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Effect of *Xanthobacter autotrophicus* on growth of *Capsicum annuum* at different doses of nitrogen and phosphate fertilizer

Abstract

Capsicum annum is an agricultural crop of high economic food and industrial value that demands nitrogen fertilization such as NH_4NO_3 and phosphate or PO_4^{-3} necessary for healthy plant growth, that applied without regulation cause a fertility problem with plant growth that affects profitable yield, as well as the low availability of PO_4^{-3} due to the narrow solubility constant (Kps) and pH of the soil. An alternative solution is to regulate the dose of both and inoculate the seed with *Xanthobacter autotrophicus*, an endophytic genus that promotes plant growth that improves the uptake of NH_4NO_3 as well as the synthesis of alkaline acid phosphatases for healthy growth of *C. annum*. The objectives of this study were: a) to analyze the effect of *X. autotrophicus* on the growth of *C. annum* at various doses of nitrogen and phosphate fertilizers, b) to evaluate the effect of various doses of nitrogen and phosphate fertilizers on the phosphatase activity of *C. annum* inoculated with *X. autotrophicus*.

To this end, *C. annum* seeds were inoculated with varying doses of nitrogen and phosphate fertilizers in soil poor in mineral nitrogen and phosphates placed in a Leonard's semi-hydroponic jar system. The response variables used were days and percentage to germination, phenology, plant height, root length, fresh and dry weight of the aboveground and root parts to seedling. All data were analyzed by ANOVA-Tukey.

The results demonstrated a positive effect of *X. autotrophicus* at various doses of nitrogen and phosphate fertilizer on the germination time and percentage of *C. annum*, as well as on the phenology and biomass, corroborating with the acid and alkaline phosphatase activity in *C. annum* improved by *X. autotrophicus* compared to the growth of *C. annum* fed with the recommended dose without inoculation with *X. autotrophicus*. This demonstrates that reducing the dose of nitrogen and phosphate fertilizer through the phytohormonal and phosphatase action of *X. autotrophicus* in *C. annum* prevents the loss of soil fertility as well as the release of N_2O to mitigate global warming.

Keywords: soil, *C. annum,* NH_4NO_3, PO_4^{-3}, *X. autotrophicus,* phytohormones, phosphatases, greenhouse gases.

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Introduction

Capsicum annum (chili) in México is one of the main agricultural crops of dependent on of chemical fertilizers applied without regulation.[1–3] The production system of *C. annum* a problem is the restitution of the minerals uptake by *C. annum*, under specific chemical and physical soil conditions,[2,4,5] causes loss of the productive capacity of the soil, due to the rapid decrease of organic matter, the imbalance of the carbon: nitrogen ratio (C: N), that includes the low availability of PO_4^{-3} (phosphates) in the soil, that compromises the health of *C. annum*, for a forecast of profitable yield with the risk of release of greenhouse gases: nitrogen oxide (N_2O) due to overfertilization.[1–3,5] An alternative of reducing the dose of NH_4NO_3 and PO_4^{-3} and inoculation of the *C. annum* seed with *Xanthobacter autotrophicus* an endophytic plant growth promoting bacteria that optimizes the uptake of both.[1–3,5,6] Therefore, the objectives of this work were: i) to analyze the growth of *C. annum* with *X. autotrophicus* with different doses of NH_4NO_3 and PO_4^{-3} ii) activity of acid and alkaline phosphatases of *X. autotrophicus* in steam and root *C. annum* at different doses of NH_4NO_3 or (NIF) and PO_4^{-3} or (POF).

