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Effect of *Azospirillum* spp. and *Azotobacter* spp. on the growth and yield of strawberry (*Fragaria vesca*) in hydroponic system under different nitrogen levels

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ABSTRACT

It is well established fact that bacterial species promotes plant growth. This growth enhancing activity was believed to be through different mechanisms such as synthesis of phytohormones, nitrogen-fixing, and biological control. For this reason, in this present investigation we have isolated and identified *Azotobacter* spp. and *Azospirillum* spp. through macromorphologically and micromorphologically in order to assess its effect on growth and yield of strawberry (*Fragaria vesca*) an Albion variety in hydroponic system. The inoculation and co-inoculation of bacterial culture was performed in combination with three nitrogen levels (50, 100 and 150 ppm), growth parameters such as plant height, root length, fresh and dry weight of root and aerial parts, leaf area, chlorophyll content, nutrient content, solid soluble, caliber, yield/plant. It is observed that in T8 (co-inoculation in100 ppm N) group showed significantly increase in plant height (18.57cm), chlorophyll content (48.57 Soil Plant Analysis Development-SPAD), fresh root weight (25.82g) and dry root weight (5.93g), while in treatment group T5 (*Azotobacter* spp. 100ppm of Nitrogen) and T6 (*Azotobacter* spp. 150ppm of Nitrogen) showed significant increase in root length, leaf area, dry and fresh weights of aerial parts. The N content of leaf for all treatments was in the ranges of 2.42 - 2.83 % that is suitable for cultivation. Similarly, the treatment group T5 and T6 showed increase in yield per plant and soluble solids content. So, *Azotobacter* and Nitrogen treatment has growth related benefits in strawberries under hydroponic system.

INTRODUCTION

In Ecuador, the cultivation of strawberry (*Fragaria vesca*) is economically important with an annual growth between 20-30% of the planted area (MAGAP 2011). However, due to the large number of fruits produced per unit area, it requires extensive use of mineral fertilizers, which increases the production cost up to 30% (Hamlet 2001). There is an extensive demand of crops and other agricultural products to full fill the need of day to day growing population. To increase the production it is necessary to depend on the chemical fertilizers. Even though chemical fertilizers are beneficial in increasing the crop yield but at the same time they are harmful to the environment. So, there is a need to implement appropriate management techniques to address potential environmental hazards caused by these fertilizers. One of the possible solutions to lower the risk of accumulation of chemical fertilizers in the environment is combining chemical fertilizers with biofertilizers (Adesemoye *et al.*, 2010). Currently, beneficial microorganisms are used to alleviate the arid conditions like problems of water stress and soil salinity. All microorganisms are the most abundant and efficient to take up this challenge. They harbor and supply nitrogen by fixing free nitrogen in the root system and in return they use carbon sources and different compounds from the plant through exudation. Interestingly, rich bacterial concentration was observed in the rhizosphere or root nodules of the plant and this is the place where the high concentration of nutrients get accumulated, enabling high

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growth rate and bacterial metabolism (Walsh 2008).

The beneficial microorganisms used in place of chemical fertilizers, are not only able to improve the plant growth but also maintain the environmental health and productivity of the soil. Recent studies confirm that a number of species of bacteria mainly associated with the rhizosphere of plants are beneficial to the growth, yield and crop quality.

These are called as Plant Growth Promoting rhizobacteria (PGPR) which include strains of the genus *Azospirillum*, *Azotobacter* and others (Esiteken *et al.*, 2009). These bacteria colonize in the roots of plants, and in doing so promote growth and in some cases protects from diseases. Depending on the bacterial strain their mode of action is either direct or indirect. Indirect mode of action, bacteria provide plant the compounds synthesized by themselves and promote growth.

These compounds may be nitrogen, growth hormones and certain nutrients such as iron or phosphorus, from the natural environment. In case of indirect mode of action, bacteria protect plants from diseases by protecting from different phytopathogenic microorganisms. The implementation PGPR is a potential alternative method of biological control and at the same time a biotechnological tool to reduce the adverse effects of chemicals and allow a suitable and sustainable land management.

