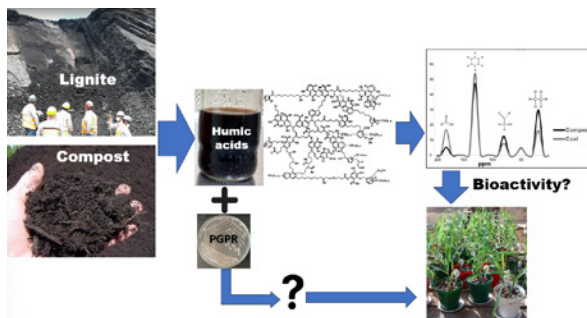


Biostimulant activity of humic acids derived from goat manure vermicompost and lignite in relation to their structure and interaction with a PGPR strain under semiarid conditions

Actividad bioestimulante de ácidos húmicos derivados de vermicompost de estiércol caprino y lignito en relación con su estructura e interacción con una cepa PGPR en condiciones semiáridas



NELSON VALERO^{1, 3}
YEISON USTATES¹
MANUEL PANTOJA-GUERRA²

Photos: the authors

ABSTRACT

The use of humified organic matter (HOM)-based plant biostimulants and plant growth-promoting rhizobacteria (PGPR) has emerged as a promising approach to enhance agricultural productivity in arid and semiarid environments. However, the bioactivity of humic stimulants varies based on their chemical composition, and the synergistic effects of co-applying these biostimulants remain to be fully elucidated. In this research, we investigated the structural and bioactive characteristics of humic acids derived from goat manure vermicompost (HAVC) and lignite coal (HAC). Additionally, we explored the plant growth-promoting effects of each humic acid (HA) in conjunction with the *Bacillus mycoides* strain BSC25 (Bm) on corn plants in arid conditions. To assess the relationship between structure and bioactivity, we determined the supramolecular composition of the HAs and evaluated their effectiveness through a corn coleoptile elongation test. Subsequently, we conducted biostimulation tests on maize seedlings in a growth chamber and performed a field-based biostimulation test in a semi-arid region. Notably, HACs exhibited coleoptile elongation at lower concentrations (25-50 mg LC) compared to HAVCs, which required higher concentrations (100-200 mg LC) to achieve the same effect. These outcomes correlated with the supramolecular composition of HAs. The bioactivity of HACs was linked to their oxygen content, aromatic and carboxylic groups, whereas HAVCs'

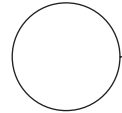
¹ Universidad de la Guajira, Facultad de Ciencias Básicas y Aplicadas, Riohacha (Colombia). ORCID Valero, N.: <https://orcid.org/0000-0001-9186-6245>; ORCID Ustates, Y.: <https://orcid.org/0000-0002-3342-2897>

² Unilasallista Corporación Universitaria, Facultad de Ciencias Agropecuarias, Caldas (Colombia); ORCID Pantoja-Guerra, M.: <https://orcid.org/0000-0002-0485-7605>

³ Autor correspondiente. nvalerov@uniguajira.edu.co

bioactivity was associated with their carbohydrate, aliphatic carbon, and hydrogen content. The application of both HAs, together with Bm, resulted in enhanced corn leaf biomass production in the growth chamber and under field conditions. This effect can be attributed to the hormone-like actions of HA and the PGPR activity of Bm. Interestingly, despite foliar application, HAs displayed bioactivity at the root level, as evidenced by increased root biomass in the field. These results indicate a PGPR effect of Bm that remained unaltered with co-application of HAVC. However, the joint application of Bm-HAC and Bm-HAVC reversed the positive effect of Bm on corn production under field conditions. This outcome likely relates to the hormone-like effects of HA and potential additive effects following Bm inoculation.

Additional keywords: bioactive compounds; *Zea mays*; *Bacillus mycoides*; dryland management; food insecurity; biotechnology.

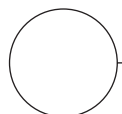


RESUMEN

El uso de bioestimulantes vegetales a base materia orgánica humificada (MOH) y PGPR's es una tecnología en vía de consolidación para promover la productividad agrícola bajo condiciones marginales. Sin embargo, la bioactividad de los estimulantes húmicos varía de acuerdo con su naturaleza química y aun es necesario explicar los efectos sobre la aplicación conjunta de estos dos agentes bioestimulantes. En este trabajo se analizó la relación entre la estructura y bioactividad de ácidos húmicos obtenidos de lombricompost de estiércol caprino (HAVC) y de un carbón tipo lignito (HAC), así como el efecto promotor del crecimiento vegetal de cada uno de estos ácidos húmicos (AH) aplicados en conjunto de la cepa *Bacillus mycoides* BSC25 (Bm), sobre plantas de maíz, bajo condiciones de aridez. Para ello se determinó la composición supramolecular de los HA, su bioactividad fue evaluada mediante el test de elongación de coleóptilos de maíz, se desarrollaron ensayos de bioestimulación sobre plántulas de maíz en cámara de crecimiento, finalmente se desarrolló un ensayo de bioestimulación en maíz en condiciones de campo en una zona semiárida. Los HAC causaron elongación de coleóptilos a una concentración inferior (25-50 mg CL), en comparación con los HAVC, que causaron el mismo efecto a una concentración más alta (100-200 mg CL); se encontró una asociación entre estos resultados y la composición supramolecular de los HA. La bioactividad de los HAC estuvo asociada a su contenido de oxígeno, grupos aromáticos y carboxílicos; mientras que la bioactividad de los HAVC se correlacionó con su contenido de carbohidratos, carbono alifático y de hidrógeno. La aplicación de ambos HA, Bm y la aplicación conjunta HA- Bm, promovió la producción de biomasa foliar de maíz en cámara de crecimiento y en condiciones de campo. Esto se puede atribuir a los efectos hormone-like de los HA y a la actividad PGPR de Bm. Aunque los HA fueron aplicados por aspersión foliar, mostraron bioactividad a nivel radicular. Los resultados de biomasa radicular en campo indican un efecto PGPR de Bm, el cual no se vio modificado por la aplicación conjunta con HAVC. Sin embargo, la producción de mazorcas en condiciones de campo fue promovida por la actividad de Bm, pero este efecto fue revertido por la aplicación conjunta Bm-HAC y Bm-HAVC. Estos resultados están asociados a los efectos hormonales de los HA y de posibles efectos aditivos tras la inoculación con Bm.

Palabras clave adicionales: compuestos bioactivos; *Zea mays*; *Bacillus mycoides*; manejo de tierra secas, inseguridad alimentaria; biotecnología.

Received: 08-05-2023 Accepted: 21-07-2023 Published: 18-08-2023



INTRODUCTION

A set of strategies to mitigate food insecurity through agrobiology is currently being implemented worldwide with some success. These strategies include biocontrol, biofertilization, and biostimulation

technologies, which have shown potential to mitigate the difficulties associated with agricultural practices, especially under limiting conditions in aridisols (Reeves *et al.*, 2016; FAO, 2022; Li *et al.*, 2022).

Biostimulation is based on the use of substances or microorganisms that, when applied to plants, trigger physiological responses that lead to improvements in nutritional efficiency, tolerance to biotic and abiotic stress (Van Oosten *et al.*, 2017), stimulate the metabolism, and generally favor the performance of crop plants (Nardi *et al.*, 2016). Biostimulants include inputs based on microorganisms, fractions of humified organic matter, hydrolysates of proteins, amino acids and other nitrogenous compounds (Nardi *et al.*, 2016), oligosaccharides, inorganic compounds, green algae extracts, and botanicals (Du Jardin, 2015).

On the other hand, humic acids are a fraction of humified organic matter, whose supramolecular structure is related to its bioactivity and determines its biostimulatory action. This comprises a series of physiological effects that lead to proliferation, elongation, and modifications in the root architecture, increase in the absorption and transport of ions, efficiency of primary metabolism (Aguar *et al.*, 2018), regulation of molecular processes, and induction of secondary metabolites synthesis (Shah *et al.*, 2018), as well as systemic responses to stressors (da Piedade Melo *et al.*, 2017) thanks to a “priming effect” (Canellas *et al.*, 2020). Moreover, they also induce the increase in the production of exudates (Canellas *et al.*, 2019), which affect the magnitude and activity of the rhizosphere microbial community, plant-microorganism communication, therefore, the establishment and performance of symbiotic, associative and endophytic interactions by plant growth-promoting microorganisms (Roomi *et al.*, 2018; Bulgari *et al.*, 2019; García *et al.*, 2019; Nunes *et al.*, 2019; Jindo *et al.*, 2020).

The intensification of organic agriculture in arid and semiarid zones require to adapt cultivars tolerant to adverse climatic and ecological conditions, such as high temperatures, drought and salinity; thus, it is convenient to use biostimulating agents, such as humic acids, that relieve stress and promote plant growth and development. The genus *Bacillus* comprises a wide variety of bacterial plant growth-promoting species that express several biostimulation mechanisms, including indole acetic acid (IAA) synthesis (Tejera-Hernández *et al.*, 2011; Rojas, *et al.*, 2013). In addition, characteristics such as their persistence in a broad spectrum of environmental conditions, metabolic versatility, presence of resistance structures as endospores, and the motility that facilitates plant colonization, make genus *Bacillus* very useful in agricultural biotechnology (Tejera-Hernández *et al.*, 2011). Such advantages acquire importance

in arid and semi-arid soils compared to other microorganisms commonly used as bioinoculants for agriculture. Their role has been documented as an adjuvant for the soil rehabilitation, and the adaptation of pioneering plants in desert areas in a joint application with organic amendments (Bashan *et al.*, 2012).