Materials and methods

This research at the greenhouse of the Environmental Microbiology Laboratory -Research Institute in Biology and Chemistry at Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Mich., México. The research was performed under greenhouse environmental conditions with average values: T = 23.2°C, luminosity = 450 µmol•m²/s, relative humidity = 67%. The agricultural soil was collected from 19°37'10" north latitude and 101°16'41.00" west longitude, with an altitude of 2013 meters above sea level, at a temperate climate zone called "Uruapilla" municipality of Morelia, Michoacán, México, soil physicochemical proprieties are shown in Table 1. The *X. autotrophicus* strain was kindly donated by Dr. Nocera of the Department of Chemical Biology, Harvard University, Boston, Mass, USA. While the *C. annum* seed was donated by the Ministry of Agriculture of the Government of México.

The soil used was sieved through a No. 20 mesh and solarized at 70°C/48 h to minimize pests and diseases, then 1.0 kg of soil, in Table 1 shows the properties of the agricultural soil used to grow *C. annum* in that the pH was slightly acidic with a poor concentration of organic matter of 2.27%, total nitrogen of 0.15% as well as phosphates (PO_4^{-3}) that limits the healthy growth of this vegetable, these conditions allowed to evaluate the response of *C. annum* inoculated with *X. autotrophicus* to different doses of NH_4NO_3 or NIF and PO_4^{-3} or POF. This agricultural soil was placed in the upper part of a Leonard jar, while water or nitrogen NH_4NO_3 (NIF) and phosphate (PO_4^{-3}) fertilizer (POF) were added to the lower part. Both parts of the jar were joined with a 20 cm cotton strip shown in Figure 1. While Figure 2 shown colony and microscopic morphology at Gram stain of *X. autotrophicus* when was activated on agar without nitrate and sucrose at pH of 7.0 and incubated at 30°C/48 h. Then seeds of *C. annuum* were disinfected with 5% NaClO/5 min, then rinsed 5 times with sterile tap water, disinfected in 70% alcohol/5 min, washed 5 times with sterile tap water, then for every 10 seeds of *C. annuum* were inoculated with 1.0 mL of *X. autotrophicus*, finally sown in Leonard's jars by randomized block diagram with 3 controls, 6 treatments (T) with 6 replicates: absolute control (AC) = *C. annuum* uninoculated irrigated only with water; relative control one (RC1) = *C. annuum* uninoculated fed with NIF at 100%; relative control two (RC2)= *C. annuum* uninoculated fed with NIF and POF at 25%; (T1)= *C. annuum* with *X. autotrophicus* with NIF at 50% and POF at 100%; (T-2)=*C. annuum* with *X. autotrophicus* with NIF at 100% and POF at 50%; (T-3)=*C. annuum* with *X. autotrophicus* with the NIF at 100% and POF at 50%; (T-4)= *C. annuum* with *X. autotrophicus* with the NIF at 0 % and POF at 50%; (T-5)= *C. annuum* with *X. autotrophicus* with NIF at 25 % and POF at 25%; (T-6)= *C. annuum* with *X. autotrophicus* with NIF and POF at 0%. The response variables were germination percentage and days to emergence phenology: height plant (PH), root length (RL), biomass: fresh and dry weight (AFW/RFW/ADW/RDW) parts of plant;[7–9] the experimental data obtained were analyzed by ANOVA and Tukey ($P \leq 0.05$), to establish the minimum significant difference using the Statgraphics Centurion software.[8]

Table 1 Physicochemical parameters* of agricultural soil of Uruapilla" municipality of Morelia, Michoacán, México[22,31]

Parameter*	Value and interpretation
pH (1:2)	6.68 (slightly acidic)
Electrical conductivity:2 (H_2O) (ms/cm)	0.33 (slightly saline)
Apparent density (s/mL)	0.80
Organic Matter (%)	2.27 (poor)
Texture	Loam
Apparent density of soil (g/cm^3)	0.92
Total Nitrogen (%)	0.15 (poor)
Nitric Nitrogen (ppm)	30.16 (poor)
Potassium (ppm)	368
Phosphorus (ppm)	4.65 (poor)

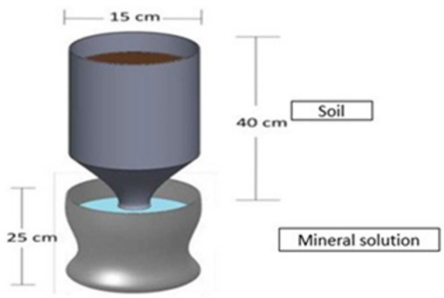

Figure 1 Leonard's jar design for agricultural soil experiment.

