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Aspergillus niger solubilizes phosphates with a positive effect on the healthy growth of *Solanum lycopersicum* and *Capsicum annuum*

Abstract

The application of chemical fertilizers to agricultural soil impacts the production costs of plant crops, and in excess, deteriorates soil quality. Phosphorus as phosphates (PO_4^{-3}) is a compounds that, at contact with the soil, can react with aluminum (Al), calcium (Ca), and iron (Fe) to form insoluble phosphates. However, there are microorganisms capable of solubilizing insoluble $Ca_3(PO_4)_2$ (phosphates) into soluble ones. Among these is the genus and species: *Aspergillus niger*, which, along with other microorganisms, promotes healthy plant growth. *A. niger* synthesizes organic acids in the rhizosphere of plants to solubilize phosphates, besides some phosphatases that are then uptake, thus promoting plant health growth and productivity. This process utilizes phosphates from the soil, based in this fact the aims of this research are: i) to determine the phosphate-solubilizing capacity of *A. niger* in liquid and solid NBRIP culture medium; ii) to analyze the effect of soluble phosphorus, spores, and mycelium of *A. niger* on the healthy growth of *Capsicum annuum* and *Solanum lycopersicum* iii) molecular identification of the phosphate-solubilizing *A. niger* isolated. For this purpose, the phosphate-solubilizing capacity was tested on NBRIP agar, where it demonstrated a solubilization index of 2.25 mm at pH 6. In liquid NBRIP, enhancing the $Ca_3(PO_4)_2$ concentration increased the concentration of soluble phosphorus generated, with a positive correlation of 0.8729 between $Ca_3(PO_4)_2$ and 72 h of agitation. It was also demonstrated that the application of soluble phosphorus generated by *A. niger* to seedling of *S. lycopersicum and C. annuum* promoted height, number of leaves, and stem diameter, and reduced phosphorus deficiency in both compared to the same plants without this treatment. *A. niger* was morphologically and molecularly identified. Therefore, it is concluded that *A. niger* is an ecological alternative for optimizing phosphate fertilizers applied to the soil for healthy plant growth.

Keywords: soil, phosphate solubilization, healthy plant growth, phosphate solubility index, Kps or solubility constant

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Introduction

In general, the soil contains reserves of insoluble phosphorus as $Ca_3(PO_4)_2$.[1] In part due to applications of phosphorus fertilizers, as well as $(NH_4)_2HPO_4$ this is PO_4^{-3}not uptake by the root system of plants, because of the soil pH and the narrow solubility product constant or (Ksp).[2,3] It is well known that one possible ecological solve for this agricultural problem, could be to apply some phosphate-solubilizing microorganisms, that by different solubilization mechanisms, releasing soluble phosphates, that could be uptake by plant roots, promoting plant healthy growth and productivity.[4,5] *Capsicum annuum* and *Solanum lycopersicum* are two commercially valuable plant species that, in besides nitrogen fertilizer, require PO_4^{-3}.[6,7] The problem of this inorganic chemical compounds is that reactions with soil cations according to pH, that increased agricultural production cost.[8–10] An ecological alternative for solving this problem is to apply or to inoculate soil or plant seed with *Aspergillus niger* or its releasing products related with PO_4^{-3} solubilization to increase its availability by acidified soil pH with organic acids, that it releases into the soil, as well as through chelation and the synthesis of acid and alkaline phosphatase to promote PO_4^{-3} uptake by plant roots of *C. annuum* and *S. lycopersicum*.[11–14] Therefore, the objectives of this research were: i) to determine the phosphate-solubilizing capacity of *A. niger* in liquid and solid NBRIP culture medium; ii) to analyze the effect of soluble PO_4^{-3} combined with *A. niger* spores and mycelium on the growth of *C. annuum* and *S. lycopersicum* and iii) to morphologically and molecularly identify the phosphate-solubilizing *A. niger* isolated.

Materials and methods

Location

This research was conducted in the Microbiology and Greenhouse Laboratory of the Parasitology Department at the Universidad Autónoma Agraria Antonio Narro, in Buenavista, Saltillo, Coahuila, Mexico 25° 22" N and 101° 02" W; 1742 m above sea level.

