Research Article

Optimization of NH₄NO₃ 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 NH₄NO₃ 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 NH₄O₃ and then optimize in P. vulgaris seed with 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% NH₄NO₃, 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% NH₄NO₃ 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% NH₄NO₃ neither CENC nor relative control (CR). The positive effect of B. thuringiensis and M. echinospora on P. vulgaris at 50% NH₄NO₃ 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 NH₄NO₃, which is essential for the healthy growth of Phaseolus vulgaris [1,2]. However, NH₄NO₃ 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 NH₄NO₃ 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 NH₄NO₃ 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 NH₄NO₃ uptake in P. vulgaris to preserve soil fertility. Based on the above, the objective of this research was to reduce and optimize NH₄NO₃ 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 H2O, 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 H2O, 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% NH4NO3 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% NaOCl for 5 min, then rinsed six times with sterile tap water; they were disinfected in 70% alcohol (v/v) /5 min 0.2% NaClO for 5 min, then rinsed six times with sterile tap water; they were disinfected with 1.0 mL of a concentration of 10 and/or 20 ppm *B. thuringiensis* and/or *M. echinospora* isolated from inside 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, 10; casein peptone, 5; yeast extract, 1.3; *K. HPO4*, 0.17; *KH2PO4*, 2.61; *MgSO4*, 1.5; *NaCl*, 0.9; *CuSO4*, 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 105 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% NH4NO3, or relative control (CR); *P. vulgaris* *B. thuringiensis* and *M. echinospora*, and 10 or 20 ppm of CENC and fed 50% NH4NO3 in a mineral solution with the following chemical composition (g/L): NH4NO3, 10; *K2HPO4*, 2.5; KH2PO4, 2.0; MgSO4, 0.5; NaCl, 0.1; CaCl2, 0.1; FeSO4, 1.0 mL/L of a microelement solution (g/L): *H3BO3*, 2.86; ZnSO4•7H2O, 0.22; MgCl2•7H2O, 1.8, pH 6.8. NH4NO3 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, 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 ≤ 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 PO4−3 (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 40–60–20% ratio (sand–silt–clay); a low apparent density and the low

| Parameters* | Value |
|-------------|-------|
| pH (1:2)    | 6.68  |
| Electrical Conductivity: (H2O) (ms/cm) | 0.33 |
| Apparent density (g/mL) | 0.80 |
| Organic material (%) | 2.27 |
| Texture | loamy |
| Bulk density of soil (g/cm3) | 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 allotropes of carbon, being nanometric in scale, improve the retention capacity and slow release of water or NH4NO3 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 (NH3) 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% NH4NO3.

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 NH3NO3 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% NH4NO3 plus 20 ppm of a CENC, this value was statistically different compared to the 93.3% germination of *P. vulgaris* with *M. echinospora* at 50% NH4NO3 and 50% NH4NO3 plus 20 ppm of CENC, and to 86.6% germination of *P. vulgaris* at 50% NH4NO3 plus 20 ppm CENC, recorded: 4.2109 g of AFW, 2.5742 g of RFW, 1.2016 g of ADW and 0.7354 g of RDW, statistical different numerical values compared to those registered in *P. vulgaris* without *B. thuringiensis* and *M. echinospora* treated at 100% NH4NO3 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 [6,10,21,22].

Table 4 shows the seedling phenology of *P. vulgaris* with *B. thuringiensis/M. echinospora* and 50% NH4NO3 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 NH4NO3 at 100% or CR. In relation to fresh and dry biomass, *P. vulgaris* with *B. thuringiensis* and *M. echinospora* fed applying NH4NO3 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 ppm CENC, and to 86.6% germination of *P. vulgaris* without *B. thuringiensis* and *M. echinospora* at 100% NH4NO3 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 [6,10,21,22].
Table 4: Phenology and biomass of Phaseolus vulgaris to seedling, plus Bacillus thuringiensis and Micromonospora echinospora at 50% NH$_4$NO$_3$ enhanced by crude extract of carbon nanoparticles.

| Treatment | Plant height (cm) | Radical long (cm) | Fresh weight (g) | Dry weight (g) |
|-----------|------------------|------------------|-----------------|---------------|
|           |                  |                  | Aeral            | Radial        |
| Absolute control | 25.11** | 13.57** | 1.9902* | 0.6136* | 0.5689* | 0.1759* |
| Relative control | 31.93* | 15.36* | 2.6471* | 0.8973* | 0.7563* | 0.2563* |
| T1 | 30.66* | 17.12** | 3.1549* | 1.3141* | 0.9014* | 0.5451* |
| T2 | 33.39* | 15.85* | 2.9773* | 0.9755* | 0.8501* | 0.2852* |
| T3 | 31.78* | 16.44* | 2.4348* | 1.7534* | 0.6957* | 0.5015* |
| T4 | 34.61* | 16.52* | 3.2135* | 2.6002* | 0.9182* | 0.7429* |
| T5 | 33.55* | 17.44* | 3.9421* | 1.9388* | 1.1262* | 0.5538* |
| T6 | 34.89* | 18.69* | 4.2109* | 2.5742* | 1.2016* | 0.7354* |

* n = 6; ** n = 8; crude extract of carbon nanoparticles (CENC). **Values with distinct letter indicate statistical difference (p < 0.05) according to ANOVA/Tukey. Absolute control + P. vulgaris irrigated only water; relative control + P. vulgaris without Bacillus thuringiensis/Micromonospora echinospora and NH$_4$NO$_3$ at 100%; T1 = P. vulgaris with B. thuringiensis and 50% NH$_4$NO$_3$ plus 10 ppm of a crude extract of carbon nanoparticles (CENC); T2 = P. vulgaris with M. echinospora and 50% NH$_4$NO$_3$ plus 10 ppm CENC; T3 = P. vulgaris with B. thuringiensis/M. echinospora and 50% NH$_4$NO$_3$ plus 10 ppm CENC; T4 = P. vulgaris with B. thuringiensis and 50% NH$_4$NO$_3$ plus 20 ppm CENC; T5 = P. vulgaris with M. echinospora and 50% NH$_4$NO$_3$ plus 20 ppm CENC.

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