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Article

Effect of the application of humic substances and rhizobacteria on raspberry fruits

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Abstract

Mexico is an important producer in the cultivation of raspberries (*Rubus idaeus* L.); nevertheless, intensive agriculture poses serious problems, such as the decrease in soil fertility due to the indiscriminate use of chemical fertilizers and pesticides, and biotechnological alternatives favorable to the environment are sought. This research aimed to evaluate the effect of a biostimulant with humic substances and rhizobacteria on agronomic and quality variables in raspberry fruits. The research was conducted in 2021 in a greenhouse of the Department of Horticulture at the Antonio Narro Autonomous Agrarian University in Saltillo, Coahuila, Mexico. The following treatments were used: 1) fulvic acids and mixture of microorganisms; 2) humic acids and *Pseudomonas fluorescens*; 3) fulvic acids and *Azospirillum*; 4) fulvic acids and *Pseudomonas fluorescens*; 5) mixture of humic acids and fulvic acids plus *Azospirillum* and the control, with two different doses: D1) humic and fulvic acids: 3 ml and 5 ml microorganism; D2) humic acid or fulvic acid 3.5 ml and 4 ml microorganism, with four replications per treatment. Plant height increased with AFyAzoz d1 by 24.3%, fruit weight was favored by AFyMM d2 by 37.8%, yield increased with AFyPF d2 by 78.2%, TSS with AFyMM d1 by 23%, vitamin C increased by 20% with the application of AFyPF d2. In the principal component analysis, there was a positive correlation between the number of fruits and plant height (r= 0.94**), yield and number of fruits (r= 0.91**). Biostimulants with rhizobacteria and humic substances are a biotechnological alternative to be applied to raspberry crops.

Palabras clave:

humic acids, fulvic acids, microorganisms, quality.

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Introduction

Raspberry (*Rubus idaeus* L.) is a strawberry of great importance in some countries as well as in Mexico; it is a shrub of the berry group and is grown in some states such as Jalisco, Michoacán, and Baja California with a production of 104 080.19, 28 895.4 and 10 221.74 t ha^{-1}, respectively (SIAP, 2020). The raspberry and berry market presents conditions in the growth of demand and consumption; it is sought to develop innovative technologies that increase quality, yield and help reduce environmental damage (García and Sommerfeld, 2016).

The extensive use of chemical fertilizers poses serious problems, such as environmental pollution, develops resistance to pests, and causes losses of soil fertility (Ye *et al*., 2020). Biostimulants offer potential to improve crop production and quality, as well as to reduce fertilizer use (Quintero *et al*., 2018). Their use is becoming a common practice in production systems due to their ability to change the physiological processes of plants related to growth, production and stress mitigation (Afonso *et al*., 2022).

Humic substances (HS) are the natural constituents of soil organic matter resulting from the decomposition of plant, animal, and microbial residues (Canellas and Olivares, 2014). These substances have considerable effects on soil fertility and crop productivity due to their physicochemical and biochemical properties; they play a vital role in establishing biotic and abiotic interactions within the plant rhizosphere (Shah *et al*., 2018). Rhizobacteria can interact with plants directly, increase the availability of essential nutrients, such as nitrogen, phosphorus, and iron, and the production of compounds involved in plant growth, such as phytohormones (Olenska *et al*., 2020).

The combined application of a microbial consortium and humic substances improved yield in blueberries, resulting in a synergistic effect when beneficial microorganisms and humic substances are combined (Schoebitz *et al*., 2016). The mechanism of humic substances that affect plant growth, development, and productivity is likely to be explained by their connection to microorganisms in the rhizosphere and their activation by the root secretions of plants treated with humic preparations (Bezuglova and Klimenko, 2022).

Plant growth-promoting bacteria and humic substances are promising options for reducing the use of pesticides and mineral fertilizers (Da Silva *et al*., 2021). Therefore, this study aimed to evaluate the effect of a biostimulant based on humic substances and rhizobacteria on agronomic variables and quality in raspberry fruits.

Materials and methods

Study area

The research was conducted in a tunnel-type greenhouse of the Department of Horticulture of the Antonio Narro Autonomous Agrarian University in Saltillo, Coahuila, Mexico, which is located between the geographical coordinates 25° 22’ north latitude and 101° 02’ west longitude and at an altitude of 1 742 m. Raspberry (xz28) plants were used and planted in February 2021 at an age of two months in three soil ridges 12 m long and 1 wide and a drip irrigation system was used, which had tapes with drippers 20 cm apart.

