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Research Article

Optimization of NH_4NO_3 in *Phaseolus vulgaris* with *Bacillus thuringiensis* and *Micromonospora echinospora* plus crude extract of carbon nanoparticles

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Summary

Nitrogenous fertilizer (NF) such as NH_4NO_3 is required to maintain the healthy growth of *Phaseolus vulgaris*, but when NF is applied indiscriminately, it causes hyperfertilization of the soil. One option is to reduce $NH4_NO_3$ and then optimize in *P. vulgaris* seed with s *Bacillus thuringiensis* and *Micromonospora echinospora* genus and species of endophytic bacteria plus a crude carbon nanoparticle extract (CENC). Under greenhouse conditions, *P. vulgaris* seeds were inoculated with *B. thuringiensis* and *M. echinospora*, then applied a CENC and fed at 50% NH_4NO_3, the response variables were germination and seedling phenology/biomass. All numerical data of the experimental were validated by ANOVA/Tukey ($p < 0.05$). The results showed a healthy growth of *P. vulgaris* with *B. thuringiensis* and *M. echinospora* at 50% NH_4NO_3 plus 20 ppm of CENC according to the percentage of germination, phenology and seedling biomass, including all numerical values have a statistical difference compared to those registered in *P. vulgaris* without *B. thuringiensis* and *M. echinospora*, at 100% NH_4NO_3, neither CENC nor relative control (CR). The positive effect of *B. thuringiensis and M. echinospora* on *P. vulgaris* at 50% NH_4NO_3 was enhanced by CENC to maximize the optimization of NF without loss of soil fertility or risk of environmental contamination.

Introduction

In agriculture is important to apply nitrogenous fertilizer (NF) as an NH_4NO_3, which is essential for the healthy growth of *Phaseolus vulgaris* [1,2]. However, NH_4NO_3 is applying in not regulate concentration according to plant nutritional real demands, part of the NF causes rapid mineralization of organic matter consequently soil fertility is lost with the risk of contamination of surface and groundwater [3,4]. An alternative solution is to reduce and optimize NH_4NO_3 in *P. vulgaris* by inoculating the seeds with *Bacillus thuringiensis* and *Micromonospora echinospora* well known as plant growth-promoting endophytic bacteria [5-8], which can convert organic compounds in seeds and roots into phytohormones [9-13]. At the same time applying a crude extract of carbon nanoparticles or CENC improves NH_4NO_3 uptake [14-16], to enhance the effect of bacterial phytohormones of *Bacillus thuringiensis* and *Micromonospora echinospora* on the root system by inducing maximum proliferation of root hairs to effectively increase NH_4NO_3 uptake in *P. vulgaris* to preserve soil fertility. Based on the above, the objective of this research was to reduce and optimize NH_4NO_3 in *P. vulgaris* with *B. thuringiensis* plus *M. echinospora* and CENC.

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Materials and methods

This research was carried out at the Environmental Microbiology Laboratory, of the Chemical-Biological Research Institute of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH), Morelia, Mich., Mexico.

Synthesis and characterization of crude extract of carbon nanoparticles from *Albizia julibrissin.*

A. julibrissin leaves were disinfected by immersion with 0.5% NaOCl for 1 min, washed with sterile deionized H_2O, then the leaves were cut into 5 cm pieces and dried at 80°C for 12 h, 30 g of *A. julibrissin* were taken and suspended in 300 mL of deionized H_2O, then heated at 70°C for 30 min, then the aqueous extract of *A. julibrissin* was filtered in Whatman No. 1, centrifuged at 4000 rpm for 10 min. The characterization of the obtained crude extract was carried out using a JEOL JSM-IT300LV scanning electron microscope (SEM) to characterize the size and morphology of the nanoparticles synthesized from *A. julibrissin*. The analysis of the qualitative and quantitative composition of the CENC was performed by energy dispersive spectroscopy (EDS) coupled with a JEOL-JSM-7600F field emission microscope [17].

Phaseolus vulgaris seed inoculated with *Bacillus thuringiensis* and *Micromonospora echinospora* at 50% NH_4NO3 and crude extract of nanoparticles carbon.

