

## Humic substances and rhizobacteria enhance the yield, physiology and quality of strawberries

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### Abstract

The strawberry fruit (*Fragaria* × *ananassa* Duch.) is appreciated for its aroma, color, texture and nutritional value. In conventional agriculture, the use of fertilizers damages the environment since it causes loss of soil fertility, salinity and its erosion, hence production alternatives, without harming the environment, are sought. The objective of this study was to evaluate the effect of a biostimulant based on humic substances and rhizobacteria, on the production and quality of the strawberry cultivar ‘San Andreas’. Strawberry plants cultivar ‘San Andreas’ were treated with fulvic acids + mixture of microorganisms, humic acids + *Pseudomonas fluorescens*, fulvic acids + *Azospirillum brasilense*, fulvic acids + *Pseudomonas fluorescens* and the mixture AH and AF + *Azospirillum brasilense* with two doses (d1, d2) in total 10 treatments were applied plus the control. Humic substances were applied every 15 days and rhizobacteria every 30 days. The results showed that the AFyAzoz d1 increased over control plants, the number of leaves in 38.3%, root volume in 42.6%, the fresh weight in 130% and dry weight in 63.8%, the number of fruits 50.0% and the yield in 59.5%. The AFyPF d1 favored Photosynthesis in 127.3%; AFyPF d1 increased TSS in 25%, AFyPF d2 vitamin C in 17.1% and MHyF + Azoz d1 increased in 20% the content of Phenols. Humic substances plus rhizobacteria are an ecological alternative to be used as biostimulant in the production and quality of strawberry plants.

**Keywords:** *Fragaria* × *ananassa* Duch; fulvic acids; humic acids; microorganisms

### Introduction

Strawberry fruit (*Fragaria* × *ananassa* Duch.) is appreciated for its nutritional value and its cultivation is one of the productive options for farmers (Medina *et al.*, 2016). The intensification of conventional agriculture

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causes pollution due to the use of synthetic products, loss of soil fertility and a decrease in biodiversity, the negative impacts of this activity are now resulting in an irreversible change for the environment (Olivares *et al.*, 2017). New production technologies are needed to reduce the damage caused to our ecosystems and at the same time they are required to be friendly to the environment, these alternatives include the use of biostimulants in agriculture (Agbodjato *et al.*, 2021).

A plant biostimulant includes organic materials and microorganisms that are supplied to plants in order to improve the absorption of nutrients, stimulate growth, improve stress tolerance and their quality (Du Jardin, 2015). There are various types of biostimulants and among them, in addition to algae extracts, it is reported chitosan, protein hydrolysates, humic substances that include humic and fulvic acids, as well as mycorrhizal fungi and rhizobacteria (Veobides *et al.*, 2018).

The use of humic substances as biostimulant in agriculture, emerges as a sustainable technology to make agricultural methods more profitable and competent, with less destructive effects on ecosystems (Canellas *et al.*, 2015). Humic and fulvic substances are the main organic elements of lignites, soil and peat, they are produced by natural decomposition of organic matter (Van Oosten *et al.*, 2017). One of the main effects of humic substances on plant growth is the reinforcement of nutrient absorption and the lengthening of root growth (Jindo *et al.*, 2020). In a study carried out by Kirschbaum *et al.* (2019), when evaluating the effect of humic and fulvic acids, the applications improved the yield and number of fruits per plant of strawberry crop. Similarly, Aghaeifard *et al.* (2015), when applying humic acids by foliar application, in strawberry cultivar 'Camarosa', reported an increase in yield, higher concentration of soluble solids, titratable acidity and vitamin C.

The use of rhizobacteria in crops reduces the negative environmental impact due to the use of fertilizers and pesticides, being an extraordinary option for producers to face the new competences of agriculture (Dos Santos *et al.*, 2020). The action of rhizobacteria improves soil fertility by mobilizing minerals, increases crop yield through the fixation of N<sub>2</sub>, induces the production of siderophores, the activity of phytohormones, solubilization of phosphorus and minerals (Naik *et al.*, 2019). The use of rhizobacteria is of consideration, particularly in the occurrence of abiotic stress since they help plants to tolerate it as well as help in the remediation of the soil by pollutants (Oleńska *et al.*, 2020).

The microorganisms most used as biostimulants are rhizobacteria belonging to the genera, *Rhizobium*, *Azospirillum*, *Azotobacter* and *Pseudomonas*; the genus *Azospirillum*, applied to plants, is the most studied (Piña *et al.*, 2016). It helps in the production of crops with the increase of aerial shoot growth and root system that causes an increase in the intake of minerals and water, colonizes the rhizosphere and uses different sources of nitrogen (Fernandes *et al.*, 2020). In a strawberry cultivation investigation by Marcondes *et al.* (2019), when performing inoculations of *Azospirillum brasilense* strains in combination with nitrogen, they responded significantly by increasing the length of the root, the dry weight of the root, the length of the aerial shoots, the dry weight of the aerial part, the number of leaves and total dry weight.

The rhizobacteria of the genus *Pseudomonas* exert a productive result, through the synthesis of phytohormones and vitamins, stimulate the germination of seeds and emergence of seedlings, solubilize phosphorus and give systemic resistance to pathogens (Cano, 2011). In an experiment carried out by Haggag and Abo (2012), when using a strain of *Pseudomonas fluorescens* and being applied to the strawberry crop, significant increases were observed in growth parameters, as well as dry weight and yield.

Humic substances and rhizobacteria are among the most infallible procedures that use natural, biologically active and ecological substances that favor plant growth (Ekin, 2019). An alternative to make efficient the use of nutrients in crops, is the combination of humic substances as a vehicle and carbon source for rhizobacteria, resulting in an increase in crop yield (Olivares *et al.*, 2017). Based on the above, the objective of this study was to evaluate the effect of a biostimulant based on humic substances and rhizobacteria, on the production and quality of the strawberry cultivar 'San Andreas'.

## Materials and Methods

### *Study area*

The research work was carried out in a tunnel-type greenhouse in the Horticulture Department at the Antonio Narro Autonomous Agrarian University, in Saltillo, Coahuila, Mexico, which is located between the geographic coordinates of 25° 22' north latitude and 101° 02' west longitude and at an altitude of 1742 meters above sea level, with semi-dry climate with hot summer, rains in summer, annual average temperatures of 12 to 18 °C.

### *Genetic material and plantation*

Two months old strawberry plants of the San Andreas cultivar, with a neutral photoperiod, were planted on July 25th, 2020. In the plantation, 3 beds of soil with 12 m in length and 1 m wide and 80 cm between beds were used, the roots of the plants before transplantation were submerged in Captan® solution at a rate of 1 g/L. The distances between plants were 30 cm and a drip irrigation system was used with drippers separated 20 cm. The physical and chemical characteristics of the soil used were: pH 8.01, electrical conductivity 1.84 (dS/m), organic matter 4.4%, cation exchange capacity 20.4 (Meq/100 g), loamy soil texture and the mineral elements available in the soil are shown in Table 1.

