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Comparative study of the growth parameters of cowpea bean plants inoculated with *Azotobacter* sp. and urea.

Mercedes Puca-Pacheco (mpucap@hotmail.com)
Universidad San Ignacio de Loyola https://orcid.org/0000-0002-2939-8054
Erika Yovana Gonzales Medina
Elizabeth Ernestina Jayos Rios
Kelva Nathally Llanos Miranda

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Abstract

Aims

There is a growing interest in developing ecological agriculture in order to achieve cleaner production and pollution reduction related to the use of chemical fertilizers. The present research goal is to compare the effects of chemical fertilizer such as urea, in comparison to biofertilizer based on bacterium *Azotobacter* sp., on the growth of cowpea bean (Vigna unguiculata L. Walp).

Method

A small block experimental design was used, in which the concentrations of chemical fertilizer such as urea and biological fertilizer *Azotobacter* sp. The dosages of the fertilizers were varied to evaluate the dependent variables such as the length of the aerial part (LAP), root length (RL), fresh weight of the aerial part (FWAP), dry weight of the aerial part (DWAP), root fresh weight (RFW), root dry weight (RDW) and stem thickness (ST).

Results

It was demonstrated that the optimal dosage was $1.9x10^9$ total cells contained in 1 mL of biofertilizer *Azotobacter* sp getting an average length of aerial part (LAP) of 16.333 ± 0.757 cm. While with urea, we have obtained length values of aerial part of 14.267 ± 0.850; 15.767 ± 0.987; 15.567 ± 1.041cm for fertilizer dosage volumes of 1, 2 and 3 mL respectively. A control test without fertilizers was carried out for comparison.

Conclusions

In conclusion, the replacement of urea with the biofertilizer *Azotobacter* sp. is feasible, being a viable alternative to reduce soil and environmental pollution.

Introduction

Chemical fertilizers are used in various formulations to improve soil fertility. However, many of them are not fully assimilated by plants. In the case of urea, approximately 50% volatilizes, so the use of alternative sources of nitrogen and less polluting for the environment is recommended (EPA, 2016).

At an international level, researchers join efforts to use new environmentally sustainable alternatives (Hungary et al. 2016). In the case of *Azotobacter* or *Rhizobium*, they have the ability to fix atmospheric nitrogen (N_2) and convert it into nitrogenous forms that can be assimilated by plants and are considered Rhizobacteria that promote plant growth (PGPR). They are bacteria that stimulate growth improving nitrogen fixation and phosphate solubilization (Glick 2012; Bonilla et al. 2013; Verma et al. 2010; Lluch &

Ligero 1992). *Pseudomonas, Bacillus, Azospirillum, Rhizobium, Arthrobacter Azotobacter, Micrococcus* and *Enterobacter* bacteria are considered PGPR. Their application, as bioinoculants to crops, improves the productivity and quality of crops and they are more ecological than chemical products that are a serious threat to ecosystems (Verma et al. 2010).

PGPRs can be applied to legumes and non-legumes, grasses, barley, and wheat (Verma et al. 2010). In addition to fixing N_2 and promoting growth, *Azotobacter species* have antifungal activity against fungi *Fusarium*, *Aspergillus, Helmintosporium, Alternaria, Cephalosporium, Rhizoctonia, Sclerotiun rolfsii* and *Cladosporium oxysporum* (Jnawali et al. 2015).

The species *Vigna unguiculada* L. Walp, known as "cowpea or Castilla bean", is a legume of great nutritional value in Peru, because it is a source of protein, fiber, minerals and vitamins. According to Deppa et al. (2016), cowpea bean seeds contain 54.5% carbohydrates, 24.1% protein, and 0.1% fat. Similarly, Aramendiz-Tatis et al. (2016) mention that *V. unguiculada* L. Walp has a high protein content between 21.2 and 27.9% carbohydrates 52 g per 100 g and iron 68 mg per 100 g, among other compounds of nutritional value.

