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THE HUMIC ACIDS FROM VERMICOMPOST PROTECT RICE (*Oryza sativa* L.) PLANTS AGAINST A POSTERIOR HIDRIC STRESS

Los ácidos húmicos de vermicompost protegen a plantas de arroz (*Oryza sativa* L.) contra un estrés hídrico posterior

Fernando Guridi Izquierdo[1]✉, Andrés Calderín García[2], Ricardo L. Louro Berbara[2], Dariellys Martínez Balmori[1] and Mayelín Rosquete Bassó[1]

ABSTRACT. The humic acids (HA) from two different vermicompost were extracted, isolated, purified and partially characterized, to evaluate their possible protection in rice (*Oryza sativa* L.) plants against an hydric stress. Differences in elemental composition, as the coagulation threshold value and E_4/E_6 relation in their UV-Vis spectra were found. Two concentrations (40 and 60 mg L^{-1}) of both HA were included in the nutritive solutions for rice plants in controlled conditions. It was verified that the previous treatment with the HA during six days stimulated the root biomass production. Later the HA were excluded and was an hydric deficit induced by adding polietilenglicol (PEG-6000) in the initially treated plants and in a group of those used as control. After 96 hours of this final condition the net radical biomass, the photosynthetic pigments content and the root membrane permeability were evaluated. In the plants previously treated with HA (at the concentration 60 mg HA L^{-1}), the root membrane permeability, the net radical biomass production and the "a" chlorophyll content had no differences when compared with those without stress. It was concluded that the previous treatment with the HA protected the rice plants against a posterior hydric stress that was induced.

Key words: radical biomass, permeability, photosynthetic pigments, biological productivity

RESUMEN. Los ácidos húmicos (AH) de dos diferentes vermicompost fueron extraídos, aislados, purificados y parcialmente caracterizados, para evaluar su posible efecto protector en plantas de arroz (*Oryza sativa* L.) ante un estrés hídrico. En los AH se encontraron diferencias en la composición elemental, en el valor del umbral de coagulación y en la relación E_4/E_6 de sus espectros UV-Vis. Dos concentraciones (40 y 60 mg L^{-1}) de ambos AH se incluyeron en disoluciones nutritivas para el cultivo de plantas de arroz en condiciones controladas. Se verificó que el tratamiento previo con los AH durante seis días, estimuló la producción de biomasa radical. Después se excluyeron los AH y tanto en las plántulas inicialmente tratadas, como en un grupo de las utilizadas como control, se indujo un déficit hídrico adicionando polietilenglicol (PEG-6000) en la disolución nutritiva. Luego de 96 horas de establecida esa condición final se evaluaron la producción neta de biomasa radical, el contenido de pigmentos fotosintéticos foliares y la permeabilidad de la membrana de las raíces. En las plantas previamente tratadas con AH (a la concentración de 60 mg AH L^{-1}), la permeabilidad de la membrana de las raíces, la producción neta de biomasa en este órgano, así como el contenido de clorofila "a" no manifestaron diferencias al compararlas con aquellas sin estrés. Se concluye que el tratamiento previo con los AH consiguió proteger a las plantas de arroz ante el estrés hídrico posterior que se indujo.

Palabras claves: biomasa radical, permeabilidad, pigmentos fotosintéticos, productividad biológica

INTRODUCTION

Humic substances are the fraction where the organic matter carbon of the soil is retained, and they intervene in multiple properties of the soil-plant system (1).

[1] Universidad Agraria de La Habana (UNAH). Autopista Nacional km 23 ½ y Carretera de Tapaste. San José de las Lajas. Mayabeque. Cuba

[2] Universidade Federal Rural do Río de Janeiro (UFRRJ). BR 465 km 7. CEP 23890-000, Seropédica, RJ. Brasil

✉ fguridi@unah.edu.cu

At present there is no comprehensive explanation that can justify the possible relationship between the structure of these substances and the direct effects they cause in plants (2).