Origin and identification of phosphate-solubilizing *A. niger*

An *A. niger* isolated was used, recovered from the rhizosphere of *Muhlenbergia macroura* (grassgrass), and morphologically identified based on mycelium type and microscopic reproduction. It was found to be capable of solubilizing $Ca_3(PO_4)_2$ in NBRIP (National Botanical Research Institute Phosphate Growth Medium) described in literature[15–19] with the following chemical composition (g/L): $Ca_3(PO_4)_2$ 5, $MgCl_26H_2O$ 5, $MgSO_4$ $7H_2O$ 0.25, KCl 0.2, $(NH_4)_2SO_4$ 0.15, dextrose 10, agar 18, a medium specifically designed for the isolation of phosphate-solubilizing microorganisms.[3,4] Species confirmation was performed by sequencing the internal transcribed regions ITS1 and ITS4 of the rDNA amplified by rtPCR[12,17]

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Determination of the phosphate-solubilizing capacity of *Aspergillus niger* in liquid and solid NBRIP

This test was performed in Petri dishes with NBRIP agar at pH values of 4, 5, 6, and 7; each pH value was considered a treatment. A 5 mm diameter portion of culture medium containing 48 h of *A. niger* mycelial growth was inoculated into the center of the NBRIP. The NBRIP dishes were incubated at 28 °C with a 12:12 light/dark photoperiod.[18,19] Solubilization was measured using a digital vernier caliper.[20] The solubilization halo of $Ca_3(PO4)_2$ in *A. niger* was measured in mm every 24 h until the entire surface of the Petri dish was covered.[21–24] A completely randomized design was used with four pH values and three replicates per treatment or pH value. The numerical values were subjected to an analysis of variance (ANOVA) by the statistical software SAS version 9.4. The mean values of $Ca_3(PO4)_2$ solubilization efficiency were compared and stratified according to Tukey's test ($\alpha = 0.05$). At the end of the experiment (168 h), the solubilization index (SI) for each pH value was determined using the following formula: SI = microorganism diameter + solubilization halo / microorganism diameter.[23–25] For the determination of phosphate solubilization in liquid NBRIP, 2000 ml baffled Erlenmeyer flasks were used, containing 700 ml of $Ca_3(PO_4)_2$ with the following concentrations: T1 = 1.0 g/L, T2 = 2.0 g/L, T3 = 3.5 g/L, and T4 = 5.0 g/L. Each flask was sterilized and inoculated with a 5 mm diameter portion of *A. niger* mycelial culture medium with 72 h of growth. The flasks were shaken at 150 rpm for 72 h at 30°C and a 12:12 light: dark photoperiod. A completely randomized design with four treatments or concentrations was used.[23–25] The determination of $Ca_3(PO4)_2$ was carried out with the Hanna Instruments H1706 colorimetric phosphorus high range kit, with a 10 ml sample of each concentration of $Ca_3(PO4)_2$: by the colorimetric reaction that determines the solubilization of $Ca_3(PO4)_2$ generated by *A. niger* in the culture medium with a Thermo Spectronic Spectrophotometer (Figure 1).[12,16,18]

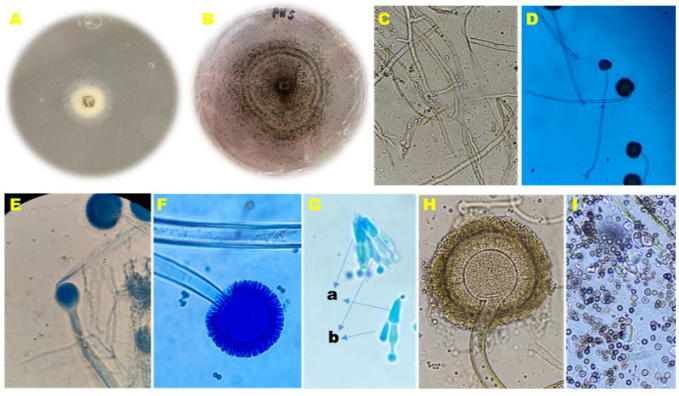

Figure 1 Morphological structures of *A. niger:* A) yellow mycelial growth after 48 h, B) black mycelial growth after 168 h, C) septate mycelium, D) long conidiophores. E) vesicle. F) vesicle surrounded by metulae and phialides, G) a- metulae and b- phialides, H) vesicle with metulae, phialides, and spores, I) dark brown spores.