Previous studies showed that PGPR stimulate growth and increases yield of different fruits such as apple, cherry and peach (Aslantas *et al.*, 2007, Karlıdag *et al.*, 2007). Of the PGPR group *Azospirillum* spp. is a most studied plant growth promoting bacteria because of its ability to colonize in roots of different plant species and most of the plants are of agricultural importance (Cessán *et al.*, 2008). Another genus belongs to this group is Azotobacter a free-living bacteria that fix atmospheric nitrogen, which belongs to Gammaproteobacteria group, can grow well in the media without nitrogen.

These bacteria use atmospheric nitrogen for the synthesis of the cellular proteins. Cellular protein is mineralized after the death of the cell, thus contributing to the availability of nitrogen for wild plants and crops (Agronet 2012).

Through literature survey we found that there is no information available on the use of these microorganisms in the cultivation of strawberries. *Azotobacter* spp. and *Azospirillum* spp. treatments were the best economic options for strawberry production in a hydroponic system. Therefore we decided to investigate the growth promoting effect of inoculation of *Azotobacter* spp. and *Azospirillum* spp. and co-inoculation of both to strawberry plant under hydroponic system with three levels of nitrogen in the nutrient solution.

MATERIALS AND METHODS

The laboratory studies were conducted at the Autonomous National Agricultural Research Institute INIAP, and field studies were performed at Chaquibamaba, Pichincha province, Quito, Ecuador.

Isolation and morphological characterization of bacteria

Azospirillum spp.

One gram of strawberry roots were placed in an individual test tube containing 9 mL of sterile distilled water (10^{-1} dilution), it was vortexed for 20 sec. and serially diluted to $1x10^{-7}$. For isolation of *Azospirillum* spp. a semisolid nitrogen free NFB medium was used. On the medium through spreader 0.3 ml of bacterial dilution was placed and incubated for 7 days at 32°C. A white bacterial pellicle was formed 2 to 10 mm below the surface of the medium was considered positive (REDCAI 2010). To further confirm the presence of *Azospirillum* spp. Positive samples were taken and streaked on petri dishes containing Malic Acid Red Congo specific medium and incubated for 7 days at 32°C. For further purification colonies turned dark red or scarlet were selected and seeded onto other Petri dishes containing the same media.

On theses colonies biochemical tests such as Catalase, Nitrate reduction, Urease, Indole production, Fructose test and Arabinose test were performed as mentioned by Mac Faddin (1993).

Azotobacter sp.

For isolation *Azotobacter* spp. Ashby (Mannitol 5 g, Dipotassium Phosphate 0.2 g, MgSO4.7H2O 0.2 g, NaCl 0.2 g, $CaSO_4$ 0.1 g, Ca CO_3 0.1 g, H_2O1000 mL) selective culture media which is free nitrogen was used; and Bacteriological media (Molasses, Sulpomag, rock phosphate, yeast, water and agricultural lime) was used in this study. Pinch of soil was sprinkled on the culture medium and inoculated for 7 days at 30°C. Creamy and transparent colonies thus formed around the soil grains were selected.

The bacteria were further subcultured on the petri dishes containing same media. The resulted colonies were further subjected to different biochemical tests such as glucose test, sucrose test, catalase test, nessler test, indole test, glycerol test, starch hydrolysis test and production of 3- indole acetic acid (Tejera *et al.*, 2005).

Field experimental design (Factors under study)

The first factor in the study was the biofertilizers *Azospirillum* spp. and an *Azotobacter* spp. strain used individually (inoculation) or in combination with both (coinoculation) and the second factor was nitrogen used in three different concentrations (50, 100 and 150 ppm). All the field experiments were performed under hydroponic system.

Inoculation

Plant stolons prior to being planted were inoculated and co-inoculated with biofertilizer. These bacterial inocula was prepared with a liquid molasses by immersing roots for 30 minutes and drained for 5 seconds (Table 1) (Zargar 2008, Pedraza 2009).

Table 1: Details of inoculation and co-inoculation treatment.

Symbol	Description
B1	*Azospirillum* spp.
B2	*Azotobacter* spp.
B3	*Azotobacter* spp. + *Azospirillum* spp.

Nutrient solutions

Three different levels of nitrogen concentration (50, 100 and 150 ppm) containing nutrient solutions were prepared and adjusted to pH 5.5 - 5.8 and delivered to plants for irrigation exuding tapes were used (Table 2).

Table 2: Nitrogen levels.

