RESEARCH ARTICLE

Changes in root development of *Arabidopsis* promoted by organic matter from oxisols

L.B. Dobbss[1], L.O. Medici[2], L.E.P. Peres[3], L.E. Pino-Nunes[3], V.M. Rumjanek[4], A.R. Façanha[5] & L.P. Canellas[1]

1 Laboratório de Solos, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil

2 Departamento de Ciências Fisiológicas, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil

3 Departamento de Ciências Biológicas, Escola Superior de Agricultura Luiz de Queiroz, Piracicaba, São Paulo, Brazil

4 Departamento de Química, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil

5 Laboratório de Biologia Celular e Tecidual, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil

Abstract

The presence of ramified and abundant lateral roots is fundamental to plant growth on highly weathered soils. In this work, the effects of humic acid (HA), fulvic acid and hexanic–methanolic (HM) extract, after alkaline extraction from topsoil of seven different oxisols, on the development of roots of *Arabidopsis thaliana* was evaluated. Furthermore, we used another emergent plant model, that is, micro-tom (MT) tomatoes with size similar to that of *Arabidopsis* to test the effects of HA. It was observed that both humic fractions and the HM extract were able to change the root development, improving the number of lateral roots and their development in comparison to control plants. The promotion of root growth by the three organic matter fractions was higher than that observed by 10^{-6} mol L^{-1} indole acetic acid. The treatment of MT tomato mutant, less sensitive to auxin, with HA did not promote the emergence of lateral roots, being an additional indication of auxin-like activities of HA. However, some organic matter fractions exhibited, besides promotion of lateral roots number, increase in the length of principal root, which is not a typical auxin effect, indicating that these substances could contain other physiologically active substances.

Introduction

The activity of root systems is essential for plant environmental adaptation. Changes in the root architecture and physiology affect water and nutrient absorption, especially in oxisols with low cation exchange capacity (CEC) (Hodge, 2004). Humic substances (HS) are the major component of soil organic matter in high weathering soils, and their importance has been largely recognised as it improves physical, chemical and biological properties of soil (Whitehead, 1963; Nardi *et al.*, 2004; Arancon *et al.*, 2005). The tropical agriculture is widely dependent on the organic matter level in soils, and its decline can result in low productivity and environmental degradation (Singh, 2000). In addition, organic fertilisation could reduce environmental pollution and increase quality of agricultural products (Atiyeh *et al.*, 2002; del Amor, 2006). Furthermore, HS can directly influence plant physiology, mainly root systems (Vaughan & Malcolm, 1985; Chen & Aviad, 1990; Nardi *et al.*, 2002a). Several works have been carried out to study the effects of HS on root growth (O'Donnell, 1973; Nardi *et al.*, 1988, 1997, 2002b; Canellas *et al.*, 2002), using water-soluble or low molecular weight HS that can form soluble complexes with metal ions (Pinton *et al.*, 1999; Chen *et al.*, 2003; Garcia-Mina *et al.*, 2004) or, alternatively, show hormone-like activity (O'Donnell, 1973; Pizzeghello *et al.*, 2001; Canellas *et al.*, 2002; Quaggiotti *et al.*, 2004; Zandonadi *et al.*, 2007) including expression induction of plasma membrane H^+-ATPase in plants (Canellas *et al.*,

2002; Quaggiotti *et al.*, 2004). However, there are relatively few studies that deal with biological stimulation produced by tropical soil organic matter (Canellas & Façanha, 2004).

The small crucifer *Arabidopsis thaliana* (L.) Heynh is a model plant widely used in genetic and physiology studies as it has a relatively small and simple genome with five chromosomes and a short life cycle lasting approximately 6 weeks (Estelle & Somerville, 1986). Curiously, it is not frequently used to study physiological effects of HS. In the present work, the HS effects were studied in *Arabidopsis* and tomato (*Solanum lycopersicum* L.) plants. Tomato is an economically relevant crop worldwide and is a plant model for physiological and genetic studies (Tanksley *et al.*, 1992). Furthermore, the morphogenetic pattern different from that of *Arabidopsis* qualifies tomato plant as an additional model for comparative studies (Pratt *et al.*, 1997).

The aim of this work was to evaluate the effect of humic acid (HA), fulvic acid (FA) and hexanic–methanolic (HM) extracts after alkaline organic matter extraction from topsoil of seven different Brazilian oxisols on *Arabidopsis* root development. To investigate whether auxin-signalling mechanisms are involved in HS effects on lateral root development, we investigated the HS biological activity on the diageotropica (*dgt*) mutant of a tomato miniature cultivar [micro-tom (MT)].

Materials and methods

Soil samples and chemical characterisation

Soil samples were collected from the superficial horizon of seven different profiles of oxisols [previously described by EMBRAPA (1980; 1999)] from different regions of Brazil: Xanthic Hapludox (P1) – Campos dos Goytacazes, Rio de Janeiro State, geographical coordinates: 21°45′S and 41°19′W Greenwich (Gr); Haploperox (P2) – Vacaria, Rio

Grande do Sul State, geographical coordinates: 28°30′S and 50°56′W Gr; Rhodic Humic Hapludox (P3) – Nova Lima, Minas Gerais State, geographical coordinates: 20°04′S and 43°58′W Gr; Sombrihumox (P4) – Nova Friburgo, Rio de Janeiro State, geographical coordinates: 22°16′S and 42°31′W Gr; Rhodustalf (P5) – Santo Angelo, Rio Grande do Sul State, geographical coordinates: 28°17′S and 54°15′W Gr; Hapludox (P6) – Mendes, Rio de Janeiro State, geographical coordinates: 22°31′S and 43°43′W Gr; Rhodic Hapludox (P7) – Brasília, Federal District, geographical coordinates: 15°47′S and 47°55′W Gr.

The main soil characteristics were determined according to the EMBRAPA (1979) soil handbook, and the results are shown in Table 1. The localisation of soils and the schematic representation of area under oxisols in Brazil are shown in Fig. 1.