To determine the acid and alkaline phosphatase activity of *Xanthobacter autotrophicus*, the leaves, stem and root of *C annuum* with different doses of NIF and POF was carried out as follows: the plant organs were disinfected with 5% NaClO/5 min, then rinsed 5 times with sterile tap water, disinfected in 70% alcohol/5 min, washed 5 times with sterile tap water, then the tissues were macerated in a previously sterilized mortar with 10 mL of 0.85% detergent saline solution, the liquid was recovered and the different macerates were labeled. In total there were 6 different macerates with three replicates. From each of the macerates, 5 mL were taken with 45 mL of sterile distilled water, transferred to 20 mL of universal buffer adjusted to pH 5.5 and 9.0 for the determination of acid and alkaline phosphatases, respectively. The preparations were homogenized at 800 rpm/30 sec, 3 mL of the suspension was recovered and 1.0 mL of p-nitrophenyl phosphate 0.025 M was added, incubated for 37°C/3h, and again centrifuged at 2000 rpm/10 min, 0.5 mL of the supernatant was taken with 4.5 mL of NaOH 0.5 M. The released p-nitrophenol was measured in spectrophotometer at 410 nm.[3,10–13] The experimental data obtained were analyzed by ANOVA and Tukey ($P \leq 0.05$), to establish the minimum significant difference.[8]

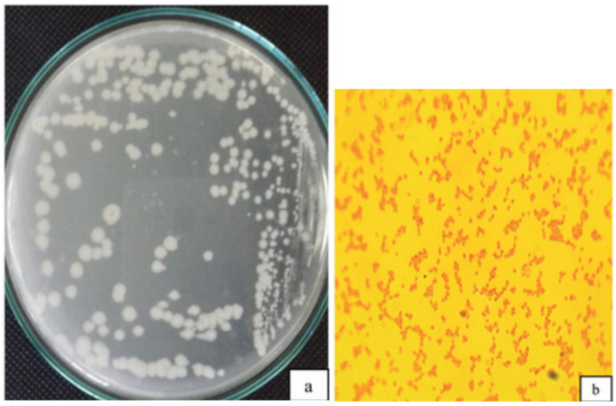

Figure 2 Colonial (a) and microscopic morphology of the (b) Gram of *Xanthobacter autotrophicus* grown on agar without nitrogen or carbon for 48 h at 30°C.

Results and discussion

Table 2 shows the positive effect of *X. autotrophicus* on *C. annuum* with various doses of NH_4NO_3 and PO_4^{-3} fertilizer, on the days to emergence and germination percentage, especially with 100% NH_4NO_3 and 50% PO_4^{-3} as well as when without applying NH_4NO_3 and 50% PO_4^{-3} a germination time of 10 days was registered with 100% germination of the *C. annuum* seed indicating that *X. autotrophicus* recognized the seed exudates invaded it and then colonized the interior of the young *C. annuum* roots by converting organic compounds from the root metabolism *X. autotrophicus* synthesized phytohormones to accelerate germination time and increase the germination percentage of *C. annuum*, the positive effect of accelerating germination and increasing the germination percentage was registered when *X. autotrophicus.* It was inoculated into the seed of *C. annuum,* that demonstrates the capacity of this genus and endophytic species to promote plant growth;[2,5–7;12–26] compared to when was registered with the recommended dose of NH_4NO_3 and PO_4^{-3} at 100% or RC1 which was used with the 25% dose of both fertilizers or RC2 was used. This supports why it is advisable to inoculate *C. annuum* with *X. autotrophicus* at reduced doses of PO_4^{-3}.[19,20,27–32]

