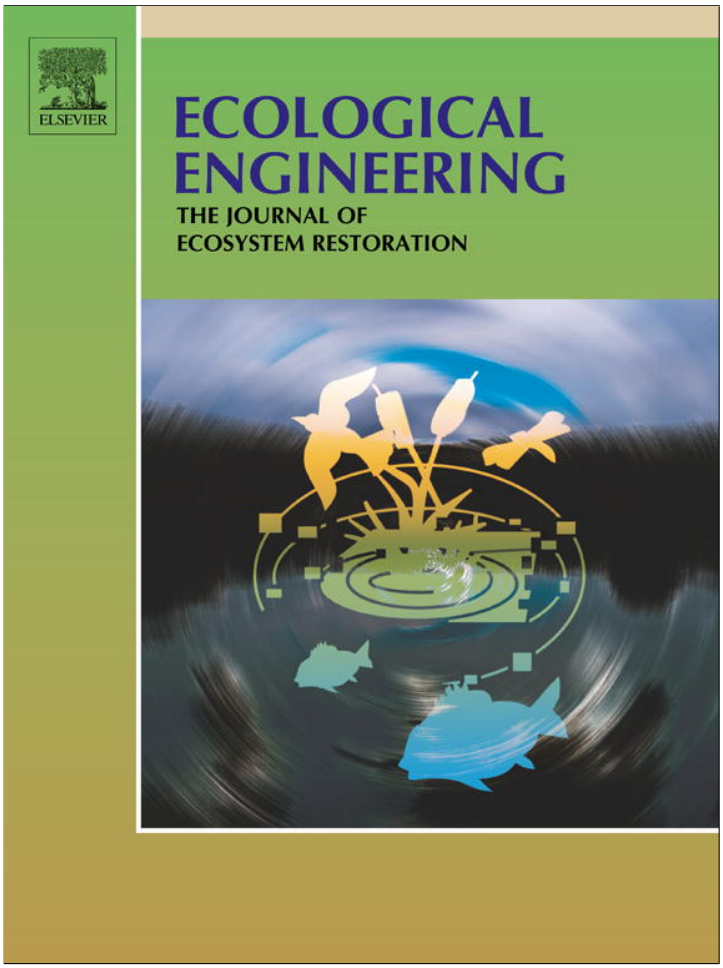

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Short communication

Vermicompost humic acids as an ecological pathway to protect rice plant against oxidative stress

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ABSTRACT

Humic substances (HS) contained in the vermicompost, are environmentally friendly materials that restore the chemical and physical properties of soils and improve plant growth. The work described herein was aimed at studying the HA–root interactions and their relationship with some components of antioxidative metabolism in rice (*Oryza sativa* L.). These studies encompassed aspects ranging from the activity of enzymes in antioxidative metabolism and the relationship between these enzymes and the reactive oxygen species (ROS) content in rice to the expression of genes encoding rice tonoplast intrinsic proteins (OsTIP), a sub-family of aquaporins. The HA–root interactions were also characterized. The results from these studies demonstrate that the interaction of HA with the radicular system in plants activates antioxidative enzymatic function, thus controlling the ROS content and modifying OsTIP expression. Microscopic and spectroscopic techniques confirmed the interactions between HA fragments of lower structural complexity and the radicular system. It appears that HA act in plants via a specific form of stress that is detected by anti-stress defense systems in plants. These HA applied to plants can protect against water stress in degraded soils.

1. Introduction

The adition of compost to soils exerts benefitial effects for the restoration of their physical property (Sparke et al., 2011) and humic acids (HA) extracted from vermicompost can protect plants in water deficient-soils. Despite diverging views, great progress has been made toward understanding the action of humic substances (HS) in plants (Singh and Agrawal, 2010). Recently, a new mode of action for HA suggests that HA can cluster in roots to affect transpiration and, therefore, the hydraulic conductivity of the roots via colloidal stress (Asli and Neumann, 2010). Other authors have observed effects on antioxidative defense mechanisms, reporting the stimulation of catalases (CAT) and the generation of reactive oxygen species (ROS) that act as intermediaries in plant growth (Cordeiro et al., 2011).

