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Characterization of Bioactive Compounds in Blueberry and Their Impact on Soil Properties in Response to Plant Biostimulants

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ABSTRACT

Vaccinium corymbosum L. (Ericaceae) is a highly valued fruit crop and the most common type of blueberry grown in Chile. Plant growth and yield crop production are affected by agricultural practices and different soil parameters including chemical and biological properties. We performed a field assay to assess the effect of the inoculation with a mixture of 10 microorganisms and the addition of humic substances on the growth of blueberry, quality of fruit and soil chemical and biological properties. Two years after planting, the microbial consortium was more effective than the addition of humic substances recording a 35% increase in shoot dry weight, 70% increase in root dry weight and 104% on total fruits yield compared to the control plants. Total polyphenols and ferric reducing antioxidant power (FRAP) in fruit were increased on humic substance treatment. The combination of both factors increased by 18% organic matter and 60% cation exchange capacity. Soil respiration, microbial biomass C and enzyme activities (dehydrogenase, phosphatase, β-glucosidase, urease, and protease) to a greater extent than individual application. The combined treatment, involving microbial inoculant and humic substances, had an additive effect on improving the biochemical and microbiological quality of the soil.

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Introduction

Plant growth and yield crop production are affected by different soil parameters, including chemical and biological properties. Chemical fertilization, which is one of the methods of enhancing nutrient quantity and availability for plant utilization, is a fast way of providing the plant with the necessary nutrients (Miransari 2011). The use of chemical fertilizers to improve soil quality and crop production can often result in leaching and runoff of nutrients, especially N. The low efficiency of fertilizers and their continuous long-term use can account for this situation (Adesemoye and Kloepper 2009). Different studies have been conducted in order to improve the efficiency of the use of fertilizers, including the inoculation with beneficial soil microorganisms. In fact, the use of microorganisms has a potential role in developing sustainable systems for crop production (Couillerot et al. 2013; Pérez-García, Romero, and de Vicente 2011). Plant growth-promoting rhizobacteria (PGPR) are important part of the soil microbiota, which are known for their capacity to increase the root surface area and improve plant growth (Bashan et al. 2014). Application of PGPR *Pseudomonas fluorescens* to roots of blackberries (*Rubus* sp.) is part of an optimized cultivation practice to improve yields and quality of

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fruit (Garcia-Seco et al. 2015). In this regard, PGPR may have a potential role in the plant yield of blueberry seedlings under field conditions in volcanic soils.

The beneficial effects of humic substances on plant growth may be related to increased fertilizer efficiency, reducing soil compaction, enhancing plant biomass (Canellas et al. 2015) and increasing soil microflora (Puglisi et al. 2013). Humic substances do not only have a positive effect on soil fertility, resulting in a higher nutrient availability for plant growth, but they also seem to positively influence metabolic and signaling pathways involved in plant nutrient uptake (Kolodziej, Sugier, and Bielinska 2013). In some cases, a better crop response was observed using humic-like substances obtained from compost instead of humic extracts from leonardite (Canellas et al. 2015). Many commercial products containing humic substances, most commonly humic acid, are currently being used in commercial vegetable production; these products are frequently applied into the soil. Although the potential activity of products containing humic acids has been well documented, there are serious limitations recorded in the existing scientific literature (i.e., highly dependent on plant genotype and application rate) (Hartz and Bottoms 2010).

Some recent studies have described the beneficial effects of humic acid combined with inoculated beneficial bacteria. A study conducted by Olivares et al. (2015) showed beneficial effects on the chemical soil properties and plant yield when humic acid was applied in combination with *Herbaspirillum seropedicae* as plant growth-promoting bacteria. Furthermore, Canellas and Olivares (2014) have studied the basic mechanisms and benefits of the combined application of humic substances and plant growth-promoting bacteria in different types of crops, reporting findings that provide evidence that humic acids can promote beneficial soil microbial growth. The effect of humic substances combined with bacteria in foliar applications has been studied in maize grown under field conditions, resulting in a 65% increase in grain production compared to the untreated control (Canellas et al. 2015).