Humic substances

The humic and fulvic substances were extracted from Leonardite, an organic mineral compound, provided by the mining company DHD de México, located in Sabinas, Coahuila. The extraction was carried out with López *et al*. (2014) methodology and potassium hydroxide (KOH) 1 N was used; for separation, acetic acid was used until a pH of 4 was obtained; after 24 h, by decantation, fulvic acids with a golden yellow coloration were obtained, while humic acids with a soil-like appearance and dark brown remained at the bottom of the container.

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Microbiological material and preparation of bacterial strains

The rhizobacteria strains of *Azospirillum brasilense*, *Pseudomonas putida* and *Pseudomonas fluorescens*, discovered by Johana Dobereiner, Ananda Mohan Chakrabarty and Migula, respectively, were provided by the National Collection of Microbial Strains and Cell Cultures of the Center for Research and Advanced Studies of the National Polytechnic Institute. The rhizobacterium *Azospirillum brasilense* was reactivated in NRCB culture medium and *Pseudomonas* species were seeded in King's B medium. In preparation, the bacteria were cultured in nutritious broth and in constant stirring at 150 rpm for 48 h at 25 °C ±5 °C, where bacterial growth became evident by turbidity in the medium. The bacterial cell concentration was 109 cel ml^{-1} according to Mc Farland's (Mc Farland, 1907) turbidity scale.

Treatments

Five different mixtures were applied with two doses, resulting in 10 treatments plus a control with four replications each, applied in a completely randomized design. The following treatments were used in the experiment: 1) fulvic acids + mixture of microorganisms dose 1 (AFyMM d1); 2) fulvic acids + mixture of microorganisms dose 2 (AFyMM d2); 3) humic acids + *Pseudomonas fluorescens* dose 1 (AHyPF d1); 4) humic acids + *Pseudomonas fluorescens* dose 2 (AHyPF d2); 5) fulvic acids + *Azospirillum brasilense* dose 1 (AFyAzoz d1); 6) fulvic acids + *Azospirillum brasilense* dose 2 (AFyAzoz d2); 7) fulvic acids + *Pseudomonas fluorescens* dose 1 (AFyPF d1); 8) fulvic acids + *Pseudomonas fluorescens* dose 2 (AFyPF d2); 9) mixture of humic acids and fulvic acids + *Azospirillum brasilense* dose 1 (MHyF+Azoz d1); 10) mixture of humic acids and fulvic acids + *Azospirillum brasilense* dose 2 (MHyF+Azoz d2) and 11) control: Steiner nutrient solution was applied (Steiner, 1961).

The following were used in the mixture of microorganisms: *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Azospirillum brasilense*. Dose 1) humic or fulvic acids: 3 ml and 5 ml microorganism; dose 2) humic and fulvic acids: 3.5 ml and 4 ml microorganism; the treatments were applied directly to the soil near the base of the roots of the plant, humic substances every 15 days and rhizobacteria every 30 days.

Variables evaluated

Agronomic variables were evaluated at the end of the crop cycle, 180 days after transplanting. Plant height was measured with a tape measure from the base of the crown to the apex of the highest leaf; the basal diameter of the stem was measured with a Steren digital vernier caliper, model HER-411. Fruit weight was recorded with a TJ gram scale, model MH-500. The polar and equatorial diameters of the fruit were measured with a Steren digital vernier caliper, model HER-411; the number of fruits was recorded per plant and the total number of fruits per plant was weighed for the yield and reported as g plant^{-1}.

In the quality variables, total soluble solids (TSS) were measured by placing a drop of raspberry fruit juice on the lens of the Hanna digital refractometer, model 96-801 and the readings were expressed in °Brix. The potential of hydrogen (pH) was measured with a HI98130 digital potentiometer (Hanna Instruments). Titratable acidity was determined by colorimetry according to the AOAC (2000). Vitamin C in raspberry fruits was determined by Padayatt *et al*. (2001) methodology.

Statistical analysis

The data were analyzed by means of an analysis of variance (Anova); the statistical package of Infostat version 2020 was used. Fisher's LSD test ($p\leq 0.05$) was used for the separation of means. The principal component analysis was performed with the computational package of Minitab 16, 2009.