The chemical composition of humic acids varies according to their source; thus, certain supramolecular characteristics are linked to variations in their bioactivity. Bioactivity patterns associated with specific structural configurations have been previously described. For instance, labile and functionalized humic fractions are related to root proliferation, whereas less functionalized and more recalcitrant fractions are associated with root elongation (García *et al.*, 2016a). However, these patterns are not regular, and the bioactivity of humic acids concerning their supramolecular structure is still a subject of discussion (García *et al.*, 2019).

Variable results have been observed in the joint application of humic acids and plant growth promoting rhizobacteria (PGPR). Eventually, this combination could have not the best effect on plant performance compared to the separate application of each biostimulant (Ahmad *et al.*, 2016; Valero *et al.*, 2016; Bacilio *et al.*, 2017; Bratkova *et al.*, 2021). Some authors have hypothesized that these results are generated by modulating effects of a “hormonal balance” in microorganism-plant communication (Blouin, 2018). Therefore, it is important to evaluate the bioactivity of humic acids whose chemical nature varies according to their origin, as well as the effects of their interaction with PGPR.

This work aims to evaluate the relationship between the bioactivity and the structural characteristics of humic acids obtained from two locally available raw materials in the semi-arid La Guajira peninsula, Colombia: vermicompost made with goat manure—goats are the main livestock activity in the region—and the lignite coal generated as a by-product of open-pit coal mining—it is also a large-scale economic activity in the area. Additionally, we sought to analyze the effect of treating maize plants, under controlled conditions and field conditions, with each humic acid separately and together with the strain *Bacillus mycoides* BSC25. The latter has been evaluated previously due to its performance as a PGPR under the limiting conditions that derive from the aridity of this territory (Valero-Valero *et al.*, 2021; Beleño-Carrillo *et al.*, 2022).

MATERIALS AND METHODS

Extraction of HA

Two raw materials common in La Guajira peninsula were used as humic acids (HA) source: a lignite generated as a by-product of coal mining, and goat manure vermicompost. 1 kg of each material was taken and oven-dried at 70°C until to obtain constant weight. Subsequently, the total humic extract was obtained by applying the classic extraction method with alkaline solutions (1N NaOH), following the standard methodology described by the International Humic Substances Society for each type of sample (Chang *et al.*, 2014). The organic matter was extracted from the samples with 1 N NaOH (10 h, 60°C) and subsequently, the extract obtained was allowed to stand for 24 h and was filtered using Whatman paper No. 40 to separate the humic and fulvic acids from the insoluble residue or humins. The HAs in the extracts were separated from the fulvic acids (FAs) by acidifying the solution to pH 2 using 1N HCl. This fraction was collected and taken to successive washes with distilled water and subsequent centrifugation; then, the HA extract was freeze dried and stored in plastic tubes at -4°C.

Chemical nature of HA

The chemical characterization of the two AH types (HAC: humic acid-coal; HAVC: humic acid-vermicompost) comprised: 1) elemental composition (C, H, O, N, S) using ASTM D5373 with an EAI CE-440 Elemental Analyzer (Pantoja-Guerra and Valero-Valero, 2020); 2) analysis through Fourier transform-Infrared spectroscopy (FT-IR), the protocol described by Spaccini and Piccolo was followed to obtain the IR spectrum of each HA sample (Spaccini and Piccolo, 2009); and 3) nuclear magnetic resonance analysis (¹³C-CP-TOSS (¹H)-NMR), NMR spectroscopy was performed in solid state. As proposed by Zhang *et al.* (2017), samples were processed on a Bruker Avance III HD Ascend 400 MHz (9.4 T) spectrometer. For the interpretation of the spectra, the chemical shift recorded between 0 and 200 ppm was divided into the following resonance regions: C-alkyl (0-45 ppm), C-methoxy and N-C (45-60 ppm), C-O-alkyl (60-110 ppm), C-aromatic (110-160 ppm), C-carboxy and carbonyl, aldehydes, and ketones (160-200 ppm) (Spaccini and Piccolo, 2009). The integration of the area under the curve was subsequently performed at each range. In this way, quantitative data of the relative

percentage of ¹³C in each group were obtained. Finally, the aromaticity index of each HA sample was determined with the quantitative data (Spaccini and Piccolo, 2009).

Evaluation of humic acid bioactivity in plant tissue

HA bioactivity was assessed by the coleoptile elongation test (Valero *et al.*, 2018a). Corn seeds (variety ICA-109) were germinated on agar-agar plates at 28°C. Once the coleoptiles emerged, 5 mm segments were cut from the base. The segments were immersed in Petri dishes with 10 mL of HAC or HAVC solutions in 2 mM CaCl₂ at concentrations of 10, 25, 50, 75, 100, 150, and 200 mg carbon per liter of solution (mg C L⁻¹). As a control treatment, 2 mM CaCl₂ solution was used, and 10 repetitions were made per treatment. The coleoptiles were shaken in the dark in the different solutions for 48 h at 150 rpm.

After the stirring period, the coleoptiles were photographed with a digital camera next to a pattern of known length. The change in the length of the coleoptiles was determined by digital image analysis by the ImageJ software, Fiji version.

Association between the chemical structure of HA and their bioactivity

The coleoptile length data, the elemental composition of HA, and their chemical composition by functional groups (¹³C-CP-TOSS (¹H)-NMR) were analyzed by principal component analysis (PCA) multivariate statistics using the PAST statistical package (v. 4) to compare the bioactivity of both types of AH and find a possible association between the bioactivity and the chemical nature of their structure.

Effect of humic acid application and inoculation of *B. mycoides* BSC25 on corn plant growth

Growth chamber tests

Corn seeds, variety ICA-109, were disinfected in a 2.5% NaClO solution for 1 min; then, they were rinsed 5 times with sterile distilled water. Subsequently, they were placed in a humidity chamber, and after 4 d of germination they were transferred to pots with 300 g of soil obtained from a semi-arid area in the Alta Guajira (municipality of Uribia: 11°35'38.8" N and 072°19'27.9" W). The soil characteristics are presented in table 1.

Table 1. Main characteristics of the soil used for growth chamber and field tests.

Corn crop	Texture				pH	OM	Ca	Mg	K	Na	CEC	P	S	Fe	Mn	Cu	Zn	B	N		
	Sand %	Silt %	Clay %	Class		%	cmol _c kg ⁻¹					mg kg ⁻¹									%
	78	18	4	AF		7.9	0.84	7.4	1.3	6.7	0.12	9.5	127	9	7	3	1	2	1	0.06	

OM: Organic matter

Six treatments were applied under a completely randomized experiment: HAC, HAVC, *Bacillus mycooides* BSC25 (Bm) (Valero *et al.* 2014) HAC+Bm, HAVC+Bm, and control. Each treatment had five replicates. The treatments were applied at two times: 1) at planting time seedlings, in the pots; 2) 15 d after the first application. The applied Bm doses were 10 mL of bacterial suspension at 1·10⁸ CFU concentration applied directly to the root. For the HA treatment, 5 mL doses were applied by foliar spray at a concentration of 100 mg C L⁻¹, in 2 mM CaCl₂ solution.

The plants were kept in a growth chamber under the following conditions: photoperiod 12 h, relative humidity near to 60-70%, and temperature between 28 and 30°C. Irrigation was done with sterile distilled water every 2 d. After 28 d, the plants were harvested, taking care not to damage the roots. Plants were washed and dried for 72 h at 65°C; then, the dry weight was determined; the root and shoot were weighed separately.

Field test

The seeds were disinfected and germinated in a humidity chamber as explained above. This experiment was carried out under a completely randomized design. For this, rows of 12×1 m were built. The plants were separated 80 cm from each other. The assay consisted of 6 treatments: HAC, HAVC, Bm, HAC+Bm, HAVC+Bm, control (distilled water). Three replicates per treatment were evaluated, each one consisting of a group of 8 plants. Sowing and other crop activities were carried out according to the tradition of local producers, without agrochemical application.

The crop beds were prepared and the soil was tilled, the seeds were placed in germinators with forest soil saturated with water. One week after seedlings emergence, a 4 mL dose of HAC or HAVC solution at 100 mg C L⁻¹ was applied to the plants according to each treatment. After 12 d, eight plants were transplanted

from the germinators to the soil; 2 d later, a second 30 mL dose of the HA solution, or 30 mL of Bm suspension were applied to the corresponding treatments. When flowering began, a last dose of each treatment was applied. It was monitored for 82 d, observing developmental variables such as number of fruits per plant. Eighty-two days later planting, two plants were harvested randomly from each repetition of each treatment, carefully avoiding damaging the roots. They were washed carefully; then, dry weight of roots and shoot were determined.

Statistical analysis

All data were analyzed by ANOVA. Mean comparisons were made with the Dunnett test using the R Studio package v 2022.12.0+353. Multivariate analysis of the association between bioactivity and supra-molecular characteristics of HA was performed by the PAST package v 4.

RESULTS AND DISCUSSION

Extraction and characteristics of HA

The extraction yield of total humic extract (THE) was 44.68±7.3% for coal and 32.31±7.5% for vermicompost; however, the percentage of the fraction of HA that can be separated from the THE of the two raw materials is similar: 7.39±1.1 and 6.26±0.96% for coal and compost, respectively.