Therefore, the purpose of this research was to determine in what concentration the biofertilizer *Azotobacter* sp. can be used to achieve the same growth (LAP) produced in cowpea plants, when urea is used. Therefore, the use of *Azotobacter* is an ecological alternative, since the excessive use of urea, despite containing a high nitrogen content (46% nitrogen) and its low cost compared to other chemical fertilizers, its prolonged use produces water pollution by nitrates, eutrophication and emission of gases into the atmosphere.

Materials And Methods

Biological samples and culture media

The strain of *Azotobacter* sp. was cultivated in Winogradsky medium, free of nitrogen, which consisted in a solution composed of dipotassium phosphate (KH_2PO_4), 0.25g; magnesium sulfate ($MgSO_4 .7H_2O$), 0.125g; sodium chloride (NaCl), 0.125g; Sodium Molybdate ($Na_2Mo.5H_2O$), 0.005g; manganese sulfate ($MnSO_4 .4H_2O$), 0.005g; Calcium Carbonate ($CaCO_3$), 0.1g; glucose, 10g and agar, 15g; all of them were dissolved in water until reaching a final volume of 1L and the solution had a pH equal to 7. Bacterial growth was evaluated at incubation temperature (28°C) for 48 h. Bacterial cells were gram-stained and then observed under a compound microscope with a Leica camera.

Sowing, transplanting and inoculation.

The germinated plants were transplanted into the pots with the help of a clamp, one seed per pot. After 48 h, different dosages were inoculated near the root (See Table 1). Germination, sowing, inoculation and growth were carried out during March. The plants grew in a greenhouse located in Lima, with relative

humidity between 65% and 75% and controlled temperature between 20°C (night) and 30°C (day). All the pots were simultaneously irrigated with the same amount of water.

Experimental design

A small block experimental design was used, in which the concentrations of chemical fertilizer such as urea and biological fertilizer *Azotobacter* sp. were varied to evaluate dependent variables such as RFW, RDW, FWAP, DWAP LAP, RL and ST. Table 1 shows the dosage volumes of 1, 2 and 3 ml used for each fertilizer and, on the other hand, the control test was carried out in which no fertilizer was used.

Table 1
Treatments applied to the cowpea bean crop

Fertilizer Name	Concentration (%m/V)	Dosing volume (ml)	Fertilizer Type
Urea (U)	0.1	1, 2, 3	Chemical
*Azotobacter (*Azo)	$1.9x10^{9}$ *	1, 2, 3	Biological
Water	0	-	-
*cells Source: own elaboration			

Characterization of farmland

Table 2 shows the methods used for the characterization of the farmland content in the pots for each treatment and without treatment.

Table 2
Methods used for the characterization of the soil (farmland).

Parameters	Method
pH	Potentiometer
EC (Electrical Conductivity) dS /m	Conductivity meter
$CaCO_3$ (%)	Gaseous-volumetric method
OM (Organic Matter) (%)	Walkey and Black method
P(ppm)	Olsen's method
K(ppm)	Acetate extraction
Textural class	hydrometer method
CEC (Cation Exchange Capacity) meq /100g	Saturation with ammonium acetate
Ca^{+2}	flame photometry
Mg^{+2}	flame photometry
K^{+}	flame photometry
Na^{+}	flame photometry
Al^{+3}	Yuan Method
N%	Micro-Kjeldahl method
Source: Own elaboration.	

Results

Determination of cell size and morphological characterization of the bacterium *Azotobacter* sp. In the morphological characterization, transparent colonies were obtained, with an entire border, round shape, aqueous texture and convex elevation, translucent appearance and with mucus (See Fig. 1 and Table 3). According to the description of León et al. (2017) *Azotobacter* colonies are transparent and translucent on Winogradsky culture medium, coinciding with the results obtained in the present investigation, showing that the colonies on Winogradsky medium had a diameter of less than 1mm.