Composting and vermicomposting are widely used procedures for the production of renewable organic matter, to be applied as an improver of the soil degraded by agricultural activity and also represent very useful sources for the extraction of soluble humic substances. In addition, it has been found (3) that the insoluble residual solid obtained, after extraction of the soluble humic substances containing vermicompost, has a considerable capacity to retain heavy metal cations in aqueous medium.

The structural characteristics and properties of the soluble humic substances obtained from composting or vermicomposting materials depend on the original organic source, the conditions used for its processing (mainly temperature, time and biological transforming agent), as well as of the procedure used in the extraction (4-7).

Some recent research is directed to the study of the influence that soluble humic substances (especially humic acids) have on the stimulus for the adaptation of plants to abiotic stresses such as salinity (8-11), the presence of heavy metals in Toxic levels (12) and water deficiencies (13-15), always using them simultaneously or after the stress implementation, as well as in the stimulation of growth and development in different crops, for which different implementation alternatives have been used (16-18).

The rice cultivation (*Oryza sativa* L.) requires an optimal water management given the considerable volumes consumed of this resource, which may not always be available in the necessary quantities. For this reason, it is important to carry out studies that allow us having alternatives to mitigate the adverse effects of water limitations.

There are not enough reports in the literature, referring to the durability of the protective effect that humic acids could exert in the face of water stress when they are no longer present in the culture medium.

Based on the above, the main objective of this research was to evaluate the effects of the previous application of humic acids (AH) obtained from two different vermicompost, on the biological productivity of rice plants, as well as their protective action in inducing water stress.

MATERIALS AND METHODS

The activities described below were carried out in the Department of Soils of the Agronomy Institute of the Federal Rural University of Rio de Janeiro, Brazil, between September and November. 2014.

Extraction, isolation, purification and partial characterization of HA

The procedures for the extraction, isolation, purification and partial characterization of HA were carried out in the laboratory of organic matter of the Agronomy Institute of the Federal Rural University of Rio de Janeiro.

As sources of humic acids the following vermicomposting were used:

- Vermicomposting of bovine manure processed by African red worms (*Eudrilus eugeniae* spp.), in piles above ground, according to the procedures established by the Technical Manual for Organoponics 2007, with a maturation time of 70 days. The manure was supplied by the cattle ranches of the farma “El Guayabal” (San Jose de las Lajas municipality, province Mayabeque, Cuba) with coordinates 22º 59’55,95’’ Latitude North and 82º 10’ 10,27’’ West Longitude
- Vermicomposting of bovine manure, processed by California redworms (*Eisenia andrei* Bouché) in gutters with a ripening time between 45 and 50 days. The vermicomposting was produced at the “Fazendinha” Farm of the EMBRAPA (Seropédica municipality, State of Rio de Janeiro, Brazil) with coordinates 22º 45’ 42,51’’ Latitude South and 43º 40’ 31,85’’ Longitude West.

In both vermicomposting, the HA were extracted, isolated and purified according to established by the International Society of Humic Substances[A]. Basically the procedure consists in the use of a extraction solution of sodium hydroxide (NaOH) 0,1 mol L^{-1} in dinitrogen atmosphere; followed by centrifugation to separate the insoluble fraction and isolation of HA from the aqueous alkaline medium by coagulation by acidification to pH <2 with a 6 mol L^{-1} hydrochloric acid (HCl) solution. The recovery of the clotted was achieved by centrifugation and subsequently washed with distilled water until no chloride ions were present. Purification was effected by redissolving the HA obtained and the coagulation, centrifugation and washing were performed as described. Finally the HAs were lyophilized.

[A] IHSS. *International Humic Substances Society* [en línea]. , [Consultado: 2 de marzo de 2017], Disponible en: <http://www.humicsubstances.org/>.

Hence, HA obtained from vermicompost from Cuba was identified by HAC and from vermicompost from Brazil as HAB.