Phosphate solubilizing capacity of *A. niger* on NBRIP agar

Effect of soluble phosphorus, spores, and mycelium of *A. niger* on the growth of *C. annuum* and *S. lycopersicum.*

Seeds of *S. lycopersicum* variety Floradade and *C. annuum* hybrid Platino were sown in 200-cell polystyrene trays with a 3:1 mix of peat-moss and perlite. Eighteen days after germination, 16 seedlings of each variety, reaching a height of 5 cm, were selected and transplanted to a 60-cell polystyrene tray containing a 1:3 mix of peat-moss and soil for the phosphate-solubilizing experiment; with a completely randomized experimental design with three treatments and an absolute control: T1= soluble phosphorus of $Ca_3(PO_4)_2$ solubilized by *A. niger*, T2= spores of *A. niger* 2 x 107 x ml, T3= mycelium of *A. niger*, T4= absolute control or water; with four repetitions per treatment, total of 16 experimental units shown in Table 1, for both *C. annuum* and *S. lycopersicum*. Treatments 1 to 3 were applied 10 days after transplanting (DAT), and repeated at 20, 30 and 40 DAT. The following phenological response variables were evaluated: plant height, stem width, number of leaves and number of leaves with PO_4^{-3} deficiency, using a completely randomized block design. The experimental results were subjected to ANOVA and Tukey's mean comparison test (P=0.05) with the statistical program InfoStat version 2019.1.2.0.[15,16,18]

Table 1 Experimental design to analyze the effect of soluble phosphorus, conidia and mycelium of *A. niger* on the growth of *C. annuum* and *S. lycopersicum*

T2	T1	T4	T3
T1	T2	T3	T4
T4	T3	T2	T1
T3	T4	T1	T2

Results & discusion

In Table 2, it was registered that the solubilization of $Ca_3(PO_4)_2$, was directly related to the growth of *A. niger*, represented by uppercase letters, while lowercase letters indicate the type of treatment. At 48 h, the numerical values showed a statistically difference. In T3, the greatest solubilization of $Ca_3(PO_4)_2$, was registered with 12.43 mm. At 120 h and 168 h of incubation, there was no statistically difference. The treatment with the largest solubilization halo was A. *niger* T3, shown in Figure 15C with 66.56 mm, in Table 2 with an SI of 2.25 mm, followed by *A. niger* or T4 with a diameter of 65.48 mm registered in Table 2 with an SI of 2.24 mm observed in Table 3 and Figure 15D. In third place, *A. niger* or T1 with a diameter of 61.78 mm shown in Table 2 with an SI of 2.09 mm, in Table 3 and Figure 15 A and finally *A. niger* or T2 with a diameter of 60.87 mm registered in the table with an SI of 2.08 mm shown in Table 3 and Figure 15 B, these results were similar to those reported in literature[3,7,12] with a strain of *A. niger* with a growth of 15 mm in diameter and a $Ca_3(PO_4)_2$, solubilization halo with an SI of 4.3 mm in 14 days, unlike this research an SI of 2.25 shown in Table 2, in 7 days, of *A. niger* isolated from the rhizosphere of a wild grass that had a greater growth than that reported.[18,22–25] The growth of A. niger and the diameter of the $Ca_3(PO_4)_2$ solubilization halo were related to the genetic capacity to solubilize $Ca_3(PO4)_2$ phosphate, which depends on the synthesis of organic acids released from the sugars they use as a source of carbon and energy. Another genetically induced mechanism is the formation of acid and alkaline phosphatases that depend on the pH of the environment or the culture medium. These are produced to release $Ca_3(PO_4)_2$from other substances that prevent them from using it as a source of P for energy production, as well as for the growth of the fungus, especially when inoculated in a plant culture where the soil does not allow the uptake of $Ca_3(PO_4)_2$ for healthy plant growth (Figure 2).[26–28]

Table 2 Phosphorus solubilization of $Ca_3(PO_4)_2$ by *A. niger* at different pH in NBRID Agar

	Treatments (mm)													
Hours	**T1**			**T2**			**T3**			**T4**			**P>F**	**C.V**
24	5.73	ab	G	5.70	b	G	5.75	ab	E	7.77	a	E	0.01	6.19
48	11.39	ab	F	10.93	ab	F	12.43	a	E	9.83	b	E	0.01	6.08
72	18.76	b	E	18.92	ab	E	22.32	a	D	18.88	a	D	0.03	6.19
96	31.62	cb	D	29.88	c	D	38.77	a	C	32.33	b	C	0.001	2.37
120	43.79	a	C	45.04	a	C	47.38	a	CB	49.88	a	B	0.07	5.16
144	49.42	a	B	52.40	a	B	52.90	a	B	54.04	a	B	0.06	3.24
168	61.78	a	A	60.87	a	A	66.56	a	A	65.48	a	A	0.46	7.66
Pr>F	0.001			0.001			0.001			0.001				
C.V.	3.19			4.22			9.25			4.54				

