Humic Acid Effect on Catalase Activity and the Generation of Reactive Oxygen Species in Corn (Zea mays)

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Humic acids (HAs) have positive effects on plant physiology, but the molecular mechanisms underlying these events are only partially understood. The induction of root growth and emission of lateral roots (LRs) promoted by exogenous auxin is a natural phenomenon. Exogenous auxins are also associated with HA. Gas nitric oxide (NO) is a secondary messenger produced endogenously in plants. It is associated with metabolic events dependent on auxin. With the application of auxin, NO production is significantly increased, resulting in positive effects on plant physiology. Thus it is possible to evaluate the beneficial effects of the application of NO as an effect of auxin. To investigate the effects of HA the parameters of root growth, Zea mays was studied by evaluating the application of 3 mM CL−1 of HA extracted from Oxisol and 100 μM SNP (sodium nitroprusside) and the NO donor, subject to two N−NO3−/C0, high dose (5.0 mM N−NO3−) and low dose (5.0 mM N−NO3−). Treatments with HA and NO were positively increased, regardless of the N−NO3− taken, as assessed by fresh weight and dry root, issue of LRs. The effects were more pronounced in the treatment with a lower dose of N−NO3−. Detection of reactive oxygen species (ROS) in vivo and catalase activity were evaluated; these tests were associated with root growth. Under application of the bioactive substances tested, detection of ROS and catalase activity increased, especially in treatments with lower doses of N−NO3−. The results of this experiment indicate that the effects of HA are dependent on ROS generation, which act as a messenger that induces root growth and the emission of LRs.

Key words: humic acid; growth root; nitric oxide; reactive oxygen species

Increased tolerance of nutritional stress might be strongly related to increased activity of the antioxidant systems of plants, with high-level expression of proteins such as catalase. Substances that act as antioxidants can minimize the effects of stress. Humic substances play important roles in the quality of agricultural systems by interfering with nutrient availability to plants and are sources of energy for soil microorganisms, and thus a decisive influence on chemical, physical, and biological soil properties, and they exert direct effects on plant growth.1 The study of the action of humic substances on the metabolism and growth of plants has been focused mainly on fulvic acids, i.e., the humified fraction, considered to be of lower molecular weight. The apparent high molecular mass of humic acids (HAs) appears in compatible with their direct effects in plant physiology. This is because it is not conceivable that a substance of mass two or three million times larger, such as humic acids (on the order of micrometers) can pass through the pores or spaces apparent in the apoplast (on the order of nanometers).2 Nitric oxide (NO) is a reactive gas easily formed and rapidly diffused into a biological system. It operates in developing roots,3 leaf expansion, stomatal aperture, and do on. Recently, it has been found that NO and reactive oxygen species (ROS) can substantially affect plant growth and development.4 Treatment of an NO donor and ROS, for example SNP and H2O2 respectively, can enhance germination and break seed dormancy.5 NO and ROS can interact with each other, and this can be cytotoxic or cytoprotective, depending on their concentrations and situation.6 Enzymatic ROS scavenging mechanisms in plants include catalase (CAT).7 ROS can influence the expression of a number of genes and signal transduction pathways, suggesting that cells have evolved strategies to utilize ROS as signals that control various biological programs.8 Nitrogen is an element required in large quantities and frequently limits plant growth. About

Abbreviations: CAT, catalase; C, carbon; HAs, humic acids; NO, nitric oxide; HSs, humic substances, H2DCFDA, 2,7ʹ-dichlorodihydrofluorescein diacetate; SNP, sodium nitroprusside; ROS, reactive oxygen species
Humic Acids Produced Reactive Oxygen Species in Corn

90% of total plant nitrogen is in organic form, and it plays of important roles as a structural constituent of macromolecules, enzymes, proteins, nucleic acids, and many other cellular constituents, including membranes and several plant hormones. Plants absorb N from solution both in organic form as amino acids and citrate, and in inorganic form, such as ammonium (NH$_4^+$) and, nitrate (NO$_3^-$), which is important in soil aerobics. Hence on objective was to evaluate the influence of HA and NO on CAT activity and the generation of ROS in maize seedlings (Zea mays cv Bandeirantes), subjected to nitrate at two levels.