The study was carried out in a greenhouse under the following microclimatic conditions: temperature of 23.2 °C, luminosity of 450 µmol $m^{-2}s^{-1}$, and relative humidity of 67%. The seeds of *P. vulgaris* var. *black turtle* were disinfected with 0.2% NaClO for 5 min, then rinsed six times with sterile tap water; they were disinfected in 70% alcohol (v/v) /5 min and rinsed six times with sterile water, then the *P. vulgaris* seeds were inoculated with *B. thuringiensis* isolated from inner of roots of *Zea mays var mexicana* (teosinte) and/or *M. echinospora* isolated from inside of roots of *Medicago* sp. While *B. thuringiensis* was grown on nutrient agar (g/L): meat extract, 3.0; meat peptone, 5.0; agar, 18.0; pH 7.0; incubated at 30°C for 24 h, while *M. echinospora* was grown in agar avocado pit (g/L): avocado pit, 10.0; casein peptone, 5.0; yeast extract, 1.3; K_2HPO_4, 0.17; KH_2PO_4, 2.61; $MgSO_4$, 1.5; NaCl, 0.9; $CuSO_4$, 0.05; bromothymol blue, 10 ppm; 10% detergent, 2.5 mL/L; trace element solution, 1 mL/L; agar 18.0; pH 7.5, then incubated at 30°C for 72 h [18]. Subsequently, in plastic bags of 250 g for every 10 *P. vulgaris* seeds, they were inoculated with 1.0 mL of *B. thuringiensis* and/or *M. echinospora* in a 1:1 (v/v) ratio equivalent to a concentration of 1 x 10^6 CFU /mL, obtained by viable plate count on nutrient agar and avocado bone agar, then treated with 1.0 mL of a concentration of 10 and/or 20 ppm of the CENC suspended in a 0.85% NaCl solution with 0.5% Roma™ detergent (w/v). The seeds with *B. thuringiensis* and/or *M. echinospora* and the CENC were shaken at 200 rpm for 30 min at 28°C to ensure the entry of both (bacteria and CENC). Seeds were sown in 100 g of agricultural soil previously sifted with a No. 20 mesh and solarized to prevent pests and plant diseases, in a greenhouse container as described in Table 1 of the experimental randomized block design with two controls, six treatments, and six repetitions: *P. vulgaris* without *B. thuringiensis* and *M. echinospora* irrigated only with water or absolute control (AC); *P. vulgaris* without *B. thuringiensis* and *M. echinospora* fed with 100% NH_4NO_3 or relative control (CR); *P. vulgaris B. thuringiensis and M. echinospora*, and 10 or 20 ppm of CENC and fed 50% NH_4NO_3 in a mineral solution with 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; $FeSO_4$ and 1.0 ml/L of a microelement solution (g/L): H_3BO_3 2.86; $ZnSO_4 \cdot 7H_2O$, 0.22; $MgCl_2 \cdot 7H_2O$ 1.8, pH 6.8. NH_4NO_3 was applied at a volume of 5 mL every 3 days for one month to ensure 80% field capacity. The response variables used were: germination percentage, phenology: plant height (PH) and root length (RL); biomass: aerial and radical fresh weight (AFW/RFW) and aerial and radical dry weight (ADW/RDW) at seedling. All results were validated using the ANOVA analysis of variance through Tukey's comparative test of means ($p \leq 0.05$) with the statistical program Statgraphics Centurion [19,20].

Results and discussion

Table 2 shows the physical-chemical properties of the agricultural soil, where a slightly acidic pH of 6.68 was detected, which determines the solubility of PO_4^{-3} (phosphates), with an average organic matter content of 2.27%, indicating an evident imbalance in the C: N ratio; with a loamy texture in a 40-40-20% ratio (sand-silt-clay); a low apparent density and the low

Table 1: Experimental design to analyze the optimization of NH_4NO_3 at 50% in *Phaseolus vulgaris* plus *Bacillus thuringiensis* and *Micromonospora echinospora* and crude extract of carbon nanoparticles.