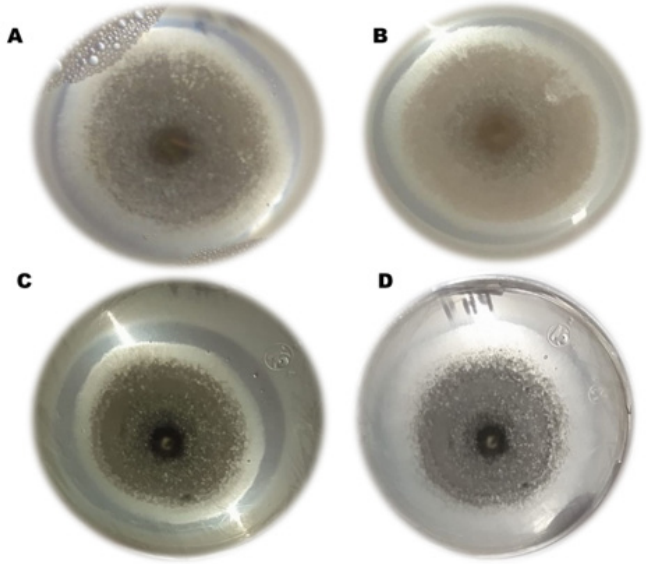

Figure 2 Solubilization test of $Ca_3(PO_4)_2$ by *A. niger* after 168 h of incubation in NBRIP at different pH values. A) T1, NBRIP at pH 4, B) T2, NBRIP at pH 5, C) T3, NBRIP at pH 6 and D) T4, NBRIP at pH 7.

Table 3 shows that the growth of *A. niger* in T1 and T2 showed greater mycelial growth with 56.46 mm and 56.04 mm respectively, greater than the growth of *A. niger* in T3 and T4, which reached greater IS with 53.01 mm and 52.78 mm with similar phosphate solubility indices by *A. niger* associated with the synthesis of carboxylic acids derived from the organic carbon source, the concentration and organic or inorganic type of nitrogen source, the pH and the initial amount of $Ca_3(PO_4)_2$ in the solid or liquid culture medium.[3,4,12]

Table 3 Mycelial growth and $Ca_3(PO_4)_2$ solubilization index of *A. niger* in NBRIP after 168h of incubation.

	Mycelial growth (mm)	Solubility index (SI)
T1	56.46	2.09
T2	56.04	2.08
T3	53.01	2.25
T4	52.78	2.24

*Means with a common letter were not statistically different (p >0.05).

In Figure 3 it shown standard curve of soluble phosphorus where colorimetrically was quantify the concentration of soluble phosphorus generated by *A. niger* when grown in NBRID with $Ca_3(PO_4)_2$, a standard curve of soluble phosphorus was initially prepared using a spectrophotometer with K_2HPO_4 at different phosphorus concentrations, As observed in the colorimetric analysis of phosphorus, a proportionality was registered the determination, indicated by a correlation coefficient greater than 0.92, suggesting a correlation between the phosphorus concentration in solution and the color detected by the spectrophotometer at 880 nm.

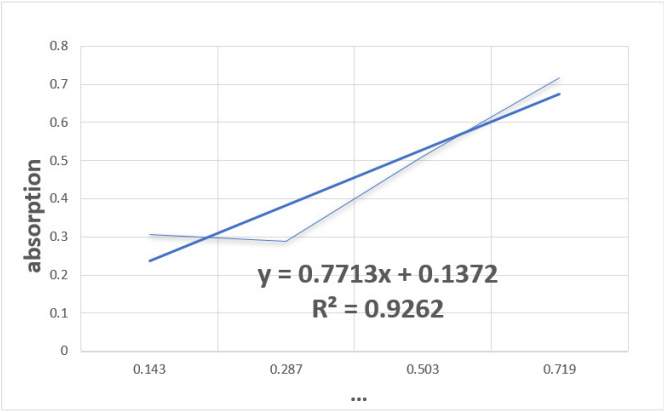

Figure 3 Standard curve for the determination of soluble phosphorus.