Organic matter extraction

Alkaline-soluble HS were extracted with 0.1 mol L^{-1} NaOH, 1:10 (v/v), under N_2 atmosphere for 4 h followed by centrifugation (3000 g). The extraction procedure was repeated until the extracts presented zero absorbance at 280 and 465 nm. The separation of HA from the alkaline extract was obtained by acidification to pH 1 with 6 mol L^{-1} HCl. Dissolution and precipitation was repeated three times. HA were treated for 8 h with 200 mL of a mixture of HF and HCl (5 mL conc. HCl plus 5 mL conc. HF in 1 L of water). After centrifugation, HA were washed with water until a negative test against $AgNO_3$ and were dialysed (cut-off 14 kDa, Thomas Sc., Rancho Domingues, CA, USA) and freeze-dried. FA were obtained with XAD-8 resin (36 g mL^{-1} of FA solution). The column was washed with water (two volumes), and the retained FA were eluted with 0.01 mol L^{-1} NaOH. The salt content was decreased with the use of Amberlite IAR 120 H^+ resin (Rohm and Haas Company, Philadelphia, PA, USA). The FA were dialysed against ultra pure water

Table 1 Some pertinent characteristics of topsoil from oxisols used to isolate organic matter fractions

Oxisols	pH	Cation Exchange Capacity (cmol kg^{-1})	Sand (g kg^{-1})	Lime (g kg^{-1})	Clay (g kg^{-1})	Ki[a]	C (g kg^{-1})	C_{FA} (g kg^{-1})	C_{HA} (g kg^{-1})	C_{HM} (g kg^{-1})
P1	5.1	3.24	600	20	380	2.05	8.64	0.13	0.09	2.0
P2	5.1	8.56	50	230	720	1.77	43.00	0.61	0.05	9.7
P3	5.8	2.13	430	290	280	0.11	12.84	0.10	0.02	4.3
P4	4.8	3.03	140	110	750	1.62	29.49	0.32	0.08	2.6
P5	6.8	7.43	130	250	620	1.97	10.70	0.28	0.06	3.5
P6	5.3	6.50	400	110	490	1.53	12.50	0.16	0.06	4.1
P7	5.1	5.19	350	160	490	0.72	25.42	0.40	0.07	5.6

C, total organic carbon; C_{FA}, organic carbon as fulvic acids; C_{HA}, organic carbon as humic acids; C_{HM}, organic carbon as hexanic–methanolic extract; P1, Xanthic Hapludox; P2, Haploperox; P3, Rhodic Humic Hapludox; P4, Sombrihumox; P5, Rhodustalf; P6, Hapludox; P7, Rhodic Hapludox.

[a]$Ki = \frac{\frac{SiO_2\%}{60}}{\frac{Al_2O_3\%}{102}}$.

Figure 1 Schematic representation of area under oxisols in Brazil and localisation of different surface profiles used in this study. P1, Xanthic Hapludox; P2, Haploperox; P3, Rhodic Humic Hapludox; P4, Sombrihumox; P5, Rhodustalf; P6, Hapludox; P7, Rhodic Hapludox.

(SpectraPure, 700 Da cut-off; Thomas Sc.). After the alkaline extraction, the residual soil samples were washed three times with water and centrifuged. Water was discarded and the soil samples were put on a Soxhlet apparatus and extracted with a mixture of hexane and methanol (1:1, v:v) at 45°C for 7 days. Afterwards, the solvent was evaporated at low temperature with a rotary evaporator. Finally, these three fractions, HA, FA and HM extract, were used for the biological assays. The HA and FA fractions were characterised by elemental composition with a CHN analyser (Perkin-Elmer 1483; Perkin-Elmer, Norwalk, CT, USA). E_4/E_6 ratios were determined with 20 mg C L^{-1} of $NaHCO_3$ 0.05 mol L^{-1}. The absorbances at 465 and 665 nm were measured on a Shimadzu spectrophotometer DMS 8000 (Tokyo, Japan). The ratio of the absorbance values gave the E_4/E_6 ratio. The intensity of fluorescence was determined with the same solution of HA and FA fractions, and the emission spectra were obtained with fixed excitation at 465 nm, 5 nm slit, scan velocity 120 nm min^{-1} and automatic spectra. Total fluorophors intensity in the spectra was obtained by automatic electronic integration by the spectrometer software. The HM extract was characterised only by their elemental composition.

Arabidopsis assay

Seeds of *A. thaliana* ecotype Columbia 4 were surface sterilised with 95% (v/v) ethanol for 5 min and with 0.5% (w/v) NaClO for 7 min. After rinsing five times with distilled water, seeds were germinated and grown in a mini-hydroponic system that we have developed, in which the roots grow between layers of different materials measuring 6 × 5 cm. A layer of thin white synthetic fabric is placed on a layer of germinating paper. These two layers are wet with distilled water, and 10 seeds are placed along the 5-cm margin on the synthetic one, then a layer of transparent plastic is placed on the seeds. A black plastic cover, previously folded, is placed around the three layers, leaving the 5-cm borders opened. This plate of layers is left overnight at 10°C, and then, each plate is placed vertically in one cell of an ice tray with distilled water (Fig. 2). These ice trays are placed in a plant growth cabinet with a photoperiod of 14 h of light and 8 h of darkness, a light intensity of 90 μmol m^{-2} s^{-1} and temperature of 22–24°C. After 5 days in the cabinet, the five best plants are left in each plate and the germinating papers are rinsed four times with 1 mL of solutions containing organic matter fractions. A minimum of distilled water is left in each ice tray cell to irrigate the plates by capillarity. After 3 days of treatment, the germinating papers are rinsed four times with 1 mL of a modified Hoagland & Arnon (1950) solution with 1 mmol L^{-1} of N, as ammonium nitrate, described by Medici *et al.* (2004). This low N level is used to avoid the root branching by nitrate (Zhang *et al.*, 1999; Lea & Azevedo, 2006). Three days later, this rinsing with nutrient solution was repeated. After 2 weeks of growth, the black covers and the germinating papers were taken off the plates and the roots, kept between the two other layers, were stained with toluidine blue (0.05%) and the images were digitalised by scanner (300 d.p.i.) for the root analysis by the Delta-T scan software (Delta-T Devices, Cambridge, UK). Two plants in the central position of the plates were chosen for the evaluation of number of lateral roots, length of principal root and density and length of lateral roots. Two experiments were carried out with this system. In the first, the treatments given were HA, FA and HM extract isolated from one representative oxisol (Sombrihumox) at 0, 3, 30 and 300 mg L^{-1} (pH 5.8). In this experiment, there was also a treatment with indole acetic acid (IAA; Sigma Co., St Louis, MO, USA) at 10^{-6} mol L^{-1}, which is frequently used in *Arabidopsis* assays (Parry & Estelle, 2006). In the second experiment, the treatments given were the organic matter fractions from the seven oxisols at 40, 100 and 100 mg L^{-1} for HA, FA and HM, respectively. These concentrations were expected to be enough to stimulate changes in plant roots and to avoid inhibition effects.