For the partial characterization of the HAs, the elemental composition was determined using the CHN Analyzer 2400 Perkin Elmer (England) apparatus. The ratio E4/E6 (ratio of absorbances at wavelengths of 465 and 665 nm) in the UV-Vis spectrum of a solution of 3 mg of HA in 10 mL of sodium hydrogen carbonate (NaHCO 3) 0,05 mol L^{-1} of pH =8 using a Shimadsu UV-1800 Spectrophotometer (Japan) and the coagulation threshold was determined by mixing the same volume of 150 mg L^{-1} solutions of HA at pH = 6 with Equal volume of 15 different concentrations (from 1,25 mmol L^{-1} to 18,75 mmol L^{-1}) of calcium chloride ($CaCl^2$) of PA quality, followed by 24 hours of rest to visually detect the lowest concentration of $CaCl_2$ caused coagulation.

Obtaining rice seedlings and treatments with HAC and HAB

Certified seeds of the "Piauí" cultivar, previously disinfected with 2 % sodium hypochlorite for 10 minutes and then washed with sufficient distilled water, were used to germinate in contact with a $CaCl_2$ (pH) concentration of 0, 5 mmol L^{-1}, being maintained in the conditions of the growth chamber of the Department of Soils of the Federal Rural University of Rio de Janeiro, which were: light cycle 12 hours of light and 12 of darkness; photonic flux of 250 µmol m^{-2} s^{-1}; relative humidity of 70 %; Daytime temperature of 28 ^{0}C and night of 24 ^{0}C.

After germination a homogeneous set of germinated seeds with the same radicle length (2 cm) was selected. In 200 mL plastic pots, Hoagland's solution (19) was added with ¼ of the ionic strength and covered with sterile gauze, on which six of the seeds selected were placed in each. After three days of adaptation to the experimental conditions of hydroponics and the growth chamber, the nutritive solution was changed to a ½ of the ionic strength, maintaining them for another 72 hours, at the end of which the application of the Treatments with HA (six pots per treatment) when renewing the culture medium, which was maintained with the same composition. In a further six pots, HA was not included for reference (control).

These last conditions remained the same for six days, with a replacement of the treatments in the third.

The treatments applied in this stage of the study are summarized in Table I.

Table I. Description of treatments

Treatment	Symbology	AHB mg L^{-1}	AHC mg L^{-1}
1	T	-	-
2	AHB 40	40	-
3	AHB 60	60	-
4	AHC 40	-	40
5	AHC 60	-	60

HAB = HA of vermicomposting from Brazil HAC = HA from vermicomposting from Cuba

At the conclusion of the previous stage (pretreatment with the HA), two seedlings were removed from each pot, forming three groups (replicates) of four seedlings per treatment, to determine the dry mass of both shoots and roots by desiccation (Venticell-707, China) at 60 ^{0}C to constant mass (Sartorius scale, precision ± 0,0001 g, Germany), in order to calculate the relationship between the two.

Application of water stress

After the described period, water stress was induced for 96 hours, including 15 % polyethylene glycol (PEG-6000) in the nutrient solutions, except in half of the pots of the seedlings where the HA (control) were not applied.

For this new stage of the study the treatments used (three pots for each treatment) are summarized in Table II.

Table II. Treatments (symbology and description) used in the stage of induction of water stress

Symbology	Description
T E	Plants not treated with AH with hydric stress
T S E	Plants not treated with AH with hydric stress
AHC40 E	Plants previously treated with AHC 40 mg L^{-1} with stress hydric
AHC60 E	Plants previously treated with AHC 60 mg L^{-1} with hydric stress
AHB40 E	Plants previously treated with AHB 40 mg L^{-1} with hydric stress
AHB60 E	Plants previously treated with AHB 60 mg L^{-1} with hydric stress

At the end of the induced water stress period, two plants of each pot were used to evaluate the permeability of the root membrane, by measuring the electrical conductivity every five minutes, after being washed with distilled water and placed in 100 mL of water Ultrapure (mili-Q) with permanent agitation. The readings were made with a pH meter-conductivity OrionStar A329 (England).

In the remaining four plants of each pot the dry and root dry masses were determined and the content of foliar photosynthetic pigments was quantified by extraction with 80 % acetone (20).

All the results obtained in the evaluations performed in the plants in the two stages of the experiment with a completely randomized design were subjected to simple classification variance analysis and the means were compared with the Tukey test for p <0,05 (21), using the statistical program Statgraphic version 5.1 (22).