**Table 1.** Mineral elements reported from the soil analysis

Elements	K	Ca	NO <sub>3</sub>	Na	Mg	Fe	Zn	Mn	B
Value (ppm)	909	2968	95.8	143	320	41.11	15.5	5.88	3.21

### *Extraction of humic substances and separation of humic acids and fulvic acids*

The humic substances were extracted from Leonardite, this mineral organic compound was provided by the DHD Company of México, located in Sabinas, Coahuila. To extract these humic substances the methodology reported by López *et al.* (2014) was used, for this, 5 grams (g) of Leonardite were weighed and placed in a 250 mL flask and 100 mL of 1N potassium hydroxide were added, it was left in a "water bath" at 60 °C for two hours and allowed to cool down. For the separation of humic and fulvic acids, 25 mL of humic substances obtained in the extraction were taken and 50 mL of acetic acid were added in order to lower the pH to 4, it was heated on a laboratory grill for 5 minutes at 70 °C and left it to rest for three days at room temperature. After this period of time humic and fulvic acids were obtained, the latter remained in the upper part with a golden yellow color, while humic acids were left precipitated at the bottom of the container with the appearance of soil and a dark brown color.

### *Obtaining and preparing bacterial strains*

Rhizobacteria of *Azospirillum brasilense*, *Pseudomonas putida* and *Pseudomonas fluorescens* were obtained by the strains provided by the National Collection of Microbial Strains and Cell Cultures, of the Center for Research and Advanced Studies of the National Polytechnic Institute (IPN). The rhizobacteria *Azospirillum brasilense* was reactivated in NRCB culture medium (yeast extract 1 g, mannitol 5 g, K<sub>2</sub>HPO<sub>4</sub> 0.7 g, KH<sub>2</sub>PO<sub>4</sub> 0.1 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 1 g, bacteriological agar 15 g, distilled water 1 L, pH, 7.0-7.2), the *Pseudomonas* species were seeded in King B medium (peptone 20 g, purified agar 12 g, K<sub>2</sub>HPO<sub>4</sub> 1.5 g, MgSO<sub>4</sub>·7 H<sub>2</sub>O 1.5 g, glycerol 15 ml, distilled water 1 L). In the preparation of the bacteria, they were cultivated in nutritive broth (with 5 g peptone and 3 g meat extract), and they were placed in constant agitation at 150 rpm

for 48 hours at  $25 \pm 5$  °C, the bacterial growth was evidenced by the turbidity in the medium. The bacterial cell concentration was 109 cells / ml according to the McFarland turbidity scale (McFarland, 1907).

### Treatments

Five different mixtures with 2 doses were applied, resulting in 10 treatments plus a control with 7 repetitions each were applied in a completely randomized design (Table 2). Dose 1 used was: 3 ml of humic substances (humic and / or fulvic acids) plus 5 mL of a mixture of microorganisms (*Pseudomonas fluorescens*, *Pseudomonas putida* and *Azospirillum brasilense*) or microorganisms alone as indicated from treatment 3 onwards. Dose 2: 3.5 mL of humic and / or fulvic acids and 4 ml of a mixture of microorganisms or microorganisms alone as indicated from treatment 3 onwards. Steiner nutrient solution (Steiner, 1961) was applied for control plants. The humic substances were applied every 15 days and the rhizobacteria every 30 days, the treatments were placed directly in the soil at the base of the roots of the plant. A total of 7 strawberry plants were used for each treatment including the control.

**Table 2.** Treatments applied to the San Andreas variety strawberry crop

Treatments	Dose	Keys
1. Fulvic Acids (3 mL) + Mixture of Microorganisms (5 mL)	Dose 1	AFyMM d1
2. Fulvic Acids (3.5 mL) + Mixture of Microorganisms (4 mL)	Dose 2	AFyMM d2
3. Humic Acids (3 mL) + <i>Pseudomonas fluorescens</i> (5 mL)	Dose 1	AHyPF d1
4. Humic Acids (3.5 mL) + <i>Pseudomonas fluorescens</i> (4 mL)	Dose 2	AHyPF d2
5. Fulvic Acids (3 mL) + <i>Azospirillum brasilense</i> (5 mL)	Dose 1	AFyAzoz d1
6. Fulvic Acids (3.5 mL) + <i>Azospirillum brasilense</i> (4 mL)	Dose 2	AFyAzoz d2
7. Fulvic Acids (3 mL) + <i>Pseudomonas fluorescens</i> (5 mL)	Dose 1	AFyPF d1
8. Fulvic Acids (3.5 mL) + <i>Pseudomonas fluorescens</i> (4 mL)	Dose 2	AFyF d2
9. Mix of Humic and Fulvic acids (3 mL) + <i>Azospirillum brasilense</i> (5 mL)	Dose 1	MHyF+Azoz d1
10. Mix of Humic and Fulvic acids (3.5 mL) + <i>Azospirillum brasilense</i> (4 mL)	Dose 2	MHyF+Azoz d2
11. Control (Steiner Solution)		Control

### Agronomic variables evaluated

At the end of the cultivation cycle, 240 days after transplantation, the height of the plant was measured with an 30 cm aluminum Ruler (Arly 3006) from the neck of the plant to the apex of the highest leaf, the number of leaves and number of crowns were evaluated manually by counting one by one, root volume was determined by volume displacement with a natural color plastic cylinder (Kimax 25301) with a capacity of 1 L, fresh weight of aerial part (leaves, petioles, crowns) and roots was obtained on a digital scale (OHAUS model CS-5000). To obtain dry weight of aerial part and root, the samples were placed in drying oven (Yamato model DX-602) at a temperature of 60 °C for 48 h or until constant weight was reached, finally weight was recorded in grams.

Physiological variables such as gas exchange parameters, including stomatal conductance ( $g_s$ ), net CO<sub>2</sub> assimilation rate ( $A_N$ ), transpiration (E) along with instantaneous water use efficiency (WUE) expressed as  $A_N / E$  (Yinn *et al.*, 2006) were determined with a Li-6800 portable open gas exchange system (Li-Cor Inc., Lincoln NE) between 12:00 and 13:00 hours of the day, totally sunny, on the most recent fully expanded leaf of the well exposed upper canopy. For the measurements, the light saturation was maintained at 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with the Li-6800 LED lamp, the CO<sub>2</sub> concentration was set at 390 ppm, the leaf temperature at 25 °C and the relative humidity it was 55-65%. The midday stem water potential (SWP) was measured in two leaves covered by the lower canopy with a pump pressure chamber (PMS Instrument Company, Albany, OR) as described by

Fulton *et al.* (2001). Chlorophyll was measured on the fully expanded leaf at 8:00 a.m. with a Konica Minolta brand SPAD-502.

Polar diameter of the fruit and the equatorial diameter of the fruit were measured with a digital calliper (STEREN model HER-411), the weight of the fruit was recorded with a digital scale (TJ model MH-500), the number of fruits was recorded per plant and the total yield of fruits per plant was reported in g / plant.