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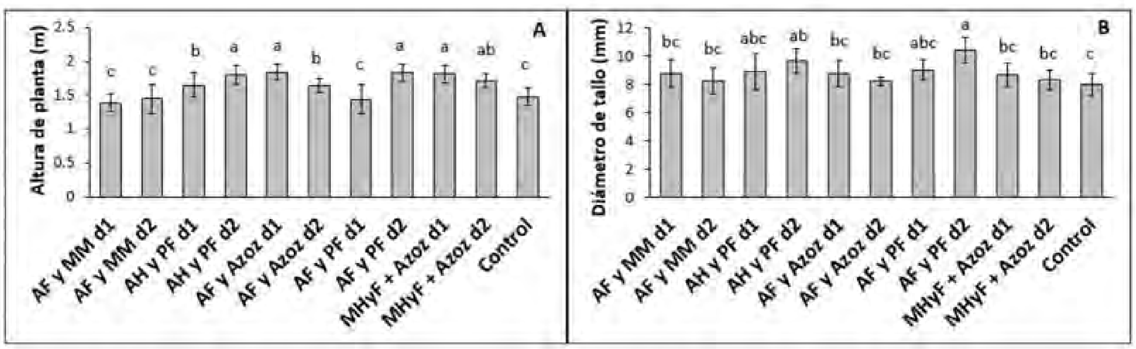
Results and discussion

Agronomic and production variables

Raspberry plant height was evaluated (Figure 1A); significant differences were observed between treatments; height benefited from the application of AFyAzoz d1, AFyPF d2, MHyF + Azoz d1, AHyPF d2, MHyF + Azoz d2, AHyPF d1, AFyAzoz d2; the increase with respect to the control was 24.3, 23.6, 22.2, 21.6, 15.5, 11.4, and 10.8%, respectively; the rest of the treatments behaved statistically the same as the control. These results are consistent with Schoebitz *et al*. (2016); when humic acids plus a bacterial consortium were applied to the blueberry crop, plant height increased by 16% compared to the control. The application of humic and fulvic acids promotes plant growth through hormone-like effects as the breakdown of these substances releases auxins (Shahrajabian *et al*., 2021).

Figure 1. Plant height (A) and basal stem diameter (B) in raspberries with applications of humic substances and rhizobacteria. Different letters between bars indicate significant differences between treatments, Fisher's LSD ($p\leq 0.05$).



In the evaluation of basal stem diameter in raspberry plants (Figure 1B), it was observed that the application of AFyPFd2 and AHyPF d2 increased stem diameter by 30.4 and 20.8%; the rest of the treatments behaved the same as the control.

In a study conducted by Protim *et al*. (2017), inoculating strains of the genus *Pseudomonas* in castor plants increased stem diameter by 10.6% compared to chemical fertilization. This observed increase was caused by the combination of humic substances and rhizobacteria; according to Ahmad *et al*. (2018), they found that the combined application of humic acid and rhizobacteria is much better than their individual effect and observed a considerable improvement in rapeseed plant growth.

Fruit weight (Figure 2A) was significantly favored by the application of AFyMM d2 and with AFyPF d2, where they obtained an increase of 37.8 and 24.9%; however, the latter treatment was statistically equal to the control, like the rest of the treatments. A study by Zejak *et al*. (2021), when evaluating the production and quality of raspberry fruits, reported fruit weight values between 3.4 and 4.7 g, which were similar to the findings in this work.