Symbol	Description
N1	50 ppm
N2	100 ppm
N3	150 ppm

Effect on the growth and yield of strawberries under hydroponic system

The present study involved 10 treatment groups (Table 3) including the control (Table 3). In every treatment a morphological assessment of growth (plant height, root length, leaf area, fresh and dry weight of root and aerial part) and performance (size, soluble solids and yield/plant) were made; further the amount of chlorophyll in degrees SPAD and analysis of the nutrient content at leaf level were measured.

Table 3: The details of ten treatment groups involved in the present study.

Treatments	Interaction	N_2Levels (ppm)	Biofertilization
T1	B1N1	50	*Azospirillum* spp.
T2	B1N2	100	*Azospirillum* spp.
T3	B1N3	150	*Azospirillum* spp.
T4	B2N1	50	*Azotobacter* spp.
T5	B2N2	100	*Azotobacter* spp.
T6	B2N3	150	*Azotobacter* spp.
T7	B3N1	50	*Azospirillum*spp. + *Azotobacter* spp.
T8	B3N2	100	*Azospirillum* spp. + *Azotobacter* spp.
T9	B3N3	150	*Azospirillum* spp. + *Azotobacter* spp.
T10 Control	---	---	----

Statistical analysis

As the present investigation was Completely Randomized Design (DCA) a factorial arrangement 3x3 + 1 system was used. Further Coefficient of variation was also calculated. Functional analysis was performed by Duncan test at 5% for Biofertilization and Nitrogen treatments in general.

RESULTS AND DISCUSSION

Morphological identification of *Azotobacter*

Bacterial colonies appeared on the culture medium showed similar morphological characters as that of *Azotobacter* spp. such as cream colored colonies, Gram negative bacilli, large and short, in pairs or in chains (Figure 1). These results were similar to as described by Jimenez (2007), who found gram-negative bacilli, large and short and the colonies of the same coloring and shape.

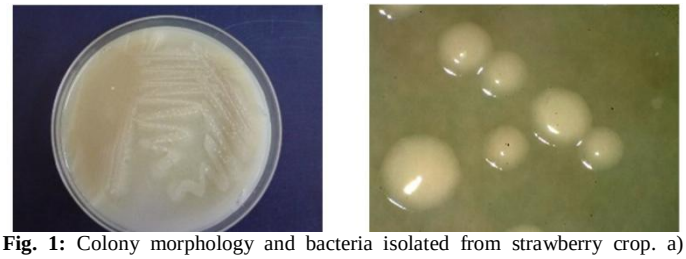

Fig. 1: Colony morphology and bacteria isolated from strawberry crop. a) *Azotobacter* in ashby colonies. b) Cream color, irregular and bright colonies. Ashby (Mannitol 5 g, Dipotassium Phosphate 0.2 g, MgSO4.7H2O 0.2 g, NaCl 0.2 g, CaSO4 0.1 g, Ca CO3 0.1 g, H2O 1000 mL) selective culture media.

Biochemical profile of isolated strains of *Azotobacter* spp.

The biochemical profiles of bacterial isolates were presented in Table 4. A four bacterial isolates were compared with the positive bacterial control. The C1 and C3 colonies were found to be positive for all tests, which showed that these two isolates belong to *Azotobacter* spp.and the same was used inoculation.

Table 4: Biochemical tests of four bacterial isolates including positive control.

Test	C1	C2	C3	C4	Control
Glucose	+	-	+	+	+
Sucrose	+	-	+	+	+
Catalase	+	+	+	+	+
Nessler	+	+	+	-	+
Indole	+	-	+	-	+
Glycerol	+	+	+	+	+

C1-C4 are different isolates.

Morphological identification of *Azospirillum*

Bacterial colonies appeared on the culture medium showed similar morphological characters as that of *Azospirillum* spp. such as colonies with flat morphology, glossy, large, scarlet-red coloring, with circular and regular edges. Bacteria are Gram-negative individual or in chains (Figure 2). Further, confirmed by comparing the results obtained by Perez and Casas (2005).

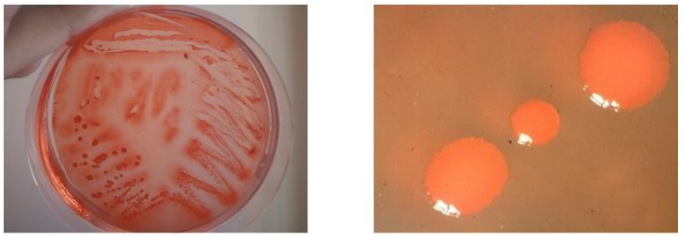

Fig. 2: Morphology of the colony in *Azospirillum* spp. isolated from strawberry crops. a) colonies scarlet, flat and bright b) Gram-negative bacilli small and thick (Malic Acid Red Congo specific medium).