This result can be explained because coal undergoes a geochemical process of transformation of organic matter from plant debris. It results in a highly condensed material (Giannouli *et al.*, 2009), where most of the original plant biomass was transformed to constitute the mobile fraction (humic substances) or the fixed fraction (carbonaceous matrix) without residues of other types of molecules originally present in the plant from which the carbonification process

took place. The compost, in turn, is obtained by a plant debris biological transformation process, not geochemical. This process occurs in a very short time scale, giving rise to humic substances by condensation and microbial polymerization of organic matter. Thus, together with the humified organic matter, another type of molecule is generated and is the result of the partial transformation of the original plant materials (Canellas *et al.*, 2010).

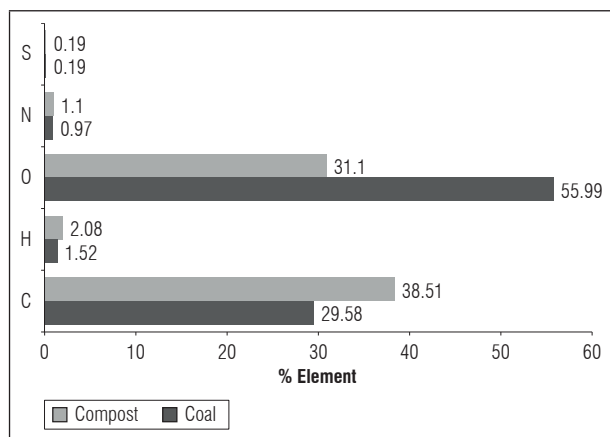


Figure 1. Elemental composition (C, H, O, N, S) of HA obtained from vermicompost and low rank coal.

Figure 1 shows the elemental composition of the two types of HA —both have low C and N content—, the differences are mainly due to a high oxygen content in the HAC and a higher carbon content in the HAVC. The higher oxygen content in the HACs is an outstanding feature that could indicate a higher content of carbonyl, carboxyl, and hydroxyl functional groups. It could confer greater reactivity to the HA supramolecule, as previously explained (Muscolo *et al.*, 2007a; Muscolo, *et al.*, 2013; Scaglia *et al.*, 2016; Fischer 2017). VCHA had a higher carbon content, which can be explained by a higher content of aliphatic structures and carbohydrates (Nebbioso and Piccolo, 2011), as evidenced in the ^{13}C -CP-TOSS (^1H)-NMR analysis (Fig. 2).

The semi-quantitative determination of the 6 types of major functional groups detected by ^{13}C -CP-TOSS(^1H)-NMR analysis (Fig. 2) revealed that the two types of HA have a high predominance of aromatic fractions; however, HAC presents a higher value. This result is consistent with the geochemical process of coalification that increases the degree of aromatic condensation (Ritchie and Perdue, 2008; García *et al.*, 2016b). Aliphatic carbon is the following fraction in relative abundance; HAVC exhibit the

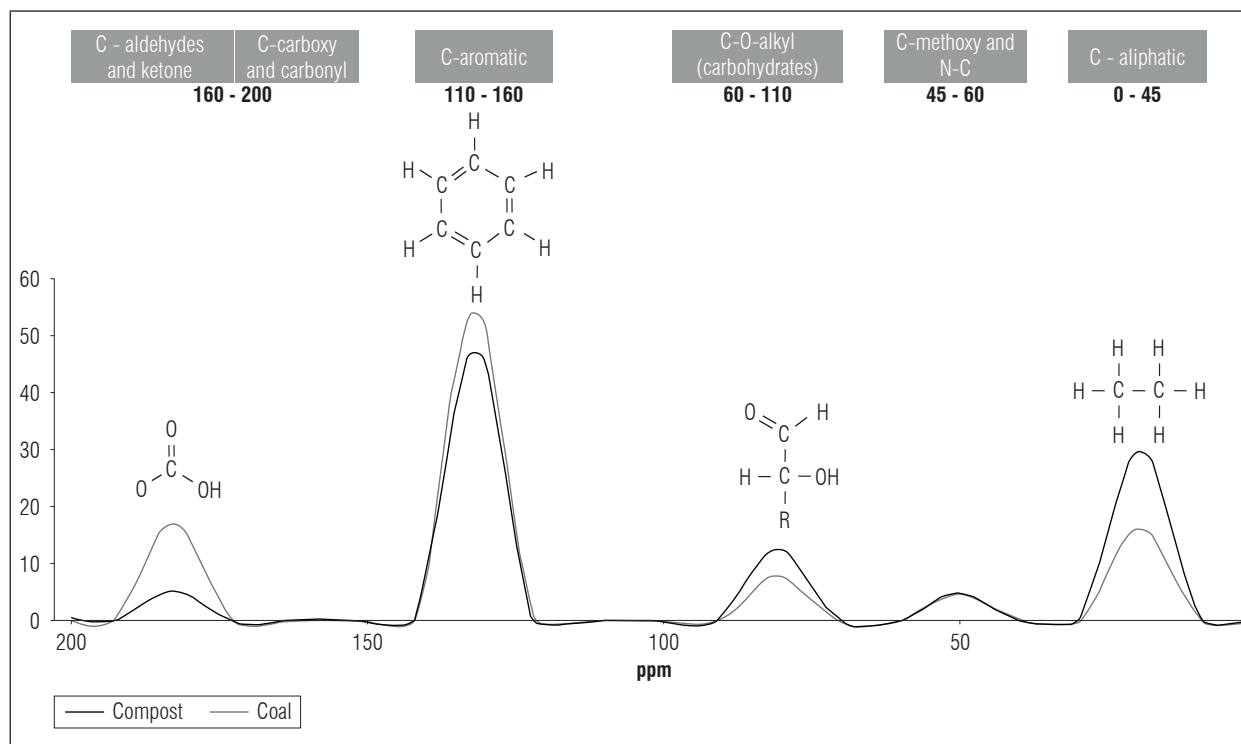


Figure 2. ^{13}C -CP-TOSS-NMR spectra of HA obtained from vermicompost and low rank coal.

highest content. This may be related to abundant carbohydrate residues due to the predominant plant residues in the manure used as raw material to obtain the vermicompost (Cavagnaro, 2014; García *et al.*, 2016b). Carboxylic and carbonylic carbon are almost twice as high in HACs, which is related to the high oxygen content observed in the elemental analysis. Similarly, the carbon is much higher in aldehydes and ketones, while the carbon in polysaccharides, alcohols and amino sugars is higher in HAVC. The carbon in methoxy and N-alkyl groups has similar values in both types of HA, which corresponds to similar values of the nitrogen content in them (Fig. 2).

From the above, we can infer a more condensed character together with the value of the aromaticity index, with high content of oxygen functional groups in HACs, and a more aliphatic character in HAVCs with presence of simpler polysaccharides. The high carboxylic content in HACs and the aliphatic character in HAVCs have been previously related to their degree of bioactivity (Valero *et al.*, 2018a). The molecular characteristics described show resonance patterns like those observed in other HAs with high bioactivity expressed in the stimulation of H⁺ATPase pumps and the proliferation of secondary roots (Canellas *et al.*, 2002; 2010; García *et al.*, 2016a). This effect has been associated with well-defined resonance signals around 56 (C-methoxy and N-C), 125-150 (aromatic), and 175 ppm (C-aldehydes, ketones and carboxy) (Aguar *et al.*, 2013; García *et al.*, 2019).

Figure 3 shows the spectra generated by the two types of HA by FTIR analysis. In general, their profile agrees with the characteristics of the functional groups typical of the humified organic matter

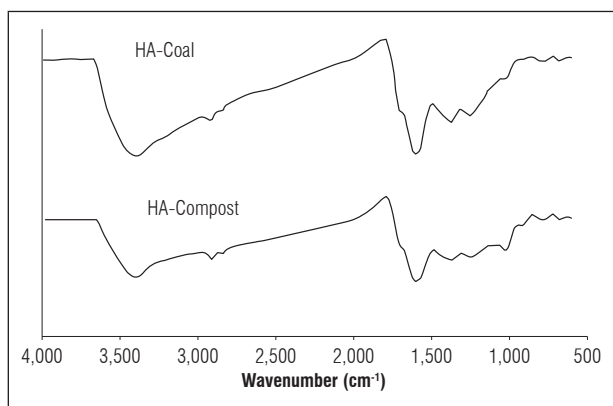


Figure 3. FTIR spectra of HA obtained from of HA obtained from vermicompost and low rank coal.

(Canellas *et al.*, 2010; Sharma *et al.*, 2016). It reflects a consistent quality parameter described by the presence of a broad band around 3,400 cm⁻¹, characteristic of OH groups in alcohols, free phenols, and carboxylic groups; a subtle narrowing band around 2,920 cm⁻¹; and a bending band towards 1,380 cm⁻¹. Together, these correspond to the C-H bond in aliphatic groups; a narrowing band corresponding to the C=O bond around 1,700 cm⁻¹; a narrowing band at 1,717 cm⁻¹ corresponding to aromatic rings; and a bending band corresponding to OH or to the C=O narrowing around 1,280 cm⁻¹ (Valero *et al.*, 2018b). The only appreciable difference between the two types of HA corresponds to two bands of moderate intensity around 1,100 cm⁻¹ in the HAC spectrum that can be given by the presence of alkyl C-O groups and indicate that these are richer in carbohydrate fractions (Valero *et al.*, 2018b).