The production of ROS in superior plants is inherent to photosynthesis and respiration (Gill and Tuteja, 2010) and is stimulated in response to the majority of the most important biochemical processes, such as stress, hormonal signaling and both polarized and gravitropic growth (Mori and Schroeder, 2004). Hydrogen peroxide (H_2O_2) is one of the most stable ROS in plants that diffuses across membranes (Henzler and Steudle, 2000). In its role as a signaling molecule, low concentrations of H_2O_2 result in a plant's adaptation to various abiotic and biotic stresses (Quan Li-Juan et al., 2008) and affect the plant's growth and developmental processes (Foreman et al., 2003).

Aquaporins play a fundamental role during intercellular regulation due to their action in both turgor and osmotic pressure, membrane permeability and cell osmotic balance (Hohmann et al., 2000). Specifically, tonoplast intrinsic proteins (TIPs) direct the flow of water and solutes between the cytoplasm and the vacuolar compartments (Kaldenhoff and Fischer, 2006), and their role in the transport and flux of intracellular H_2O_2 has been demonstrated (Bienert et al., 2007). Our work has the following objectives: (i) investigate HA–root interactions via a chemical–spectroscopic characterization, (ii) evaluate whether these interactions are perceptible at the cellular level in antioxidative defense mechanisms via an analysis of aquaporin expression and (iii) obtain visual information regarding HA–root interactions and the diverse effects they produce in plants.

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2. Methods

2.1. Extraction and characterization of HA

The HA were obtained from a vermicompost of bovine manure following the methods of the IHSS. Infrared spectroscopy and ^{13}C-MNR spectroscopy were performed using a VERTEX 70 FTIR series spectrometer Bruker equipped with a diffuse reflectance detector and Bruker spectrometer UltraShield NMR at 500 MHz, respectively.

2.2. Growth conditions of plants, HA application and collection of vegetal samples

The plants rice (*Oryza sativa* L. cv. Nipponbare) were grown in growth chambers (luminosity cycle: 12/12 h (light/dark), photosynthetic photon flux: 250 μmol m^{-2} s^{-1}, relative humidity: 79% and temperature: 28 °C/24 °C (day/night). A Hoagland solution was used after germination (Hoagland and Arnon, 1950). Three HA concentrations were prepared (20 mg (C) L^{-1}, 40 mg (C) L^{-1} and 80 mg (C) L^{-1} (HA20, HA40 and HA80, respectively). The HA were dissolved and supplied with the nutrient solution every three days. The plants were collected 28 days after their transfer into the growth pots and collected at both 8 h and 24 h after the last interation of HA supplementation.

2.3. Evaluation of the activity of antioxidative system enzymes and metabolite content

The activity of peroxidases (POX) (*EC 1.11.1.7*) was determined according to the methodology developed by Li (2000). The CAT activity (*EC 1.11.1.6*) was determined using the methods of Beers and Sizer (1952). The APOX activity (*EC 1.11.1.11*) was evaluated using the methods of Nakano and Asada (1981). SOD activity (*EC 1.15.1.1*) was determined using the methods of Becana et al. (1986). The H_2O_2 content was estimated according to the method of Rao et al. (1997), and the production of the superoxide anion radical ($O_2^{\bullet -}$) was measured using the methods of Jiang and Zhang (2002). Malondialdehyde (MDA) content was determined using the methods of Dhindsa and Matowe (1981), and the protein content assays were performed using the Bradford method described by Bradford (1976). $O_2^{\bullet -}$ formation was visualized in vivo and photographic images were acquired with a digital camera connected to a microscope (Olympus HX-200) (Frahry and Schopfer, 2001; Liskay et al., 2004).

2.4. Evaluation of OsTIP expression

The total RNA was extracted using Gao et al. (2001) methods. PCR reactions were performed in duplicate on synthesized cDNA templates using the SYBR® Green PCR Master Mix kit (Applied Biosystems, Inc.). The expression of the β-actin gene (NM_001057621.1) was used as an endogenous housekeeping control (Jain et al., 2006). The primers used in the RT-PCR to evaluate the gene expression levels are listed: *OsTIP 1;1* (LOC_Os03g05290), *OsTIP 1;2* (LOC_Os01g74450) and *OsTIP 4;1* (LOC_Os05g14240).