To analyse the influences of HS and HM extract, the treatments were randomised with eight repetitions. The data from each organic matter fraction (HA, FA and HM) were analysed by the statistical procedure analysis of variance from SAEG software systems from Universidade Federal de Viçosa, and the means were tested by least significant
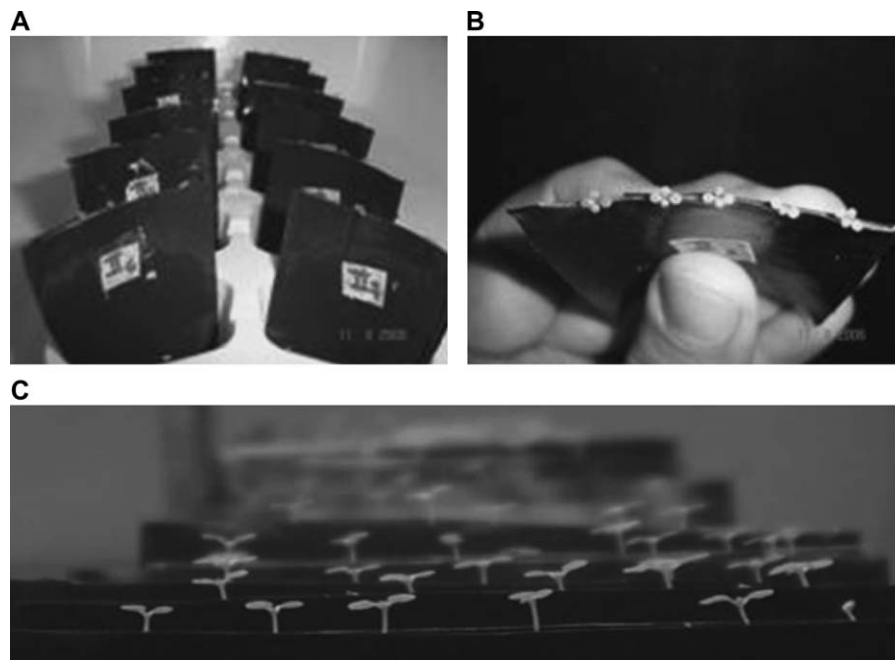
Figure 2 Aspects of the experiment. (A) Mounted experiment. (B and C) Visual aspect of the plants.

difference (LSD), $P < 0.05$ test. The means were normalised with respect to control plants (control = 100%) to compare with IAA results.

Diageotropic (*dgt*) assay

The *dgt* tomato mutant *Solanum lycopersicum* L. is characterised by horizontal shoot and root growth, thin stems, hyponastic leaves and lack of lateral roots and is insensitive to exogenous IAA (Kelly & Bradford, 1986). The mutation is in a single gene and was introgressed into dwarf tomato (MT). Thus, MT-*dgt* is a model to test if a given substance acts like auxin. Seeds of MT-*dgt* were surface sterilised with 95% (v/v) ethanol for 5 min and with 0.7% (w/v) NaClO for 15 min. These seeds were washed and inoculated into Murashige–Skoog medium (Murashige & Skoog, 1962) with half of macronutrient concentration, 0.3% saccharose and 0.8% agar. HA from P1, P7 and P4 were used at 50 mg L^{-1}, and the plates were incubated for 10 days at 25°C and 16 h of photoperiod. After incubation, the number of emerged lateral roots was evaluated. The experiment was entirely randomised, with 10 repetitions for each treatment. The MT tomato without the *dgt* mutation was also used as a control and is referred in the text as wild type.

Results

Organic matter was extracted from oxisols with different chemical characteristics (Table 1). The Ki index (i.e. the ratio between Si and Al on molar basis) ranged from 0.11 to 2.05, an indicative of high weathering degree of these oxisols. The clay content also varied widely (280–750 g kg^{-1}), but because of their low activity, the CEC is very low (2.13–8.56 cmol C kg^{-1}) and pH values are found in an acidic range (from 4.8 to 6.8). The surface chemical properties of these soil samples are affected by C content that varied widely (8.64–43.0 g C kg^{-1}). Despite the relative high C content of soil samples, the soluble humified organic matter comprises less than 10% C (Table 1).

Soil organic matter from these oxisols is characterised by low HA/FA ratio, that is, the major alkaline-soluble humic fraction is FA. However, the highest content of soluble C was found in HM extract (Table 1). Chemical characterisation of HA and FA fractions is shown in Table 2. HA present higher C content, lower E_4/E_6 ratio and higher intensity of fluorescence at 456 nm than FA. The HM extract has low C content but high N content, affording low C/N ratios (Table 3). Despite the accentuated chemical differences among HA, FA and HM extract isolated from different oxisols (Tables 2 and 3), all organic fractions promote changes in *Arabidopsis* root development.