**Phaseolus vulgaris*	CENC (ppm)	*Bacillus thuringiensis*	*Micromonospora echinospora*
Irrigated just water or absolute control (AC)	-	-	-
NH4NO3 at 100% or relative control (RC)	-	-	-
(T1) NH_4NO_3 at 50%	10	+	-
(T2) NH_4NO_3 at 50%	10	-	+
(T3) NH_4NO_3 at 50%	10	+	+
(T4) NH_4NO_3 at 50%	20	+	-
(T5) NH_4NO_3 at 50%	20	-	+
(T6) NH_4NO_3 at 50%	20	+	+

*Number of repetitions (*n*) = 6; treatment (T); crude extract of carbon nanoparticles (CENC); added (+); not added (-)

Table 2: Physiochemical parameters of agricultural soil for the growth of *Phaseolus vulgaris.*

Parameters*	Value
pH (1:2)	6.68
Electrical Conductivity: (H_2O) (ms/cm)	0.33
Apparent density (s/mL)	0.80
Organic material (%)	2.27
Texture	loamy
Bulk density of soil (g/cm^3)	0.92
Total, nitrogen (%)	0.15

*Physical-chemical parameters for agricultural soils according to the NOM-021. RECNAT-2000.

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total N, this due to the constant use of the soil for agricultural production and therefore are limiting factors for the healthy growth of *P. vulgaris* according to NOM-021-SEMARNAT-2000.

Figure 1 shows the SEM micrograph that provides the morphology and size of the CENC synthesized from *A. julibrissin*, where some spherical shapes with a size of less than 200 nm were recorded. Furthermore, some nanoparticles tended to form aggregates. There were also some irregular carbon shapes that could be attributed to amorphous carbon. Based on these results, it is possible that these allotropic forms of carbon, being nanometric in scale, improve the retention capacity and slow release of water or NH_4NO_3 according to the need of *P. vulgaris* with *B. thuringiensis* and *M. echinospora* [21,22]. Since authors such as Vithanage, et al., 2017 [23], mentioned that some carbon nanostructures improve the uptake of nitrogen (N) from ammonia (NH_3) and release hydrogen (H^+) ions, which improves the uptake of water and nutrients necessary to maintain the healthy growth of *P. vulgaris* with *B. thuringiensis* and *M. echinospora* at 50% NH_4NO_3.

Figure 2 shows the qualitative and quantitative EDS analysis of the elements in the CENC; There, an atomic percentage of carbon of 63.34% was recorded, as the main element in the formation of nanoparticles, followed by oxygen with 28.36% and other elements such as magnesium (Mg), phosphorus (P), chlorine (Cl) and potassium (K) between 0.84-3.90% that can be attributed to the precursor *A. julibrissin* as the only carbon source used, which contains these elements that help to improve uptake of NH_4NO_3 by *P. vulgaris* with *B. thuringiensis* and *M. echinospora* [22].

Table 3 shows the 100% germination rate of *P. vulgaris* seeds inoculated with *B. thuringiensis* and *M. echinospora* and 50% NH_4NO_3 plus 20 ppm of a CENC, this value was statistically different compared to the 93.3% germination of *P. vulgaris* with *M. echinospora* with 50% NH_4NO_3 and 20 ppm of a CENC; compared to 90% germination of *P. vulgaris* with *B. thuringiensis* and *M. echinospora* with 50% NH_4NO_3 and 20 ppm CENC; compared to 90% germination of *P. vulgaris* with *B. thuringiensis* and *M. echinospora* plus 50% NH_4NO_3 and 10 ppm CENC, and to 86.6% germination of *P. vulgaris* without *B. thuringiensis* and *M. echinospora*, at 100% NH_4NO_3 with no CENC treatment or relative control (RC). The increase in *P. vulgaris* seed germination supports the fact that CENCs enhanced water retention [22] then induced starch hydrolysis with the release of organic compounds, which both *B. thuringiensis* and *M. echinospora* transformed into phytohormones that improved the germination rate [6,10,21,24].