The main objective of our study was to assess the effect of the combined application of a single dose of humic substances and microbial consortium on the growth performance of blueberry seedlings and soil properties under field conditions. . Humic substances in combination to microbial consortium become more susceptible to interact with soil microorganisms. We hypothesize that the effect of the combined application of microbial consortium and humic substances into the soil can effectively improve soil properties, improve yields and quality of blueberries.

Materials and methods

Study site and plant characterization

The experiment was located at El Nogal Experimental Station of the University of Concepción in Chillán (36°35′43.2" S, 72°04′39" W; 144 m a.s.l.), Chile. The climate at this location is classified as temperate Mediterranean, with an annual rainfall of 980 mm per year, concentrated in winter, with a potential evapotranspiration of 818 mm. The annual mean temperature is 13°C, with an average temperature of 7°C in July and 20°C in January. Annual mean RH is 74% and the frost-free period is 6-mo (Del Pozo and Del Canto 1999). Soil is classified as medial, amorphic, thermic Humic Haploxerands, derived from a volcanic ash-derived soil (Andisol) (Soil Survey Staff 2006). The chemical properties of the soil are: pH 6.7, organic matter 5.8%, NO_3 11.6 mg kg^{-1}, available P 77 mg kg^{-1}, and extractable K 406 mg kg^{-1}.

Highbush blueberry *Vaccinium corymbosum* L. cultivar 'Legacy', which belongs to the Ericaceae family, was used in this study. Legacy is a mid-season cultivar, high production, medium-sized light blue fruit and adapted to acidic soil. Seedlings were grown in ProPlant nursery (Chillán, Chile) with soil as substrate for 18-mo prior to experimental procedures. At planting, *V. corymbosum* was 70.5 ± 8.2 cm high, with a shoot dry weight of 6.8 ± 1.2 g, root dry weight of 8.0 ± 1.0 g, diameter 4.9 ± 0.1 mm, N total 32 mg $plant^{-1}$, P total 3 mg $plant^{-1}$ and K total 2.6 mg $plant^{-1}$.

Microbial consortium and humic substances

Microbial consortium Oiko bac 174 (Oikos Chile Ltda., Santiago, Chile) was evaluated in terms of plant yield and plant nutrient uptake. This consortium contained soil beneficial microorganisms belonging to bacteria and fungi. The beneficial microbial consortium was a mixture of 10 soil microorganisms: *Bacillus subtilis, B. licheniformis, B. megaterium, B. polymyxa, B. macerans, Pseudomonas fluorescens, P. putida, Nocardia corallina, Saccharomyces cerevisiae* and *Trichoderma viride*, being the cell concentrations of 10^8 CFU g^{-1}. Therefore, 50 g microbial consortium and 100 g saccharose were dissolved in 10 L tap water (based on the manufacturer's recommendations). Bioestimulants were applied to soil spraying locally around the stems. One liter of microbial consortium and 15 g humic substances were applied at planting and reapplied from August 3, 2015; October 5, 2015; December 7, 2015, and March 1, 2016. Biosolve (Oikos Chile Ltda.), which is a commercial product containing humic acid (derived from leonardite shale), was used as humic substance for the soil (70% humic acid, 15% fulvic acid, and 10% K_2O w/w).

Experimental design

A full-factorial design was established with two factors and fivefold replicates in a split-plot design. The first factor was the inoculation or not of blueberry seedlings with the microbial consortium and the second was the addition or not of humic substances into the soil.

Twenty seedlings were transported to the experimental field in April 2014, where planting holes 30 × 30 cm wide and 30 cm deep were dug manually. The seedlings were planted at least 1 m apart between holes and rows were spaced 3 m apart. At planting, an amount of 120 g elemental S was applied into the soil in all of the treatments to reduce soil pH. The irrigation was applied as needed and weeds were controlled manually and no pesticide and fertilizer was applied.

Samples were collected by March 2016. Five plants per treatment (20 plants in total), including root systems and rhizosphere soil, were harvested and placed in polyethylene bags to be transported to the laboratory. Rhizosphere soil samples were separated into two subsamples before chemical and microbiological analyzes. Root system was washed and measured. Fruits were collected by January 2016.