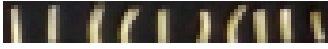


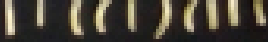
Humic acid bioactivity

Bioactivity refers to some hormonal effects of HA on plants, which activate biochemical mechanisms that modulate plant growth (Nardi, *et al.*, 2021). These mechanisms have not yet been fully elucidated (García *et al.*, 2019); however, they have been partially characterized through morphological, histological, biochemical, and transcriptomic approaches. Auxin-like effect is the hormonal mechanism of humified organic matter with more available evidence (Muscolo *et al.*, 1999; Canellas *et al.*, 2002; Nardi *et al.*, 2002; Muscolo *et al.*, 2007b; Trevisan *et al.*, 2010; Canellas *et al.*, 2011; Canellas and Olivares, 2014; Mora *et al.*, 2014; Nardi *et al.*, 2018; Canellas *et al.*, 2020).

Table 2 presents the effect of the application of both types of HA on the coleoptiles corn elongation. A bioactivity pattern is observed according to the origin of the samples, it is also associated with the structure of the HA. HAC exhibited a coleoptile elongation effect at low concentrations (25-50 mg L⁻¹), while HAVCs exhibited coleoptile elongation ability at higher concentrations (100-200 mg L⁻¹).

HACs exhibited higher bioactivity, since lower concentrations were required to cause elongation of the coleoptiles. ¹³C-CP-TOSS-NMR and FTIR analyses indicate that these HAs have a higher content of aromatic carbon, carboxylic, and carbonyls. This is consistent with the elemental composition analysis that showed a higher oxygen content (Fig. 1) and indicates greater bioactivity (Muscolo *et al.*, 2020). Some polar HA small fractions occluded in hydrophobic

Table 2. HA bioactivity by the coleoptile elongation test.

HA source	HA concentration (mgCL)	Average n = 10	P value (Dunnett test)		
			0***	0.001**	0.01*
Low rank coal (lignite)	Control	6.94			
	10	8.06	0.93		
	25	10.18	0.01*		
	50	10.03	0.02*		
	75	8.93	0.38		
	100	9.26	0.21		
	150	9.38	0.17		
	200	9.39	0.16		
Vermicompost	Control	8.1			
	10	7.96	1.00		
	25	8.69	0.97		
	50	9.52	0.40		
	75	9.80	0.23		
	100	11.30	0.002**		
	150	12.00	<0.001***		
	200	12.02	<0.001***		

polyaromatic domains can disaggregate themselves from their suprastructure, cross some plant tissues, interact with the cell membrane through hydrogen bonds, and enter the cell (García *et al.*, 2019). Both the intracellular access of HA fractions, as well as adsorption interactions with the membrane, trigger hormonal effects in plant tissues (Ojwang' and Cook 2013; García *et al.*, 2014). The presence of IAA and other molecules with auxin-like activity within the humic suprastructure has been widely reported (Canellas *et al.*, 2002; Valero *et al.*, 2018b; Pizzeghello *et al.*, 2020).

The highest concentrations of HAVC were found to stimulate the elongation of the coleoptiles. García *et al.* (2019) suggested that HAs rich in aromatic and aliphatic (recalcitrant) structures require higher concentrations for effective plant growth stimulation. Despite HAC having a slightly higher aromatic content, HAVCs exhibited a significantly higher aliphatic content (Fig. 2). This observation is consistent with the overall higher total carbon content found in HAVC. Notably, these results deviate from the

typical behavior of this type of HA (Ait Baddi *et al.*, 2003), possibly due to the high lignin content of the composted feedstock.

The vermicompost came from goat manure and the tropical dry forest litter in the middle Guajira, this includes woody tree species such as *Aspidosperma polyneuron*, *Platymiscium pinnatum*, *Tabebuia billbergii*, among others (Gualdrón, 2011). Moreover, the diet of the goats in this area is mainly based on low-growing semi-woody plant species such as *Tabebuia billbergii*, *Mimosa arenosa*, and *Bignoniaceae* (Gualdrón, 2011). Therefore, the advanced stage of lignification in the composted raw material is a plausible explanation for the high aromatic content of HA.

Principal component analyses (Fig. 4 and 5) make it possible to better explain these inferences. The bioactivity of HACs, expressed as coleoptile elongation, is observed in low concentrations (10-25 mg C L⁻¹) and is associated with the aromatic, carboxylic, carbonyl, and oxygen content in their supramolecular structure. In contrast, HAVC bioactivity is expressed

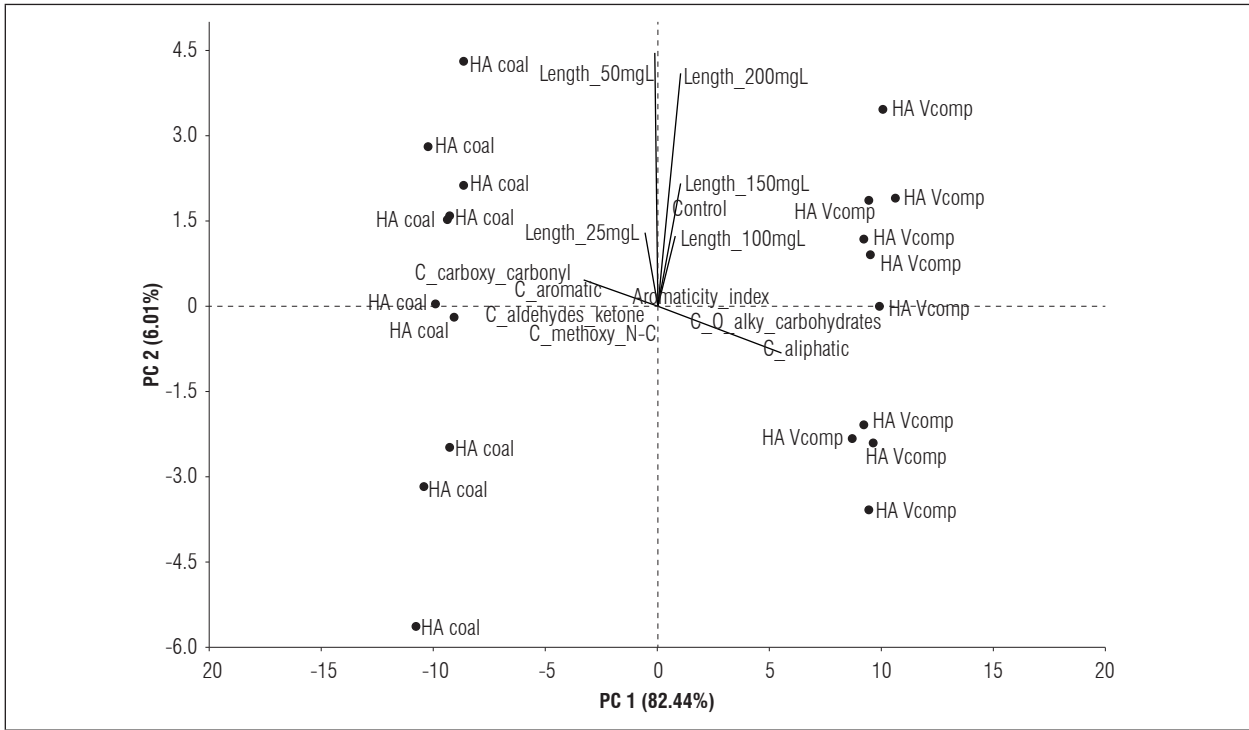


Figure 4. PCA showing the relationship between the data resulting from the quantification of carbon types based on the ¹³C-CP/MAS-NMR spectrum of HAC and HAVC, and the bioactivity (elongation coleoptile test).