Total soluble solids content (TSS) was assessed by placing a drop of fruit juice on the lens of digital refractometer (Hanna Instruments model 96-801), the readings were expressed in brix degrees (°B). Firmness was evaluated with a manual penetrometer, (WAGNER Instruments, model FDK) applying the force in the central part of the fruit. The strawberry fruit was macerated and the pH was evaluated with a digital potentiometer (Hanna Instruments, model HI98130).

Fruit vitamin C determination was achieved using the methodology reported by Padayatt *et al.* (2001), where 20 g of fresh fruit were macerated using a porcelain mortar with 10 mL of HCl at 2% , then 100 ml of deionized water were added and filtered with an absorbent gauze placed in a funnel over a flask, then a proportion of 10 mL was taken from the total volume of the filtrate and the titration was achieved with 2-6 dichlorophenolindophenol with the help from a glass burette (EISCO, CH0240G), until a constant pink color was observed.

$$\text{Vitamin C (mg 100 g FW)} = \frac{(\text{mL 2.6 dichlorophenolindophenol}) (0.088)}{(\text{total volume}) (100)/(\text{aliquot volume}) (\text{sample weight})} \quad (1)$$

Titrate acidity was determined by colorimetry according to the AOAC, (2000), for this, 10 g of fresh strawberry fruit were weighed, it was macerated homogeneously and then 100 mL of deionized water was added and filtered, from the total volume of the filtrate 10 mL were taken and added 2-3 drops of phenolphthalein were added and it was titrated with NaOH (0.1N) until obtaining a pink color. Titrable acidity was expressed as% citric acid. Where: VNaOH = Spent volume of NaOH to titrate, NNaOH = NaOH Normality, Meq citric acid = 0.064

$$\% \text{ acid} = \frac{(VNaOH \times NNaOH \times \text{Meq citric acid} \times 100)}{\text{sample volume}} \quad (2)$$

The content of total phenols was determined with the methodology reported by Yu and Dahlgren (2000), with modifications, 2 g of fresh strawberry fruit were weighed and 5 mL of solution water: acetone (1:1) were added, then it was centrifuged at 15,000 revolutions per minute for 15 min, the supernatant was extracted, stored at 4 °C in the dark, then a 50 µL aliquot of the extract was taken and added 200 µL of Folin-Ciocalteu reagent, 500 µL of 20 sodium carbonate and 5 mL of distilled water, then samples were placed in a water bath at 45 °C for 30 minutes, finally the absorbance was read at 750 nm in a Bio-145025 BIOMATE 5 Thermo Electron Corporation spectrophotometer. A calibration curve made with gallic acid was used for quantification. The results were expressed in mg EQ of Gallic Acid \* 100 g of PF-1.

#### *Statistical analysis*

All data obtained were analyzed by one way ANOVA with the statistical package Infostat version (2020). The Fisher's Least Significant Difference (LSD) test ( $P \leq 0.05$ ) was used for mean separation.

## **Results and Discussion**

### *Agronomic variables in strawberry cultivation*

Biostimulants are substances that, when applied to plants through the soil, cause changes in vital and structural processes to contribute to the development of plants (Feitosa and Garófalo, 2019). The use of humic substances increased the number of leaves (NL) that showed significant differences between treatments (Table 3). With applications of AFyAzoz d1 it increased by 38.3% with respect to the control that showed less leaf

development. With the AHyPF d2 treatment, although the number of leaves increased by 15% more than the control, it was not significantly different. The rest of the treatments were all statistically equal to the control.

Rätsep *et al.* (2015), when using humic acids to increase plant development and productivity of strawberry plants, reported that they obtained an increase of 5% with respect to their control. The increase found in our study was higher than that informed by Rätsep *et al.* (2015), and this may be due to the fact that the doses used in our experiment were higher than those applied by them.

In plant height (PH) no differences were observed among treatments (Table 3). Our results coincide with those reported by Shehata *et al.* (2011), where they used humic acids and compost in strawberry plants, they did not find significant differences in plant height with the applied treatments. In contrast, our findings differ from those registered by Álvarez *et al.* (2018), who evaluated the effect of beneficial microorganisms of the genus *Bacillus* on the growth of plants in strawberry and reported significant differences in plant height with an increase of 43% with respect to the control plants. This can possibly be attributed to the fact that the bacterial strain they used in their experiment adapted more efficiently to the prevailing conditions at the experiment site such as the nature of the soil and environmental conditions.

The number of crowns (NC) of the strawberry plants increased significantly by 38% with the application of AFyAzoz d1, followed by the plants treated with AFyMM d1 which increased it by 28.5% compared to the control plants. The other treatments behaved statistically equal as the control plants.

**Table 3.** Agronomic variables in strawberry cultivation with application of humic substances and rhizobacteria

Treatments	NL	PH (cm)	NC	RV (cm <sup>3</sup> )	FWAP (g)	DWAP (g)	FWR (g)	DWR (g)
AFyMM d1	48.7b	21.0a	5.4ab	41.7f	200de	57.7fg	50.0f	9.7e
AFyMM d2	55.5b	21.9a	5.1abc	57.0bc	285b	74.7bc	72.0ab	14.2bc
AHyPF d1	57.2b	21.7a	5.1abc	54.0cd	282b	72.2bcd	62.7bcde	11.7de
AHyPF d2	60.4ab	21.9a	5.2abc	55.0cd	273bc	80.5ab	63.5bcd	14.2bc
AFyAzoz d1	72.2a	21.7a	5.8a	64.2a	359a	87.7a	76.2a	16.7a
AFyAzoz d2	53.4b	19.8a	4.2bc	46.2ef	246bcd	64.5def	57.2def	10.7de
AFyPF d1	56.7b	20.7a	4.7bc	46.2ef	205cde	65.5def	58.5cdef	12.7cd
AFyF d2	54.2b	19.0a	4.7abc	62.0ab	240bcd	68.5cde	68.5abc	16.2ab
MHyF+Azoz d1	51.0b	20.8a	4.2bc	49.5de	186de	60.5def	55.7def	11.2de
MHyF+Azoz d2	53.7b	19.8a	5.0abc	63.7a	224bcd	63.7efg	71.2ab	16.5ab
Control	52.2b	18.0a	4.2c	45.0ef	136e	52.0g	53.0ef	11.7de
MSD	13.03	2.9	1.03	14.9	90.9	22.9	19.4	4.8
ANVA ( $P \leq$ )	0.0499	0.1701	0.0492	0.0001	0.0001	0.0001	0.0001	0.0001

NL= Number of leaves, PH= Plant height, NC = Number of crowns, RV = Root volume, FWAP = Fresh weight of aerial part, PSPA = Dry weight of aerial part, FWR = Fresh weight of root, DWR = Dry weight of root. Values with the same letter within columns are statistically equal (LSD test,  $P \leq 0.05$ ). MSD = Minimum significant difference. ANVA= Variance analysis

These results suggest that fulvic acids positively influenced the increase in the number of crowns since, as mentioned by Aminifard *et al.* (2012a), fulvic acid influences the growth and plant development of plants by increasing the rate of cell division. The increase in NC was possibly due to the fact that by applying *Azospirillum brasilense* and the mixture of rhizobacteria in combination with fulvic acids, plant growth in the strawberry plant was enhanced, help improve the availability of plant nutrients.