Table 4 shows the seedling phenology of *P. vulgaris* with *B. thuringiensis/M. echinospora* and 50% NH_4NO_3 enhanced with 20 ppm CENC; where 34.89 cm of PH and 18.69 cm of RL were registered, both numerical values have a statistical difference compared to those registered in *P. vulgaris* without *B. thuringiensis/M. echinospora* or neither CENC, only fed with NH_4NO_3 at 100% or CR. In relation to fresh and dry biomass, *P. vulgaris* with *B. thuringiensis* and *M. echinospora* fed applying NH_4NO_3 at 50% and 20 ppm of CENC recorded: 4.2109 g of AFW, 2.5742 g of RFW, 1.2016 g of ADW and 0.7354 g of RDW, statistically different numerical values compared to the 2.6471

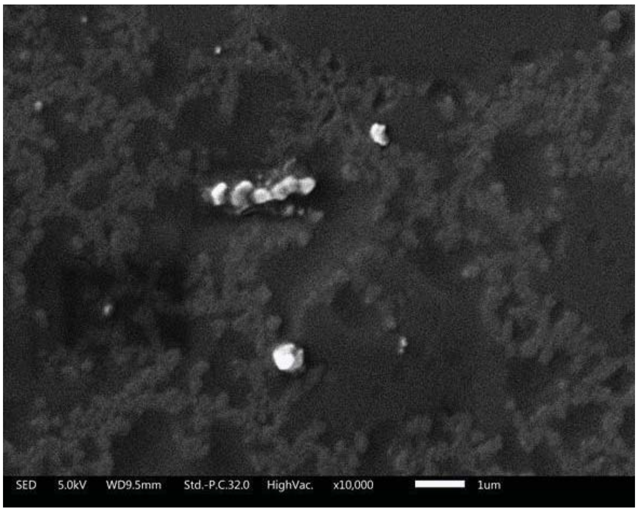
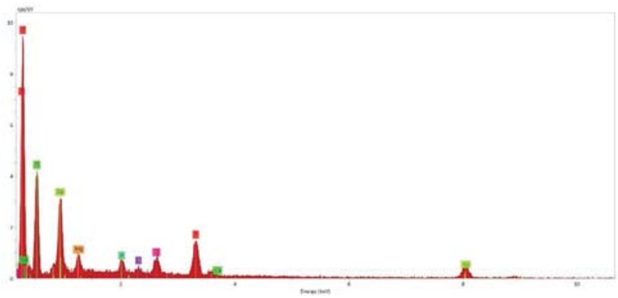


Figure 1: SEM micrograph of carbon nanoparticles extract from *Albizia julibrissin*.



Element	Atomic (%)
carbon	63.34
oxygen	28.36
magnesium	2.38
phosphorus	0.84
Chlorine	1.15
Potassium	3.90

Figure 2: EDS analysis of the extract of carbon nanoparticles synthesized from the leaves of *Albizia julibrissin*.

Table 3: Germination percentage of *Phaseolus vulgaris* plus *Bacillus thuringiensis* and *Micromonospora echinospora* at 50% NH_4NO_3 plus a crude extract of carbon nanoparticles.

**Phaseolus vulgaris*	Germination (%)
Irrigated only water or absolute control (AC)	80d**
With NH_4NO_3 at 100% or relative control (RC)	86.6^c
Bacillus thuringiensis with NH_4NO_3 a 50% plus 10 ppm CENC	83.3^d
Micromonospora echinospora with NH_4NO_3 at 50% plus 10 ppm CENC	90^b
B. thuringiensis/M. echinospora con NH_4NO_3 at 50% plus 10 ppm CENC	90^b
B. thuringiensis fed NH_4NO_3 at 50% plus 20 ppm CENC	86.3^c
M. echinospora fed NH_4NO_3 at 50% plus 20 ppm CENC	93.3^b
B. thuringiensis/M. echinospora with NH_4NO_3 |at 50% plus 20 ppm CENC	100^a

**n* = 6; crude extract of carbon nanoparticles (CENC). **Values with letter indicate statistical difference (p > 0.05) according to ANOVA/Tukey.