Table 3 shows the effect of inoculation of *X. autotrophicus* on *C. annuum* with various doses of NH_4NO_3 or NIF and PO_4^{-3} or POF at the seedling level. It was evident that by colonizing the radical system of *C. annuum* specifically with NIF at 100% and POF at 50% the greatest plant height (PH) was observed with 16.66 cm the maximum root length (RL) with 12.33 cm an aerial fresh weight (AFW) of 1.49 g a radical fresh weight (RFW) of 0.436, an aerial dry weight (ADW) of 0.243 and a radical dry weight (RDW) of 0.109 g as a

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consequence of *X. autotrophicus* from within the radical, conduction system converting compounds released by the plant both the root and the stem generating phytohormones, that optimized the uptake of both fertilizers;[21,23–25] that allowed *C. annuum* to achieve the best aerial and radical growth,[27–29] with numerical values statistically different from the values of *C. annuum* uninoculating with *X. autotrophicus,* at the maximum doses of NIF and POF recommended for *C. annuum.* The positive effect of *X. autotrophicus* on *C. annuum* was also observed in most of the doses of NH_4NO_3 and PO_4^{-3}, except when the concentration of 50% NIF and 100% PO_4^{-3} was applied due to phytohormonal effect,[30,33,35–37] that shows that in that case a response of *C. annuum* was registered uninoculated with *X. autotrophicus* with 100% NH_4NO_3 and PO_4^{-3}, indicating that these doses are too much for the radical capacity of *C. annuum*, that can cause loss of soil fertility due to excess NIF and POF,[28] that can +-also cause water contamination by POF and generation of N_2O by excess NH_4NO_{3*}.[9,10,31] Based on these results it was evident that the dose of PO_4^{-3} should be regulated and inoculated with *X. autotrophicus* to ensure healthy growth of *C. annuum,*[12,15,29] without risk of damaging the environment when the soil is poor in mineral nitrogen as shown by the physicochemical analysis of this agricultural soil.[14]

Table 2 Effect of *Xanthobacter autotrophicus* on seed germination of *Capsicum annum* at different doses of NH_4NO_3 and PO_4^{-3} fertilizer

Treatment (T) **Capsicum annuum***	**Days of emergence**	**Germination percentage (%)**
(AC) Water or absolute control uninoculated non fed with of NH4NO$_3$ and PO4$_{-3}$ fertilize	14^{b**}	50^c
(RC1) NH_4NO_3 (NIF) and PO_4^{-3} fertilizer (POF) at 100% uninoculated or relative control-1	14^b	50^c
(RC2) NIF y POF at 25%, uninoculated or relative control 2	14^b	50^c
T-1 *X. autotrophicus* + NIF at 50% and POF at 100%	10^a	75^b
T-2 *X. autotrophicus* + NIF at 100% and POF at 50%	10^a	100^a
T-3 *X. autotrophicus* + NIF at 50% and POF at 50%	10^a	68.75^b
T-4 *X. autotrophicus* + NIF at 0% and POF at 50%	10^a	100^a
T-5 *X. autotrophicus* + NIF at 25% and POF at 25%	10^a	93.73^a
T-6 *X. autotrophicus* + NIF at 0% and POF at 0%	10^a	62.2^b

*Number of replicates (n) = 6. **Different letters indicate statistical difference by ANOVA/Tukey (P ≤ 0.05).

Table 3 Effect of *Xanthobacter autotrophicus* on phenology and seedling biomass of *Capsicum annuum* at different doses of NH_4NO_3 and PO_4^{-3} fertilizer

*n =6. **Different letters indicate statistical difference by ANOVA/Tukey (P ≤ 0.05).