The increase in root growth is one of the major effects of HS, and it also depends on plant species and HS source and concentration (Vaughan & Malcolm, 1985). To find an optimal humic concentration, a dose–response curve of HA, FA and HM extract versus number of lateral roots emerged was designed for HS from Sombrihumox (Fig. 3). A quadratic model describes HA and FA effects

Table 2 Elemental composition, E_4/E_6 ratio and intensity of fluorescence of humic and fulvic fractions isolated from different oxisols

Oxisols	C (g kg^{-1})	H (g kg^{-1})	N (g kg^{-1})	O (g kg^{-1})	Ash (g kg^{-1})	E_4/E_6	Intensity of Fluorescence
Humic acids							
P1	562.5	64.0	52.5	536.7	106.0	7.2	107
P2	542.0	64.1	58.9	524.1	66.0	6.9	158
P3	503.1	51.1	49.9	605.8	116.0	6.7	81
P4	432.0	56.6	57.8	653.7	86.0	4.0	150
P5	468.0	60.9	46.6	608.7	66.0	6.4	82
P6	504.8	61.1	68.5	526.8	38.0	5.4	115
P7	536.5	62.7	52.9	540.1	82.0	8.9	114
Mean (SD)	507 (45)	60 (5)	55 (7)	571 (51)	80 (26)	6.5 (2)	115 (30)
Fulvic acids							
P1	535.3	66.5	86.0	509.8	170	9.5	28
P2	286.7	52.4	60.9	797.6	160	13.2	16
P3	271.0	57.7	89.6	779.3	100	11.8	36
P4	498.1	60.1	78.0	561.4	240	9.3	31
P5	253.4	44.7	48.5	851.0	130	10.8	40
P6	328.4	44.6	60.7	764.0	180	9.3	42
P7	462.7	63.6	82.6	588.7	150	10.0	19
Mean (SD)	376 (78)	55.7 (9)	72 (16)	693 (135)	164 (44)	10.6 (1)	30 (10)

P1, Xanthic Hapludox; P2, Haploperox; P3, Rhodic Humic Hapludox; P4, Sombrihumox; P5, Rhodustalf; P6, Hapludox; P7, Rhodic Hapludox; SD, standard deviation.

but not the HM extract effects. The maximum effects of the three organic matter fractions obtained by first derivative of these quadratic equations were higher than the effect of IAA at 10^{-6} mol L^{-1} (Fig. 4).

The biological activity of soil organic matter fractions, such as the HS, may be evaluated by plant growth response, as a result of direct effects of HS on plant metabolism (Vaughan & Malcolm, 1985; Nannipieri *et al.*, 1993). In this work, we found that humic fractions extracted from soils from the same soil group have similar bioactivity as shown by the plant root development (Figs 5–8). Nevertheless, HS can influence plant growth by mechanisms not yet very clear. Here, the different HS effects can be explained by the high chemical variability among the humic fractions of the oxisols (Table 1).

Table 3 Elemental composition and ash content of hexanic–methanolic extracts isolated from different oxisols

Oxisols	C (g kg^{-1})	H (g kg^{-1})	N (g kg^{-1})	Ash (g kg^{-1})
P1	99	15	8	18
P2	191	45	48	27
P3	79	8	7	14
P4	168	31	48	26
P5	185	39	42	31
P6	123	17	12	25
P7	99	16	8	17
Mean (SD)	135 (46)	24 (14)	25 (20)	22 (6)

P1, Xanthic Hapludox; P2, Haploperox; P3, Rhodic Humic Hapludox; P4, Sombrihumox; P5, Rhodustalf; P6, Hapludox; P7, Rhodic Hapludox; SD, standard deviation.

The visual aspects of root systems treated with HA, FA and HM extract from different oxisols are shown in Fig. 5, and their results are shown in Figs 6–8. The HA1 yields the highest number of lateral roots and HA5 the lowest, but both exhibited statistically higher values than control plants. Curiously, HA5 promotes the largest enhancement on lateral root length (Fig. 6B). Smaller lateral root lengths were observed for plants treated with HA1, HA2, HA3 and HA4, but this effect was not observed when HA from P5, P6 and P7 were used. Lateral root density (i.e. number of lateral roots per millimetre of principal root) was affected by HA treatment (Fig. 6D). The HA isolated from P1 affords the highest number of lateral roots by principal root length unit.

The FA treatments also affect the number of lateral roots and root length (Fig. 7), with the largest effects exhibited by FA2, FA5 and FA7. Primary root growth was strongly inhibited by FA isolated from P3 and P6. This effect was not observed with FA from P1, P2 and P7. An increase of two to three times of lateral root density was observed with the FA and HA treatments. FA extract from P2 promotes the highest stimulation on number of lateral roots followed by FA from P5 and P7. The smallest stimulation was observed with FA6 and FA3, which were nevertheless larger than that observed in control plants. The major effects of FA were on length of lateral roots, with FA4 and FA5 promoting an 11-fold stimulation with respect to control plants (Fig. 7B), while the smaller stimulation (twofold) was found in FA2 and FA7.
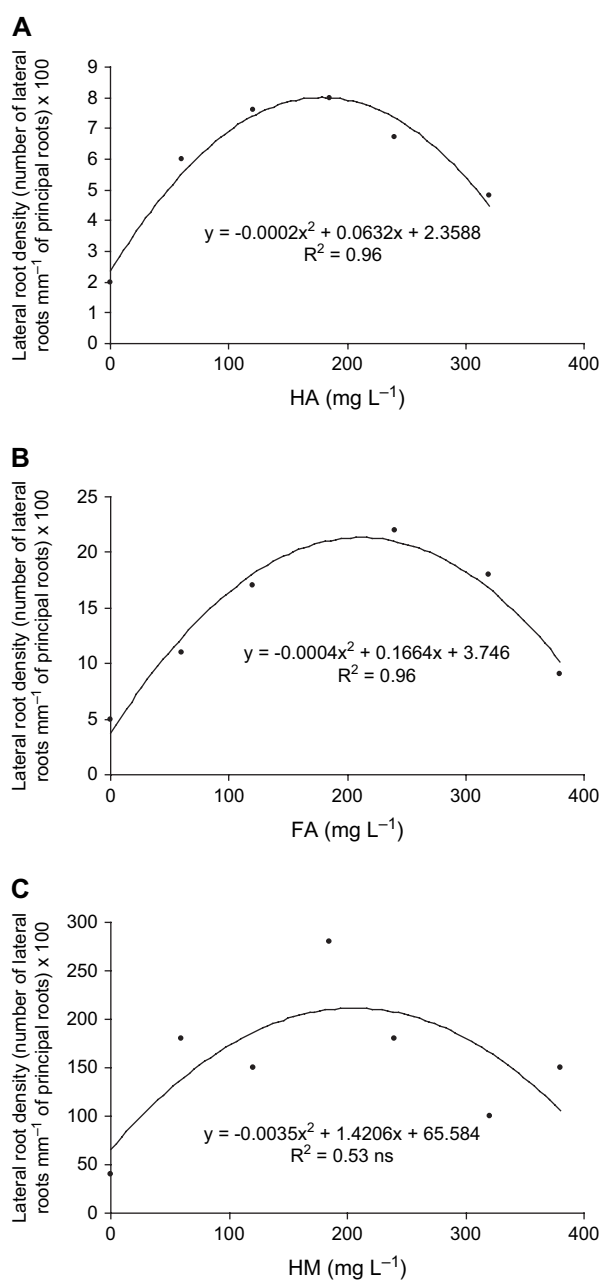
A