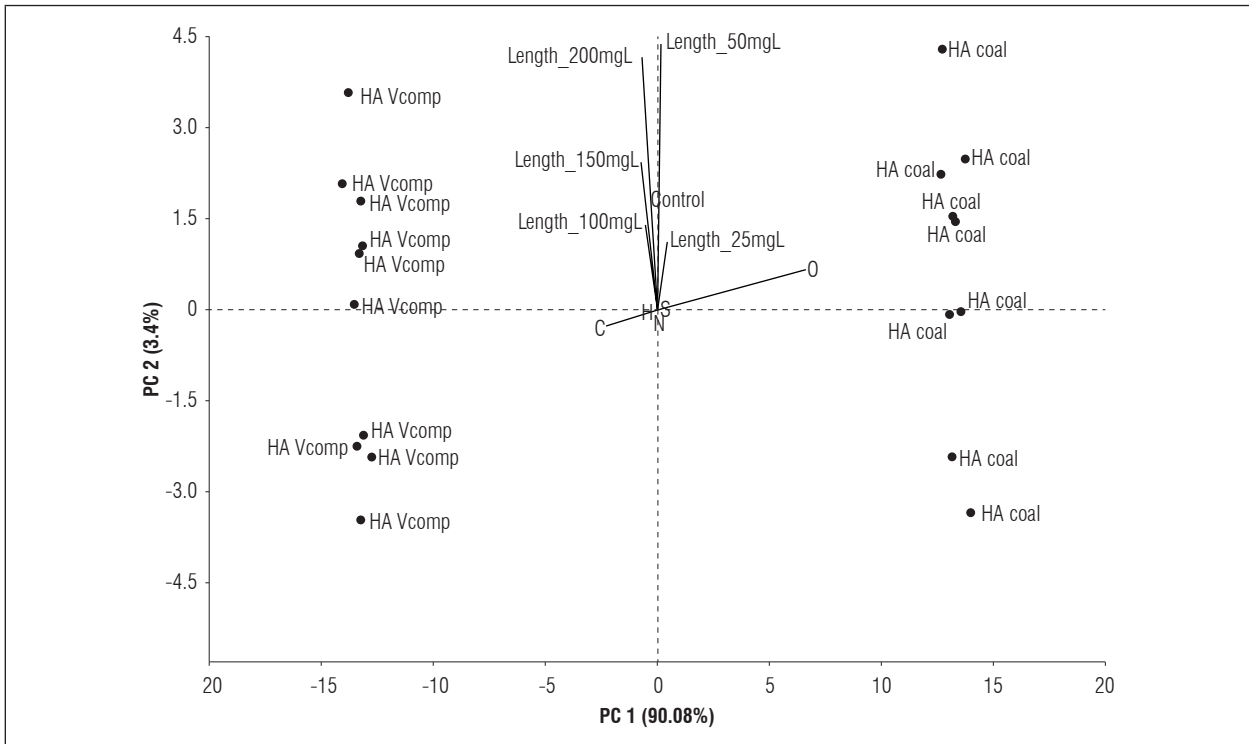


Figure 5. PCA showing the relationship between the data resulting from the elemental analysis of HAC and HAVC, and the bioactivity (elongation coleoptile test).

at high concentrations (100-200 mg C L⁻¹) and can be explained by its content of aliphatic carbon, alkyl, and carbohydrates.

These bioactivity patterns associated with chemical characteristics of HAs, as well as the origin of the samples, are consistent with previous reports (García *et al.*, 2016a, b; García *et al.*, 2018; García *et al.*, 2019; Araújo *et al.*, 2021). However, it is unlikely that fixed and predictive molecular patterns of bioactivity can be defined; on one hand because the bioactivity mechanisms of HA have not been fully elucidated (García *et al.*, 2019; Nardi *et al.*, 2021); on the other hand, the high variability in the sources of HAs makes it difficult to predict their bioactivity. In this case, the type of composted organic matter gave rise to HAs particularly rich in aromatic and aliphatic carbon.

Effect of HAC/HAVC application and inoculation of *B. mycooides* BSC25 on maize plants growth

Figure 6 shows the HAC and HACV application and inoculation with *B. mycooides* BSC25 results under growth chamber and field conditions. The treatments led to different results in foliar biomass and root biomass.

In the growth chamber assay, all treatments except HAC induced increases in foliar biomass. The contact type of HAs with plant tissues is different depending on whether they are applied at the shoot or root level. At the foliar level, the entry of small molecules derived from the humic superstructure is unlikely; therefore, bioactivity will depend on the contact of the molecules' surface with the cells of the leaf tissues (García *et al.*, 2019; Nardi *et al.*, 2021). So, it is reasonable to assume that HA with high content of alkyl carbon and carbohydrates—which are more reactive (García *et al.*, 2016b; Nardi *et al.*, 2021)—better stimulate the biomass of the leaves when applied by leaf spraying. This bioactivity type may be attributed to several types of like-hormone mechanisms, such as auxins (Canellas *et al.*, 2002; Trevisan *et al.*, 2010; Canellas *et al.*, 2011; Nardi *et al.*, 2018; Canellas *et al.*, 2020), cytokinins (Pizzeghello *et al.*, 2013; Mora *et al.*, 2014), abscisic acid-ABA (Olaetxea *et al.*, 2019; Canellas *et al.*, 2020), alkamydes (Zandonadi *et al.*, 2019), NO (Mora *et al.*, 2014), gibberellic acid (de Sanfilippo *et al.*, 1990; Nardi *et al.*, 2002; Canellas *et al.*, 2020), and biotic and abiotic stress response enzymes involved in the homeostasis of reactive oxygen species (catalases, superoxide dismutase, and peroxidases)

(García, *et al.*, 2016a; García *et al.*, 2019; Canellas *et al.*, 2020).

On the other hand, in this same test (growth chamber), all treatments except HAVC showed increases in root biomass. Although the application of HA was done in a foliar manner, root stimulation was also observed, as has been previously described (Canellas and Olivares, 2014; Canellas *et al.*, 2020). This occurs because HA stimulation at the foliar level also triggers signaling and transcriptional responses involving root development such as auxin response factors (ARF) (Elmongy *et al.*, 2020), ABCB polar auxin transporters (Tahiri *et al.*, 2016), calcium-dependent membrane receptors (Canellas *et al.*, 2020), small auxin up-regulated RNA (SAUR) genes (Canellas *et al.*, 2020), and H⁺ATPase. This evidence suggests that HA stimulation at the root level is primarily due to auxin-like effects (Elmongy *et al.*, 2020; Araújo *et al.*, 2021). This is consistent with the results obtained in this work (Tab. 2); the HAC sample, which caused increases in root biomass, was rich in oxygen, carboxylic, and aromatic groups (Fig. 4 and 5). These chemical characteristics have been associated with auxin-like effects (García *et al.*, 2016a; García *et al.*, 2019).

In the growth chamber, Bm treatment exhibited PGPR activity and showed increases in shoot and root biomass. Previous work had provided evidence of PGPR traits associated with this strain, including biological nitrogen fixation, inorganic phosphorus solubilization, and IAA production (Brito-Campo *et al.*, 2022). Under these conditions, the PGPR activity of Bm was not affected by co-treatment with HA. This could be explained by three possible factors: 1) the possible PGPR mechanisms of Bm are not necessarily biostimulation; therefore, they do not affect signaling pathways that affect the plant hormonal balance. 2) Bm's IAA production is low ($\mu\text{g}\cdot\text{mL}^{-1}$) compared to other PGPR strains (Brito-Campo *et al.*, 2022); therefore, the HA combination might not represent an additive effect that increases auxin-like activity to a deleterious level to the plant (Pantoja-Guerra *et al.*, 2023). 3) The used plant model (corn) is a monocotyledonous plant, usually resistant to exogenous auxin activity (McSteen, 2010; Nelissen *et al.*, 2016; Conklin *et al.*, 2019); thus, in the presence of a bacterial strain with low IAA production, higher doses are likely required to express an additive effect that reverses the PGPR activity of the strain.

In field experiments, the shoot biomass results were similar to those obtained under growth chamber

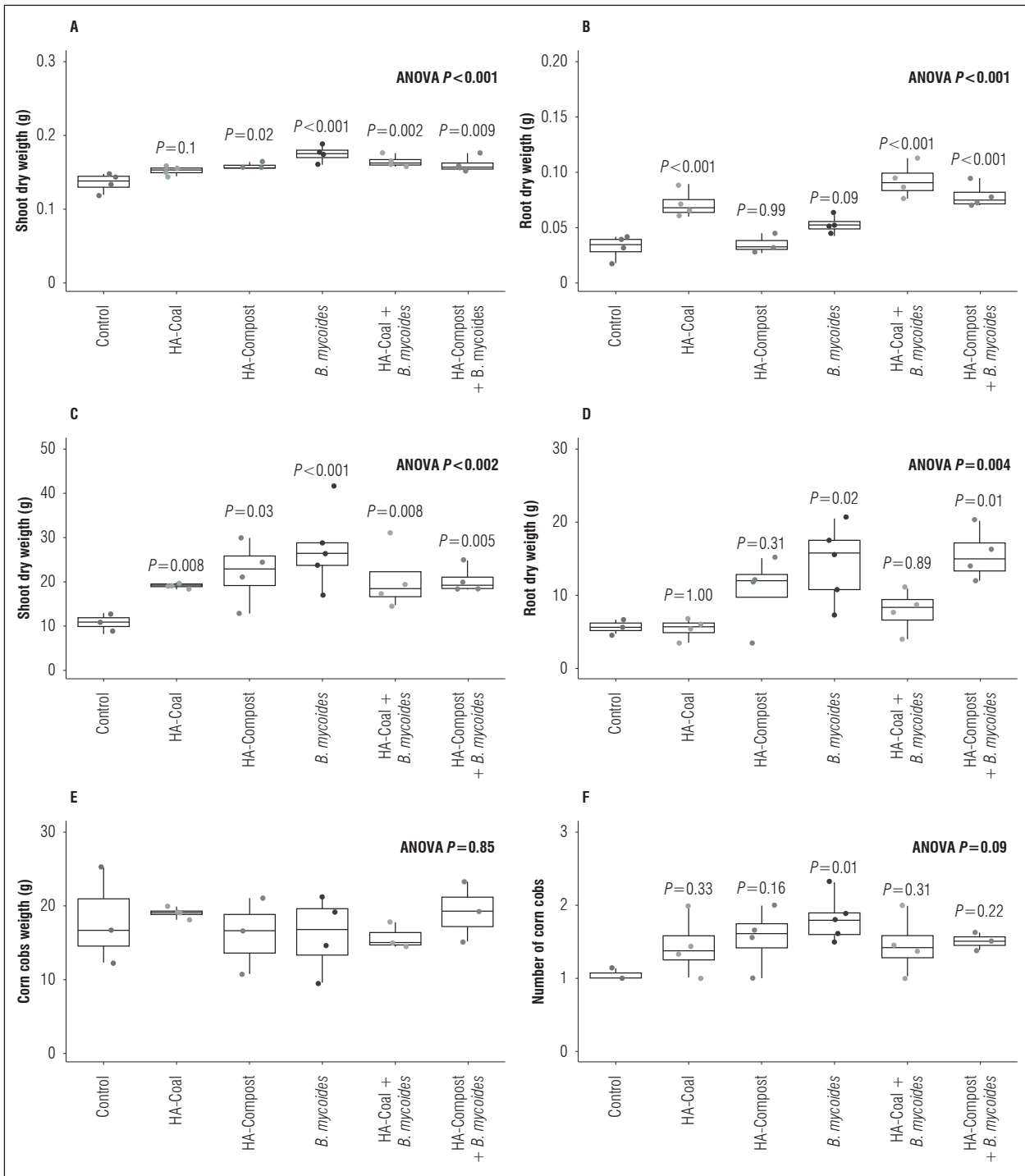


Figure 6. Effect of applying HAs and inoculating *B. mycooides* BSC25 on maize plants, in the growth chamber (A and B) and in the field (C, D, E and F). Dunett test on boxplots.