The size of the bacteria ranged between 1.35 and 1.81 µm, with an average value of cell size of 1.49 µm and its morphology is Gram negative bacillary (See Fig. 1). This size is very similar to that reported by Kennedy et al. (2005) who maintain that “the bacterial cells of *Azotobacter* sp. They can be rod-shaped with blunt edges to ellipsoidal and coccoid and can measure from 1.6 to 2.7 µm in diameter and from 3.0 to 7.0 µm in length”. The bacteria *Azotobacter* sp. are Gram negative, non-symbiotic, aerobic and

diazotrophic. They grow optimally in nitrogen-free media, with phosphate, magnesium, calcium, molybdenum, iron and carbon (Heidari et al., 2018). The culture medium Winogradsky, inoculated with the bacteria, was kept in a shaking water bath (28°C/48 h).

Table 3
Characteristics of the bacterial colonies after 48 h of growth.

culture medium	Characteristics of the colonies after 48 h of growth					
	Diameter	Appearance	Shape	Edge	Texture	Elevation
Winogradsky (flask)	< 1mm < 0.5mm	translucent	round	Whole	watery	convex
Winogradsky (plate)	1mm	translucent	round	Whole	watery	convex
† Samples stored refrigerated at 4°C in flask and plate. Source: Own elaboration.						

Bacterial concentration was performed after 48 h of growth and registered 1.8 x 10^{9} cells/mL, which is similar to the number 6 according to the Mac Farland scale (See Table 4). This result coincides with Zúñiga (2012), who maintains that inoculants under laboratory conditions require an approximate concentration of 10^{8} to 10^{9} cells/mL for successful inoculation in plants.

Table 4
Mac Farland scale used for bacterial quantification.

No.	$BaCl_2$ 0.048M V(mL)	H_2SO_4 0.36M V(mL)	Vf (mL)	Number of cells
0.5	0.05	9.95	10	$0.15x10^9$
1	0.1	9.90	10	$0.30x10^9$
2	0.2	9.80	10	$0.60x10^9$
3	0.3	9.70	10	$0.90x10^9$
4	0.4	9.60	10	$1.20x10^9$
5	0.5	9.50	10	$1.50x10^9$
6	0.6	9.40	10	$1.80x10^9$
7	0.7	9:30	10	$2.10x10^9$
8	0.8	9.20	10	$2.40x10^9$
9	0.9	9.10	10	$2.70x10^9$
10	1.0	9.00	10	$3.00x10^9$

Germination and its evaluation.

A germination test was carried out to verify the viability of the seeds. In the germination process, 100 cowpea bean seeds were used, after disinfection with a solution 4% sodium hypochlorite for 5 min, then they were washed with cold water and submerged in 500 mL of water for 1 h. In a tray, it was placed with double moistened paper towel, the seeds separated from each other by 1 cm were placed for their germination. They were sprayed with distilled water every 6 hours for 2 days so that the seeds remain moist, and 99% germination was obtained, with an average root length of 10.34 mm. On the third day, they were planted in pots for growth and subsequent evaluation.

Soil Evaluation.

The soil used for planting contained organic matter, 0.97%; electrical conductivity, 0.51dS/m; phosphorus, 7.5 ppm; potassium, 143 ppm and CEC, 16.00. It was a loam soil with 21% clay, 30% silt and 49% sand. The average soil pH was 7.50. These results are reported in Tables 5 and 7. The soil from the farm was placed in pots with a capacity of 1 kg, previously aerated one day before sowing.

The pH (potential of hydrogen) determines the degree of adsorption of ions (H^+), and is the main indicator of the nutrient availability in plants, influencing the solubility, mobility, availability and other constituents and inorganic contaminants present in the soil. In this experiment, the pH in all the treatments has been maintained at a value close to 7.5, considering it slightly basic (See Table 5).