Treatments (T)/	**Plant height**	**Root length**	**Fresh weight (g)**		**Dry weight (g)**	
C. annuum*	**(cm)**	**(cm)**	**Aerial**	**Radical**	**Aerial**	**Radical**
(AC) Water or absolute control	5.0^{d**}	4.33^c	0.03^d	0.001^c	0.001b	0.002^c
(RC1) NH_4NO_3 (NIF) and PO_4^{-3} (POF) fertilizer at 100% uninoculated or relative control 1	6.3^d	5^c	0.10^b	0.013^b	0.013^b	0.004^d
(RC2) NIF y POF at 25% uninoculated or relative control 2	5.6^d	6.33^c	0.10^b	0.017^b	0.013^b	0.003^d
T-1 *X. autotrophicus* + NIF at 50% and POF at 100%	7^d	6.66^c	0.16^b	0.025^b	0.026^b	0.004^d
T-2 *X. autotrophicus* + NIF at 100% and POF at 50%	16.66^a	12.33^a	1.49^a	0.436^a	0.243^a	0.109^a
T-3 *X. autotrophicus* + NIF at 50% and POF at 50%	10.33^b	7.33^b	0.30^b	0.06^b	0.050^b	0.015^b
T-4 *X. autotrophicus* + NIF at 0% and POF at 50%	11.66^b	9.33^b	0.61^b	0.157^{ab}	0.092^b	0.030^b
T-5 *X. autotrophicus* + NIF at 25% and POF at 25%	9.66^c	9.0^b	0.10^b	0.025^b	0.059^b	0.007^d
T-6 *X. autotrophicus* + NIF at 0% and POF at 0%	8.66^c	6.33^{bc}	0.08^c	0.019^b	0.015^b	0.006^d

Figure 3 shows the phenology of *C. annuum* inoculated with *X. autotrophicus* at different doses of NH_4NO_3 or NIF and PO_4^{-3} or POF. Where it was observed that *C. annuum* with *X. autotrophicus* and 100% NIF with 50% POF, reached the greatest plant height (PH) and root length (RL), an intense green color and wide leaves,[3,4,10–13] with an evident statistical difference compared to *C. annuum* uninoculated with *X. autotrophicus,* 100% NIF and POF. In general, it was observed that except in the case of *C. annuum* plus *X. autotrophicus* with NIF and 50% POF, the response of *C. annuum* to *X. autotrophicus* at different doses of both fertilizers. *C. annuum* had an intense leaf color, a greater number of leaves, greater PH and RL.[8,12,14,16,29] That supports that *X. autotrophicus* when colonizing the conduction system of *C. annuum,* converted root and stem metabolites into phytohormones that optimized the different doses of NIF and POF to the maximum, consequently increasing chlorophyll in *C. annuum* for healthy growth at the seedling level, that allows a favorable forecast in yield[8,9,15,16] without compromising soil fertility,[17,18] that allows mitigating global warming by avoiding the generation of N_2O, from the remaining NH_4NO_3 or PO_4^{-3} eutrophication of fresh water due to excess.[6,10,12–14]

Figure 3 Effect of *Xanthobacter autotrophicus* on seedling phenology of *Capsicum annuum* at different doses of NH_4NO_3 and PO_4^{-3} fertilizer.

AC= *C. annuum* uninoculated irrigated only with water or absolute control; RC1= *C. annuum* uninoculated fed with of NH_4NO_3 (NIF) and PO_4^{-3} (POF) fertilizer at 100% or relative control; RC2= *C. annuum* fed with NIF and POF at 25% or relative control 2; T1= *C. annuum* + *X. autotrophicus* with NIF and POF at 50%; T2= *C. annuum* + *X. autotrophicus* with NIF and POF at 50%; T3= *C. annuum* + *X. autotrophicus* + NIF at 100% and POF at 50%; T4= *C. annuum* + *X. autotrophicus* with NIF at 100% and POF at 50%; T5= *C. annuum* + *X. autotrophicus* with NIF at 100% and POF at 50%; T3 = *C. annuum* with *X. autotrophicus* with NIF and POF at 50%; T4 = *C. annuum* + *X. autotrophicus* with NIF at 0% and POF at 25%; T5 = *C. annuum* + *X. autotrophicus* with NIF and POF at 25%; T6 = *C. annuum* with *X. autotrophicus* with NIF and POF at 0%.