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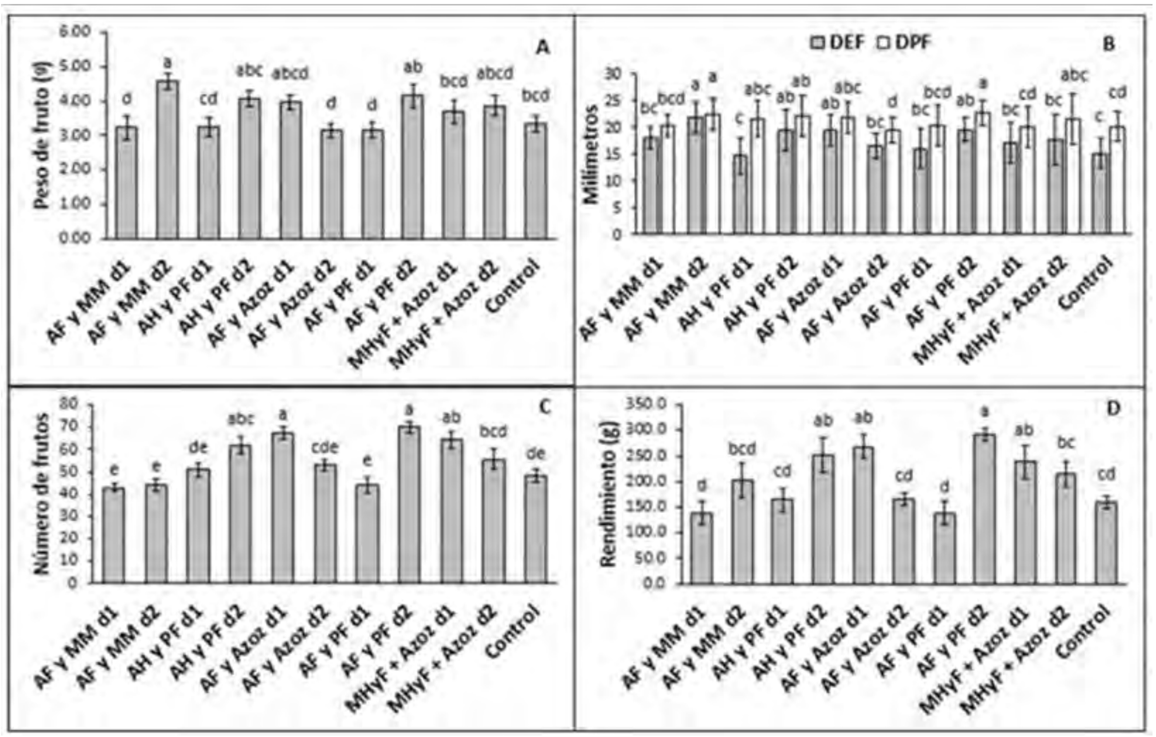
Figure 2. Fruit weight (A); fruit equatorial diameter (DEF) and fruit polar diameter (DPF) (B); number of fruits (C) and yield (D) in raspberry, with applications of humic substances and rhizobacteria. Different letters between bars indicate significant differences between treatments, Fisher's LSD ($p \leq 0.05$).



Fruit weight was increased by fulvic and humic acids; in this regard, Kumar *et al*. (2019), when applying fulvic acids derived from different sources and applied to the corn crop, obtained a significant growth in grain weight of 4% compared to the control; the increase found in the research is due to the fact that fulvic acids were extracted from Leonardite and not from plant extracts, such as those used by Kumar *et al*. (2019) in their research.

The evaluation of the fruit equatorial diameter (DEF) is observed in Figure 2B; the growth observed in this variable is with the application of AFyMM d2, AFyPF d2, AFyAzoz d2, and AHyPF d2, with increases of 44.5, 29.8, 28.6, and 28.4%, respectively, compared to the control; the other treatments applied behaved the same as the control. The fruit polar diameter (DPF) in Figure 2B was favored by the AFyPF d2, AFyMM d2, and AFyAzoz d1 treatments by 13.3, 11.8, and 8.4%; the other treatments were statistically the same as the control.

In a study by Abd El-Razek *et al*. (2020) with applications of humic acids and organic matter in olive crops, when evaluating the fruit, the DPF benefited by 30.2% and the DEF by 28.9%. Abd El-Rheem *et al*. (2017) reported that applications of fulvic acids with a dose of 2 ml L^{-1} in persimmon tree (*Diospyros kaki* L.) increased the DPF by 5.3% and the DEF by 8.8%; this lower increase was caused by the application of lower doses of fulvic acids than the one used in the study, which ranged from 3 to 3.5 ml L^{-1} plus the use of rhizobacteria. Espinosa *et al*. (2017) applied rhizobacteria, such as *Pseudomonas lini*, with different substrates in tomato crops and in the evaluation of the fruit, they found that DPF increased by 6.1% and DEF by 4% compared to the control without rhizobacteria. Nevertheless, Andrade-Sifuentes *et al*. (2020), when bioinoculating with *Azospirillum brasilense* in tomato crops, found no differences between treatments when evaluating DPF and DEF.