2.5. Statistical analysis

A statistical analysis was performed using simple analysis of variance (ANOVA) tests. The significance levels were evaluated using a Tukey's multiple comparison test ($p < 0.05$) whenever the factors under study showed significance.

Table 1
H_2O_2 and MDA levels in the foliage and roots at 8 h and 24 h after final HA treatment. The different letters represent the statistical significance between mean values (Tukey's test, $p < 0.05$).

H_2O_2 (μmol mg^{-1} protein)					
Time/tissues		noHA	HA20	HA40	HA80
8 h	Leafs	0.0141^{c}	0.0184^{b}	0.0228^{a}	0.0144^{c}
	Roots	0.0182^{c}	0.0395^{a}	0.0215^{b}	0.0184^{c}
24 h	Leafs	0.0187^{b}	0.0189^{b}	0.0341^{a}	0.0185^{b}
	Roots	0.0188^{d}	0.0739^{a}	0.0312^{b}	0.0275^{c}
MDA (μg g^{-1} FW)					
8 h	Leafs	0.1523^{c}	0.3848^{a}	0.1593^{c}	0.1743^{b}
	Roots	0.5564^{b}	1.3673^{a}	0.5615^{b}	0.5633^{b}
24 h	Leafs	0.1678^{c}	0.3513^{a}	0.1776^{b}	0.1729^{b}
	Roots	0.1992^{b}	0.2014^{a}	0.1998^{b}	0.1993^{b}

3. Results

3.1. Enzymes and metabolites in the antioxidative defense system

POX activity was stimulated after 8 h by the action of the HA at all three concentrations and in both roots and leaves. After 24 h, the POX activity assay demonstrated that in the foliage, the HA80 treatment was the only superior treatment with respect to the noHA treatment. APOX activity was further stimulated after 8 h by the HA80 and HA40 treatments on foliage and roots, respectively. After 24 h, the highest stimulation of APOX activity was observed in the HA40 and HA20 treatments in the foliage and roots, respectively. CAT activity was stimulated by all three treatments at both 8 and 24 h and for both tissues relative to the controls. However, SOD activity was not induced by HA in the foliage at 8 h, whereas in the roots, it was superior for all three treatments with respect to controls. After 24 h, the SOD activity in both foliage and roots was elevated relative to that of the controls (Fig. 1A).

After 8 h, the HA40 and HA20 treatments resulted in the highest H_2O_2 contents in the foliage and roots, respectively. Nonetheless, at 24 h, only the HA40 treatment resulted in the highest H_2O_2 contents in foliage, whereas roots in all treatment groups displayed values superior to the controls. The MDA content at 8 h was lowest for the HA80 and HA40 treatments in the foliage and roots, respectively, whereas at 24 h, only the HA20 treatment was superior to the controls in both the foliage and the roots (Table 1).

3.2. Gene expression of OsTIP aquaporins

In the foliage, *OsTIP 1;1* gene expression decreased in all treatment groups with respect to the controls; *OsTIP 1;2* gene expression in the HA treatment group was also lower with respect to the controls. The *OsTIP 4;1* expression decreased only in the HA40 and HA80 treatment groups with respect to the control. The HA20 treatment exhibited statistically equivalent gene expression to that of the controls (Fig. 1B). In the roots, the *OsTIP 1;1* gene expression decreased following the HA40 and HA80 treatments with respect to the controls, whereas it remained equivalent to that of the controls following the HA20 treatment. *OsTIP 1;2* exhibited a decrease in expression following the HA40 treatment, whereas its levels after HA20 treatment remained equivalent to those of the controls. In contrast, *OsTIP 1;2* gene expression was induced by the HA80 treatments with respect to the controls. *OsTIP 4;1* also displayed lower expression levels, although the results of the HA80 treatment were not statistically significant from those of the controls (Fig. 1C).