$y = -0.0002x^2 + 0.0632x + 2.3588$
$R^2 = 0.96$

B

$y = -0.0004x^2 + 0.1664x + 3.746$
$R^2 = 0.96$

C

$y = -0.0035x^2 + 1.4206x + 65.584$
$R^2 = 0.53$ ns

Figure 3 Response of *Arabidopsis* lateral root density at different concentrations of (A) humic acids (HA), (B) fulvic acids (FA) and (C) hexanic–methanolic (HM) extract from Sombrihumox soil.

The HM extract obtained from oxisols after alkaline extraction also promotes *Arabidopsis* root growth, with enhancements from 150% to 264% for number of lateral roots and from 405% to 2280% for length of lateral roots (Fig. 8A and Fig. 8B). This dramatic effect on root growth is observed mainly for the HM3 extract followed by HM4 and HM5 extracts. However, the effects on length of principal roots are dependent on the source.
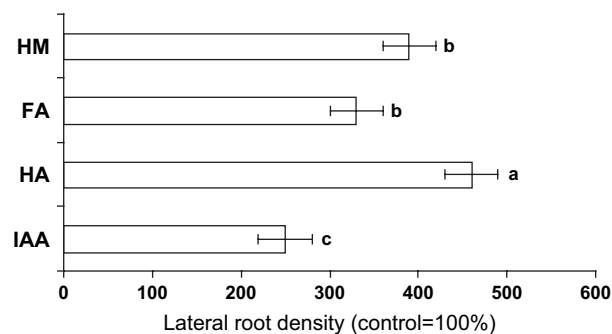
Figure 4 Increase in the lateral root density relative to control at the concentration of 100% of the stimuli, obtained by the first derivative of the quadratic regression of the effect of concentration of humic acids (HA; 190 mg L^{-1}), fulvic acids (FA; 220 mg L^{-1}), hexanic–methanolic extract (HM; 100 mg L^{-1}) and indole acetic acid (IAA; 10^{-6} mol L^{-1}). Means followed by different letters are statistically different by the LSD $P < 0.05$ test.

For example, HM2, HM3 and HM7 extracts enhance the length of the principal root, while HM1, HM4 and HM5 extracts shorten the principal root length.

The HA isolated from P1, P7 and P4 do not have the ability to induce lateral root formation in *dgt* mutant, which have a defective gene for auxin response, while the wild-type tomato exhibited lateral root development after treatment with these HA isolates (Fig. 9).

Discussion

Root architecture changes with hormonal and environment stimulations, and the amount of lateral roots is influenced by a diverse and very complex series of endogenous and exogenous signals (Torrey, 1986; López-Bucio *et al.*, 2003; Sorin *et al.*, 2005; Zandonadi *et al.*, 2007). For a very long time, substances with physiological activity have been found in the humified organic matter, which are able to improve root growth (Bottomley, 1917; O'Donnell, 1973). The physiological activity of organic matter from a predominant Brazilian soil class was studied in the present work.

The process of oxisols formation is characterised by intense transformation of parental matter by chemical weathering and consequently intense removal of SiO$_4$. Thus, Moniz (1972) indicated that SiO$_2$/Al$_2$O$_3$ molar ratio (Ki) is a good index to evaluate weathering degree, and according to this index, the studied oxisols have the following weathering sequence: P1<P5<P2<P4<P6< P7<P3. The lower E$_4$/E$_6$ ratio and higher intensity of fluorescence at 456 nm exhibited in HA than in FA is in accordance with the general theory of humification processes that suggests that HA is a more condensed fraction in comparison with FA (Stevenson, 1994).
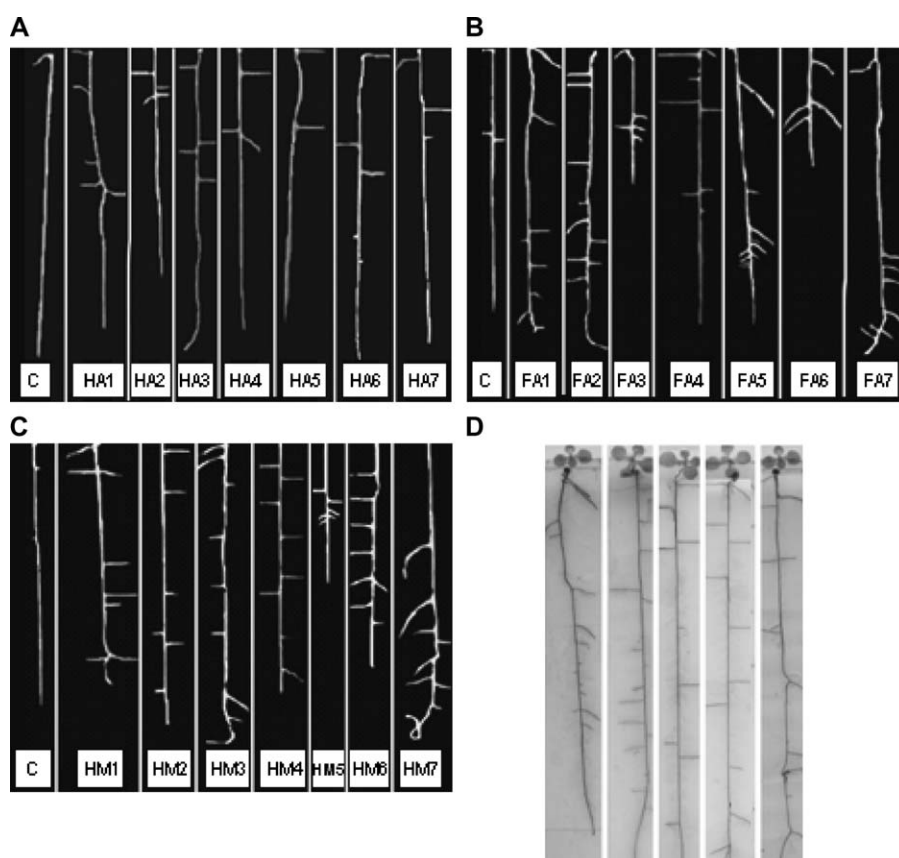
Figure 5 Roots of *Arabidopsis* treated with (A) humic acids (HA; 40 mg L^{-1}), (B) fulvic acids (FA; 100 mg L^{-1}) and (C) hexane–methanolic extract (HM; 100 mg L^{-1}) from oxisols after the extraction of the alkali-soluble humic fraction. (D) Roots of *Arabidopsis* treated with toluidine blue (0.05%). C, control; 1, Xanthic Hapludox; 2, Haploperox; 3, Rhodic Humic Hapludox; 4, Sombrihumox; 5, Rhodustalf; 6, Hapludox; 7, Rhodic Hapludox.