Root Volume (RV) increased significantly with applications of AFyAzoz d1 in 42.6%, MHyF + Azoz d2 in 41.5% and with AFyF d2 in 37.7% followed by the treatments with AFyMM d2, AHyPF d2 and AHyPF d1 with 26.6, 22.2 and 20% more in VR compared to control plants. The rest of the treatments were not different from the control, except for the plants treated with AFyMM d1 in which a statistically lower value of

7.3% than the control plants was obtained. The greatest increase in RV was favored with the applications of fulvic acids in combination with the rhizobacterium *Azospirillum brasilense*, coinciding with Marcondes *et al.* (2019) who reported that inoculations with strains of *Azospirillum brasilense* significantly increased the size of the roots and therefore its volume. Likewise, it coincides with that mentioned by Saidimoradi *et al.* (2019), who applied humic substances to reduce salinity stress in strawberry cultivation, and reported an increase of 41.0% in root volume with respect to the control. In contrast, these results differ with a study effected out by Ortiz *et al.* (2016), where they obtained only a 1.8% increase in root volume in strawberry plants with respect to their control when used phosphorus solubilizing rhizobacteria such as *Pseudomonas tolassi*, *Paenibacillus polymyxa* and *Bacillus pumilus*. This suggests that for increasing the volume of the root *Azospirillum brasilense* in combination with humic substances is more efficient than the phosphorus-solubilizing rhizobacteria. The plants treated with AFyMM d1 that produced a statistically lower 7.9% in RV when compared to the control plants, could be associated with the fact that the inoculum of microorganisms at certain concentrations inhibits the development and growth of roots and therefore the volume (Bashan, 1990).

The fresh weight of the aerial part (FWAP) including crown, petiole and lamina, increased significantly in 163% with the application of AFyAzoz d1 followed by the treatments AFyMM d2, AHyPF d1 and d2, AFyAzoz d2, AFyF d2 and MHyF + Azoz with an increase of 109, 107, 100, 80, 76.4 and 64.7% respectively with respect to the control, while the other treatments were not different from the control. The dry weight of the aerial part (DWAP) had a similar behavior to the fresh weight of the aerial part and increased significantly with applications of AFyAzoz d1 in 67.6% and with the treatment of AHyPF d2 in 54.8% followed by AFyMM d2, AHyPF d1, AFyF d2, AFyAzoz d2 and MHyF + Azoz d1 with 43.6, 38.8, 31.7, 25.9, 24 and 16.3% more respectively, this compared to the control plants. These effects coincide with that found by Haggag *et al.* (2012), who applied a strain of *Pseudomonas fluorescens*, in strawberry cultivation to optimize nutritional conditions and biomass production, they obtained an increase of 25% and 28.5% in fresh and dry weight respectively with respect to the control. With the treatments applied in our experiment, the differences found were greater than that reported by these authors, which could be due to the fact that in addition to *Pseudomonas fluorescens*, humic substances were applied; also, one possibility is that the strawberry varieties were different.

The fresh weight of root (FWR) increased significantly with the application of AFyAzoz d1 in 43.7%, being the highest value, followed by the treatments AFyMM d2, MHyF + Azoz d2, AFyF d2 and AHyPF d2 with 35.8, 34.3, 29.2 and 19.8% respectively with respect to the control. Likewise, the dry weight of root (DWR), increased mostly with the AFyAzoz d1 treatment in 42.7%, being the treatment that caused the highest dry weight, followed by the MHyF + Azoz d2, AFyPF d2, AFyMM d2 and AHyPF d2 treatments in 41, 38.4, 21.3 and 21.3% respectively compared to the control plants. The other treatments were not different from the control, however, the plants treated with AFyMM d1 obtained a lower value than the control in 7.3% FWR and 17% less in DWR. The FWR and DWR were favored with fulvic acids plus the rhizobacteria *azospirillum brasilense*, this coincides with Pedraza *et al.*, (2010), who reported an increase in strawberry roots due to the effect of *Azospirillum*. In other crops such as tomato, Ribaudó *et al.* (2006) informed an increase in root hair length and root fresh weight. However, it is different from that reported by Castañeda-Saucedo *et al.* (2013), who applied a strain of *Azospirillum brasilense* on strawberry Albión and did not find significant differences between treatments in root dry weight, this could be due to the fact that in this particular study the authors applied the rhizobacteria alone without chemical fertilization. Naiman *et al.*, (2009) in other crops such as wheat, when using plant growth promoting bacteria (PGPB) alone, did not find significant statistical differences and in our study the treated plants were fertilized and also humic substances were added as a carbon source, which suggests that the combination of them applied in our study favored and caused a greater root increase. With respect to the plants treated with AFyMM d1 that caused a lower value than the control in 7.3% in PFR and 17% less in PSR, could be caused by an over dose of microorganisms as indicated by Bashan, (1990), who reported that when the inoculum of microorganisms is  $> \log 8$  at  $\log 10$  CFU / ml, they inhibit root

development and growth. In our study, this particular treatment included the three Rhizobacteria, so it could have that high concentration when applied and caused the inhibition of roots and consequently the decreases reported in this study compared to the control plants.

#### *Physiological variables*

Even though, it has been found that biostimulants are effective to improve the resistance of plants to adverse effects caused by stress from environmental conditions, in this study the stomatal conductance values were not significantly affected by any treatment applied (Table 4). These effects are different from those found by Arikan *et al.* (2020), who applied in calcareous soil a strain of the genus *Pseudomonas* to evaluate the effects of salinity in strawberry plants, they obtained an increase of 162% in stomatal conductance in comparison to the control plants; the increase could be caused due to the fact that control plants were possibly affected by salinity and by calcareous soil conditions, a situation of stress; these authors reported that the strains of the genus *Pseudomonas* can be used for situations of stress due to salinity and calcareous soil conditions in strawberry cultivation.