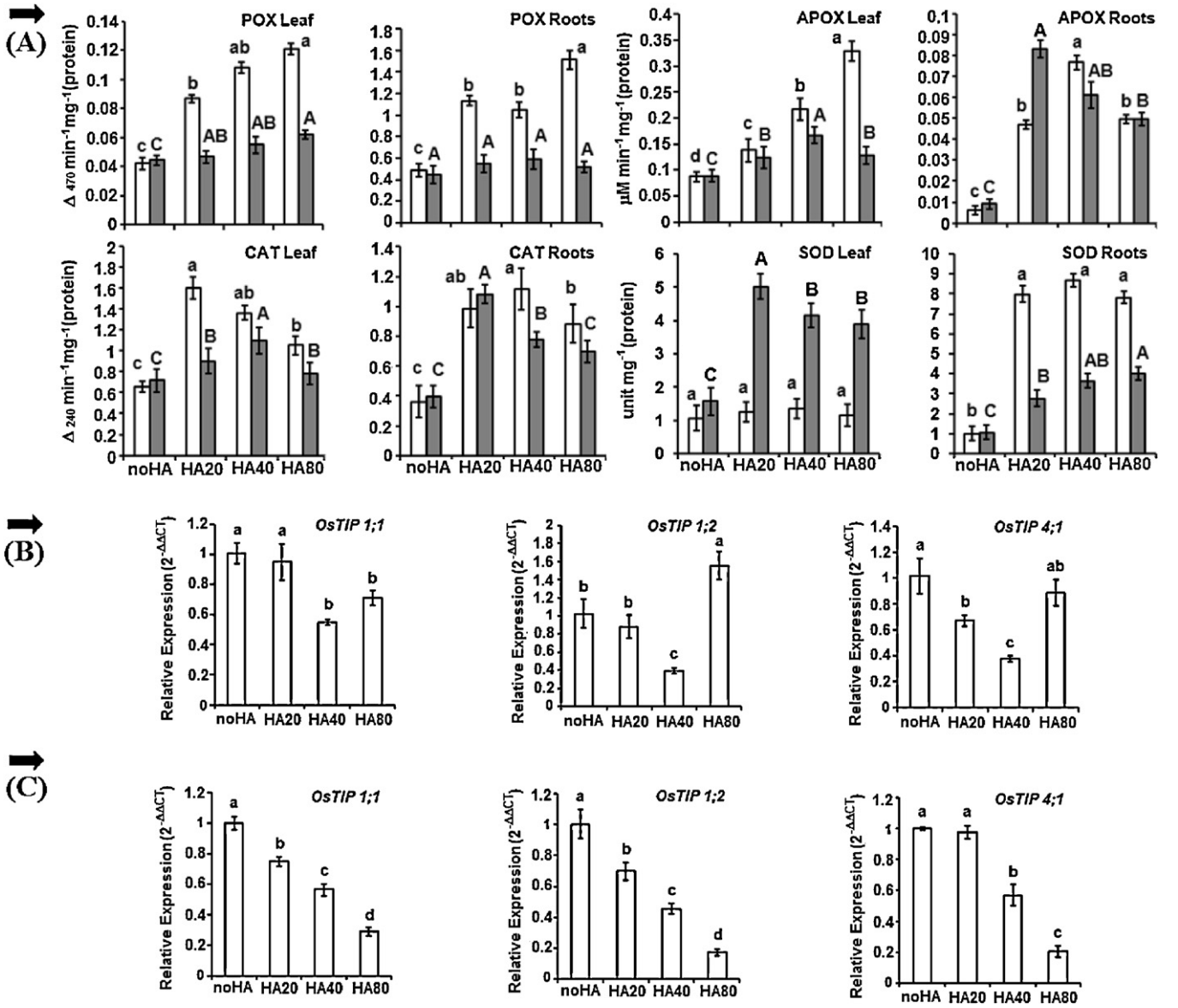

Fig. 1. (A) Activity of enzymes from the antioxidative defense system (POX, APOX, CAT and SOD) in the foliage and roots at 8 h (white bars) and 24 h (gray bars) after the final HA treatment. (B and C) Relative gene expression of *OsTIPs* aquaporins at 8 h after the final HA treatment. (A) Relative expression in the foliage; (B) relative expression in the roots. (C) Relative expression in the foliage. The different letters represent the statistical significance between the mean values: lowercase letters correspond to the statistical test at 8 h; capital letters correspond to the statistical test at 24 h (Tukey's test, $p < 0.05$). The error bars correspond to the mean value ± S.E.M. of three replicates.