Overall, the effects on *Arabidopsis* indicate the presence of hormone-like substances in the organic matter from oxisols. The increase in number of lateral root, exhibited mainly by HA1, HA2, HA3, HA6, FA2, FA5, FA7, HM1, HM3, HM4, HM6 and HM7, indicates the presence of auxin-like substances (Dong *et al.*, 2006). The inhibition of principal root length, exhibited mainly by HA2, FA3, FA6 and HM5, is also a typical behaviour of exogenous addition of higher IAA concentration. The observed results from *dgt* tomato mutant also support the putative presence of auxin-like substances in HA isolated from P1, P7 and P4. Some authors employing different methods determined the presence of structural fragments similar to auxins in HS (Muscolo *et al.*, 1998; Canellas *et al.*, 2002). Thereafter, Canellas *et al.* (2002) and Zandonadi *et al.* (2007) found that HA interacts with cell membranes similarly to auxin. However, the presence of atypical auxin responses such as stimulation of growth of principal roots observed with HA2, FA3, FA6 and HM5 was previously reported by Zandonadi *et al.* (2007) who also observed that exogenous IAA application at very low con-

centration (10^{-15} M) induced H$^+$ pump activation and elongations of principal root axis in initial stage of root development. However, it is possible that the presence of other physiologically active substances, such as alkamides, enhances principal root growth, but the ability of the root system to respond to it was found to be independent of auxin signalling (Ramírez-Chavez *et al.*, 2004).

It is interesting that *Arabidopsis* treated with some organic matter fractions exhibited a kind of compensation between number and length of lateral root, as these organic fractions increase the number of lateral root and decrease the lateral root length, as observed in HA1, HA2, HA3, FA2, FA7, HM6 and HM7. As the plants were grown under low nitrate level, this compensation between number and length of lateral roots is in agreement with the model presented by Zhang *et al.* (1999).

The effect on root development is obtained with low concentration of organic matter, and, in general, when the concentration is high, an inhibition effect is observed. The HS dose–response curve is bell shaped, typical of hormone assays (Vaughan & Malcolm, 1985). The maximum effects
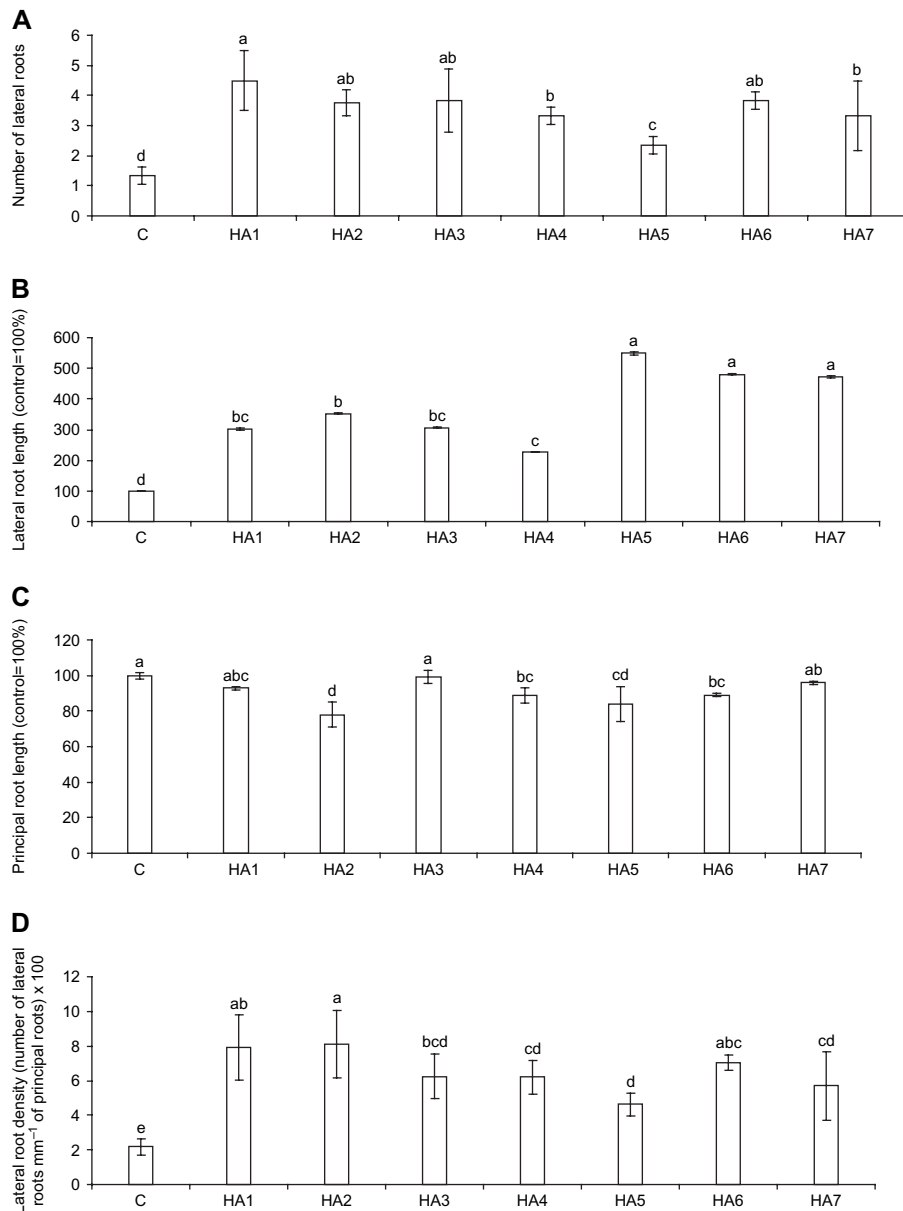
Figure 6 Effect of humic acids (HA; 40 mg L^{-1}), isolated from different Brazilian oxisols, on *Arabidopsis thaliana* root development. (A) Number of lateral roots, (B) lateral root length, (C) principal root length and (D) lateral root density are shown. Data represent means of eight plants ± standard deviation. Lateral root length and principal axis length are normalised in relation to control (100%). Means followed by different letters are statistically different by the least significant difference *P* < 0.05 test. C, control; 1, Xanthic Hapludox; 2, Haploperox; 3, Rhodic Humic Hapludox; 4, Sombrihumox; 5, Rhodustalf; 6, Hapludox; 7, Rhodic Hapludox.