**Table 4.** Physiological variables in strawberry cultivation with application of humic substances and rhizobacteria

Treatments	Stomatal conductance (gs) (mmoles/m <sup>2</sup> s <sup>-1</sup> )	Transp. (E) (mol m <sup>-2</sup> s <sup>-1</sup> )	CO <sub>2</sub> (A <sub>N</sub> ) (μmol m <sup>-2</sup> s <sup>-1</sup> )	Intrinsic efficiency (A <sub>N</sub> /E)	Stem water potential (MPa)	Chl (SPAD)
AFyMM d1	407.7a	2.1cd	11.2ef	7.3a	-0.82a	52.0a
AFyMM d2	483.7a	1.9d	10.0fg	5.4abcd	-0.80abc	52.3a
AHyPF d1	347.a	2.3bcd	13.4cde	5.9abc	-0.66abc	52.5a
AHyPF d2	350.6a	4.2a	15.3bc	3.6cd	-0.71abc	52.3a
AFyAzoz d1	405.7a	2.5bcd	15.6bc	5.4abcd	-0.61c	51.5a
AFyAzoz d2	448.8a	1.9d	16.6ab	4.3bcd	-0.62bc	51.8a
AFyPF d1	493.2a	2.1bcd	18.9a	6.8ab	-0.69abc	49.6a
AFyF d2	401.2a	3.2abc	12.8de	2.9d	-0.80ab	48.7a
MHyF+Azoz d1	332.7a	3.2abc	15.3bcd	4.9abcd	-0.84a	49.9a
MHyF+Azoz d2	412.6a	3.3ab	15.8bc	3.6cd	-0.80ab	50.8a
Control	357.2a	2.0cd	8.3g	4.2bcd	-0.71abc	50.6a
MSD	120.02	1.14	2.47	2.7	0.160	2.94
ANVA ( <i>P</i> ≤)	0.1785	0.0033	0.0001	0.0384	0.0460	0.1599

Transp= Transpiration, CO<sub>2</sub> = CO<sub>2</sub> assimilation rate, Intrinsic efficiency= Intrinsic efficiency of the water, Chl= Chlorophyll. Values with the same letter within columns are statistically equal (LSD test, *P*≤0.05). MSD = Minimum significant difference. ANVA= Variance analysis

Transpiration increased significantly in plants treated with AHyPF d2, MHyF + Azoz d2 by 110 and 65% respectively compared to the control, the rest of the treatments were statistically equal to the control. Humic acids caused an increase of transpiration in strawberry plants. These results coincide with an experiment carried out by Soppelsa *et al.* (2019), who applied biostimulants to improve production and yield in strawberry, when using humic acids (HA), they reported an increase in the transpiration rate by 25%. However, the increase found in our experiment was higher than that reported by Soppelsa *et al.* (2019), which can possibly be attributed to the fact that they used humic acids alone and, in our experiment, they were used in combination with microorganisms and also the doses of these were higher. In contrast, the results found in our study were different from those reported by Reina and Peluzio (2017), who inoculated with the rhizobacterium *Azospirillum brasilense* in soybean cultivation and did not obtain any significant increase in the transpiration rate.

The CO<sub>2</sub> assimilation rate ( $A_N$ ) increased significantly with the application of AFyPF d1 and AFyAzoz d2 in 127 and 100% respectively, in relation to the Control plants. Other treatments that increased  $A_N$  with respect to the control were MHyF + Azoz d2, AFyAzoz d1, MHyF + Azoz d1, AHyPF d1, AFyPF d2, AHyPF d2, and AFyMM d1 and the increase was 90.3, 87.9, 84.3, 84.3, 61.4, 54.2 and 39.2% respectively, the AFyMM d2 treatment behaved statistically the same as the control. In an experiment carried out by Gamboa *et al.* (2019), found similar results when applied treatments with nitrogen fixing rhizobacteria such as *Azospirillum* in strawberry plants, reporting an increase in gas exchange by 76.9%. However, Soppelsa *et al.* (2019), found lower values in  $A_N$  for plants treated with humic acids applied 57 days after transplantation, they reported a 33.3% increase, this could be due to the fact that the doses used by these authors were smaller.

The intrinsic efficiency of the water in the plant increased significantly with the application of AFyMM d1 with an increase of 73.5% with respect to the control plants, the rest of the treatments used were statistically equal to the control. In a study carried out by Bulegon *et al.* (2016) where they used a strain of *Azospirillum brasilense* in soybean plants, the inoculations increased the intrinsic efficiency by 22.9% compared to the control. The increase in our experiment could be due to the use of a mixture of microorganisms (*Azospirillum brasilense*, *Pseudomonas fluorescens* and *putida*) that helped strawberry plants to have a better water use efficiency. As mentioned by Medrano *et al.* (2007) that plant water use efficiency can be understood as the volume of water that plants need to consume (transpiration) to incorporate a certain amount of carbon into their biomass (photosynthesis).

Regarding water potential, the treatments applied did not affect strawberry plants as the values reported in a range from -0.61 to -0.84 MPa were all statistically equal to the control (Table 4), however, numerically the treatments AHyPF d1, AFyAzoz d1 and d2 and the treatment AFyPF d1 showed averages values of -0.66, -0.61, -0.62 and -0.69 MPa which were a little lower than control plants which had an average value of -0.71 MPa. According to Klamkowski and Treder (2006), in a study to examine water deficiency in growth and physiological responses in strawberry plants, they reported water potential values for well-watered unstressed plants in a range similar to the ones reported here and for stressed plants they reported values from -1.58 to -1.81 MPa. In another study by Jensen *et al.* (2009) when making measurements of water potential in strawberry plants, he obtained averages of -0.58 MPa throughout the experiment indicating that the plants grew under low levels of stress. In an investigation effected out by Saeed *et al.* (2016) they reported significant differences between their treatments when applying an *Azospirillum* strain in the canola crop, with an increase of 11% compared to the control, this increase reported by these authors could be due to the fact that They used a control without inoculation with the rhizobacteria (-11.1 MPa) and plants exposed to drought (-10.0 MPa), while in our work no drought stress was used in the control and all the plants were under the same irrigation conditions.

For the SPAD units, no significant differences were observed with the application of treatments to strawberry plants. These results coincide with Palencia *et al.* (2016), in a study carried out to evaluate the quality in strawberry cultivation, when measured the plants, there were no significant differences in SPAD readings among their treatments. However, these results differ from what was found by Lovaisa *et al.* (2015), using strawberry plants cultivar Fortuna, which were inoculated with *Azospirillum brasilense*, when evaluating the chlorophyll content in SPAD units, a positive effect was observed in leaves from inoculated plants; they reported a 32% increase compared to the non-inoculated control. It is also different from that reported by Erdogan *et al.* (2016), when using rhizobacteria *Pseudomonas fluorescens* and *putida* to moderate the impacts of drought stress in strawberry plants, they observed an increase of chlorophyll in SPAD readings, of 24.5 and 22.7% respectively, compared to the control. In the same way Karlidag *et al.* (2013) with the application of plant growth promoting bacteria (PGPB) in strawberry plants under saline field conditions, the chlorophyll content in SPAD units increased significantly with the use of rhizobacteria compared to the non inoculated control; the increase observed by these authors may be due to the fact that they used both drought and salt stress that interfered in the plants cell function, ionic imbalance and disorders in metabolic activities.