conditions. All treatments promoted foliar biomass accumulation. As previously explained, this result is attributed to the hormone-like effects of HA and the PGPR activity of Bm. However, in this test, the HAC

treatment did induce increases in shoot biomass. It should be noted that although the concentration of HAs evaluated in the field was the same than that in the growth chamber, the doses (sprayed volumes)

were higher in the field. We speculate that an increase in the dose generated a “compensating effect” that allowed the aromatic and carboxyl groups —associated with reactivity in the HAs from coal (García *et al.*, 2016a) to be sufficient to express bioactivity.

The root biomass production results in the field test indicate a PGPR effect of Bm, and it was not affected by the co-application of HAVC. These HAs did not promote the development of roots by themselves, neither under growth chamber nor in field conditions. Supramolecular analysis indicates that these HAs have a lower content of carboxylic and aromatic groups associated with low auxin-like activity (García *et al.*, 2016a; Araújo *et al.*, 2021). This is consistent with the result of the coleoptile elongation test, in which HAVC bioactivity was only observed at the highest concentrations. Therefore, it is reasonable to infer that the combination of Bm with these HAs is not negative for root production in corn, because the “sum of auxin-like effects” is not deleterious under these conditions (Pantoja-Guerra *et al.*, 2023).

Under field conditions, Bm had a positive effect on the number of cobs per plant. However, this effect was reversed by the co-application of HAC and HAVC, thus suggesting that biostimulation caused by both types of HA has a possible additive effect that affects Bm’s PGPR activity. This could be associated with several HA-related hormonal effects.

CONCLUSION

Bioactivity patterns associated with the structural differences of humic acids were observed. HAC presented higher oxygen content, while HAVC presented higher carbon content. On the other hand, the ¹³C-CP-TOSS-NMR and FTIR analyses show spectral patterns for each sample type, which indicates differences in the composition of functional groups. HAC presented higher aliphatic and aromatic content, while HAVC presented higher aromatics and carboxylic groups content.

Supramolecular composition patterns were correlated with the auxin-like effect of both HA types. The coleoptile elongation test showed differential bioactivity patterns according to the dose. This correlation suggests a possible causal association between HA bioactivity and their supramolecular configuration, as has been reported by other researchers.

Regarding the effect in-planta (growth chamber and field conditions), the HA bioactivity modulated the PGPR effect of the *Bacillus mycooides* BSC25 strain depending on the sample origin. These results suggest that HA bioactivity should be estimated for the joint application of AH and PGPR strains.

Conflict of interests. The manuscript was prepared and reviewed with the participation of the authors, who declare that there exists no conflict of interest that puts at risk the validity of the presented results.

Acknowledgements and Funding: This work is part of the results of the project “Evaluación de ácidos húmicos obtenidos a partir de dos materias primas locales, como bioestimulantes para plantas de interés agrícola en La Guajira (Colombia)”, financed by the University of La Guajira in conjunction with the support of the Ministry of Science and Technology-Colombia (Grant number 757 of 2016-Colciencias National Doctorates (Researcher granted: Manuel Pantoja-Guerra).

Author contributions

NV: Original idea of the research, structure of the manuscript, determination of chemical nature of HA, development of biostimulation assays in the laboratory and field. YU: Participation and follow-up in the development of tests in laboratory and field conditions. MP-G: Evaluation of humic acid bioactivity in plant tissue, statistical analysis, Participation in the structure and preparation of the manuscript.

BIBLIOGRAPHIC REFERENCES

- Aguiar, N.O., E.H. Novotny, A.L. Oliveira, V.M. Rumjanek, F.L. Olivares, and L.P. Canellas. 2013. Prediction of humic acids bioactivity using spectroscopy and multivariate analysis. *J. Geochem. Explor.* 129, 95-102. Doi: <https://doi.org/10.1016/j.gexplo.2012.10.005>
- Aguiar, N.O., F.L. Olivares, E.H. Novotny, and L.P. Canellas. 2018. Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids. *PeerJ* 6, e5445. Doi: <https://doi.org/10.7717/peerj.5445>
- Ahmad, P., S. Rasool, A. Gul, S.A. Sheikh, N.A. Akram, M. Ashraf, A.M. Kazi, and S. Guzel. 2016. Jasmonates: multifunctional roles in stress tolerance. *Front. Plant Sci.* 7, 813. Doi: <https://doi.org/10.3389/fpls.2016.00813>

- Ait Baddi, G., M. Hafidi, V. Gilard, and J.-C. Revel. 2003. Characterization of humic acids produced during composting of olive mill wastes: elemental and spectroscopic analyses (FTIR and ^{13}C -NMR). *Agronomie* 23(7), 661-666. Doi: <https://doi.org/10.1051/agro:2003042>
- Araújo, K.V., M. Pittarello, P. Carletti, A.R.M. Campos, and L.B. Dobbss. 2021. Structural characterization and bioactivity of humic and fulvic acids extracted from preserved and degraded Brazilian Cerrado Biomes Soils. *Eurasian Soil Sci.* 54(1), 16-25. Doi: <https://doi.org/10.1134/S1064229322030024>
- Bacilio, M., M. Moreno, D.R. Lopez-Aguilar, and Y. Bashan, 2017. Scaling from the growth chamber to the greenhouse to the field: demonstration of diminishing effects of mitigation of salinity in peppers inoculated with plant growth-promoting bacterium and humic acids. *Appl. Soil Ecol.* 119, 327-338. Doi: <https://doi.org/10.1016/j.apsoil.2017.07.002>
- Bashan, Y., B.G. Salazar, M. Moreno, B.R. Lopez, and R.G. Linderman. 2012. Restoration of eroded soil in the Sonoran Desert with native leguminous trees using plant growth-promoting microorganisms and limited amounts of compost and water. *J. Environ. Manage.* 102, 26-36. Doi: <https://doi.org/10.1016/j.jenvman.2011.12.032>
- Beleño-Carrillo, J., L. Gómez-Gómez, and N.O. Valero-Valero. 2022. *Bacillus mycoides* y ácidos húmicos como bioestimulantes de fríjol caupí bajo estrés por salinidad. *Rev. U.D.C.A Act. Div. Cient.* 25(2), e1974. Doi: <https://doi.org/10.31910/rudca.v25.n2.2022.1974>
- Blouin, M. 2018. Chemical communication: An evidence for co-evolution between plants and soil organisms. *Appl. Soil Ecol.* 123, 409-415. Doi: <https://doi.org/10.1016/j.apsoil.2017.10.028>
- Bratkova, S., K. Nikolova, P. Genova, and A. Angelov. 2021. Use of plant growth-promoting bacteria and humic acids for phytostabilization of acid-generating mining wastes. *Res Sq. Preprint v1*. Doi: <https://doi.org/10.21203/rs.3.rs-550365/v1>
- Brito-Campo, H.L., M.F. Ayala-Santamaría, K.J. Barros-Escalante, J.G. Cubillos-Hinojosa, M.F. Pantoja-Guerra, N.O. Valero, and L. Gómez. 2022. PGPR activity of coal solubilizing bacteria. *Rev. Fac. Agron.(LUZ)* 39(2), e223932. Doi: [https://doi.org/10.47280/Rev-FacAgron\(LUZ\).v39.n2.10](https://doi.org/10.47280/Rev-FacAgron(LUZ).v39.n2.10)
- Bulgari, R., G. Franzoni, and A. Ferrante. 2019. Biostimulants application in horticultural crops under abiotic stress conditions. *Agronomy* 9(6), 306. Doi: <https://doi.org/10.3390/agronomy9060306>
- Canellas, L.P., N.O.A. Canellas, L.E.S.S. Irineu, F.L. Olivares, and A. Piccolo. 2020. Plant chemical priming by humic acids. *Chem. Biol. Technol. Agric.* 7(1), 12. Doi: <https://doi.org/10.1186/s40538-020-00178-4>
- Canellas, L.P., D.J. Dantas, N.O. Aguiar, L.E.P. Peres, A. Zsögön, F.L. Olivares, L.B. Dobbss, A.R. Façanha, A. Nebibioso, and A. Piccolo. 2011. Probing the hormonal activity of fractionated molecular humic components in tomato auxin mutants. *Ann. Appl. Biol.* 159(2), 202-211. Doi: <https://doi.org/10.1111/j.1744-7348.2011.00487.x>
- Canellas, L.P., A. Piccolo, L.B. Dobbss, R. Spaccini, F.L. Olivares, D.B. Zandonadi, and A.R. Façanha. 2010. Chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid. *Chemosphere* 78(4), 457-466. Doi: <https://doi.org/10.1016/j.chemosphere.2009.10.018>
- Canellas, L.P. and F.L. Olivares. 2014. Physiological responses to humic substances as plant growth promoter. *Chem. Biol. Technol. Agric.* 1(1), 3. Doi: <https://doi.org/10.1186/2196-5641-1-3>
- Canellas, L.P., F.L. Olivares, N.O.A. Canellas, P. Mazzei, and A. Piccolo. 2019. Humic acids increase the maize seedlings exudation yield. *Chem. Biol. Technol. Agric.* 6(1), 3. Doi: <https://doi.org/10.1186/s40538-018-0139-7>
- Canellas, L.P., F.L. Olivares, A.L. Okorokova-Façanha, and A.R. Façanha. 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+ -ATPase activity in maize roots. *Plant Physiol.* 130(4), 1951-1957. Doi: <https://doi.org/10.1104/pp.007088>
- Cavagnaro, T.R. 2014. Impacts of compost application on the formation and functioning of arbuscular mycorrhizas. *Soil Biol. Biochem.* 78, 38-44. Doi: <https://doi.org/10.1016/j.soilbio.2014.07.007>
- Chang, R.R., R. Mylotte, M.H.B. Hayes, R. McInerney, and Y.M. Tzou. 2014. A comparison of the compositional differences between humic fractions isolated by the IHSS and exhaustive extraction procedures. *Naturwissenschaften* 101(3), 197-209. Doi: <https://doi.org/10.1007/s00114-013-1140-4>
- Conklin, P.A., J. Strable, S. Li, and M.J. Scanlon. 2019. On the mechanisms of development in monocot and eudicot leaves. *New Phytol.* 221(2), 706-724. Doi: <https://doi.org/10.1111/nph.15371>
- Da Piedade Melo, A., F.L. Olivares, L.O. Médiçi, A. Torres-Neto, L.B. Dobbss, and L.P. Canellas. 2017. Mixed rhizobia and *Herbaspirillum seropedicae* inoculations with humic acid-like substances improve water-stress recovery in common beans. *Chem. Biol. Technol. Agric.* 4(1), 6. Doi: <https://doi.org/10.1186/s40538-017-0090-z>
- De Sanfilippo, E.C., J.A. Argüello, G. Abdala, and G.A. Orioli. 1990. Content of auxin-inhibitor-and gibberellin-like substances in humic acids. *Biol. Plant.* 32(5), 346-351. Doi: <https://doi.org/10.1007/BF02898497>
- Du Jardin, P. 2015. Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3-14. Doi: <https://doi.org/10.1016/j.scienta.2015.09.021>
- Elmongy, M.S., X. Wang, H. Zhou, and Y. Xia. 2020. Humic acid and auxins induced metabolic changes and