Materials and Methods

The study was conducted in hydroponic pots in a growth chamber, Soil Science Department in UFRJ/Seropédica-Rio de Janeiro-Brazil.


detection of ROS in vivo. Sample incisions of maize roots were treated with 15 μM of fluorescent probe 2,7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Calbiochem, Germany), a marker of plasma membrane permeable ROS. In DMSO. The final concentration of DMSO was 0.1% v/v. The specimens were incubated in the respective treatments for 2 h at 25 °C under shaking in the dark. After the incubation period, the roots were washed 3 times with the medium prepared for incubation, and then were mounted on slides for viewing under a microscope. The samples were visualized using a fluorescence microscope Axiosplan- Zeiss (Carl Zeiss, Germany) adapted with a digital camera, AxioCam MRc5 (Carl Zeiss, Germany). We used filters with a wavelength of excitation at 495 nm and emission at 515 nm for the indicator, H$_2$DCFDA. Images were acquired using computerized image capture and analysis equipped with software version 4.8 AxioVision LE (Carl Zeiss, Germany) and processed using the same software. The fluorescence intensity of root tips was determined using the AxioVision LE software version 4.8 (Carl Zeiss, Germany), expressed in number of pixels per area.

Results and Discussion

According to elemental composition analysis of HA (Table 1), the values for total carbon, oxygen, nitrogen, hydrogen, and ash were 50.3%, 40.2%, 4.2%, 4.3%, and 0.61% (dry weight basis) respectively. The carboxyl and phenolic groups accounted for 342 and 211 centimol charge kg$^{-1}$ of total acidity (553 cmol$_{k}$ kg$^{-1}$). These results are consist with the normal levels found for oxisol. Atomic ratios O:C, H:C, and C:N are often used to identify humic substances from different sources and to follow their structural changes in different environments. The O:C ratio is considered an indicator of the carbohydrate and carboxylic contents of humic substances, and can be used to compare HAs. The O:C ratio is also representative of the degree of humification. A decrease in ratio commonly suggests an increase in aromatic condensation. A progressive decrease in aromaticity suggests the maturity of HS in this soil. The values of the H:C ratios shows the degree of maturity of humic substances, since indirectly it reflects the presence of more condensed aromatic ring or replaced ring structures. E$_2$/E$_6$ ratios were determined by sample absorbance at 465 and 665 nm. Despite considerable controversy, the significance of the optical properties of humic substances is not yet fully understood, but considerable evidence indicates that lower E$_2$/E$_6$ ratios values are associated with more mature humic acid fractions, characterized by more abundant aromatic components and higher degrees of condensation.

<table>
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<th>Content, %</th>
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Fig. 1. Fresh Mass.
Maize root growth without, and at low (0.5 mM) and a high dose (5.0 mM) of N–NO$_3^-$, Effects of 3 mM CL$^{-1}$ humic acid (HA) and 100 μM sodium nitroprusside with nitric oxide donor (NO). Error bars indicate the standard error of the mean. Asterisks indicate a significant difference by respect to the control (low N–NO$_3^-$) at p < 0.05 by ANOVA and Dunnett tests.

Fig. 2. Dry Weight.
Maize root growth without, and at low (0.5 mM) and a high dose (5.0 mM) of N–NO$_3^-$, Effects of 3 mM CL$^{-1}$ humic acid (HA) and 100 μM sodium nitroprusside with nitric oxide donor (NO). Error bars indicate the standard error of the mean. Asterisks indicate a significant difference by respect to the control (low N–NO$_3^-$) at p < 0.05 by ANOVA and Dunnett tests.