Biochemical profile of isolated strains

The biochemical profiles of bacterial isolates were presented in Table 5. A four bacterial isolates were compared with the positive bacterial control. The C1 and C3 colonies were found to be positive for all tests, which showed that these two isolates belong to *Azospirillum* spp. and used for inoculation.

Table 5: Biochemical tests in four bacterial isolates including positive control.

Test	C1	C2	C3	C4	Control
Catalase	-	+	+	+	+
Nitratereduction	+	-	+	+	+
Ureasereduction	-	+	+	+	+
Indoleroduction	-	+	-	+	+
Fructose	+	-	+	+	+
Arabinose	-	+	+	+	+

C1 - C4 are different isolates.

Plant height

The plant height was measured at the interval of 30, 60, 90 and 120 days. The higher plant heights were observed in co-inoculated treatments (B1 *Azospirillum* spp. + B2 *Azotobacter* sp.) in combination of high doses of nitrogen and in treatment group T6 B2N3. This supports the finding of Zargar *et al.,* (2008) who reported that greater heights were obtained by increasing the amount of nitrogen in conjunction with the inoculation of *Azotobacter* in the strawberry crop (Figure 3).

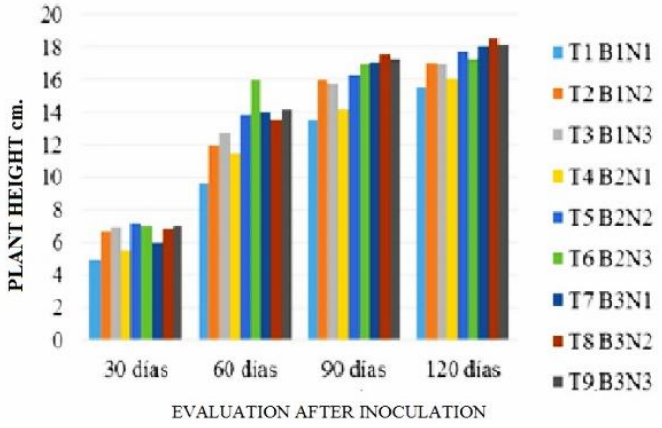

Fig. 3: Effect of treatments on strawberry plant height in four evaluations.

Fig. 4: Root Length of strawberry T6 (left) and control (right).

Root length and weight

After comparing root length, fresh weight and dry root weight from inoculated treatments to control group it was observed that the biofertilizer and nitrogen treatment significantly increased the length and weight of root in hydroponic strawberry (Fig. 4). The treatment groups such as T5 B2N2, T6 B2N3 and T7 B3N1 to 120 days are among them, which showed a root length of 12.91, 14.65 and 17.13 cm respectively.

Similarly, the treatment groups with co-inoculation and nitrogen concentration of 50 and 100 ppm showed significant increase in fresh root weights with respect to control group. Treatment groups such as T7 B3N1and T8 B3N2 reported highest dry root weights. Further, treatment group T6 did not differ statistically from the aforementioned treatments which are at an interval of 60 and 120 days. The overall average of dry weight and fresh root weight in hydroponic strawberry were 5.18, 13.08 and 18.74g at 60, 90 and 120 days respectively, with variation coefficients between 14.17 and 18.02 %. This increase in root length, fresh weight and dry root weight in an inoculated treatments compared to control, was because of *Azotobacter* spp. *Azospirillum* spp.

As they are an efficient nitrogen fixers this promoted the root growth (Lozada 2010, Borda 2009) and also responsible for an increase in dry matter.

Leaf area

A significant increase in leaf area was observed at an interval of 60 and 120 days in the treatment group T5 B2N2 and T6 B2N3(Figure 5). Interestingly, the control surpassed the treatments containing 50 ppm of nitrogen in the nutrient solution. The bacterial inoculation showed improvement in leaf area, fresh and dry weight of the aerial part this is due to the enhanced nitrogen-fixing, better absorption of nutrients especially N, secretion of growth promoting substances. This is in agreement with the results obtained by Umar *et al.,* (2009) that better results were obtained when consortium of *Azotobacter* and nitrogen fertilization were used. Whereas, according to Lata *et al.,* (2013) the leaf area even further increased if combination of *Azospirillum* spp. And *Azotobacter* spp. with nitrogen fertilizer and manure was used.