Plant and soil properties

To evaluate the response to microbial consortium inoculation and humic substances, the following growth parameters were considered and values were recorded before the chemical analysis: stem diameter, plant height, and shoot and root dry weights (70°C; 24 h). Foliar concentrations of N, P, K; Ca, Mg were determined according to the methodology described by Sadzawka et al. (2007). Foliar samples were taken in harvest time (March 2016). The N content in the soil was measured using dry combustion in a total elemental analyzer (LECO, TruSpec CN, Saint Joseph, Michigan, USA). Soil pH was measured in a 1:2.5 (w/v) aqueous solution. Available N (NO_3 and NH_4), extractable K and Ca, cation exchange capacity (CEC) and organic matter (%) were determined as proposed by Sadzawka et al. (2006).

Water-soluble carbohydrates were determined by the method of Brink, Dubar, and Lynch (1960). Soil respiration was calculated as the amount of CO_2 emitted during a 24 h incubation period: 10 g dry soil was placed in an incubation vessel; moisture was adjusted to 45% of water-holding capacity and a vial containing 2 mL KOH (0.1 g KOH in 50 mL distilled water) was placed inside the incubation vessel for retention of the evolved CO_2. Soil microbial biomass C was evaluated by fluorescein diacetate (FDA) method. FDA (0.1 mL) was added to the samples in proportions of 2 mg mL^{-1} acetone, except to the blank. The tubes were subjected in vortex and brought to the thermostatic bath by incubating at 20°C for 1 h and cooled in an ice bath. Ten mL acetone was added to all tubes (samples and blanks), stirred and filtered. After obtaining the filtrate, absorbance of the

samples and targets in the spectrophotometer (Rayleigh – Model UV1601 UV/VIS BRAIC, Beijing, China) at 490 nm was read against the reactive blank. The results were expressed as μg F g^{-1} dry soil (Alef and Nannipieri 1995; Green, Stott, and Diack 2006).

Dehydrogenase activity was determined according to Garcia, Hernandez, and Costa (1997). For this, 1 g soil at 60% of its field capacity was exposed to 0.2 mL 0.4% iodonitrotetrazolium chloride (INT; 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22°C in darkness. The iodonitrotetrazolium formazan (INTF) formed was extracted with 10 mL methanol by shaking vigorously for 1 min and filtering through a Whatman Nr 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Urease and *N*α-benzoyl-L-arginine amide (BAA) hydrolyzing protease activities were determined in 0.1 M phosphate buffer at pH 7; 1 M urea and 0.03 M BAA were used as substrates, respectively. Aliquots of 2 mL buffer and 0.5 mL substrate were added to 0.5 g sample followed by incubation for 90 min at 30°C (urease) or 39°C (protease). Both activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al. 1980).

Alkaline phosphatase activity was determined using 0.115 M *p*-nitrophenyl phosphate disodium (PNPP) as substrate. For the assay, 2 mL 0.5 M sodium acetate buffer adjusted to pH 11 using acetic acid (Naseby and Lynch 1997) and 0.5 mL substrate was added to 0.5 g soil and incubated at 37°C for 90 min. The reaction was stopped by cooling at 0°C for 10 min. Then, 0.5 mL 0.5 M $CaCl_2$ and 2 mL 0.5 M NaOH were added and the mixture centrifuged at 4,000 rev min^{-1} for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremmer 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

β-Glucosidase was determined using 0.05 M *p*-nitrophenyl-β-D-glucopyranoside (PNG) as substrate. For this assay, based on the release and detection of PNP, 2 mL 0.1 M maleate buffer at pH 6.5 and 0.5 mL substrate were added to 0.5 g sample and incubated at 37°C for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethano (THAM) according to Tabatabai (1982). The amount of PNP was determined by spectrophotometry at 398 nm (Tabatabai and Bremmer 1969).