According to the edaphoclimatic requirement, the cowpea bean is considered a rustic plant and according to Arias et al. (2007) who reported that the cowpea grows better in soils with a loamy texture and does not tolerate soils with poor drainage. Its optimal pH is in the range of 6.0 to 7.5 grows in humid tropical climates and has optimum growth at temperatures between 20°C and 35°C. As stated above, the experiment carried out in this investigation had favorable conditions for the growth of cowpea beans.

According to Table 5, it is observed that using urea produced an increase in electrical conductivity from 0.51 dS/m to an average electrical conductivity of 0.97 dS/m, which is due to the hydrolysis of urea for the presence of water during irrigation and is catalyzed by the enzyme urease and because the pH of the farmland was greater than 6.3. The hydrolysis reaction occurs according to Eq. 1.

$$CO(NH_2)_2 + H^+ + 2H_2O \rightarrow NH_4^+ + (HCO_3)^- \quad (1)$$

The ammonium Ion (NH_4^+) released in the hydrolysis of urea is in dynamic equilibrium with the ammonia in the atmosphere, as shown in Eq. 2:

$$NH_4^+ \text{ (adsorbed)} \leftrightarrow NH_4^+ + OH^- \text{ (sol. soil)} \leftrightarrow NH_3 + H_2O \text{ (sol. soil)} \leftrightarrow NH_3 \text{ (atmosphere)} \quad (2)$$

The hydrolysis of urea generates a significant increase in pH around the urea granule as it consumes protons. The increase in pH displaces the balance of ammonium and ammonia, favoring the volatilization of NH_3 into the atmosphere, with significant volatilization due to the slightly alkaline ($pH \approx 7.50$). Therefore, the increasing of conductivity is due to the rising salinity in soil. The main cations that give rise to salinity are: sodium, calcium, magnesium and potassium and the main anions are: sulfates, chlorides, carbonates and bicarbonates.

In the treatment with urea, the decreasing of the Cation Exchange Capacity (CEC) was obtained from 16.00 to a value of 15.20 meq/100g and this is due to the fact that the ammonium released from the hydrolysis of urea is retained, in the exchange sites and there is less availability of the cation to be volatilized (See Table 7).

Moreover, when *Azotobacter* sp. electrical conductivity increases from 0.51 dS/m to 0.66 dS/m, being the smallest increase compared to the use of urea (See Table 6). It is observed that it passes from a slightly saline medium to a saline medium and this is due to the increasing of soluble salts in soil. The CEC increase from 16.00 to a value of 17.88 meq/100g was obtained.

The bacterium *Azotobacter* sp. is a nitrogen-fixing PGPR-type bacterium, which allows the transformation of N_2 into bioavailable nitrogen by means of the enzyme called nitrogenase (Annan et al. 2012). This type of bacteria has the ability to solubilize phosphates (Rodriguez et al. 2006), which is why there is an

increase in phosphorus from 7.5 ppm to an average phosphorus content of 8.7 ppm (See Table 5). The bacterium *Azotobacter* sp. has the ability to solubilize phosphates from inorganic or organic compounds, using enzymes such as non-specific phosphatases, phytases, phosphonatases and CP lyases (Lugtenberg and Kamilova 2009).

Table 5
Analysis of pH, EC, $CaCO_3$, OM, P, K, textural class and nitrogen in the soil at the end of its evaluation of each treatment after 1 month of growth of cowpea bean.

Sample	pH (1:1)	EC (1:1) dS/m	$CaCO_3$ (%)	OM %	P ppm	K ppm	Sand (%)	Slime (%)	Clay (%)	Textural class	N %
U1	7.51	0.97	0.10	1.22	8.1	146	49	28	23	Frank	0.05
U2	7.49	0.98	0.10	1.20	8.0	145	49	28	23	Frank	0.05
U3	7.50	0.96	0.10	1.22	8.2	146	49	28	23	Frank	0.05
Azo1	7.46	0.65	0.10	1.22	8.8	143	47	30	23	Frank	0.05
Azo2	7.55	0.60	0.10	1.00	8.7	143	47	30	23	Frank	0.04
Azo3	7.46	0.66	0.20	1.02	8.6	143	47	30	23	Frank	0.05
TC	7.50	0.51	0.10	0.97	7.5	143	49	30	21	Frank	0.04
Source: Own elaboration.											

