6 **Values with different letters had statistical difference (P<0.05) according to ANOVA-Tukey.

In relation to biomass such as fresh and dry aerial and radical weight (AFW/RFW/ADW/RDW), the highest was registered in; *P. vulgaris* with *A. vinelandii/B. licheniformis* and NH_4NO_3 at 50% plus 10 ppm of MWCN, were registered 1.59 g of AFW 0.57 g of RFW, 0.17 g of ADW and 0.05 g of RDW values with statistical difference compared to the values of *P. vulgaris* with NH_4NO_3 at 100% uninoculated or neither MWCN with: 1.38 g of FAW, 0.52 g of RFW, 0.14 g of ADW and 0.04 g of RDW; these values were statistically different from those registered in *P. vulgaris* with *A. vinelandii/B. licheniformis* with NH_4NO_3 at 50% non- MWCN with: 1.39 g of AFW, 0.53 g of RFW, with 0.14 of ADW and 0.04 of RDW, these values were also statistically different to *P. vulgaris* uninoculated at 50% NH_4NO_3 plus 10 ppm MWCN, 1.34 g AFW, 0.50 g RFW with 0.13 g ADW and 0.03 g RDW. It was evident that all these numerical values were different from those registered in *P. vulgaris* uninoculated, neither NH_4NO_3, non- MWCN irrigated only with water with 1.21 g AFW, 0.34 g RFW as well as 0.11 ADW and 0.02 RDW.

These results support several obvious facts. First is that the uptake capacity of NH_4NO_3 by *P. vulgaris* roots for the 100% dose is limited, or excessive, because even 50% of NH_4NO_3 is required for the beneficial phytohormonal activity, in *P. vulgaris* roots of *A. vinelandii* in synergic action with *B. licheniformis* to be optimized (4,5:10,11), and avoid the release of NO_3 with the possibility of generating N_2O (1,2:12,13). Second is that MWCN have a positive effect, on the water and mineral uptake mechanism that initiates germination and favors the uptake of NH_4NO_3 at 50%[14,16,23], according to the concentration of the MWCN a positive effect better with 10 ppm than with 20 ppm MWCN [23-25], that is associated with the possible toxicity in the metabolism of seeds and roots of *P. vulgaris* [21,22]. Third is that if the seeds are inoculated with *A. vinelandii*/B. l*icheniformis* it is induced, the growth promotion effect on the germination of *P. vuglaris*, is accelerated and improved, allowing the optimization of the 50% NH_4NO_3 dose [26-28], to prevent the release of NO_3 into the soil to avoid the generation of N_2O, as well as the loss of organic matter, as well as the contamination of surface water and groundwater [1,2,10]. The results registered on the germination and growth of *P. vulgaris* with 50% NH_4NO_3, support the potential of MWCN, to solve problems associated with the dynamics of NH_4NO_3 in plant roots [29,30], as well as the real possibility that MWCN, can be used as part of an integral strategy for sustainable agriculture with plant growth promoting bacteria [26,27].

Conclusion

It is concluded that the combination of *A. vinelandii*/B. l*icheniformis*, at dose of 50% NH_4NO_3 in *P. vulgaris*, can be improved with MWCN, under the premise of finding the minimum concentration sufficient, to optimize the uptake of NH_4NO_3, without risk compromising the healthy growth of the legume, or the environment by preventing the loss of soil fertility, the contamination of surface water and groundwater, as well as the release of N_2O to mitigate global warming due to cultivation of *P. vulgaris*.

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Conflicts of Interest

The authors declare no conflicts of interest.

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