Fruit analysis

Some quality aspects of the fruit were analyzed such as diameter or yield. In addition, antioxidant properties of the blueberries were evaluated. The extraction of antioxidants was performed using 0.5 g blueberries from each treatment and treated with 20 mL methanol/water acidified with 2 N HCl (50:50 v/v, pH 2). In each case, the mixture obtained was shaken with a magnetic stirrer for 1 h and centrifuged at 3500 rpm for 15 min. The supernatant was collected. A volume of 20 mL acetone/water (70:30 v/v) was added to the residue and the mixture was stirred again for 60 min. After stirring, the mixture was centrifuged for 15 min more at 3500 rpm and the supernatant was combined with the supernatant previously removed. These extracts were used to determine total polyphenols, total anthocyanins, and antioxidant capacity.

Total polyphenols were determined using the Folin Ciocalteu method (Singleton and Rossi 1965). The samples were prepared adding 750 μL 1 N Folin Ciocalteu reagent, 750 μL 20% sodium carbonate and 500 μL extract and maintained for 2 h in the dark. Total polyphenols were measured by absorbance on a spectrophotometer (OPTIZEN 3220UV; Mecasys, Yuseong-gu, Daejeon, Korea) at 760 nm and the polyphenol content was expressed as gallic acid equivalent (mg GAE 100 g^{-1} FW).

Total anthocyanins were determined by a differential pH technique. A buffer of 0.025 M potassium chloride at pH 1 and another buffer of 0.4 M sodium acetate at pH 4.5 were used. Extracts of blueberry fruits (0.3 mL) were placed with 2.7 mL potassium chloride buffer pH 1 and 2.7 mL sodium acetate buffer, respectively. The absorbance was recorded at 510 and 700 nm in an OPTIZEN 3220UV spectrophotometer. The content of anthocyanins was expressed as mg cyanidin-3-glucoside 100 g^{-1} FW.

The ferric reducing antioxidant power (FRAP) method was used (Benzie and Strain 1996) to determine the antioxidant capacity of the blueberry samples. A volume of 1800 µL FRAP reagent was prepared and then mixed with 180 µL distilled water and 60 µL sample. The mixture was incubated at 37°C for 30 min and then the absorbance was measured at 595 nm. The results were expressed as µmol Trolox g^{-1} FW. Also, the free radical scavenging activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH)·method as described by Mena et al. (2011). This method consists of determining the antioxidant activity by measuring the variation in absorbance at 515 nm after a reaction time of 30 min with the DPPH radical. The results were expressed as percentage. All the assays were performed using an OPTIZEN 3220UV spectrophotometer and three replicates were performed per sample.

Statistical analysis

Values were log- and arcsine-transformed to achieve normality. The values indicating the effects exerted by both humic substances (HS) and microbial consortium (MC) treatments, either alone or in combination, were analyzed by a two-way ANOVA and a post-hoc mean separation was performed by Duncan's multiple range test, calculated at $P \leq 0.05$. All statistical analyzes were performed using SPSS software (version 19.0 for Windows; IBM, Armonk, New York, USA).

Results

Effect of the inoculation with microbial consortium and humic substances on plant growth

The inoculation with microbial consortium (MC) promoted an increase in shoot and root dry weights, with 35% and 70% increases, respectively, compared to control treatment. On the other hand, the combined treatment (MC+HS) mediated a 20% increase in shoot dry weight and a 33% increase in root dry weight compared to the control plants (Table 1). The ANOVA shows a significant effect of MC on root and shoot biomass. The humic substances (HS) treatment did not show a significant effect on shoot or root dry weights. The experimental factors tested did not significantly affect basal stem diameter and plant height (Table 1).

Foliar nutrient uptake of blueberry and soil chemical properties

The experimental treatments did not significantly affect the foliar nutrient uptake, compared with control plants (Table 2). The ANOVA shows that MC significantly affected pH, organic matter, nitrate, Ca and CEC. Humic substance had a significant effect on organic matter, K and CEC, while the MC×HS interaction affected K extractable. In many cases, the Duncan test confirmed a significant increase in the values for these treatments compared with the control (Table 3). Only for soil nitrate was recorded a decrease after the MC and HS application. The pH values increased with MC and MC+HS. With regard

Table 1. Effect of inoculation with microbial consortium (MC) and humic substances (HS) on shoot and root biomass, diameter, and plant height measured 2 years after planting.