Table 4 shows the activity of acid and alkaline phosphatases of *C. annuum* inoculated with *X. autotrophicus* and the dose of NH_4NO_3 at 100% or NIF and PO_4^{-3} at 50% or POF at the stem level and roots at the seedling level.[10,11,15] Where it was evident that the optimization of PO_4^{-3} or POF was associated with the ability of *X. autotrophicus* to colonize both the stem and the root to increase, the activity of alkaline phosphatase with 138 µg/mL and 81.90 µg/mL, compared to the low activity of both acid and alkaline phosphatase of *C. annuum* uninoculated with *X. autotrophicus*, at the 100% dose of NIF and POF, in the stem and even lower in the root, it was clear that acid and alkaline phosphatase participate little in the uptake of PO_4^{-3}, naturally that makes it even more necessary to use *X. autotrophicus* as an inducer of alkaline phosphatase activity, to optimize both the uptake of PO_4^{-3} from the soil or POF, as a biological strategy;[2–5] to avoid the euphorification of fresh water by excess PO_4^{-3}, that is not uptake by the plant, the Kps of PO_4^{-3} and the soil pH.[10,16,24,29] The sum of these factors contributes to the loss of soil fertility and contamination of surface water, which is why *X. autotrophicus* is an endophyte, that is convenient to use for the optimization not only of NH_4NO_3, but also a sustainable agriculture and mitigation of global warming.[33,35–37]

Table 4 Acid and alkaline phosphatase activity of *Xanthobacter autotrophicus* from *Capsicum annuum* with NH_4NO_3 (NIF) and PO_4^{-3} (POF) fertilizer

Treatments/stem and root of *Capsicum annuum**	**Type of phosphatase**	**Concentration of released p-nitrophenol (µg/mL)**
(AC) Absolute control (saline solution)	Acid	-
	Alkaline	-
(RC1) Stem of *C. annuum* uninoculated with 100% nitrogen (NIF) and phosphate (POF) fertilizer.	Acid	1.36c**
	Alkaline	1.29 c
(RC-1a) Root of *C. annuum* uninoculated with NIF and POF at 100%.	Acid	0.64c
	Alkaline	0.33c
T-3 Stem of *C. annuum* with *Xanthobacter autotrophicus* with NIF at 100% and POF at 50%.	Acid	38.70a
	Alkaline	81.90b
T-3 Root of *C. annuum* with *Xanthobacter autotrophicus* with NIF at 100% and POF at 50%.	Acid	0.73b
	Alkaline	138.33ª

*Number of replicates (n) = 3. **Different letters indicate statistical difference by ANOVA/Tukey ($P \leq 0.05$).

Conclusion

It was evident that inoculating *C. annuum* seeds with *X. autotrophicus* prior to applying NH_4NO_3 or NIF at doses between 50 and 100% of the recommended dose allows for maximum NIF uptake to prevent loss of soil fertility while also avoiding the generation of N_2O, a greenhouse gas, thereby mitigating global warming. At the same time, *X. autotrophicus*, with its ability to invade the plant from the root through the conduction system, improves PO_4^{-3} or POF uptake through the synthesis of phosphatases, especially alkaline and less so acidic, for healthy *C. annuum* growth without the risk of POF not being uptake due to the restricted availability of POF in the soil, combined with the pH, causing eutrophication in surface water near the agricultural area where *C. annuum* is cropping. The plant growth promotion activity of *X. autotrophicus* on *C. annuum* constitutes an ecological tool for cropping of this vegetable.

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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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