of organic substances from soil Sombrihumox (P4) on number of lateral roots were higher than the effect of 10^{-6} M IAA. *Arabidopsis* plants treated with this IAA level exhibited higher number of lateral roots than the control plants, which is in agreement with the report of Dong *et al.* (2006).

Arabidopsis is a well-studied plant, and a genetic investigation identified genes with auxin-signalling function,

and two large protein families were extensively identified: auxin response factor (22 identified genes) and Aux/IAA (29 identified genes) (Parry & Estelle, 2006). Gray *et al.* (2001) have shown that auxin could act by promoting the degradation of the Aux/IAA transcriptional repressors through the action of the ubiquitin protein ligase SCF^{TIR1}. Recently it was reported that TIR1 is an auxin receptor that mediates Aux/IAA degradation
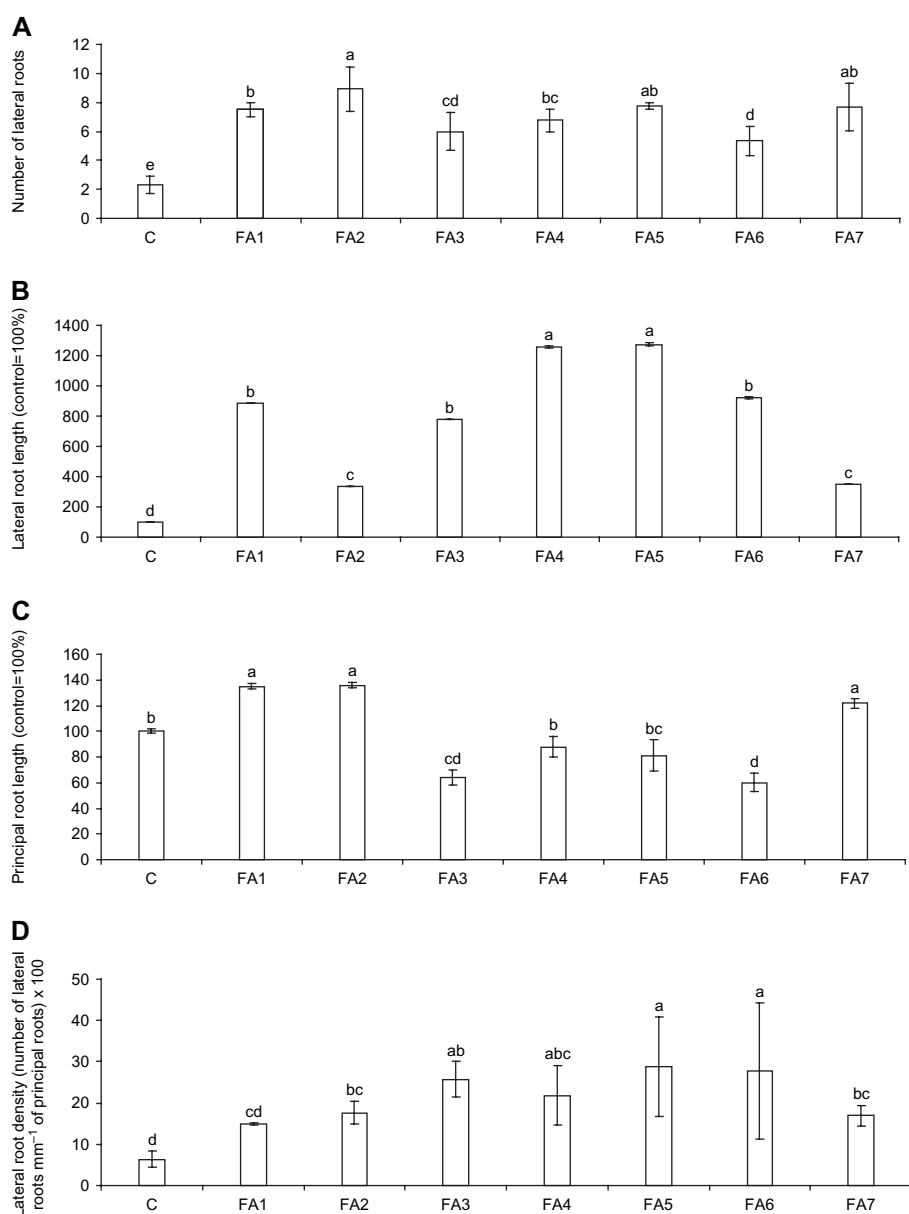
Figure 7 Effect of fulvic acids (FA; 100 mg L^{-1}), isolated from different Brazilian oxisols, on *Arabidopsis thaliana* root development. (A) Number of lateral roots, (B) lateral root length, (C) principal root length and (D) lateral root density are shown. Data represent means of eight plants ± standard deviation. Lateral root length and principal axis length are normalised in relation to control (100%). Means followed by different letters are statistically different by the least significant difference *P* < 0.05 test. C, control; 1, Xanthic Hapludox; 2, Haploperox; 3, Rhodic Humic Hapludox; 4, Sombrihumox; 5, Rhodustalf; 6, Hapludox; 7, Rhodic Hapludox.

and auxin-regulated transcription (Dharmasiri *et al.,* 2005; Kepinski & Leyser, 2005).