	BSD	Plant height	Root	Shoot
	mm	cm	g DW	
Control	6.9 ± 0.5a	76 ± 5a	77 ± 8a	51 ± 3ab
MC	8.5 ± 0.4a	81 ± 4a	131 ± 7c	69 ± 2c
HS	7.7 ± 0.7a	79 ± 4a	90 ± 7ab	47 ± 3a
MC+HS	7.8 ± 0.9a	78 ± 5a	103 ± 8bc	61 ± 4bc
ANOVA, *P* values				
MC	1.441 (0.253)	0.303 (0.592)	17.546 (< 0.001)	20.397 (< 0.001)
HS	0.000 (0.992)	0.000 (0.993)	0.255 (0.623)	3.492 (0.086)
MC×HS	1.523 (0.241)	0.566 (0.466)	6.437 (0.026)	0.249 (0.627)

Table 2. Foliar nutrients of blueberry seedlings in response to the addition of microbial consortium (MC) and humic substances (HS) measured 2 years after planting.

	N	P	K	Ca	Mg
			mg gDW^{-1}		
Control	207 ± 27ab	46 ± 2a	346 ± 52a	357 ± 21a	125 ± 9a
MC	354 ± 49b	56 ± 15a	582 ± 111a	376 ± 98a	142 ± 20a
HS	187 ± 14a	40 ± 2a	370 ± 9a	329 ± 20a	109 ± 10a
MC+HS	264 ± 90ab	52 ± 18a	423 ± 120a	451 ± 135a	147 ± 11a
ANOVA, *P* values					
MC	4.100 (0.066)	0.078 (0.785)	2.416 (0.146)	0.044 (0.838)	0.044 (0.838)
HS	1.769 (0.208)	0.160 (0.696)	0.601 (0.453)	0.088 (0.772)	0.088 (0.772)
MC×HS	0.770 (0.397)	0.027 (0.873)	1.807 (0.204)	0.417 (0.531)	0.417 (0.531)

to organic matter, the greatest value was observed after the addition of the combined treatment (19% increase). The K underwent a significant increase after the HS and the application of the combined treatment (3.8 and threefold higher with respect to the control, respectively). Ca values were increased by the combined treatment. CEC also increased with all the treatments, especially the combined treatment (by 60%, with respect to the control).

Soil biochemical and microbiological properties

The ANOVA shows that both factors and their interaction significantly affected urease, protease, and β-glucosidase enzyme activities. Microbial consortium had a significant effect on dehydrogenase and phosphatase, while also the MC×HS interaction affected phosphatase activity (Table 4). Phosphatase activity was improved by the combined treatment, the MC+HS yielding an increase of 43%. The activity of β-glucosidase showed an increase with MC and MC+HS treatments; the highest value was achieved with the combined treatment (a 90% increase, with respect to the control). Urease activity also increased with MC and MC+HS treatments, especially the combined treatment (by 10-fold higher with respect to the control). Protease activity was improved by the combined treatment, the MC+HS yielding an increase of 116%. Dehydrogenase activity was improved by the application of MC and the combined treatment, obtaining its maximum value with the latter (a rise of 177% relative to the control) (Table 4).

The HS significantly affected the microbial biomass. The MC and the MC×HS interaction significantly affected soil respiration (Table 5). The Duncan test confirmed increases in microbial biomass on HS and MC+HS treatments. Microbial biomass reached its highest value (50% higher than the control) with the MC+HS. The highest increases in soil respiration were recorded after the MC and the addition of HS (84% and 140%, respectively, compared with the control) (Table 5).

Production and fruit quality

The diameter of the berries did not differ significantly between treatments (Table 6), whereas the total yield fruit (g $plant^{-1}$) was significantly increased by the MC treatment and MC+HS combination.

The HS treatment significantly improved polyphenol contents by 65% with regard to the control. However, regarding the anthocyanins, there were nonsignificant differences between the treatments.

The HS treatment significantly increased the antioxidant capacity of blueberries when evaluated through FRAP assay, whereas DPPH assay showed a significantly higher antioxidant capacity for both HS and the combined MC+HS treatments.