*Strawberry production and yield variables*

The Polar diameter of fruit (PDF) increased statistically with the application of MHyF + Azoz d1 and d2 in 6.6 and 6.1% respectively compared to the control (Table 5) the rest of the treatments were statistically equal to control plants. The Equatorial diameter of fruit (EDF) increased with the application of MHyF + Azoz d1 in 5.5% with respect to the control, while the other treatments behaved statistically the same as the control. Castañeda *et al.* (2013) who inoculated with *Azospirillum brasilense* strawberry plants and reported values of DPF and EDF by 4 and 2% respectively than the control. In other crops such as tomato, Andrade *et al.*, (2020) with applications of *Azospirillum brasilense* did not find any significant differences between treatments when evaluating PDF and EDF. In contrast, Aghaeifard *et al.* (2015) with the utilization of humic acids in the strawberry crop they reported increases in PDF and EDF by 36 and 60.6% respectively, this increase was possibly due to the different doses of humic acids they applied in their experiment compared to ours.

Fruit weight was favored by the AFyAzoz d1 treatment with an increase of 12.8% compared to the control, while the rest of the treatments applied were statistically equal to the control. However, these results differ from those registered by Castañeda *et al.* (2013) who inoculated strawberry plants with *Azospirillum brasilense* and obtained a fruit weight decrease of 8.3% with respect to the control. In orange crop Samra *et al.* (2017) applied fulvic acids alone and in combination with humic acids and they did not find significant differences between the applied treatments. In corn crop Kumar *et al.* (2005), applied fulvic acids derived from different sources of organic matter and obtained a significant increase of 0.4% in grain weight with respect to the control. The increase found in our experiment could be due to the fact that the Fulvic acids used, extracted from Leonardite, were combined with a rhizobacteria to potentiate its benefits.

The number of fruits increased with the application of AFyMM d2, AFyAzoz d1, AFyAzoz d2 and AFyMM d2 in 51.0, 40.2 and 38.5 33.1% respectively, the rest of the treatments were statistically equal to the control. These results were different from those reported by Kirschbaum *et al.* (2019), who applied humic and fulvic acids in strawberry San Andreas, and obtained an increase of 6.3% in number of fruits compared to their control. Suh *et al.* (2014), found similar results when applying fulvic acids via foliar spray in tomato crop reporting an increase of 58% in the number of fruits compared to the control. In contrast, Castañeda *et al.* (2013) inoculated with *Azospirillum brasilense* and did not find any significant differences for the number of fruits per plant with any of the treatments applied. The increase in our study can be possibly attributed to the fact that rhizobacteria were used in combination with humic and fulvic substances.

**Table 5.** Production variables in strawberry cultivation with application of humic substances and rhizobacteria

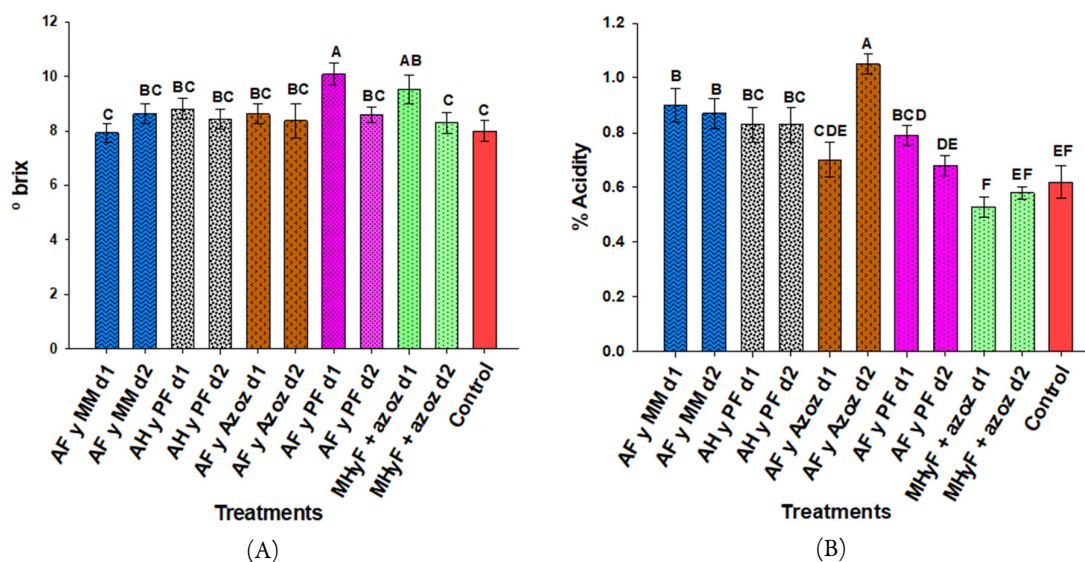
Treatments	PDF (mm)	EDF (mm)	Fruit weight (g)	Number of fruits	Yield (g / plant)
AFyMM d1	41.6abc	30.6bcd	16.0abcd	24.5ab	394.4ab
AFyMM d2	41.3abc	31.1abcd	16.1abcd	27.8a	448.1a
AHyPF d1	40.3c	30.9abcd	15.6bcd	24.1abc	378.7abc
AHyPF d2	40.5c	29.9d	15.0d	23.4abc	352.9abc
AFyAzoz d1	42.4abc	31.9ab	17.6a	25.8ab	454.6a
AFyAzoz d2	42.6abc	31.3abcd	17.3ab	25.5ab	440.2a
AFyPF d1	42.1abc	31.5abc	16.9ab	23.8abc	412.9ab
AFyF d2	40.7bc	30.3cd	14.8d	21.4bc	322.7bc
MHyF+Azoz d1	43.0ab	32.2a	17.9a	20.1bc	360.7abc
MHyF+Azoz d2	43.2a	31.3abcd	16.7abcd	18.5c	309.6bc
Control	40.5c	30.5bcd	15.6bcd	18.4c	285.0c
MSD	2.3	1.4	1.9	5.7	106.8
ANVA ( $P \leq$ )	0.0322	0.0393	0.0252	0.0268	0.0206

PDF = Polar diameter of fruit, EDF = Equatorial diameter of fruit. Values with the same letter within columns are statistically equal (LSD test,  $P \leq 0.05$ ). MSD = Minimum significant difference. ANVA = Variance analysis

The fruit yield (Table 5) increased significantly with the application of AFyAzoz d1, AFyMM d2, AFyAzoz d2, AFyPF d1 and the treatment AFyMM d1 in 59.5, 57.2, 54.4, 44.8 and 38.3% respectively compared to the control, while the rest of the treatments were significantly equal to the control. The yield was favored with fulvic acids, and the rhizobacteria *Azospirillum brasilense* and *Pseudomonas fluorescens*, the results were slightly similar to that reported by Pirlak and Kose (2009), where they used rhizobacteria of the genus *Pseudomonas* and *Bacillus* in the cultivation of strawberry variety ‘Selva’ and reported an increase by 25.7% with respect to the control. In an investigation carried out by Xudan (1986) in wheat crop where they applied fulvic acids, the yield increased by 96% with respect to the control, this could possibly be due to the fact that the applications of the fulvic acids were in foliar form. Bocanegra *et al.* (2006), mentioned that fulvic acids via foliar spray increased the mobilization and absorption of nutrients and accelerated the metabolic processes of plants that manifested themselves with an increase in yield. Turan *et al.* (2021), when applying a commercial product with fulvic and humic acids plus microorganisms, the yield significantly increased in cherry tomato.