Table 6
Classification of salinity based on electrical conductivity (Andrades and Martinez 2014).

EC (dS/m)	EC 1/5 (dS/m)	Classification
two	0.35	not saline
2–4	0.35–0.65	slightly saline
4–8	0.65–1.15	Saline
8	1.15	very saline
Source: Own elaboration.		

Table 7
Analysis of the CEC, concentration of cations and anions in the soil at the beginning (TC) and at the end of its evaluation of each treatment after a month of growth of cowpea bean.

Sample	CEC meq/100g	Exchangeable Cations					sum of cations	addition of bases
		Ca^{+2}	Mg^{+2}	K^{+1}	Na^{+1}	$Al^{+3} + H^{+}$		
	meq/100g							
U1	15.04	11.72	2.50	0.36	0.46	0.00	15.04	15.04
U2	15.12	11.88	2.45	0.35	0.44	0.00	15.12	15.12
U3	15.21	11.92	2.47	0.34	0.48	0.00	15.21	15.21
Azo1	17.88	14.65	2.33	0.35	0.55	0.00	17.88	17.88
Azo2	17.12	14.07	2.22	0.37	0.46	0.00	17.12	17.12
Azo3	17.28	14.16	2.30	0.34	0.48	0.00	17.28	17.28
TC	16.00	13.22	2.10	0.31	0.37	0.00	15.04	15.04
Source: Own elaboration.								

Final evaluation of the plants

The final evaluation of bean seedlings was carried out at the end of a month of plant growth. To eliminate humidity and find the final dry weight of the plants, an oven was used at 70°C for 48 h. Tables 8 and 9 show the results of the dependent variables such as the length of the aerial part (LAP), root length (RL), fresh weight of the aerial part (FWAP), dry weight of the aerial part (DWAP), fresh weight of the root (RFW), dry weight of the root (RDW) and thickness of the stem (ST) at different dosage volumes and type of fertilizer. Likewise, the control test was carried out without fertilizer.

Table 8
Results of the response variables RFW, RDW, FWAP, DWAP and humidity at 30 days of *evaluation.*

Sample	RFW (g)	RDW (g)	Root Humidity (%)	FWAP (g)	DWAP (g)	Aerial part Humidity (%)
U1	0.580 ± 0.040	0.097 ± 0.023	83.27	2.616 ± 0.427	0.345 ± 0.092	86.81
U2	0.692 ± 0.084	0.094 ± 0.024	86.42	2.815 ± 0.623	0.389 ± 0.089	86.18
U3	0.699 ± 0.025	0.097 ± 0.023	86.12	2.964 ± 0.440	0.406 ± 0.076	86.30
Azo1	0.507 ± 0.093	0.082 ± 0.011	83.82	2.965 ± 0.241	0.369 ± 0.043	87.55
Azo2	0.887 ± 0.186	0.141 ± 0.048	84.10	3.126 ± 0.179	0.368 ± 0.019	88.23
Azo3	0.537 ± 0.168	0.082 ± 0.024	84.73	2.808 ± 0.726	0.316 ± 0.099	88.75
TC	0.569 ± 0.069	0.093 ± 0.023	83.66	2.860 ± 0.552	0.313 ± 0.068	89.06

Source: Own elaboration

Table 9
Results of the LAP, RL and ST response variables at 30 days of growth.