Fig. 3. Lateral Root Density (LRs per centimeter).
Maize root growth without, and at low (0.5 mM) and a high dose (5.0 mM) of N–NO$_3^-$, Effects of 3 mM CL$^{-1}$ humic acid (HA) and 100 μM sodium nitroprusside with nitric oxide donor (NO). Error bars indicate the standard error of the mean. Asterisks indicate a significant difference by respect to the control (low N–NO$_3^-$) at p < 0.05 by ANOVA and Dunnett tests.

The results for the fresh weight of the roots can be seen in Fig. 1. Humic acid treatments promoted a significant increase in the fresh weight of the roots of maize as compared to control (low N–NO$_3^-$). Similar behavior was observed in the treatment with NO, but with less intensity than with HA. Treatment with the HA without nitrate, there was a slight increase in the root fresh weight of the maize. Figure 2 shows the values of dry mass of maize roots subjected to standard treatment. A behavior similar to results Fig. 1 (fresh weight of corn roots). The treatments with bioactive substances (HA and NO) led to significant increases in root dry weight as compared to the controls (rates of nitrate) tested. There were no significant differences between treatments with HA and NO at both doses of nitrate tested (Fig. 2).

HA in the treatment without the application of N–NO$_3^-$ produced significant increases in the root dry weight of the maize. This indicates that this substance acts as an elicitor of root growth, process of cell differentiation, and in promoting the formation of lateral roots. These events promote an increase in root surface and consequently higher absorption of nutrients. Thus there are increases in the fresh and dry weight of the roots. This behavior demonstrates the influence of HA on the accumulation of the fresh mass of roots. A study of the effects of HA from sewage sludge on lettuce found higher values of fresh root in treatments with HA. The average values of fresh weight of root showed that supplementation with HA and NO in the nutrient solution increased the root fresh weight. This behavior demonstrated HA bioactivity, providing growth and increases in weight of the organs. However, the biochemical mechanisms involved in this process are not clear. Substances can promote plant growth through increased efficiency in absorbing nutrients.

The density of lateral roots (LRs) is shown in Fig. 3. Treatment with HA caused significant increases in LRs, order of 25%, 125%, and 90% for treatment without N–NO$_3^-$, and with low and high N–NO$_3^-$ respectively, as compared with the control (low N–NO$_3^-$). Similar behavior was observed for treatments with NO, which increased about 90% and 25% with supplementation at low and high N–NO$_3^-$, respectively, compared to control (low N–NO$_3^-$). This small increase (a 25% increase in LRs as compared to control) in treatment NO + 5.0 mM N–NO$_3^-$ might be related to increased levels of endogenous NO, and perhaps with the application of SNIP as NO donor, these increased the levels. With increases in N–NO$_3^-$, nitrate reductase activity increased, because N–NO$_3^-$ acted as a positive regulator of this enzyme. Thus endogenous levels of NO are increased and with the application of exogenous NO, this promoted the antagonistic effect of the stimulus caused by this gas. It has been reported that NO mediates auxin LR development, and the hypothesis of a NO signal downstream of auxin signaling during LR formation has been strongly supported. This indicates that maize root architecture is modified by HA and by SNIP, in similar ways, leading to increases in the number of LRs, and a higher root density. Evaluating humic substances extracted from different sources, molecules of indole acetic acid (IAA) were found by gas chromatography/mass spectrometry (GC/MS) and detection by immunoassay. This indicates that the
bioactivity of HAs can be explained at least in part by the presence of auxin. The phytohormone auxin is a key regulator of lateral root development.\textsuperscript{30}

In the treatments supplemented with HA (Fig. 4), the roots showed higher catalase activity than under other treatments. At the lower dose of N–NO$_3^-$ with HA, the catalase activity was higher than control, by about 400%, whereas at the higher dose with N–NO$_3^-$ this increase was 80% as compared to control. This behavior reflects the ability of HA to act as an antioxidant and to promote auxin activity, as evidenced by the higher fresh weight of the roots. H$_2$O$_2$ had an important physiological function, especially in the oxidative metabolism of the cell-wall, because these reactive species indicate the activity of the enzymes that promote cell wall loosening, such as cellulases, hemicellulases, glucanases, and pectinases, acting on the growth of plant cells. Thus catalase activity is indicative of the differentiation and growth of root cells.