#### Quality variables in strawberry cultivation

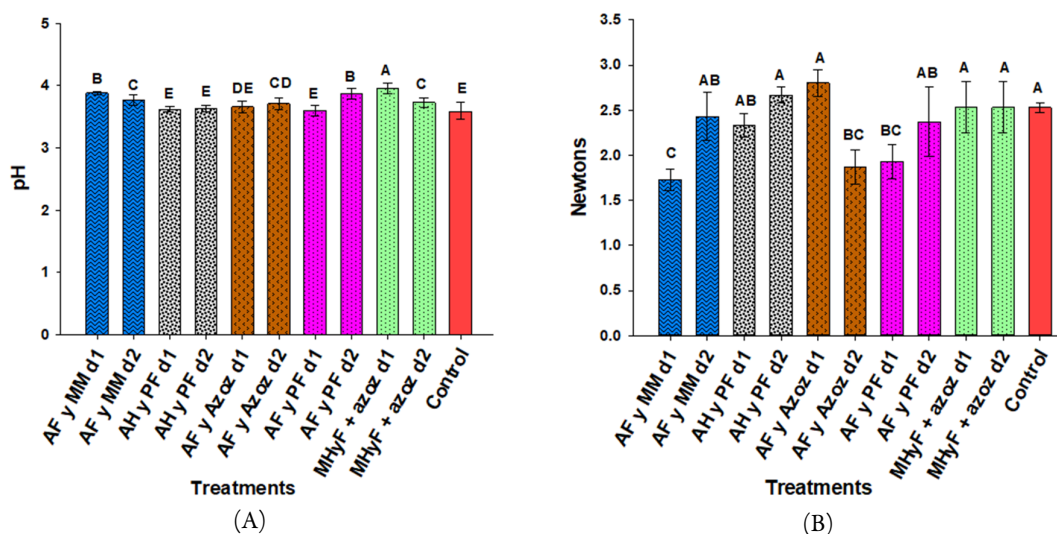
Total soluble solids (TSS) measured in °Brix are shown in Figure 1A, the treatment AFyPF d1 and MHyF + Azoz d1 significantly increased the TSS in 25.8 and 19.0 % respectively compared to control. The rest of the treatments applied to the plants were statistically equal to the control. TSS were favored with fulvic and humic acids as well as *Azospirillum brasilense* and *Pseudomonas fluorescens*, these results agree to the ones reported by Aghaeifard *et al.* (2015), where they assessed the impact of humic acid alone applied to strawberry plants and obtained an increase in SST of 13.9%. In a study by Hosseini *et al.* (2013), when using humic acids alone in strawberry cultivation, TSS increased by 6.7% compared to the control. In addition, Ortiz *et al.* (2016), when using rhizobacteria alone of the genus *Pseudomonas*, they reported increases in TSS of 1.2% in the strawberry fruit. The increase registered in our experiment in the total content of TSS with the application of rhizobacteria and humic substances may be possibly attributed to the fact that they were used in combination. Tripathi *et al.* (2016) mentioned that the application of these biostimulants accelerates the metabolic process of starch in soluble compounds due to the enzymatic activity and increase the transport of sugars from the leaves to the strawberry fruits.



**Figure 1.** Effect of applications of humic substances and rhizobacteria in (A) Total Soluble Solids (TSS) and (B) Titratable Acidity of strawberry fruit ‘San Andreas’ variety. Values with the same letter within columns are statistically equal Fisher’s (LSD) test ( $P \leq 0.05$ ).

For the titratable acidity of the fruit (Figure 1B), the treatments AFyAzoz d2, AFyMM d1 and d2, AHyPF d1 and d2 and the AFyPF d1 significantly increased the acidity in 69.3, 45.1, 40.3, 33.8, 33.8 and 27.4 % compared to the control, the other treatments were statistically equal to the control. These results coincide with the ones reported by Aghaeifard *et al.* (2015) when using humic acids in strawberry cultivation, they reported an increase of 58.4% with respect to the control. However, it is different from results found by Ullah *et al.* (2017), where they studied the influence of humic acid on the growth and yield of strawberry ‘Chandler’, they reported a 2.3 % lower titratable acidity than the control. In a study carried out by Todeschini *et al.* (2018), where they used strains of the genus *Pseudomonas* in combination with mycorrhizal fungi, they found a 2.2% increase in titratable acidity in strawberry fruits with respect to control plants. While in other plants, González *et al.* (2018), when applying *Pseudomonas lini* in tomato cultivation, the acidity of the fruit increased by 18.9% with respect to the control without inoculation. The increase in titratable acidity in strawberry fruits observed in our work, could possibly be due to the fact that different concentrations and doses of humic substances were used. Organic acids are used during fruit respiration, being essential components in the respiratory cycle of tricarboxylic acids (Kays, 2004). Hence, they contribute greatly to flavor, in a typical relationship between sugars and acids in different fruit species such as strawberry (Wills *et al.*, 1998).

Fruit pH (Figure 2A), increased with applications of in MHyF + Azoz d1, AFyMM d1, AFyPF d2, AFyMM d2, MHyF + Azoz d2 and AFyAzoz d2 in 10.3, 8.0, 7.7, 5.0, 3.8 and 3.3% respectively compared to the control, the other treatments were statistically equal to the control. The fruit pH increased with *azospirillum* and *pseudomonas*, this coincides with what was reported by Castañeda *et al.* (2013) where they inoculated with *Azospirillum brasilense* and found an increase of 3.3% in strawberry fruit pH compared to the control. However, Pirlak and Kose (2009), did not find significant differences in fruit Ph when applying rhizobacteria of the genus *Pseudomonas* and *Bacillus* in strawberry cultivation. In another study carried out by Zhang *et al.* (2021) when applying fulvic acids in the tomato crop via foliar spray, the pH of the fruit increased by 1%. These increases in fruit pH found in this research are possibly explained by what Rodríguez *et al.* (2004) reported, indicating that, the pH increases as the fruit reaches maturity, thus providing the characteristic flavor of the fruit, according to the species. In this research, the fruits of then treatments indicated above with higher pH were harvested earlier than the fruits of control plants, as mentioned by Torres *et al.* (2012), that the increase in pH is influenced by the increase in the state of maturity in certain fruits.