3.3. Characterization of added HA and their clusters in roots

DRIFT spectra (Fig. 3A) exhibit better definition in the bands associated with structures containing aliphatic features (2926–2927 cm^{-1}; 2850–2851 cm^{-1}; 1421–1461 cm^{-1}) and oxygenated functional groups (3432–3439 cm^{-1}; 1644–1649 cm^{-1}; 1714–1717 cm^{-1}) in the HA clustered in the roots; this might indicate a lower level of structural complexity in these HA relative to the HA added during treatment. The peaks in the spectra ^{13}C-NMR (Fig. 3B) of the clustered HA are better resolved, indicating the presence of less complex structures. HA spectra added during the HA20, HA40 and HA80 treatments did not exhibit any peaks corresponding to olefinic or non-substituted aromatic carbons (region between 110 and 130 ppm); these spectra also revealed that the peaks with the highest resolution occurred in regions related to oxygen-associated carbonated structures (145–160 ppm, attributed to aromatic carbons substituted by oxygen and nitrogen, and 160–200 ppm, characteristic of carboxylic acids, esters and amides).

4. Discussion

4.1. Influence of HA on antioxidative defense mechanisms

An elevated $O_2^{\bullet -}$ level is the first step in the chain reaction of antioxidative functions in cells (Gill and Tuteja, 2010). We observed peculiarities regarding the relationship between $O_2^{\bullet -}$ levels and SOD activity, which is the enzyme responsible for the conversion of $O_2^{\bullet -}$ into H_2O_2 (Fig. 2). Despite higher $O_2^{\bullet -}$ levels in all of the HA-treated samples relative to the control samples, the SOD activity in the foliage after 8 h indicated that the dismutation reaction to form H_2O_2 does not appear to have high priority. These H_2O_2 levels and the SOD activity exhibited dependence on the HA concentration, the identity of the tissue and the time. Thus, we assume that the