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Table 4: Phenology and biomass of *Phaseolus vulgaris* to seedling, plus *Bacillus thuringiensis* and *Micromonospora echinospora* at 50% NH_4NO_3 enhanced by crude extract of carbon nanoparticles.

*Treatment	Plant height (cm)	Radical long (cm)	Fresh weight(g)		Dry weight (g)	
			Aeral	Radical	Aeral	Radical
Absolute control	28.51[c**]	13.57[d]	1.9902[d]	0.6136[d]	0.5689[e]	0.1759[d]
Relative control	31.93[b]	15.36[c]	2.6471[c]	0.8973[c]	0.7563[c]	0.2563[c]
T1	30.66[b]	17.12[ab]	3.1549[b]	1.3141[b]	0.9014[b]	0.5451[b]
T2	33.39[a]	15.85[c]	2.9773[c]	0.9755[c]	0.8501[c]	0.2852[c]
T3	31.78[b]	16.10[ab]	2.4348[c]	1.7534[b]	0.6957[d]	0.5015[b]
T4	34.61[a]	18.52[a]	3.2135[b]	2.6002[a]	0.9182[b]	0.7429[a]
T5	33.55[a]	17.44[a]	3.9421[a]	1.9385[ab]	1.1262[a]	0.5538[b]
T6	34.89[a]	18.69[a]	4.2109[a]	2.5742[a]	1.2016[a]	0.7354[a]

*n = 6; n = 6; crude extract of carbon nanoparticles (CENC). **Values with distinct letter indicate statistical difference ($p > 0.05$) according to ANOVA/Tukey. Absolute control = *Phaseolus vulgaris* irrigated only water; relative control = *P. vulgaris* without *Bacillus thuringiensis/Micromonospora echinospora* and NH_4NO_3 at 100%: T1 = *P. vulgaris* with *B. thuringiensis* and 50% NH_4NO_3 plus 10 ppm of a crude extract of carbon nanoparticles (CENC); T2 = *P. vulgaris* with *M. echinospora* and 50% NH_4NO_3 plus 10 ppm CENC; T3 = *P. vulgaris* with *B. thuringiensis/M. echinospora* and 50% NH_4NO_3 plus 10 ppm CENC; T4 = *P. vulgaris* with *B. thuringiensis* and 50% NH_4NO_3 plus 20 ppm CENC; T5 = *P. vulgaris* with *M. echinospora* and 50% NH_4NO_3 plus 20 ppm CENC; T6 = *P. vulgaris* with *B. thuringiensis/M. echinospora* and 50% NH_4NO_3 plus 20 ppm CENC.

g of AFW, 0.8973 g of RFW, 0.7563 g of ADW and 0.2563 g of RDW of *P. vulgaris* or CR. The response of P. vulgaris with the NF dose reduced to 50% inoculated with B. thuringiensis and M. echinospora plus the CENC supports that inside the root system *B. thuringiensis* and *M. echinospora* converted photosynthesis-derived metabolites into phytohormones that induced a dense root system for the maximum optimization of NH_4NO_3 at 50% that, enhanced by CENC, which improves NF uptake for the healthy growth of *P. vulgaris* [12,13]. In this regard, Chichiricò & Poma, 2015; Khodakovskaya, et al., 2013; Sanzari, et al., 2019 [25-28] reported that carbon nanomaterials cause a positive effect in legumes by being uptake through the root system of *P. vulgaris*, when B. *thuringiensis/M. echinospora* are involved. Through cell walls, via the apoplast pathway, or by endocytosis, and translocate to the different plant organs of legume to improve its growth when NF dose is reduced without risk of soil hyperfertilization or environmental contamination.

Conclusion

Healthy growth of P. vulgaris by applying *B. thuringiensis* and *M. echinospora* fed 50% reduced NH_4NO_3 and CENC demonstrates that effective optimization of NF decrease was due to enhanced phytohormonal activity of *B. thuringiensis* and *M. echinospora* by CENC within the root system of *P. vulgaris*.

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