Phosphate-solubilizing capacity of *A. niger* in NBRIP Broth

In Figure 4 is show that when *A. niger* was grown in NBRIP broth in shaken flasks with different concentrations of $Ca_3(PO_4)_2$, the concentration of soluble phosphorus in the NBRIP broth supernatant was analyzed spectrophotometrically using the standard curve with K_2HPO_4 as a reference (shown in Figure 3). The soluble phosphorus concentration increased with increasing $Ca_3(PO_4)_2$ concentration.[8,10] The highest concentration of phosphorus solubilized by *A. niger* was found in the culture medium with the highest $Ca_3(PO_4)_2$ concentration of 5 g/L, These results differed from those reported,[7,10–12] whom observed that increasing the $Ca_3(PO_4)_2$ concentration decreased the concentration of soluble phosphorus in the strain of *Pseudomonas sp P2S* culture medium.[13,14,16] While reported a similar result to that of this investigation, with a *Brevibacillus brevis* that reached a phosphate solubilization diameter in solid culture medium with a correlation

of 0.9987 where with the longer incubation time greater $Ca_3(PO_4)_2$ solubilization in a directly proportional manner.[20–23]

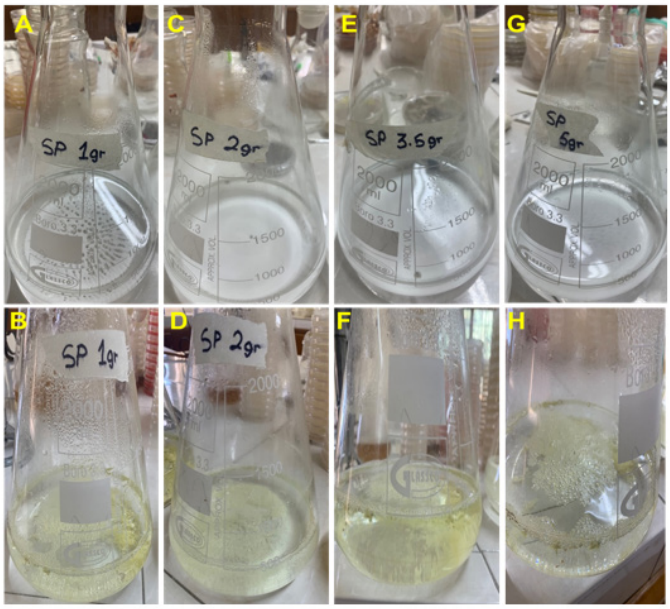

Figure 4 Liquid NBRIP culture medium with different concentrations of $Ca_3(PO_4)_2$ before and after inoculation with *A. niger*.

A) T1= 0.143 g/L before inoculation with *A. niger,* B) T1 at 72 h after inoculation with *A. niger*, C) T2= 2.0 g/L before inoculation with *A. niger,* D) T2 at 72 h after inoculation with *A. niger*, E) T3=3.5 before inoculation with *A. niger,* F) T3 at 72 h after inoculation with *A. niger,* G) T4=5 g/L before inoculation with *A. niger* H) T4 at 72 h after inoculation with *A. niger.*