RESULTS AND DISCUSSION

As for the partial characterization of HAC and HAB the results obtained with respect to their respective elemental compositions appear in Table III.

Table III. Percentages of Carbon (C), Hydrogen (H), Nitrogen (N) and Oxygen (O) in HA obtained from vermicomposting and the relationships between carbon and other elements (means of three replicates)

Humic acid	C %	H%	N%	O %	C/H	C/N	C/O
AHB	44,42	5,48	4,22	55,98	8,11	10,53	0,79
AHC	48,58	5,65	4,38	51,39	8,60	11,09	0,94

According to the above, both HA have a composition that is within the ranges found for that type of humic substance extracted from composted or vermicomposting materials (23) and those present in soils (24).

It was noticed that the main differences between HAC and HAB were in the contents of C and O, probably due to the conditions of obtaining the respective vermicomposting (6). From the above it was inferred that the second is slightly more hydrophilic, taking into account that in these substances oxygen is mostly hydroxyl (-OH) of carboxylic and phenolic groups, which have a high affinity for water, by the establishment of hydrogen bridges (4). According to the latter, the HAB coagulation threshold should be expected to be higher than HAC, since a higher concentration of electrolytes would be consumed to overcome the interaction with the water molecules.

The calculation of the ratio E_4/E_6 as well as the determination of the coagulation threshold of the HA obtained is shown in Table IV.

Table IV. E4/E6 ratio and coagulation threshold (mmol Ca2+ g-1 of HA) of humic acids (means of three replicates)

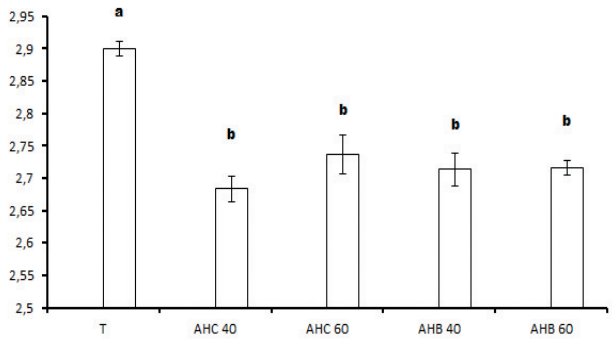
Humic acid	Ratio E_4/E_6	Threshold of coagulation
AHC	5,52	120
AHB	5,93	133

The results for this relationship, for both HAC and HAB, are within the general range (between 4 and 6) of HA extracted from different natural sources and whose value depends on the molar mass, molecular size, and grade condensation and structure aromaticity (4). These values indicate that in HAC there is a greater structural condensation when compared with HAB (23) and confirm the superior hydrophilicity of the latter, due to the oxygen content forming part of ionizable groups, in correspondence with the elemental composition found.

It is also valid to consider what was reported by other authors working with the Cu^{2+} cation as a coagulant of different HA at pH = 5 (25), who stated that the lower the aromaticity degree (equivalent to a higher E_4/E_6 value) higher coagulation threshold values were obtained.

In the scientific literature it is recognized that the biological activity of HA depends on both the structure and the elemental composition of these substances (5, 23, 26), so the differences found so far between HAC and HAB can induce different effects in their direct interaction with plants.

The evaluation of the relation between the dry mass of the aerial part and the radical in the seedlings at the end of the previous treatment with the humic acids, is represented in Figure 1.



Means of three replicates. Standard error of ANOVA = 0,032 *. Different letters represent statistically significant differences according to Tukey for p <0,05. The interval bars in the columns represent the standard deviation of the means

Figure 1. Relation between the dry masses of the aerial part and the roots (Rel PA/R) in the seedlings at the end of the previous treatment with the humic acids

The results showed that all previous treatments, including humic acids, in any of the concentrations used, led to a decrease in Rel PA/R compared to control (T treatment). This agrees with the recognized stimulation that these humic substances directly exert on the development of the radical system in different cultures in the first stages of growth (27, 28).