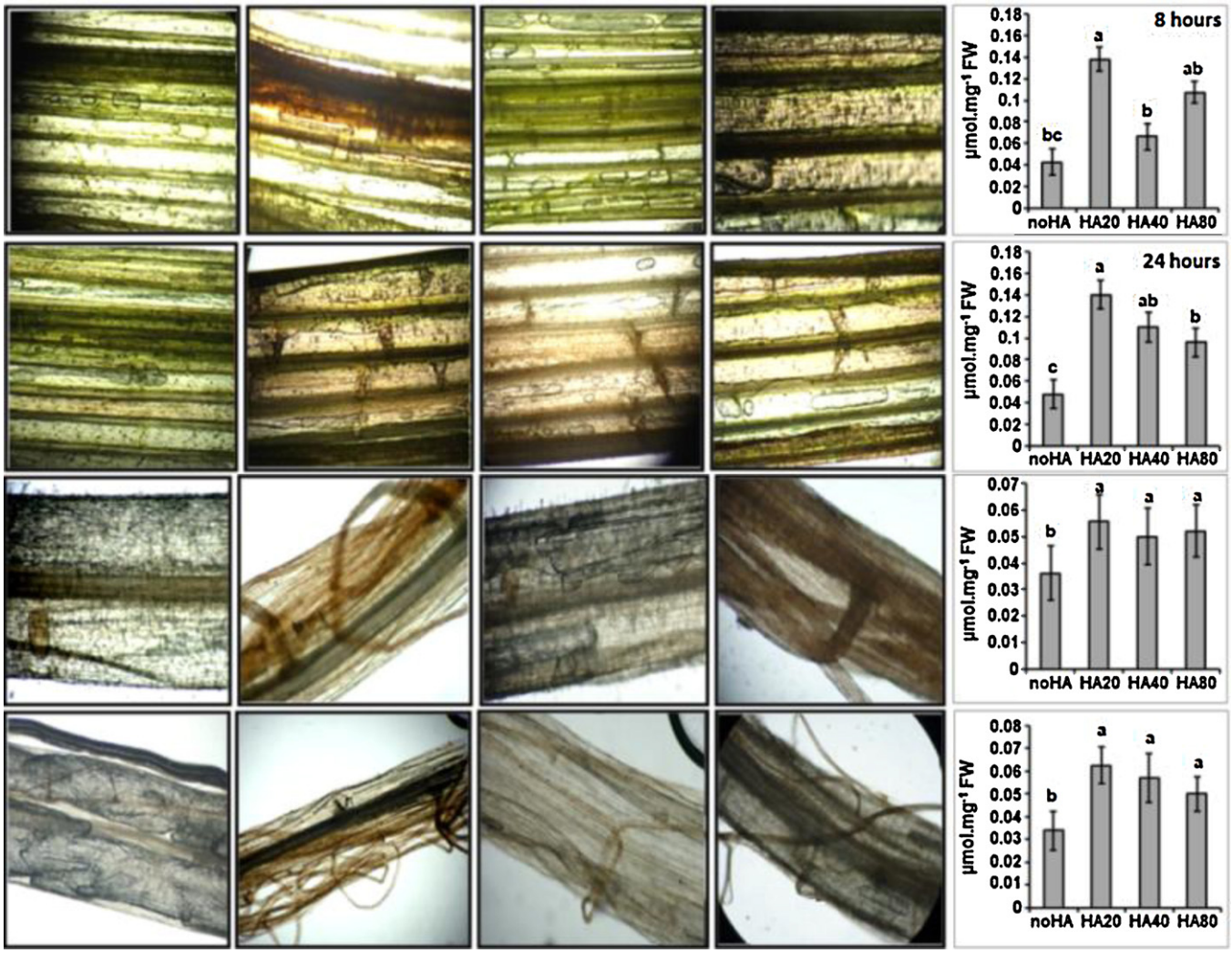

Fig. 2. $O_2^{\bullet -}$ levels in situ and in vivo in foliage and roots at 8 h and 24 h after the final HA treatment. The different letters represent the statistical significance between the mean values (Tukey's test, $p < 0.05$). The error bars correspond to the mean value ± S.E.M. of three replicates.

interaction of the HA with the roots modifies the regular function of the roots and, thus, initiates biochemical events similar to stress conditions (drought, saline, etc.), which stimulates ROS production.

In roots, plants respond within 8 h to HA20 treatments through H_2O_2 conversion catalyzed by CAT. Additionally, at 8 h, the H_2O_2 and MDA levels are high, whereas at 24 h, H_2O_2 conversion is apparently catalyzed by CAT and APOX, and the MDA contents still remain elevated. Following the HA40 treatment, roots respond at 8 h and 24 h in a similar manner to that by stimulation of H_2O_2-converting enzymes. Without the stimulatory participation of POX at 24 h, when H_2O_2 is elevated, lipid peroxidation is low in membranes, as shown by the MDA levels. In roots subjected to HA80 treatment, the activities of the H_2O_2-converting enzymes CAT and POX are elevated at 8 h, the H_2O_2 levels are low and lipid peroxidation is diminished, as shown by the MDA levels. At 24 h, the response is similar in regards to the enzymatic activity and the high levels of H_2O_2; however, lipid peroxidation remains low.

In foliage, plants responded to HA20 treatment at 8 h by the CAT-catalyzed conversion of H_2O_2, and both H_2O_2 and MDA levels are high. At 24 h, the H_2O_2 level decreases following treatment with HA40 and HA80, but the MDA level stays elevated. Following 8 h of HA40 treatment, the foliage responds by stimulating the activity of H_2O_2-converting enzymes, and, although the H_2O_2 level is elevated relative to the HA20 and HA80 treated samples, the lipid peroxidation at the membrane level is low, as indicated by the MDA levels. At 24 h, the CAT and APOX activities are stimulated, and the H_2O_2 level is elevated, but lipid peroxidation activity is low. Following HA80 treatment, the response in the foliage is different at both 8 h and 24 h compared to those observed following the HA20 and HA40 treatments. At both time points following HA80 treatments, the activities of H_2O_2-converting enzymes are stimulated, and the levels of both H_2O_2 and lipid peroxidation activity are lower than those following the HA20 and HA40 treatments.