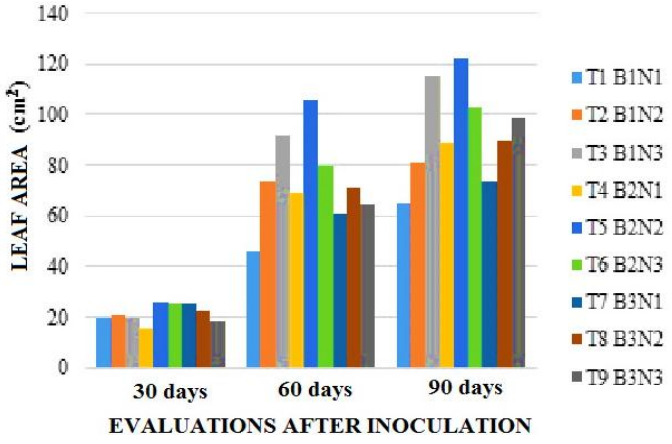

Fig. 5: The effect of biofertilizers and nitrogen treatments on the leaf area (cm^2) of strawberries under hydroponic system.

Fresh and dry weight of aerial part

A significant increase in dry weight of aerial part was observed at an interval of 120 days in the treatment group T6 B2N3 (Figure 6) but in the same group there was no much difference in dry weight was observed in first two interval i.e 30 and 60 days. The second highest dry weight was reported in treatment group T7 B3N1.

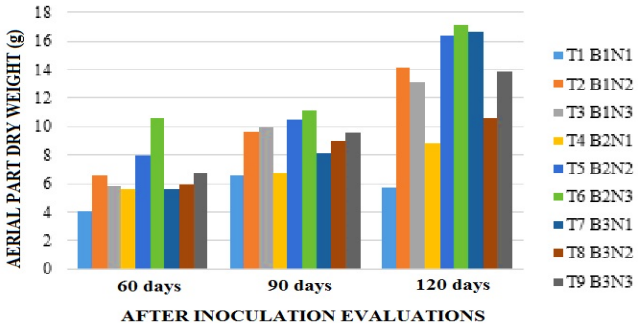

Fig. 6: The effect of treatments on the dry weight (g) of the aerial part under hydroponic cultivation of strawberries.

Estimation of chlorophyll content

As chlorophyll content was one of the parameters of growth measurement, we quantified chlorophyll from plants from different treatment groups. It was observed that treatment group T9 B3N3 showed significant increase in chlorophyll content at an interval of 60 days whereas at 90 days interval it was group T2 B1N2 showed highest chlorophyll content but after an interval of 120 days it was group T8 B3N2 which showed significant increase in chlorophyll content (Table 6).

Table 6: Effect of treatments on chlorophyll content in hydroponic strawberry.

Treatments	Evaluation after inoculation		
	60 days	90 days	120 days
T1 B1N1	45.80 ab	44.95 b	46.00 b
T2 B1N2	43.95 bc	52.90 a	51.93 a
T3 B1N3	47.57 a	46.51 ab	48.45 ab
T4 B2N1	45.62 ab	45.13 ab	48.81 ab
T5 B2N2	45.49 ab	47.70 ab	52.51 a
T6 B2N3	42.50 c	47.85 ab	49.53 ab
T7 B3N1	45.05 ab	48.11 ab	48.57 ab
T8 B3N2	42.41 c	51.15 ab	53.37 a
T9 B3N3	48.16 a	51.41 ab	51.21 ab

In the strawberry crop, Nitrogen in the leaf has great variability depending on the growing season and varieties. This element reaches its highest level in the stages of flowering and fruiting, and its lowest value at the end of the harvest (Güler *et al.*, 2006). In the first measurement date, the plants were in the vegetative stage, the second flowering and the last evaluation were fruiting plants. The SPAD readings were statistically different between treatments due to the phenological stage of the crop. However the values are within acceptable ranges, which means that there was no difference in greenness or chlorophyll content in leaves between treatments and consequently also the content of nitrogen in the leaves. The values for the control treatment were 42.13; 47.30 and 48.29 SPAD degrees beating T1 B1N1 treatment during assessments at 90 and 120 days.