- differential gene expression during adventitious root development in azalea microshoots. *HortScience* 55(6), 926-935. Doi: <https://doi.org/10.21273/HORTSCI14885-20>
- FAO. 2022. Agricultura de Conservación. In: <https://www.fao.org/3/cb8350es/cb8350es.pdf>; consulted: February, 2023.
- Fischer, T. 2017. Humic supramolecular structures have polar surfaces and unpolar cores in native soil. *Chemosphere* 183, 437-443. Doi: <https://doi.org/10.1016/j.chemosphere.2017.05.125>
- García A.C., L.G. Ambrosio de Souza, M.G. Pereira, R.N. Castro, J.M. García-Mina, E. Zonta, F.J.G. Lisboa, and R.L.L. Berbara. 2016a. Structure-property-function relationship in humic substances to explain the biological activity in plants. *Sci. Rep.* 6(1), 20798. Doi: <https://doi.org/10.1038/srep20798>
- García, A.C., L. Azevedo, F. Guridi, V.M. Rumjanek, R.N. Castro, F.S. Santos, L.G.A. Souza, and R.L.L. Berbara. 2014. Potentialities of vermicompost humic acids to alleviate water stress in rice plants (*Oryza sativa* L.). *J. Geochem. Explor.* 136, 48-54. Doi: <https://doi.org/10.1016/j.gexplo.2013.10.005>
- García, A.C., L. Azevedo, L.G.A. Souza, O.C. Huertas, A. Zonta, E.T. Martins, J.M. García-Mina, and R.L.L. Berbara. 2016b. Vermicompost humic acids modulate the accumulation and metabolism of ROS in rice plants. *J. Plant Physiol.* 192, 56-63. Doi: <https://doi.org/10.1016/j.jplph.2016.01.008>
- García, A.C., O.C. Huertas, D. Martínez, V.S. Almeida, L.P. Canellas, J.M. García-Mina, and R.L.L. Berbara. 2018. Structure-function relationship of vermicompost humic fractions for use in agriculture. *J. Soils Sediments* 18(4), 1365-1375. Doi: <https://doi.org/10.1007/s11368-016-1521-3>
- García, A.C., T.A. Van Tol de Castro, L.A. Santos, O.C.H. Tavares, R.N. Castro, R.C.L. Berbara, and J.M. García-Mina. 2019. Structure-property-function relationship of humic substances in modulating the root growth of plants: a review. *J. Environ. Qual.* 48(6), 1622-1632. Doi: <https://doi.org/10.2134/jeq2019.01.0027>
- Giannouli, A., S. Kalaitzidis, G. Siavalas, A. Chatziapostolou, K. Christanis, S. Papazisimou, C. Papanicolou, and A. Foscolos. 2009. Evaluation of greek low-rank coals as potential raw material for the production of soil amendments and organic fertilizers. *Int. J. Coal Geol.* 77(3-4), 383-393. Doi: <https://doi.org/10.1016/j.coal.2008.07.008>
- Gualdrón, R. 2011. Cerrejón: hacia la rehabilitación de las tierras intervenidas por la minería a cielo abierto. El Cerrejón, Bogota.
- Jindo, K., F.L. Olivares, D.J.P. Malcher, M.A. Sánchez-Monedero, C. Kempenaar, and L.P. Canellas. 2020. From lab to field: role of humic substances under open-field and greenhouse conditions as biostimulant and bio-control agent. *Front. Plant Sci.* 11, 426. Doi: <https://doi.org/10.3389/fpls.2020.00426>
- Li, J., J. Wang, H. Liu, C.A. Macdonald, and B.K. Singh. 2022. Application of microbial inoculants significantly enhances crop productivity: a meta-analysis of studies from 2010 to 2020. *J. Sustain. Agric. Environ.* 1(3), 216-225. Doi: 10.1002/sae2.12028
- McSteen, P. 2010. Auxin and monocot development. *Cold Spring Harb. Perspect. Biol.* 2010(2), a001479. Doi: <https://doi.org/10.1101/cshperspect.a001479>
- Mora, V., E. Bacaicoa, R. Baigorri, A.M. Zamarreño, and J.M. García-Mina. 2014. NO and IAA key regulators in the shoot growth promoting action of humic acid in *Cucumis sativus* L. *J. Plant Growth Regul.* 33(2), 430-439. Doi: <https://doi.org/10.1007/s00344-013-9394-9>
- Muscolo, A., F. Bovalo, F. Gionfriddo, and S. Nardi. 1999. Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. *Soil Biol. Biochem.* 31(9), 1303-1311. Doi: [https://doi.org/10.1016/S0038-0717\(99\)00049-8](https://doi.org/10.1016/S0038-0717(99)00049-8)
- Muscolo, A., D. Pizzeghello, O. Francioso, S. Sanchez, and S. Nardi. 2020. Effectiveness of humic substances and phenolic compounds in regulating plant-biological functionality. *Agronomy* 10(10), 1553. Doi: <https://doi.org/10.3390/agronomy10101553>
- Muscolo, A., M. Sidari, E. Attinà, O. Francioso, V. Tugnoli, and S. Nardi. 2007a. Biological activity of humic substances is related to their chemical structure. *Soil Sci. Soc. Am. J.* 71(1), 75-85. Doi: <https://doi.org/10.2136/sssaj2006.0055>
- Muscolo, A., M. Sidari, O. Francioso, V. Tugnoli, and S. Nardi. 2007b. The auxin-like activity of humic substances is related to membrane interactions in carrot cell cultures. *J. Chem. Ecol.* 33(1), 115-129. Doi: <https://doi.org/10.1007/s10886-006-9206-9>
- Muscolo, A., M. Sidari, and S. Nardi. 2013. Humic substance: relationship between structure and activity. Deeper information suggests univocal findings. *J. Geochem. Explor.* 129, 57-63. Doi: <https://doi.org/10.1016/j.gexplo.2012.10.012>
- Nardi, S., D. Pizzeghello, and A. Ertani. 2018. Hormone-like activity of the soil organic matter. *Appl. Soil Ecol.* 123, 517-520. Doi: <https://doi.org/10.1016/j.apsoil.2017.04.020>
- Nardi, S., D. Pizzeghello, A. Muscolo, and A. Vianello. 2002. Physiological effects of humic substances on higher plants. *Soil Biol. Biochem.* 34(11), 1527-1536. Doi: [https://doi.org/10.1016/S0038-0717\(02\)00174-8](https://doi.org/10.1016/S0038-0717(02)00174-8)
- Nardi, S., D. Pizzeghello, M. Schiavon, and A. Ertani. 2016. Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73(1), 18-23. Doi: <https://doi.org/10.1590/0103-9016-2015-0006>