Figure 5 Effect of soluble phosphorus, spores, and mycelium of *A. niger* on growth of *S. lycopersicum* at seedling stage. In Table 4 and Figure 5 is shown why phosphorus as PO_4^{-3} is essential for plant growth, nucleic acid synthesis during cell division, and ATP generation for all celular activity.[2–6,23,24] In Table 4 *A. niger* under different concentrations of insoluble $Ca_3(PO_4)_2$ caused some results statistically similar on the growth o*f S lycopersicum* although numerically different, *S. lycopersicum* showed greater growth than when grown with $Ca_3(PO_4)_2$ without *A. niger* or T4. Treatment T1 showed the greatest height of *S. lycopersicum* at 18.38 cm, with 30.75 leaves, similar in stem diameter to T2, that had the largest stem diameter at 3.35 mm. Meanwhile, when *S. lycopersicum* was treated with soluble P or T1, the lowest number of leaves with phosphorus deficiency symptoms was registered 0.50 (shown in Figure 5), statistically different from the leaves of *C. annum* without soluble P or *A. niger* or T4, used as a control, that registered the highest number of phosphorus deficiency leaves 6.50. The above is supported by the benefits of inoculation with phosphate-solubilizing microorganisms and application of soluble phosphate, as reported[11,12,16,23] whom inoculated *S. lycopersicum* with *A. niger*, arbuscular mycorrhizal fungi that increased phosphorus content and improved growth compared to *S. lycopersicum* uninoculated with *A. niger*. Meanwhile reported[6,18,27–29] that applying the phosphate-solubilizing genus and species of *Brevibacillus brevis* to *Physalis peruviana* increased leaf area by 3.2 cm², with a greater stem diameter, compared to *P. peruviana* uninoculated with soluble P[10,13,20,22] reported the effect of two phosphate-solubilizing rhizobacteria, *KCH3* and *TSACH2*, from the rhizosphere of *C. annum* var. *habanero* on *S. lycopersicum.*[1–4] Both bacteria increased plant height to 14.29 cm and fresh weight to 128 mg with *KCH3* and to 12.63 cm and fresh weight to 119 mg with *TSACH2*[4,7,15,25] compared to a control plant of *S. lycopersicum* uninoculated or soluble phosphorus, that had a height of 10.51 cm and a fresh weight of 90 g. Similarly reported that the use of $Ca_3(PO_4)_2$ solubilizing microorganisms promotes the growth of *Solanum tuboserum,* resulting in a greater number of stems, 3 to 5 times larger stem diameters, and more leaves without phosphorus deficiencies, compared to uninoculated *S. tuboserum.*[27–29]

Figure 5 Seedlings of *S. lycopersicum* with: A) *S. lycopersicum* seedlings with soluble phosphorus, spores and mycelium of *A. niger* 40 days after sowing. B and C) *S. lycopersicum* leaves from the absolute control, with symptoms of phosphorus deficiency due to purple discoloration of the veins

Table 4 Effect of the application of conidia, mycelium of *A. niger* and phosphorus soluble on the growth of *S. lycopersicum* to seedling stage

Treatments	Plant height (cm)	Steam diameter (mm)	Number leaves	Number leaves with P deficiency
T1- soluble P	18.38 ± 0.77^{a*}	3.16 ± 0.12^{a}	30.75 ± 2.04^{a}	0.50 ± 0.43^{b}
T2- conidia	17.30 ± 0. 77^{a}	3.35 ± 0.12^{a}	30.50 ± 2.04^{a}	1.50 ± 0.43^{b}
T3- mycelium	16.88 ± 0.77^{a}	2.91 ± 0.12^{ab}	28.75 ± 2.04^{a}	2.00 ± 0.43^{b}
T4- control	16.13 ± 0.77^{a}	2.59 ± 0.12^{b}	25.25 ± 2.04^{a}	6.50 ± 0.43^{a}

*Means with a common letter were not statistically different (p >0.05).

Effect of applying conidia, mycelium, and phosphorus solubilized by A. niger on the growth of *C. annuum* at the seedling level. In the Table 5 and Figure 6 is show the experiment applying conidia, mycelium, and phosphorus solubilized by *A. niger* to the phenology of *C. annuum* at the seedling level, all treatments: T1 to T3 were statistically similar but statistically different from the control or T4, that was neither treated not treated with phosphorus. However, there was a numerical difference, as shown in Table 5. Treatment 3, when *C. annuum* was treated with A. niger mycelium, reached the greatest height with 9.13 cm, followed by T1, that, when treated with soluble phosphorus, reached a height of 8.98 cm. Treatment T1 also showed the greatest stem diameter with 1.92 mm, the largest number of leaves 11.25, and the fewest leaves exhibiting phosphorus deficiency 0.2. While *C. annuum* with soluble P or T1 and T2 inoculated with A. niger conids were statistically and numerically different from *C. annum* used as a control without P or A. niger elements, that showed less growth and a greater number of leaves with obvious symptoms of

phosphorus deficiency,[10,15,16,24] according to Figure 6 compared to applying $Ca_3(PO_4)_2$ solubilizing microorganism in other vegetable crops such as *C. annuum* as been reported[30–32] indicate that Serratia plymuthica solubilizes phosphates in *C. annuum* var. poblano, in that promoting an increase in plant height to 9.0 cm compared to *C. annuum* uninoculated with 7.8 cm; as also has reported that Burkholderia ambifaria and B. lata, solubilizing phosphate from phosphate rock in *C. annuum* var. poblano, reduced the recommended phosphorus dose without compromising the healthy growth of the plant.33–35 In the same way it is reported that inoculating Aspergillus sp in Coffea arabica resulted in a 6.25 cm increase in plant height compared to uninoculated C. arabica with solubilizing phosphate.[15,20,23,30,32]