Table 3. Changes in chemical soil properties on blueberry seedlings in response to microbial consortium (MC) and humic substances (HS) addition.

		OM	NO_3	NH_4	K	Ca	CEC
	pH	%	mg kg^{-1}	mg kg^{-1}	cmol kg^{-1}	cmol kg^{-1}	cmol kg^{-1}
Control	6.0 ± 0.2a	5.3 ± 0.2a	2.1 ± 0.1b	5.2 ± 0.2a	0.4 ± 0.02a	5.2 ± 0.5a	7.8 ± 0.5a
MC	6.5 ± 0.1b	5.6 ± 0.2a	1.0 ± 0.1a	5.2 ± 0.3a	0.7 ± 0.07a	7.2 ± 0.6ab	10.7 ± 0.7b
HS	6.3 ± 0.2ab	5.7 ± 0.1ab	2.0 ± 0.1b	5.0 ± 0.1a	1.5 ± 0.20b	6.1 ± 1.0ab	10.5 ± 1.1b
MC+HS	6.7 ± 0.1b	6.3 ± 0.3b	1.2 ± 0.0a	5.3 ± 0.1a	1.2 ± 0.08b	7.9 ± 0.5b	12.5 ± 0.6b
ANOVA, *P* values							
MC	9.376 (0.010)	6.495 (0.026)	107.527 (< 0.001)	0.597 (0.455)	0.236 (0.636)	8.461 (0.013)	10.960 (0.006)
HS	1.798 (0.205)	8.350 (0.014)	0.682 (0.425)	0.025 (0.878)	58.792 (< 0.001)	1.194 (0.296)	8.922 (0.011)
MC×HS	0.282 (0.605)	0.163 (0.693)	4.619 (0.053)	0.633 (0.422)	5.531 (0.037)	0.020 (0.890)	0.759 (0.401)

Table 4. Changes in enzymatic activities in rhizosphere soil of blueberries in response to inoculation with microbial consortium (MC) and humic substances (HS).

	Phosphatase	β-glucosidase	Urease	Protease	Dehydrogenase
Treatments	μmol PNP g^{-1} h^{-1}		μmol NH_3 g^{-1} h^{-1}		μg g^{-1} INTF
Control	2.3 ± 0.25b	1.1 ± 0.07a	0.3 ± 0.02a	0.6 ± 0.012a	22 ± 0.9a
MC	2.8 ± 0.08bc	1.5 ± 0.13b	1.2 ± 0.07b	0.7 ± 0.05a	49 ± 1.4b
HS	1.5 ± 0.17a	1.0 ± 0.06a	0.3 ± 0.04a	0.6 ± 0.08a	22 ± 1.5a
MC+HS	3.3 ± 0.15c	2.1 ± 0.13c	3.1 ± 0.27c	1.3 ± 0.07b	61 ± 1.5c
ANOVA, *P* values					
MC	42.277 (< 0.001)	66.235 (< 0.001)	451.84 (< 0.001)	15.086 (0.002)	217.19 (< 0.001)
HS	2.673 (0.128)	6.460 (0.026)	63.37 (< 0.001)	9.349 (0.010)	2.154 (0.168)
MC × HS	13.356 (0.003)	10.003 (0.008)	50.761 (< 0.001)	7.479 (0.018)	4.369 (0.059)

Table 5. Changes in microbiological soil properties on blueberry seedlings in response to microbial consortium (MC) and humic substances (HS) addition.

	Microbial biomass C	Respiration	Water-soluble carbohydrates
	μg F g^{-1} soil	μg C-CO_2 g^{-1} soil d^{-1}	μg g^{-1}
Control	3.15 ± 0.57a	1.44 ± 0.07a	39 ± 5a
MC	4.18 ± 0.43ab	2.65 ± 0.04b	37 ± 2a
HS	4.61 ± 0.27b	1.31 ± 0.08a	41 ± 2a
MC+HS	4.72 ± 0.42b	3.45 ± 0.21c	44 ± 2a
ANOVA, *P* values			
MC	1.777 (0.207)	266.768 (< 0.001)	0.009 (0.927)
HS	4.828 (0.048)	2.540 (0.137)	2.180 (0.166)
MC×HS	1.462 (0.250)	14.020 (0.003)	0.433 (0.523)