The number of fruits (Figure 2C) was favored by the application of AFyPF d2, AFyAzoz d1, MHyF +Azoz d2, and AHyPF d2 by 45.8, 39.5, 33.3, and 29.1%, respectively, compared to the control; the rest of the treatments were statistically equal to the control. These results are consistent

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with what was reported by García-Seco *et al*. (2015); the inoculation of a plant growth-promoting rhizobacterium *Pseudomonas fluorescens* increased the number of fruits in blueberry crops by 42.8%.

In a study observed by Kamal *et al*. (2017), when using humic acids in pomegranate plants, the number of fruits increased by 38.2%. Andrade-Sifuentes *et al*. (2020) conducted an experiment where they applied the *Azospirillum brasilense* strain in tomato crops, where they increased the number of fruits per plant by 27%.

The yield observed in Figure 2D was favored by the application of the AFyPF d2, AFyAzoz d1, AHyPF d2, and MHyF+Azoz d2 treatments and showed an increase of 78.2, 66.8, 57.5, and 48.7%, respectively, compared to the control; the rest of the treatments behaved the same as the control. In works demonstrated by Hernández-Montiel *et al*. (2017), when applying strains of *Pseudomonas putida* in tomato crops, the yield increased by 20.7%. In an experiment, Andrade-Sifuentes *et al*. (2020) report that the application of the rhizobacterium *Azospirillum brasilense* increased the yield in tomato crops by 35%. Nonetheless, Rosales *et al*. (2015) applied fulvic acids in melon crops and compared three soil treatments with a control without application of fulvic acids (AF0), application of fulvic acids at pH6 (AF6), and fulvic acids at pH7 (AF7) and when evaluating the yield, they found no differences between the treatments applied.

This higher yield presented in the study resulted from the combination of rhizobacteria with humic substances that served as a carbon source. Olivares *et al*. (2017) mentions that humic substances increase the exudation of organic acids in the root, favoring the interaction of the plant with beneficial microorganisms, allowing increases in the absorption and transport of mineral nutrients.

Fruit quality variables

Quality attributes were evaluated in raspberry fruits (Table 1); total soluble solids (TSS), which were measured in °Brix, were favored by AFyMM d1 by 12.3 °Brix and by AFyPF d1 by 12.2 °Brix; the increase was 23 and 22% compared to the control; according to the statistical analysis, the other treatments behaved in the same way as the control. Dujmović *et al*. (2012), when evaluating different raspberry varieties, found in their research total soluble solids values that ranged from 9.4 to 11.5 °Brix.

Table 1. Quality variables in raspberry fruits with application of humic substances and rhizobacteria.

Treatments	TSS (°Brix)	pH	Titratable acidity (%)	Vitamin C (mg 100 g^{-1})
AF y MM d1	12.3a	3.55ab	0.96c	51.6ab
AF y MM d2	10.3b	3.57a	1.23ab	49.1abc
AH y PF d1	9.5b	3.38bc	1.21ab	50abc
AH y PF d2	9.4b	3.45abc	1.18ab	53.8a
AF y Azoz d1	9.6b	3.5abc	1.23ab	52.5ab
AF y Azoz d2	9.3b	3.35c	1.25ab	48.4abc
AF y PF d1	12.2a	3.55ab	1.08bc	43.9c
AF y PF d2	10.4b	3.52abc	1.06bc	49.5abc
MHyF + Azoz d1	9.2b	3.47abc	1.28a	46.7bc
MHyF + Azoz d2	9.7b	3.37c	1.2ab	43.9c
Control	10b	3.38bc	1.2ab	44.7c
LSD	1.21	0.18	0.19	6.22
Significance	**	*	*	**

Values with the same letter within columns are statistically equal, Fisher’s LSD ($p \leq 0.05$). LSD= least significant difference; *= significant; ** = highly significant; ns= not significant.

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Regarding the increase in TSS compared to the control, the results were different from those obtained by Aghaeifard *et al*. (2015), where they assessed the impact of humic acid applied to strawberry plants and obtained TSS growth of 13.9% compared to the control. Ortiz *et al*. (2016), when using rhizobacteria of the genus *Pseudomonas*, reported increases in TSS of 1.2% in strawberry fruits compared to the control.