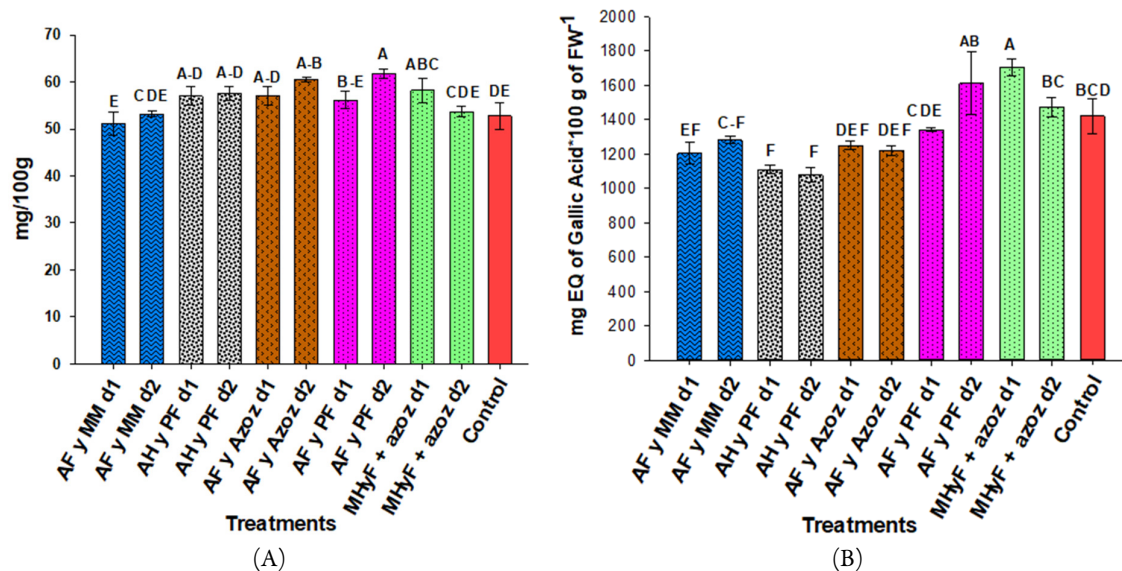


**Figure 2.** Effect of applications of humic substances and rhizobacteria in (A) pH and Firmness (B) of strawberry fruit ‘San Andreas’ variety

Values with the same letter within columns are statistically equal Fisher's (LSD) test ( $P \leq 0.05$ ).

For fruit firmness (Figure 2B) the treatments AFyMM d1, AFyAzoz d2, and AFyPF d2, were statistically lower than the control, in 43.0, 35.2 and 31.0%, the other treatments behaved significantly the same as the control. The results are different from those found by Pii *et al.* (2018), when applying the rhizobacteria *Azospirillum brasilense* they reported an increase in firmness by 16.7% with respect to the control. Esitken *et al.* (2010), in a study carried out with rhizobacteria of the genus *Pseudomonas* and bacillus did not find significant differences between their treatments when evaluating the firmness of the fruit, this could be due to the fact that in their experiment when applying the rhizobacteria *Pseudomonas* and bacillus they did not obtain either significant difference in Calcium (Ca) with respect to the control. Rincón and Martínez (2015) indicated that Ca fulfills the function of firming agent, due to the fact that calcium ions act on the pectin chains to form bridges between these, increasing the strength of the cell wall and the firmness in fruits and vegetables. Although we did not study the concentration of Ca, this could be the reason why the firmness decreased with the aforementioned treatments, coinciding with Pilanal and Kaplan (2003), when applying humic acids in high concentrations of 400 kg/ha decreased the concentration calcium in strawberry. Dominguez *et al.* (2012), in an experiment with the application of *Azospirillum brasilense* in mesquite seedlings, reported a decrease in Ca concentration compared to the control without inoculation.

Vitamin C (Figure 3A), increased significantly with the application of AFyPF d2, AFyAzoz d2 and MHyF + Azoz d1 in 17.1, 14.8 and 10.3% compared to the control, the rest of the treatments were equal to the control. These results are different from those reported by Pirlak and Kose (2009), where the increase in Vitamin C was 1.9% compared to the control when using rhizobacteria of the genus *Pseudomonas* and *Bacillus* applied to strawberry cultivation. Aminifard *et al.* (2012b), with applications of fulvic acids to the pepper crop, did not observe differences between treatments when evaluating Vitamin C. However, Eshghi and Garazhian, (2015) using humic acids in applications to the soil via drench in strawberry cultivation increased Vitamin C by 45% with respect to the control, this increase could possibly be attributed to the fact that they used different doses of humic acids.



**Figure 3.** Effect of applications of humic substances and rhizobacteria in Vitamin C (A) and Total Phenols (B) of strawberry fruit 'San Andreas' variety

Values with the same letter within columns are statistically equal Fisher's (LSD) test ( $P \leq 0.05$ ).

The total phenols (Figure 3B), increased significantly in the strawberry fruit with the MHyF + Azoz d1 treatment by 20% compared to the control, however, the AFyMM d1, AHyPF d1 and d2 treatments obtained a decrease of 17.8, 27.9 and 31.7% compared to the control, the rest of the treatments were statistically equal to the control. Soppelsa *et al.* (2019), used humic acids applied to the strawberry crop, reporting an increase in total phenols of 6.5% compared to the control. Andrade *et al.* (2020) when applying the rhizobacteria *Azospirillum brasilense* in the tomato crop they obtained an increase of 19.8% compared to the control. The treatments AFyMM d1, AHyPF d1 and d2 that decreased in total phenols with respect to the control, coincides with that reported by Aminifard *et al.* (2012b) where the applications of humic acids at high concentrations decreased on average the total phenols in cultivation of pepper with respect to the control.

## Conclusions

The biostimulants based on humic substances and rhizobacteria applied to the strawberry crop increased agronomic variables such as the number of leaves with humic acids + *Pseudomonas Fluorescens* d2, the root volume with fulvic acids + *Azospirillum brasilense* d1, fulvic acids + *Pseudomonas Fluorescens* d2 and mix of humic and fulvic + *Azospirillum brasilense* d2; in physiological variables, the CO<sub>2</sub> assimilation rate increased with fulvic acids + *Pseudomonas Fluorescens* d1 and with fulvic acids + *Azospirillum brasilense* d1; in production variables, fruit weight increased with applications of fulvic acids + *Azospirillum brasilense* d1 and mix of humic and fulvic + *Azospirillum brasilense* d1 and the yield with fulvic acids + *Azospirillum brasilense* d1 and d2, fulvic acids and mixture of microorganisms d2; in quality variables, TSS were increased with fulvic acids + *Pseudomonas Fluorescens* d1 and the mix of humic and fulvic + *Azospirillum brasilense* d1, vitamin C with fulvic acids + *Pseudomonas Fluorescens* d2, fulvic acids + *Azospirillum brasilense* with the mix of humic and fulvic d2 + *Azospirillum brasilense* d1. Biostimulants made with humic substances combined with rhizobacteria are an ecological option instead of chemical fertilizers, with its inherent reduction in soil and environmental contamination, in addition to favoring strawberry fruit production and quality.

## Authors' Contributions

Investigation: SMC, JAGF, MDDM; Methodology: MDDM, RMV, SMC; Resources: JAGF, MDDM, ARO, AHP; Supervision: JAGF, MDDM, RMV; Validation: JAGF, MDDM, DAC; Drafting: SMC, JAGF, RMV Review and editing: JAGF, MDDM. All authors read and approved the final manuscript.

## Ethical approval (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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