Sample	LAP (cm)	RL (cm)	ST (mm)
U1	14.267 ± 0.850	13.367 ± 0.551	1.294 ± 0.398
U2	15.767 ± 0.987	14.567 ± 0.907	1.340 ± 0.381
U3	15,567 ± 1,041	15.100 ± 0.854	1.305 ± 0.350
Azo1	16.333 ± 0.757	11,567 ± 1,582	1.917 ± 0.491
Azo2	15.720 ± 0.611	9,520 ± 1,636	1.690 ± 0.477
Azo3	14,420 ± 1,342	9,080 ± 1,515	1.860 ± 0.483
TC	12,467 ± 1,102	8,033 ± 1,909	2.017 ± 0.333
Source: Own elaboration			

Figure 2 shows the growth parameters such as the length of the aerial part (LAP), root length (RL), root fresh weight (RFW), root dry weight (RDW), fresh weight of the aerial part (FWAP), dry weight of the aerial part (DWAP) and stem thickness (ST) of cowpea bean, using a fertilizer dosage of 1, 2 and 3 mL in the treatments and were compared with the control (without treatment).

Discussion

In Fig. 2a, it is observed that at a dose of 1mL *Azotobacter* sp. had a greater growth (LAP) with a stem length of 16.33cm. As the dose of *Azotobacter* sp. was increased from 1mL to 3mL, there was a decrease in the length of aerial part (LAP) and this is due to the fact that increasing the concentration of *Azotobacter* sp generates an increase in phytohormones such as auxins, which cause inhibitory effects (Mantilla 2007). At a dose of 1mL of *Azotobacter* sp, similar growth is achieved than when using 2mL of urea solution, being greater than in the control test (LAP = 12.47 cm). It is important to indicate that the growth of bean plants is due to the ability of *Azotobacter* sp. to produce phytohormones and some volatile compounds, which are responsible for growth (Lugtenberg and Kamilova 2009; Bal et al. 2012). Furthermore, the increase in LAP is due to the rise in CEC from 16.00 meq/100g (without treatment) to a value of 17.88 meq/100g, when inoculated with 1 mL of *Azotobacter* sp. (See Fig. 3) (See Table 7). In the experiment carried out, the soil consisted of 23% clay, with a loamy texture and a slight increase in OM from 0.97

(without treatment) to 1.22% (with treatment) (See Table 5). Due to the low OM content, low % clay material, low CEC values were obtained, since this depends on the type of clay, texture and organic matter and this directly affects the amount and frequency of fertilizer application.

The growth of bean plants is due to the following mechanisms: 1) Attraction of the bacteria towards the rhizosphere of its host, mediated by bacterial-specific chemotaxis towards particular plant exudates (Albareda et al. 2006); 2) Adhesion and colonization to the surface of the bean root where the bacteria must have the ability to adhere to the seeds or roots, for subsequent colonization (Muñoz-Rojas & Caballero-Mellado 2003; Oliveira et al. 2009) and 3) Functionality of the associative symbiosis, which implies an effective establishment of the microorganism-plant relationship.

Figure 2b shows that when using the biofertilizer based on the *Azotobacter* sp., root lengths for doses 1, 2 and 3ml are shorter compared to Urea, and this is because *Azotobacter* sp. produces more than one type of phytohormones, such as auxins, cytokinins, ethylene, gibberellins and abscisic acid (ABA) (Vilchez and Manzanera 2011). These molecules exhibit different specific effects on plant physiology, increasing root volume, favoring a higher respiration rate of the host plant root and the flow of protons in the root membrane; consequently, the absorption of nutrients and soluble minerals is increased (Fibach-Paldi et al. 2012). Therefore, the formation of a greater number of secondary roots and a shorter length of the primary root were observed, compared to those produced in bean plants inoculated with urea.

Similar average values of fresh and dry weight of aerial part were obtained (See Fig. 2c and d), as well as similar average values of fresh and dry weight of root (See Fig. 2e and f) between the treatments with urea and *Azotobacter* sp. and the witness test. Figure 2(g) shows a greater thickness in bean plants inoculated with *Azotobacter* sp. compared to those inoculated with urea.