The pH of the fruit increased with the application of AFyMM d2, with a growth of 5.6% compared to the control. These results were close to those reported by El-Beltagi *et al*. (2022); by applying a biofertilizer based on *Azospirillum* and *Azotobacter* rhizobacteria in cherry tomato crops, the pH of the fruit juice increased by 7% compared to the control.

Nonetheless, they were different from what was mentioned by Shehata *et al*. (2011); they applied humic acids in strawberry crops and when evaluating the pH of the fruit, they did not find statistical differences between treatments. Trujano-Fragoso *et al*. (2017) evaluated raspberry fruits of the Adelita, Erika, Lupita, and Polka cultivars, with values between 3.42 and 3.83; these same pH values were obtained in this study.

In the titratable acidity of the fruit, it is observed that the treatments behaved mostly the same as the control, except for AFyMM d1 (0.96%), which presented fruits with lower acidity compared to the other treatments applied. Alvarado *et al*. (2016) studied different planting densities in raspberry crops and when evaluating titratable acidity, they reported values of 1.37 to 1.42%, which were more acidic than what was found in the research.

In a study by Todeschini *et al*. (2018), where they used strains of the genus *Pseudomonas* in combination with mycorrhizal fungi, they found an increase in titratable acidity in strawberry fruits of 2.2% compared to their control. For their part, González *et al*. (2018) applied *Pseudomonas lini* in tomato crops and the acidity of the fruit increased by 18.9% compared to the control without inoculation. Orhan *et al*. (2006), when applying rhizobacteria in raspberry crops, found no significant differences between treatments.

Vitamin C benefited from AHyPF d2 by 53.8 mg 100 g^{-1}, AFyAzoz d1 by 52.5 mg 100 g^{-1}, and from the application of AFyMM d1 by 51.6 mg 100 g^{-1}, with an increase compared to the control of 20, 17, and 15%, respectively; the rest of the treatments were statistically the same as the control. In a research, Ponder and Hallmann (2020) compared conventional and organic farming and reported that conventional raspberry fruits obtained vitamin C values of 55.4 mg 100 g^{-1} compared to organic fruits with 46.2 mg 100 g^{-1}. Aminifard *et al*. (2012), with applications of fulvic acids in pepper crops, observed no differences between treatments when evaluating vitamin C. However, Eshghi and Garazhian (2015) reported that using humic acids in soil applications via drench in strawberry crops increased vitamin C by 45% compared to the control.

Principal component analysis

A principal component analysis (PCA) was performed, which included agronomic and quality variables in raspberry crops. The analysis of the three principal components of the populations explained 85.9% of the variation in data of the 11 variables analyzed, such percentage resulted from the consecutive sum of the proportional values determined in the analysis (Table 2).

Table 2. Eigenvalues and vectors for the first three principal components of 11 variables evaluated in raspberry crops.

Valors	Principal components		
	1	2	3
Eigenvalue	4.9677	3.0595	1.416
Proportion (%)	45.2	27.8	12.9
Cumulative (%)	45.2	73	85.9
Variables		Eigenvectors	

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Valors	Principal components		
	1	2	3
Fruit weight	0.362	-0.124	0.435
TSS	-0.219	-0.478	-0.128
Acidity	0.098	0.465	0.385
Vitamin C	0.24	-0.146	-0.122
DEF	0.31	-0.27	0.394
DPF	0.342	-0.197	0.153
pH	0.083	-0.499	0.117
Stem diameter	0.269	-0.234	-0.516
Height	0.362	0.263	-0.272
Number of fruits	0.375	0.18	-0.309
Yield	0.435	0.053	-0.057

TSS= total soluble solids; DEF= equatorial diameter of fruit; DPF= polar diameter of the fruit.

According to the eigenvectors, in the first principal component, the most important original variables were fruit weight, height, number of fruits, yield, which contrast with TSS. In the second principal component, the variables with the greatest influence were acidity and height, which contrast with TSS and pH. The third component was strongly influenced by the variables: fruit weight and DEF, which are negatively related to stem diameter.

Figure 3 shows the correlations between the variables according to the angles of the vectors that represent them. There was a positive and highly significant correlation between the following variables: number of fruits and plant height (r= 0.94**), yield and number of fruits (r= 0.91**), fruit weight and DEF (r= 0.89**), and fruit weight and DPF (r= 0.85**).