A better development of the root system contributes to a more efficient use of the surrounding edaphic environment, giving the plant adaptability potential to adverse conditions, such as limitations in the availability of water or essential nutritional elements, and in situations of metallic or oxidative stress (29).

No significant statistical difference was detected between the two HA nor between their concentrations, from which it is derived that what was found in the elemental composition did not cause variations in the effect of the HA.

The determination of the photosynthetic pigments after the period of induced water stress is shown in Table V.

Table V. Contents of foliar photosynthetic pigments (g kg -1 of leaf dry mass)

Treatments	Chlo a	Chlo b	Carot	Chlo T
T E	3,01 (d)	1,71 (ab)	0,65 (b)	4,71 (c)
T SE	3,61 (c)	1,64 (b)	0,74 (a)	5,25 (b)
AHC40 E	3,45 (c)	1,78 (ab)	0,51 (c)	5,22 (b)
AHC60 E	3,75 (b)	1,65 (ab)	0,76 (a)	5,40 (b)
AHB40 E	3,56 (c)	1,25 (c)	0,60 (b)	4,81 (c)
AHB60 E	3,90 (a)	1,90 (a)	0,78 (a)	5,80 (a)
E.E.	0,042*	0,085*	0,030*	0,11*

Chlorophyll "a" (Chlo a), chlorophyll "b" (Chlo b), total chlorophylls (Chlo T), total carotenoids (Carot), after 96 hours of induced water stress. (Averages of three replicates) S.E. = standard error of ANOVA. The level of significance is indicated with asterisks. Different letters indicate statistically significant differences according to Tukey (p <0.05)

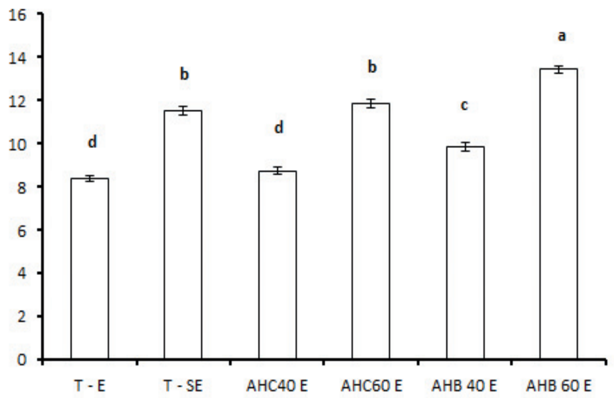
As for the individual contents of the photosynthetic pigments evaluated, the stress reduction in the "Clo a" was emphasized, which was attenuated by the previous treatments with both humic acids in the two concentrations, reaching values equivalent to and exceeding those of the seedlings (T SE) that were not subjected to the induced water deficit. It was also observed that seedlings previously treated with the highest concentrations (60 mg L^{-1}) of both HAC and HAB maintained or even exceeded those of T SE in the contents of all pigments.

The effect of water stress on photosynthetic pigments has been indicated as one of the most widespread in several crops, as indicated by studies on photosynthesis in different plants under stress conditions (27).

The results confirm what the literature shows in that humic substances have the possibility of directly influencing the metabolism of plants (2, 7, 26, 29), due to the verified activity equivalent to the phytohormones they possess, as well as well as to the stimulatory effect of enzymes related to the fixation of carbon dioxide. In addition, it has been shown that they can also induce anatomical and morphological changes (increases in leaf and root area) that have a greater effect on photosynthetic efficiency.

The fact that the HA application has attenuated the effect of water stress on the endowment of photosynthetic pigments, must have an impact on the photosynthetic efficiency in these plants and consequently on the biomass production.

The net dry mass (net of dry mass before and after stress) in the stress induction period is shown in Figure 2.