Table 5 Effect of conidia, mycelium and phosphorus solubilized by *A. niger* on the growth of *C. annuum* at seedling stage

Treatment	Plant height cm	Steam diameter mm	Number of leaves	Number leaves with P deficiency
T1- soluble P	8.98 ± 0.50[a*]	1.92 ± 0.04[a]	11.25 ± O.37[a]	0.25 ± 0.26[b]
T2- conidia	8.25 ± 0.50[ab]	1.86 ± 0.04[a]	10.25 ± 0.37[ab]	0.25 ± 0.26[b]
T3- mycelium	9.13 ± 0.50[a]	1.91 ± 0.0[a]	9.50 ± 0.37[bc]	0.50 ± 0.26[b]
T4- control	6.38 ± 0.50[b]	1.46 ± 0.04[b]	8.00 ± 0.37[c]	2.25 ± 0.26[a]

* Means with the same letter with no statistical difference (p >0.005).

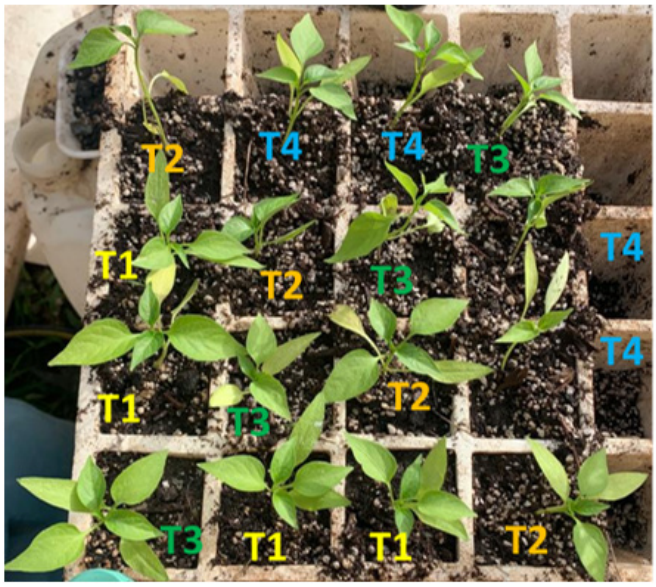

Figure 6 Effect of soluble phosphorus, spores, and mycelium of *A. niger* on growth of *C annuum* at seedling stage

T1) soluble phosphorus, T2) conidia, T3) *A. niger* mycelium and T4) absolute control with not growth.

Conclusions

The results registered with the *C. annuum* and *S. lycopersicum* treatment with soluble P (T1) reached the highest values in the phenology of the two plant species, with the exception of height in *C. annuum,* where *C. annuum* and *S. lycopersicum* with the *A. niger* mycelium (T3) were numerically lower than T1. This is most likely because when phosphorus is applied in soluble form, the plant uptakes and assimilates it quickly, compared to the other treatments. *A. niger* is adapted for the growth of compounds necessary for the solubilization of $Ca_3(PO_4)_2$ through organic acids and acid and/or alkaline phosphatases. Although *A. niger* in the soil has the advantage of efficiently solubilizing $Ca_3(PO_4)_2$ for the root system of plant crops (Cr), as in *C. annuum* or *S. lycopersicum* with *A. niger* mycelium or T3, that reached the greatest plant height compared to the single application. The soluble phosphorus in treatment T1 was effective because *C. annuum* or *S. lycopersicum* rapidly depleted it, while in treatment T3 with *A. niger*, the phosphorus was solubilized in the soil and available to *C. annuum* or *S. lycopersicum.* The importance of applying soluble phosphorus or inoculating agricultural crops with *A. niger*, a phosphate-solubilizing microbial agents lies in the fact that phosphorus is vital for the energy metabolism of *C. annuum* or *S. lycopersicum*. These high-energy phosphates are part of the chemical structure of nucleotides, that are fundamental for the growth and productivity of *C. annuum* or *S. lycopersicum*

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Conflicts of interest

The authors declare no conflicts of interest.

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