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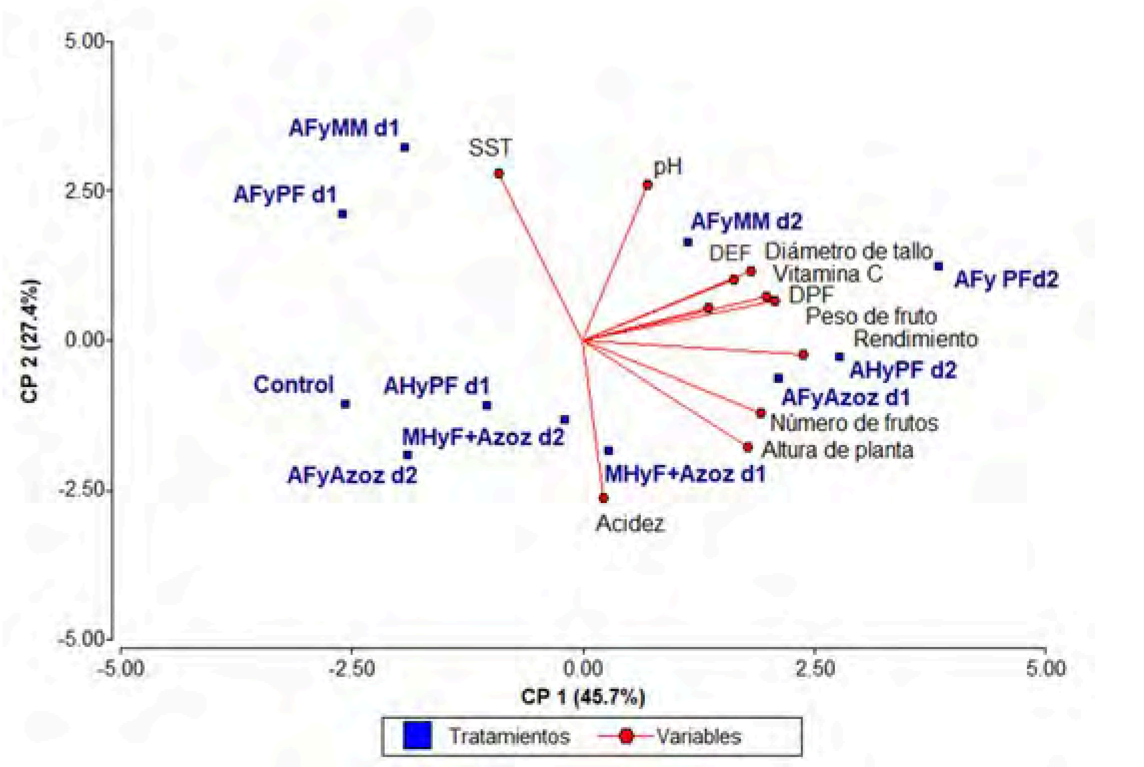
Figure 3. Biplot of treatments with humic substances plus rhizobacteria and with agronomic and fruit quality variables in raspberries. SST= total soluble solids; DEF= fruit equatorial diameter and DPF= fruit polar diameter.



This indicates that the number of fruits was favored by plant height, the yield by the number of fruits, and the weight of the fruit was influenced by the DEF and DPF. Regarding the variables that show a negative and highly significant relationship, they are fruit acidity and TSS (-0.86**). Therefore, treatments with a low percentage of acidity increased TSS in raspberry fruits.

Conclusions

Biostimulants based on humic substances and rhizobacteria applied to raspberry crops increased fruit weight with fulvic acids + mixture of microorganisms d2 and fulvic acids + *Pseudomonas fluorescens* d2; yield was favored by the application of fulvic acids + *Pseudomonas fluorescens* d2; TSS benefited from treatments of fulvic acid + mixture of microorganisms d1 and from fulvic acids + *Pseudomonas fluorescens* d1; vitamin C increased with humic acids + *Pseudomonas fluorescens* d2 and fulvic acids + *Azospirillum brasilense* d1.

There is a positive and highly significant correlation between the number of fruits and plant height, yield and number of fruits. It is recommended to continue investigating the biostimulant effect of humic substances in combination with rhizobacteria in other crops of economic importance to analyze their favorable action on agronomic and fruit quality variables, as well as to evaluate the impact of their application in unfavorable environmental conditions of water and saline stress.

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