- Nardi, S., M. Schiavon, and O. Francioso. 2021. Chemical structure and biological activity of humic substances define their role as plant growth promoters. *Molecules* 26(8), 2256. Doi: <https://doi.org/10.3390/molecules26082256>
- Nebbioso, A. and A. Piccolo. 2011. Basis of a humeomics science: chemical fractionation and molecular characterization of humic biosuprastructures. *Biomacromolecules* 12(4), 1187-1199. Doi: <https://doi.org/10.1021/bm101488e>
- Nelissen, H., N. Gonzalez, and D. Inzé. 2016. Leaf growth in dicots and monocots: so different yet so alike. *Curr. Opin. Plant Biol.* 33, 72-76. Doi: <https://doi.org/10.1016/j.pbi.2016.06.009>
- Nunes, R.O., G.A. Domociano, W.S. Alves, A.C.A. Melo, F.C.S. Nogueira, L.P. Canellas, F.L. Olivares, R.B. Zingali, and M.R. Soares. 2019. Evaluation of the effects of humic acids on maize root architecture by label-free proteomics analysis. *Sci. Rep.* 9(1), 12019. Doi: <https://doi.org/10.1038/s41598-019-48509-2>
- Ojwang' L.M. and R.L. Cook. 2013. Environmental conditions that influence the ability of humic acids to induce permeability in model biomembranes. *Environ. Sci. Technol.* 47(15), 8280-8287. Doi: <https://doi.org/10.1021/es4004922>
- Olaetxea, M., V. Mora, E. Bacaicoa, R. Baigorri, M. Garnica, M. Fuentes, A.M. Zamarreño, L. Spíchal, and J.M. García-Mina. 2019. Root ABA and H⁺ATPase are key players in the root and shoot growth-promoting action of humic acids. *Plant Direct* 3(10), e00175. Doi: <https://doi.org/10.1002/pld3.175>
- Pantoja-Guerra, M. and N. Valero-Valero. 2020. Design of a bio-conditioner prototype for the treatment of degraded soils: biomass production and lignite formulation for *Microbacterium* sp. CSB3. *Chem. Biol. Technol. Agric.* 7, 3. Doi: <https://doi.org/10.1186/s40538-019-0167-y>
- Pantoja-Guerra, M., N. Valero-Valero, and C.A. Ramírez. 2023. Total auxin level in the soil-plant system as a modulating factor for the effectiveness of PGPR inocula: a review. *Chem. Biol. Technol. Agric.* 10, 6. Doi: <https://doi.org/10.1186/s40538-022-00370-8>
- Pizzeghello, D., O. Francioso, A. Ertani, A. Muscolo, and S. Nardi. 2013. Isopentenyladenosine and cytokinin-like activity of different humic substances. *J. Geochem. Explor.* 129, 70-75. Doi: <https://doi.org/10.1016/j.gexplo.2012.10.007>
- Pizzeghello, D., M. Schiavon, O. Francioso, F.D. Vecchia, A. Ertani, and S. Nardi. 2020. Bioactivity of size-fractionated and unfractionated humic substances from two forest soils and comparative effects on N and S metabolism, nutrition, and root anatomy of *Allium sativum* L. *Front. Plant Sci.* 11, 1203. Doi: <https://doi.org/10.3389/fpls.2020.01203>
- Reeves, T.G., G. Thomas, and G. Ramsay. 2016. Save and grow in practice: maize, rice, wheat. A guide to sustainable cereal production. FAO, Rome.
- Ritchie, J.D. and E.M. Perdue. 2008. Analytical constraints on acidic functional groups in humic substances. *Org. Geochem.* 39(6), 783-799. Doi: <https://doi.org/10.1016/j.orggeochem.2008.03.003>
- Rojas, D., M. Contreras, and G. Santoyo. 2013. Mecanismos de estimulación del crecimiento vegetal en bacterias del género *Bacillus*. *Biológicas* 15(2), 36-41.
- Roomi, S., A. Masi, G.B. Conselvan, S. Trevisan, S. Quaggiotti, M. Pivato, G. Arrigoni, T. Yasmin, and P. Carletti. 2018. Protein profiling of Arabidopsis roots treated with humic substances: insights into the metabolic and interactome networks. *Front. Plant Sci.* 9, 1812. Doi: <https://doi.org/10.3389/fpls.2018.01812>
- Scaglia, B., R.R. Nunes, M.O.O. Rezende, F. Tambone, and F. Adani. 2016. Investigating organic molecules responsible of auxin-like activity of humic acid fraction extracted from vermicompost. *Sci. Total Environ.* 562, 289-295. Doi: <https://doi.org/10.1016/j.scitotenv.2016.03.212>
- Shah, Z.H., H.F. Rehman, T. Akhtar, H. Alsamadany, B.T. Hamooh, T. Mujtaba, I. Daur, Y.A. Zahrani, H.A.S. Alzahrani, S. Ali, S.H. Yang, and G. Chung. 2018. Humic substances: determining potential molecular regulatory processes in plants. *Front. Plant Sci.* 9, 263. Doi: <https://doi.org/10.3389/fpls.2018.00263>
- Sharma, H.S.S., C. Selby, E. Carmichael, C. McRoberts, J.R. Rao, P. Ambrosio, M. Chiurazzi, M. Pucci, and T. Martin. 2016. Physicochemical analyses of plant biostimulant formulations and characterisation of commercial products by instrumental techniques. *Chem. Biol. Technol. Agric.* 3, 13. Doi: <https://doi.org/10.1186/s40538-016-0064-6>
- Spaccini, R. and A. Piccolo. 2009. Molecular characteristics of humic acids extracted from compost at increasing maturity stages. *Soil Biol. Biochem.* 41(6), 1164-1172. Doi: <https://doi.org/10.1016/j.soilbio.2009.02.026>
- Tahiri, A., A. Richel, J. Destain, P. Druart, P. Thonart, and M. Ongena. 2016. Comprehensive comparison of the chemical and structural characterization of landfill leachate and leonardite humic fractions. *Anal. Bioanal. Chem.* 408(7), 1917-1928. Doi: <https://doi.org/10.1007/s00216-016-9305-6>
- Tejera-Hernández, B., M.M. Rojas-Badia, and M. Heydrich-Pérez. 2011. Potencialidades del género *Bacillus* en la promoción del crecimiento vegetal y el control biológico de hongos fitopatógenos. *Rev. CENIC Cienc. Biol.* 42(3), 131-138.
- Trevisan, S., O. Francioso, S. Quaggiotti, and S. Nardi. 2010. Humic substances biological activity at the plant-soil interface. *Plant Signal. Behav.* 5(6), 635-643. Doi: <https://doi.org/10.4161/psb.5.6.11211>

- Valero, N., L. Gómez, M. Pantoja, and R. Ramírez. 2014. Production of humic substances through coal-solubilizing bacteria. *Brazilian J. Microbiol.* 45(3), 911-918. Doi: <https://doi.org/10.1590/s1517-83822014000300021>
- Valero, N.O., L.C. Gómez, and L.M. Melgarejo. 2018b. Supramolecular characterization of humic acids obtained through the bacterial transformation of a low rank coal. *J. Braz. Chem. Soc.* 29(9), 1842-1853. Doi: <https://doi.org/10.21577/0103-5053.20180060>
- Valero, N., L.M. Melgarejo, and R. Ramírez. 2016. Effect of low-rank coal inoculated with coal solubilizing bacteria on edaphic materials used in post-coal-mining land reclamation: a greenhouse trial. *Chem. Biol. Technol. Agric.* 3, 20. Doi: <https://doi.org/10.1186/s40538-016-0068-2>
- Valero, N., J.A. Salgado, and D. Corzo. 2018a. Metodología sencilla para evaluar bioactividad de ácidos húmicos obtenidos de lignito mediante extracción alcalina y bacterias solubilizadoras de carbón. *Inf. Tecnol.* 29(4), 75-82. Doi: <https://doi.org/10.4067/S0718-07642018000400075>
- Valero-Valero, N.-O., C.M. Vergel-Castro, Y. Ustate, and L.C. Gómez-Gómez. 2021. Bioestimulación de frijol guajiro y su simbiosis con *Rhizobium* por ácidos húmicos y *Bacillus mycooides*. *Rev. Bio. Agro.* 19(2), 119-134. Doi: <https://doi.org/10.18684/bsaa.v19.n2.2021.1608>
- Van Oosten, M.J., O. Pepe, S. De Pascale, S. Silletti, and A. Maggio. 2017. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* 4(1), 5. Doi: <https://doi.org/10.1186/s40538-017-0089-5>
- Zandonadi, D.B., C.R.R. Matos, R.N. Castro, R. Spaccini, F.L. Olivares, and L.P. Canellas. 2019. Alkamides: a new class of plant growth regulators linked to humic acid bioactivity. *Chem. Biol. Technol. Agric.* 6(1), 23. Doi: <https://doi.org/10.1186/s40538-019-0161-4>
- Zhang, D., D. Duan, Y. Huang, Y. Yang, and Y. Ran. 2017. Composition and structure of natural organic matter through advanced nuclear magnetic resonance techniques. *Chem. Biol. Technol. Agric.* 4(1), 8. Doi: <https://doi.org/10.1186/s40538-017-0086-8>