Discussion

The combined application of MC and HS was the most-effective treatment for increasing chemical and biological soil properties under field conditions. Plants showed different levels of response to the MC and the addition of the HS. Thus, it was observed an increase in shoot and root biomass with MC treatment. Inoculation with plant growth-promoting MC has been shown to be a useful strategy to promote plant growth (Schoebitz et al. 2016; Thilagar, Bagyaraj, and Rao 2016). In contrast, nonsignificant differences were observed when HS alone was applied to the soil. It is well documented that rhizobacteria exert a beneficial effect on plant growth and development, and many different rhizobacteria have been commercialized for use in agriculture (Adesemoye et al., 2009; Adesemoye and Kloepper 2009; Bashan et al. 2014). Our results are in agreement with those described by Pirlak and Köse (2009), who reported an increase in the yield of strawberry plants after the inoculation with an MC. Jha and Saraf (2012) also reported that root and shoot biomass was maximized with MC compared to both control and individual trials of microorganisms. Plant growth, nutrition, yield, and mycorrhizal root colonization in the rhizosphere were found to be higher when beneficial bacteria and fungi were applied simultaneously compared to both the control and their individual inoculation (Chauhan and Bagyaraj 2015). Previous microbial studies performed without plants have indicated that some combinations allow bacteria to interact with each other synergistically, providing nutrients and stimulating each other through physical and biochemical activities that may enhance some beneficial aspects of their physiology (Jha and Saraf 2012). Microbial consortium and HS did not increase the foliar nutrient content in our experiment with blueberry seedlings. Esitken et al. (2010) conducted a field experiment adding MC to the soil and found an increase of P uptake, but not for N and K in strawberry plants 3 years after the inoculation of *Bacillus* sp. and *Pseudomonas* sp. in a combined treatment. According to Orhan et al. (2006), MC did not increase the total K in other field experiment using raspberries, but resulted in a significant increase in N content and P uptake compared to the untreated control.

Table 6. Changes in physicochemical fruit properties on blueberry seedlings in response to microbial consortium (MC) and humic substances (HS) addition.

	Fruits diameter	Total fruit yield				
	mm	g plant^{-1}	Total polyphenols[1]	Total anthocyanins[2]	FRAP	DPPH
Control	13 ± 1.0a	26 ± 2a	166 ± 12a	18 ± 2a	449 ± 55a	84 ± 5ab
MC	14 ± 0.9a	53 ± 5b	187 ± 22a	22 ± 6a	467 ± 60a	87 ± 5ab
HS	12 ± 0.4a	30 ± 5a	274 ± 19b	30 ± 5a	670 ± 40b	94 ± 1b
MC+HS	14 ± 1.3a	45 ± 5b	216 ± 33ab	20 ± 8a	586 ± 69ab	81 ± 2a
ANOVA, *P* values						
MC	1.543(0.238)	25.187 (< 0.001)	0.538 (0.477)	0.893 (0.363)	0.268 (0.614)	1.892 (0.194)
HS	0.333(0.575)	0.089 (0.771)	7.894 (0.016)	0.147(0.709)	8,814 (0.012)	0.255 (0.623)
MC×HS	0.223(0.645)	1.604 (0.229)	2.775 (0.122)	2.358 (0.151)	0.735 (0.408)	4.186 (0.063)

In terms of chemical and biological properties of the soil, we observed that the combined treatment increased pH, organic matter, K, Ca, CEC, microbial biomass, soil respiration and enzyme activities (dehydrogenase, phosphatase, β-glucosidase, urease, and protease).

Nitrate levels and nitrate in the soil did not increase in two of the treatments evaluated (MC and MC+HS), and even a decrease in nitrate content was observed in the MC and MC+HS treatment. This may be explained by the assimilation of N by the microorganisms inoculated into the soil. In the treatments involving HS, it is assumed that the addition of the amendment mediates an input on N levels, even so the total N levels in soil were not modified. MC+HS treatment increased microbial biomass, soil respiration and in particular enhanced the protease and urease activity, which is involved in the N cycle and could reveal a shift in microbial populations mediated by an increase in N assimilation by the soil microorganisms.