Soluble solids content (Brix)

We observed a positive effect on the soluble solids content in strawberries under hydroponic system after treating with the biofertilizer and different levels of nitrogen. The treatment groups T2 B1N2, T5 B2N2, T6 B2N3 borne fruits with highest soluble solids content (Figure 7), which is evident that increase in brix is achieved by increasing the amount of nitrogen in the nutrient solution and biofertlizer treatment. Under the exclusive application of *Azotobacter* spp. best results were obtained in brix values. With respect to brix values there is no much difference was observed in the treatment groups either inoculated with *Azospirillum* spp. or co-innoculated which is both *Azotobacter* spp. and *Azospirillum* spp.

The results agree with those reported by Rana and Chandel (2003) who obtained the highest content of soluble solids in strawberry fruits when treated with *Azotobacter* spp. along with 80kg N/ha compared to *Azospirillum* spp treatment. Further, studies conducted by Gotamme (2008) and Roussos *et al.*, (2012) in the report on strawberry cultivation which substantiates the same.

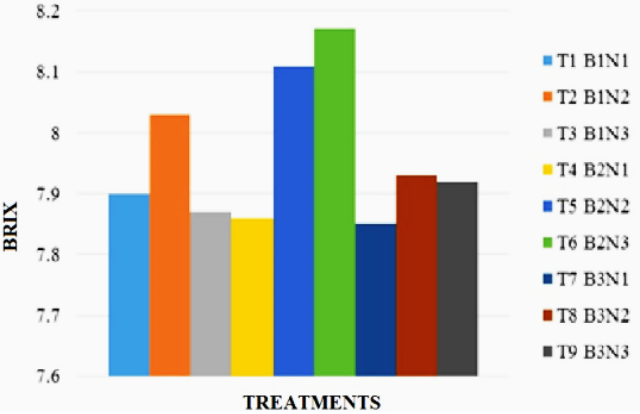

Fig. 7: Effect of treatments on strawberry fruit soluble solids.

Fig. 8: Classification of strawberry fruits by their caliber Categories III, I-II and extra from left to right.

Equatorial diameter (caliber)

After analyzing individual treatments carefully we found that the treatment group T7 B3N1 only showed the lowest average

caliber compared to other treatment groups. The fruits obtained from treatment group T8 B3N3 categorized to Class I - II and T1 B1N1 to class III. In other groups no significant differences were found (Figure 8).

Fruit size in this case the equatorial diameter (caliber) is mainly due to the accumulation of nutrients (N, P, K, Ca and Mg) in presence of ambient temperature and luminosity (Castillejo 2011, Lozada 2010). In strawberry var. Albion gauge ranges 2.1-2.9 cm were obtained when plants were inoculated with *Azospirillum* spp. this indicates that biofertlizer containing *Azospirillum* spp. promotes increase in fruit size than its counter part.

Performance or yeild/plant (g)

The treatment groups T5 B2N2 and T6 B2N3 showed the highest yields/plant. This indicates that the biofertilization i.e *Azotobacter* spp. with two different nitrogen doses (100 and 150 ppm) in nutrient solution was very efficient to increase the performance of the strawberry under hydroponic system. These results were in support with the results obtained by Dadashpour and Jouki (2012) but differ from the profits earned by Singh *et al.*, (2009) as the strawberry crop had higher values when the co-inoculation was used compared to single inoculations. Witness yields were 63.33g in Extra Class, 75.41g in categories I-II and 18.33g in category III being the lowest yields on this variable.Respect to the evaluated variables BPCP. González (2000) suggest that in addition to the effect of nitrogen fixation, these bacteria generate a large number of biostimulators substances such as auxins, gibberellins, cytokinins, phospholipids, fatty acid, indole acetic acid, as well as fungistatic substances that promote the growth of the plants and in large percentage are responsible, more than the nitrogen, its effect on germination, flowering and force of them, all of which contributes to the increase in yields (Figure 9).