Metabolomic analyses revealed that the proteins in the radicular membranes of maize and the expression of the genes encoding the proteins are indistinctly affected by HA concentration alone (Carletti et al., 2008). Authors such as Vasconcelos et al. (2009) observed that applying biostimulants with HA at concentrations between 71.3 g L^{-1} and 163.6 g L^{-1} induced SOD and APOX activity in maize but did not stimulate CAT activity. In contrast, it was recently observed (Cordeiro et al., 2011) that treating maize with HA affects ROS production and CAT stimulation.

4.2. Action by HA on aquaporin (OsTIP) expression

The gene expression responses in foliage include the down-regulation of *OsTIP*s that is directly dependent on increases in HA concentration. In the roots, this effect varies, although the

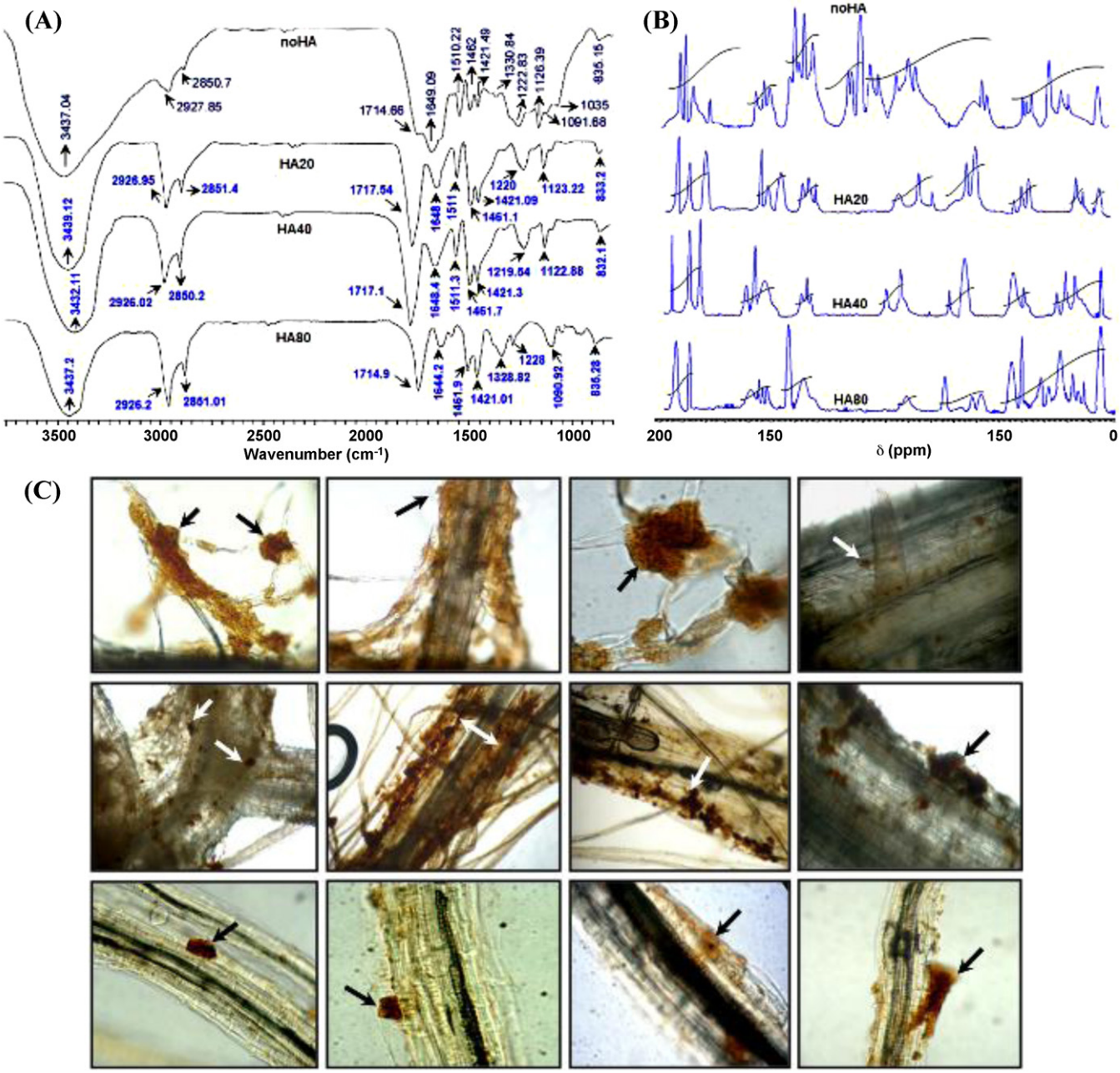

Fig. 3. (A) DRIFT spectra of added and clustered HA on roots of plants at different HA concentrations. The peaks were assigned according to Piccolo (2002). (B) ^{13}C-NMR spectra of added and precipitated HA on the roots of plants under different HA treatments assigned in accordance to Amir et al. (2010). (C) Images taken under the microscope of HA interacting with roots in plants. The images are interpreted in the context of the experimental conditions as HA clustering on the surface and/or epidermis of radicular tissues.

downregulation of the three *OsTIP*s under study is most pronounced as a result of the HA40 treatments. Only the HA80 treatments resulted in *OsTIP 1;2* overexpression. These results are in conflict with the previously observed behavior of *OsTIP*s under stress conditions, suggesting that the transcriptional response to HA action does not correspond to a typical type of stress.

The aquaporins serve as channels for H_2O_2, water and other metabolites inside cells (Maurel et al., 2002). *OsTIP*s gene expression in rice plants is elevated under conditions of stress by water deficit, cold and salinity (Liu et al., 1994). Recent works demonstrated that the *OsTIP 1;1*, *1;2* and *4;1* genes were overexpressed in the foliar and radicular tissues of rice plants that had been subjected to 15% PG-6000 and saline stress for 8 h (Li et al., 2008). Our results indicate that HA action at the cellular level relates to the antioxidative defense system with transcriptional mechanisms for aquaporins.