The combined treatments tested increased K and Ca contents in soil and cation exchange capacity (CEC) with regard to the control. Microbial consortium and HS helped plants to compensate for deficiencies of immobile nutrients such as K, which may be attributed to the mobilization of K by the soil rhizobacteria and also by the input of K provided with the addition of HS. The inoculation of soil with MC and HS can be considered an effective tool for the development of biotechnology products than can be used as a partial substitute for chemical fertilization. In that way, the introduction of microbial inoculant can improve plant nutrient availability and thereby increase the efficiency of applied manures (Adesemoye and Kloepper 2009).

Microbial biomass C, soil respiration, dehydrogenase, β-glucosidase, phosphatase, protease, and urease activities were higher in the rhizosphere soil of MC+HS treatment in comparison to control plants and these parameters have frequently been used as indicators of soil microbial activity (Caravaca, Masciandaro, and Ceccanti 2002). Enzyme activities are sufficiently sensitive to indicate changes caused by microbial inoculation (Naseby and Lynch 1997). Furthermore, MC in combination with HS may release enzymes involved in the mineralization of organic matter. Thus, a positive correlation in MC+HS treatment has been reported between enzyme activity, soil respiration and organic matter after 2 years of assay.

In relation to the studies carried out on the fruit, we have to take into account that these analyzes were not very conclusive since all the fruits were harvested at the same time, and the blueberry usually presents heterogeneity in the number of days to reach edible ripeness. In addition, these plants were young and 2 years for the blueberry may be limited, so the results obtained in fruit are considered as preliminary.

The results obtained corroborate that the production of biologically active substances or of growth regulators is one of the main mechanisms through which PGPR influences the growth of plants and development. In the same line, the use of beneficial microorganisms has great potential in increasing crop yields and in the development of sustainable agriculture (Martínez-Viveros et al. 2010) as well as a study in strawberry (*Fragaria ×ananassa* Duchesne ex Rozier) showed that the application of vermicompost and inoculants promoted a significantly higher fruit weight gain than the plants in which fertilizer (39%) was applied (Rivera-Chávez et al. 2012). This did not occur in the plants where only HS treatment was applied where there was a nonsignificant difference with respect to the control. However, primary plant metabolism and changes on secondary metabolism by HS are being documented (Canellas and Olivares 2014). The beneficial actions of HS on plant growth, resulting from improvements in nutrient uptake. This is because these effects have been linked to the ability to HS to form stable natural complexes with metals, thus increasing the solubility of relevant nutrients (Chen, De Nobili, and Aviad 2004). Besides, recent studies have shown that HS are able to improve plant yield through the activation of the main actors involved in nutrient root uptake and further transport and metabolisms within the plant (Jannin et al. 2012).

On the other hand, the research carried out by Rivera-Chávez et al. (2012) on polyphenols agrees with the results here obtained, since 29% more total phenols were recorded in the vermicompost treatment than in the fertilized treatment. Also, Rivera-Chávez et al. (2012) showed that the

application of vermicompost and inoculant increased anthocyanin content by 38% compared to fertilizer treatment. Nevertheless, we did not obtain any difference in anthocyanins. Besides, antioxidant capacity in fruit presented better results in HS or in the combination of HS+MC, showing a clear relation with the results obtained in soil.

Conclusions

The combined application of microbial consortium (MC) and humic substances (HS) improved blueberry plant yield in volcanic soils, resulting in a synergistic effect when beneficial microorganisms and HS are introduced. Regarding soil properties, the application of combined treatments increased inorganic matter, pH, K, Ca, and cation exchange capacity. Based on these data, application of the combined treatments including MC and HS seems to be the most appropriate method to improve fruits yield, stimulate biological soil properties and enzyme activities. Application of the combined treatments produced vigorous seedlings and increased growth in an Andisol. Therefore, the application of HS in combination with MC can be considered a biotechnological tool for plant growth promotion in sustainable agriculture systems.

Conflicts of Interest

The authors declare no conflict of interest.

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