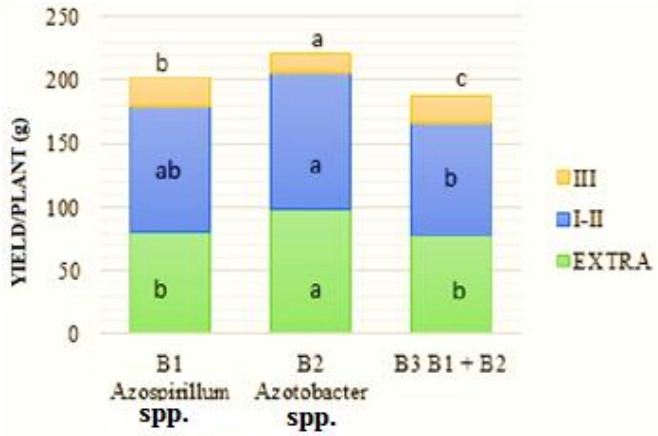

Fig. 9: Biofertilization effect on performance / strawberry plant within each of the categories.

Foliar analysis

The nutrient content of leaf from the each treatment group was measured in terms of % NPK values (table 7) which further substantiates the growth promoting benefits of biofertilizers. The nutritional content of nitrogen are corroborated with those obtained by Castillejo (2011) in which no significant difference between treatments consortium (*Azospirillum* + fertilization) treatment only with *Azospirillum* and control found uninoculated only fertilization 100% and 50%. Regarding *Azotobacter* and in relation to the nitrogen content Zargar *et al.*, (2008) also found no significant differences between treatments considering that in this study the amount of nitrogen in the present investigation was varied. This confirms that the genera *Azotobacter* and *Azospirillum* collaborate in nitrogen fixation.

Table 7: Result of foliar analysis of hydroponic cultivation of strawberries.

TREATMENTS	%		
	N	P	K
T1 B1N1	2.61	0.55	1.92
T2 B1N2	2.52	0.84	2.22
T3 B1N3	2.75	0.54	2.06
T4 B2N1	2.61	0.59	1.99
T5 B2N2	2.82	0.55	1.94
T6 B2N3	2.43	0.49	1.51
T7 B3N1	2.75	0.62	1.8
T8 B3N2	2.6	0.83	1.95
T9 B3N3	2.68	0.54	2.05
T10 Control	2.82	0.89	2.2

Treatments that showed significant growth rate.
Nitrogen: T5 B2N2; T7 and T10 B3N1 2.82; 2.85 and 2.82 respectively.
Phosphorus: T2 B1N2; B3N1 T7 and T10 with 0.82; 0.62 and 0.89% respectively.
Potassium B1N2 T2, T3 and T9 B3N3 B1N3 2.22, 2.06 and 2.05% respectively.

Economic analysis

According to Perrin *et al.*, (2008) proceeded to obtain the gross profit that corresponds to the production of yield per category for its price in the market. By placing the net benefits in order declining accompanied by their variable costs you proceeded to carry out the analysis of dominance, it was determined that the only treatments not dominated were T6 B2N3, T5 B2N2 and T4 B2N1. With treatments not dominated you proceeded to carry out the marginal analysis determined that the best economic options constitute treatments T6 B2N3 and T5 B2N2 to achieve adequate internal rates of return marginal.

CONCLUSION

Morphological and biochemical characterization of the isolates were belonged to *Azospirillum* spp. and *Azotobacter* spp. Of the different treatment groups, the combination of *Azospirillum* spp. and Azotobacter spp. with 100 ppm of N showed better results on growth parameters: height of plant, content of chlorophyll, fresh and dry root weight; nevertheless T5 B2N2 and T6 B2N3 were also evident with similar values on the mentioned variables and showed significant increase in root length, leaf area, dry and fresh weight of the aerial part. The biofertilizers *Azospirillum* spp. And *Azotobacter* spp. enhances the presence of nitrogen of leaf in the cultivation of strawberries var. Albion already presented percentages in ranges of 2.42 - 2.83 suitable for cultivation, even in the treatments with low levels of this element. The higher yields of the "extra" class and I-II were obtained with

the dose 100 and 150 ppm of nitrogen with the biofertilization on the basis of *Azotobacter* spp. manifesting itself as well as treatments for highest performance/plant 225.49 and 226.37 g, respectively. Individual application and set of *Azospirillum* spp. and *Azotobacter* spp. favored the growth and yield of crop under hydroponic strawberry in comparison the witness even when the nutrients of leaf did not differ on a large scale. T6 (*Azotobacter* spp. 150 PPM of N2) and T5 (*Azotobacter* spp. 100 PPM N2) treatments were the best economic options for strawberry production in a system hydroponic introducing the age variable costs.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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