Conclusions

Greater growth was obtained in bean plants when using a dosage of $1.9x10^9$ cells contained in 1mL of the *Azotobacter* sp. biofertilizer, reaching a length of aerial part of 16.33 ± 0.757 cm, compared to bean plants without inoculation (12.467 ± 1.102) during one month growth, inside a greenhouse.

Similar LPA values were obtained from bean plants when 1 mL of the *Azotobacter* sp. biofertilizer (16.33 ± 0.757 cm) was used, with which 2 mL of urea at 0.1% m/v (15.767 ± 0.987) were inoculated. Therefore, it is possible to consider the *Azotobacter* sp. biofertilizer as an ecological alternative to counteract the environmental damage caused by urea.

Declarations

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Conflict of interest

The authors declare no conflicts of interest regarding the publication of this paper

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Figures

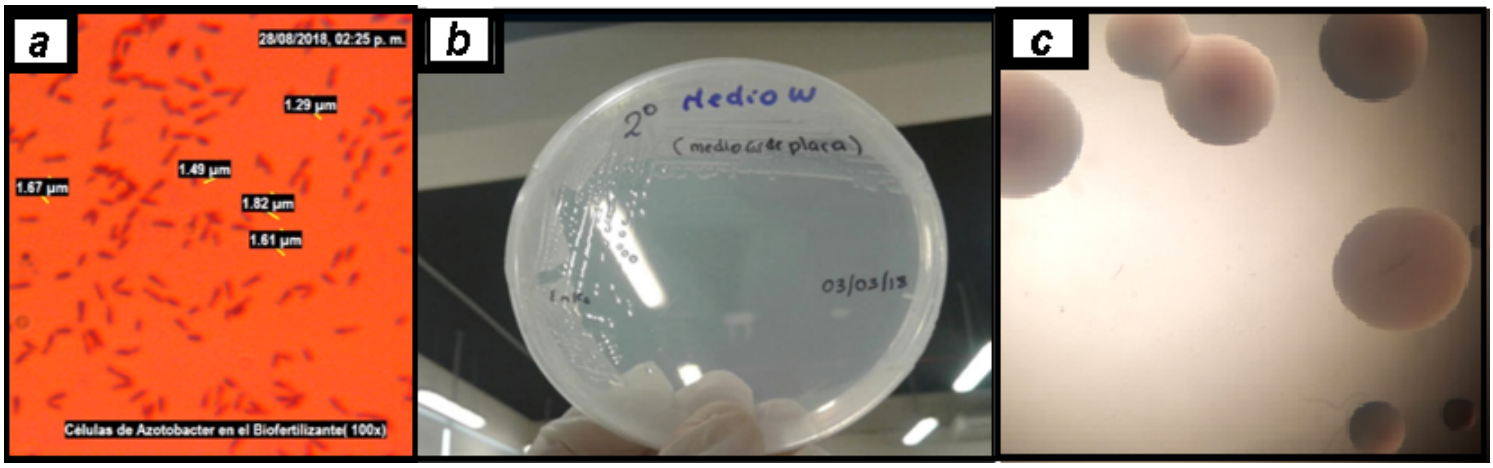

Figure 1

Azotobacter Characterization a) Bacterial cells with Gram stain, 100X; b) growth of the colonies on Winogradsky medium and c) Shape of the *Azotobacter* bacteria without staining.

Figure 2

Evaluation of growth parameters of bean seedlings at the end of a month of growth with treatment with urea, Azotobacter sp and without fertilizer (TC): a) LAP, b) RL, c) FWAP, d) DWAP, e) RFW, f) RDW and g) ST. Source: Own elaboration.

Figure 3

Photographs of the growth of cowpea bean plants after one month of treatment with 1 mL, 2 mL and 3 mL of Azotobacter sp. Source: Own elaboration.