4.3. Interactions of HA with the roots

The ability of HA to form clusters on radicular surfaces was discussed by Asli and Neumann (2010). These authors suggest that clustering occurs if the HA has an adequate molecular size to cause colloidal stress. The imagens presented in Fig. 3 support the hypothesis that HA function by inducing colloidal stress. The consequences of this stress and the metabolic adaptation of plants reafirm the notion of this physiological condition and its regulation at the

cellular level, in which adaptation is linked to a response via antioxidative mechanisms at the genetic level in the tonoplast. These spectroscopic structural findings reaffirm what was observed from the HA–root interactions. The clustered HA present less complex structures with predominantly aliphatic features and possibly an elevated degree of oxygenated substitutions. The hypothesis proposed by Asli and Neumann (2010) can be justified by these results because colloidal stress may be one explanation for these plant responses to the presence of HA via oxidative stress mechanisms. As such, the theories proposed by other authors regarding the rupture of the HA super-molecule into smaller fragments by rhizosphere acidification, as well as the entrance of HA fragments into plants that exert hormone-like effects, could further support our findings (Canellas et al., 2010; Dobbss et al., 2010; Suthar, 2010).

5. Conclusions

Our work demonstrates that there are interactions between HA and the radicular system in plants, and the HA fractions that naturally interact with plant roots have a lower complexity than those HA added exogenously by treatments. Different HA concentrations affected the activity of some enzymes in the antioxidative defense system, thus controlling the ROS levels and on the occurrence of lipid peroxidation. The mechanisms of HA action also involve the expression of genes encoding aquaporins in the tonoplast. This work indicates that HA could act according to a physiological mechanism equivalent to that functioning in plants under stress conditions. HA be applied to estimulate antioxidative stress system and protect plants in water deficient soils.

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