ROS detection indicates that treatment with HA and with NO showed a significant increase in fluorescence emission (Fig. 5A and B). This increase in ROS may have stimulated the gene expression of the enzyme catalase, resulting in increased activity of this enzyme, minimizing the oxidative effects of ROS. Studies indicate that ROS are required for cell expansion during the morphogenesis of organs such as the roots and leaves. Moreover, there is evidence that ROS are required for root growth, in which these species control the activity of the calcium channels required for polar growth. H$_2$O$_2$ is an ROS. Produced during cell growth, it can be eliminated by the action of catalase, which transforms it into O$_2$ and H$_2$O.\textsuperscript{31}

**Fig. 4.** Catalase Activity.

Maize root growth without, and at low (0.5 mM) and a high dose (5.0 mM) of N–NO$_3^-$ effects of 3 mM CL$^{-1}$ humic acid (HA) and 100μM sodium nitroprusside with nitric oxide donor (NO). Error bars indicate the standard error of the mean. Asterisks indicate a significant difference by respect to the control (low N–NO$_3^-$) at $p < 0.05$ by ANOVA and Dunnett tests.

**Fig. 5.** Endogenous Levels of ROS (A) and Relative Fluorescence Intensity, Expressed as Relative Number of Pixels (B), in Apex of Maize Roots, without, and at Low (0.5 mM) and a High Dose (5.0 mM) of N–NO$_3^-$.

Effects of 3 mM CL$^{-1}$ humic acid (HA) and 100μM sodium nitroprusside with nitric oxide donor (NO). Images obtained with a dark-field microscope, scale bar = 200μm, using fluorescent probe H$_2$DCFDA, specific to H$_2$O$_2$. Treatments: a, 0.5 mM N–NO$_3^-$; b, 0.5 mM N–NO$_3^-$ + HA; c, 0.5 mM N–NO$_3^-$ + NO; d, 5.0 mM N–NO$_3^-$; e, 5.0 mM N–NO$_3^-$ + HA; f, 5.0 mM N–NO$_3^-$ + NO; g, without N–NO$_3^-$ + HA. Error bars indicate the standard error of the mean. Asterisks indicate a significant difference by respect to the control (low N–NO$_3^-$) at $p < 0.05$ by ANOVA and Dunnett tests.
ROS are influenced by phytohormones and are required for cell growth.\(^3\) For example, in response to the gravitropism of corn roots, stimulated by auxin, promotes the production of ROS in the outer epidermis of maize coleoptiles, and ROS stimulate stretching and consequent growth.\(^3,3^6\) This suggests that the accumulation of ROS is required for root growth. They act as secondary messengers in hormonal signaling.\(^1,1^5,3^5\) Recent studies show that ROS, particularly H\(_2\)O\(_2\), are sensitive to supplemental N, and that ROS involved in root growth are dependent on the level of N–N\(_2\)O\(_3\).\(^3^5\) The root growth, coupled with the generation of ROS observed in treatments with bioactive substances (Fig. 5), is indicative of the fundamental role of these species in promoting growth, since ROS are responsible for the cleavage of polysaccharides, resulting in cell-wall relaxation and consequent increase in size. The metabolism of ROS has a decisive role in cellular differentiation and development. It is therefore possible that the H\(_2\)O\(_2\) produced due to oxidative imbalance can act as a second messenger in the expression of genes responsible for the induction of morphogenetic processes, culminating in the development of LRs.

**Conclusion**

The experiments described in this paper indicate that growth caused by the application of HA was mediated by ROS production, evidenced primarily by increased fresh and dry weight of roots of LRs. The increase in ROS production may have induced the expression of antioxidant genes such as catalase, decreasing the oxidative effects of the ROS necessary for root growth.

**References**