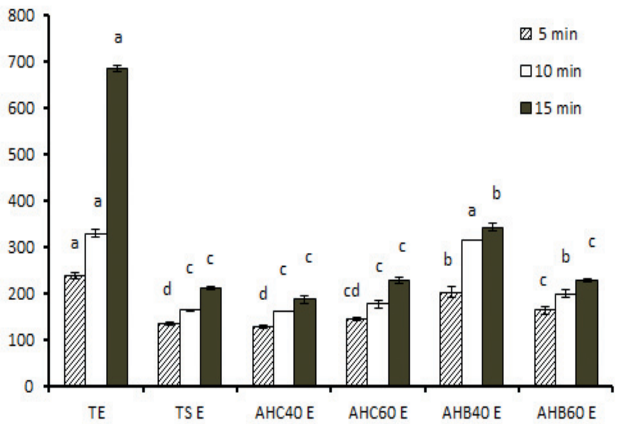
Means of three replicates. Standard error = 0,147*. Different letters represent statistically significant differences according to Tukey for p <0,05. The bars in the columns represent the standard deviation of the means

Figure 2. Net production of root dry mass during the period of water stress induction (96 hours) in mg/plant

It was evidenced that the plants that had the previous treatments with the highest concentrations of HAC and HAB, managed to at least equal the dry mass production that was registered in those that were not subjected to water stress. In plants previously in contact with the concentration of 40 mg L^{-1} HAB there was a favorable response compared to the stressed seedlings, but did not achieve the production generated in those that were not exposed to stress.

If the results on the pigment content at the end of the induced water stress are associated with the net dry mass production in that period, it is noteworthy that the plants previously treated with HA (with which it was possible to maintain a suitable composition of pigments), were in better conditions so that the photosynthesis happened without significant affectations, that prevented to generate the necessary metabolites to guarantee the production of biomass.

The measurements of the electrical conductivity in the pure water in contact with the intact roots at different time intervals, for the evaluation of membrane permeability are presented in Figure 3.



Means of three replicates. Different letters indicate statistically significant differences according to Tukey test for p <0,05 for each interval. (The S.E.s were 6,81 *, 5,92 * and 6,65 * for the ANOVA performed at 5, 10 and 15 minutes respectively. The bars in the columns represent the standard deviation of the means)

Figure 3. Electrical conductivity in the medium (mS g^{-1} of dry mass) at different time intervals after 96 hours of established water stress

In general, the protective effect durability of the HA previous application on the permeability of the root membranes was demonstrated. In the case of plants from the two concentrations of HAC, the values had no differences with those of the non-stressed (T SE) and much lower than those of ET, in all evaluations over time. This means that with this treatment the membrane permeability was persisted even after 96 hours of stress.

In the case of plants treated with HAB, a value similar to the T SE treatment was obtained for the concentration of 60 mg L^{-1} in the last evaluation, although they were always lower than those registered in the stressed seedlings that had no previous contact with HA (TE treatment).

At the HAB 40 E treatment plants, the conductivity at T SE was not equaled at any time and only lower than TE at the initial and final times.

What we found in this determination confirms what other authors have pointed out that the direct effects of HA on the physiological response of plants depend both on the concentration and its structural characteristics (26, 28) and that they do so by interfering in various physiological mechanisms (29, 30).

In relation to the structural characteristics of the HA, it was revealed that the elemental composition is insufficient information to explain the differences in their biological activity, which requires a greater depth in this aspect, such as the distribution of molecular sizes and functional carbon groups.

CONCLUSIONS

According to the above results it is possible to make the following general conclusion:

Humic acids isolated from two vermicomposting obtained under different conditions showed differences in the structural characteristics and properties evaluated, but in both cases, especially the 60 mg L^{-1} concentration, they exerted a previous protective effect on rice plants. Later they were exposed to a water deficit for 96 hours, being no longer present in the medium. This protection was verified in the net production of radical biomass, the photosynthetic pigments and the membrane permeability of the roots.

RECOMMENDATIONS

Evaluate the possibility of using an extract of soluble humic substances contained in vermicompost (without isolating humic acids) as a practical procedure in rice cultivation to achieve a more efficient use of water resources.

Evaluar la posibilidad de emplear un extracto de las sustancias húmicas solubles contenidas en el vermicompost (sin aislar los ácidos húmicos) como un procedimiento práctico en el cultivo del arroz para lograr un uso más eficiente del recurso agua.

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