Despite the increasing physiological and molecular information on lateral root formation, the hormonal mechanism that controls lateral root initiation and orientation remains poorly understood (Aloni *et al.,* 2006; Parry & Estelle, 2006). According to Aloni *et al.* (2006),

the primary signal for cell differentiation and formation of lateral roots is promoted by auxin, which induces differentiation by a descending movement from shoot to root apex through the vascular cylinder (pericycle or differentiated xylem). Cytokinin inhibits lateral root emission on the root apex, and ethylene (C_2H_4) is locally produced in the cells of the protoxylem, which determines
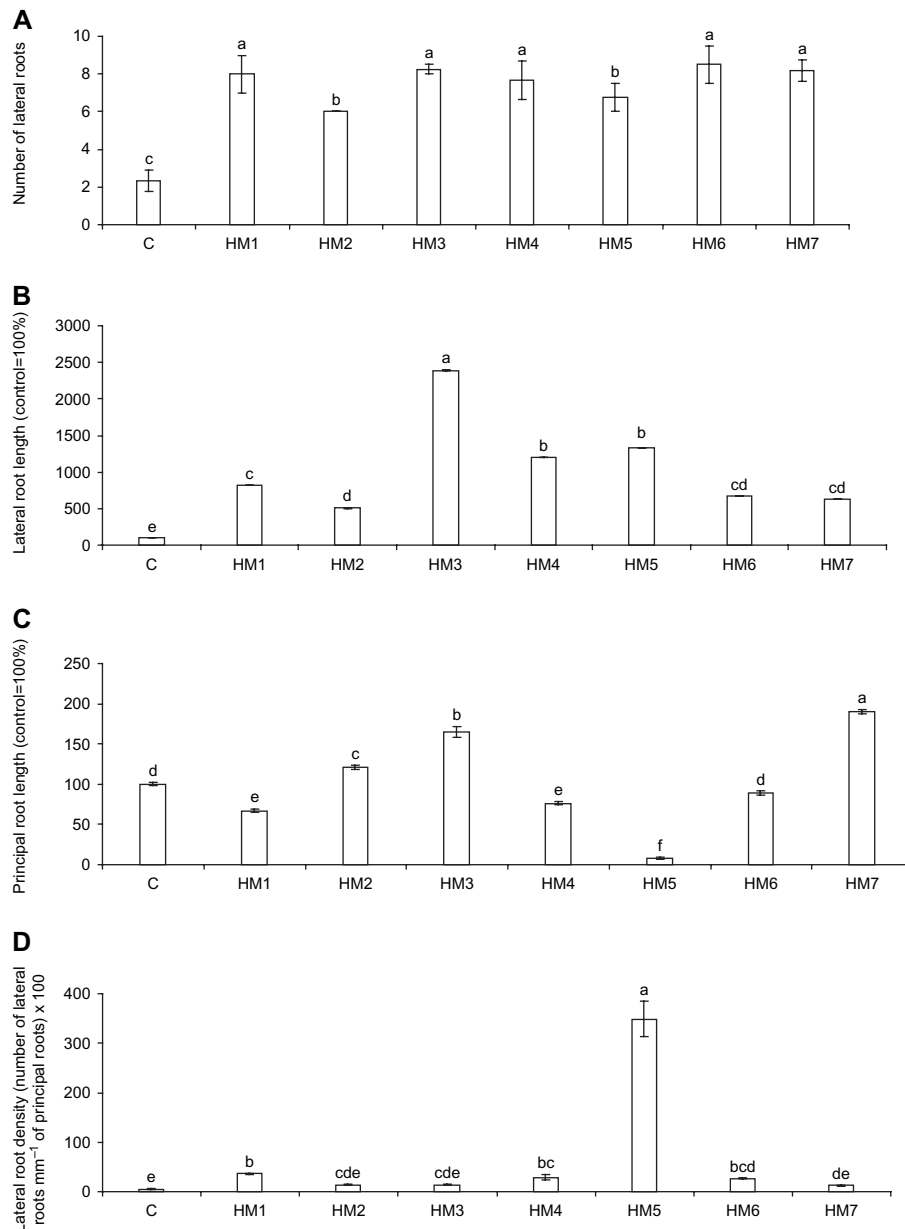
Figure 8 Effect of the hexanic–methanolic (HM) extracts from different Brazilian oxisols on *Arabidopsis thaliana* root development. (A) Number of lateral roots, (B) lateral root length, (C) principal root length and (D) lateral root density are shown. Data represent means of eight plants ± standard deviation. Lateral root length and principal axis length are normalised in relation to control (100%). Means followed by different letters are statistically different by the least significant difference *P* < 0.05 test. C, control; 1, Xanthic Hapludox; 2, Haploperox; 3, Rhodic Humic Hapludox; 4, Sombrihumox; 5, Rhodustalf; 6, Hapludox; 7, Rhodic Hapludox.

the exact location of mitosis sites for cell initiation. Ethylene production is a result of a high concentration of auxin in the protoxylem cells.

Probably, the complex, heterogeneous, natural, humified organic matter may act as a buffer, either absorbing or liberating signalling molecules according to modifications in the rhizosphere, such as the acidification brought about by the activity of plasma membrane H⁺-ATPase (Canellas

et al., 2002; Zandonadi *et al.*, 2007) or exudation of organic acids (Façanha *et al.*, 2002), thus behaving as a regulator of hormonal balance with respect to lateral root emergence.

In this work, we show that relatively high (HA > 14 kDa) and low (FA < 700 Da) molecular weight HS can induce changes in *Arabidopsis* root development, and according to Muscolo *et al.* (2006), HS have very
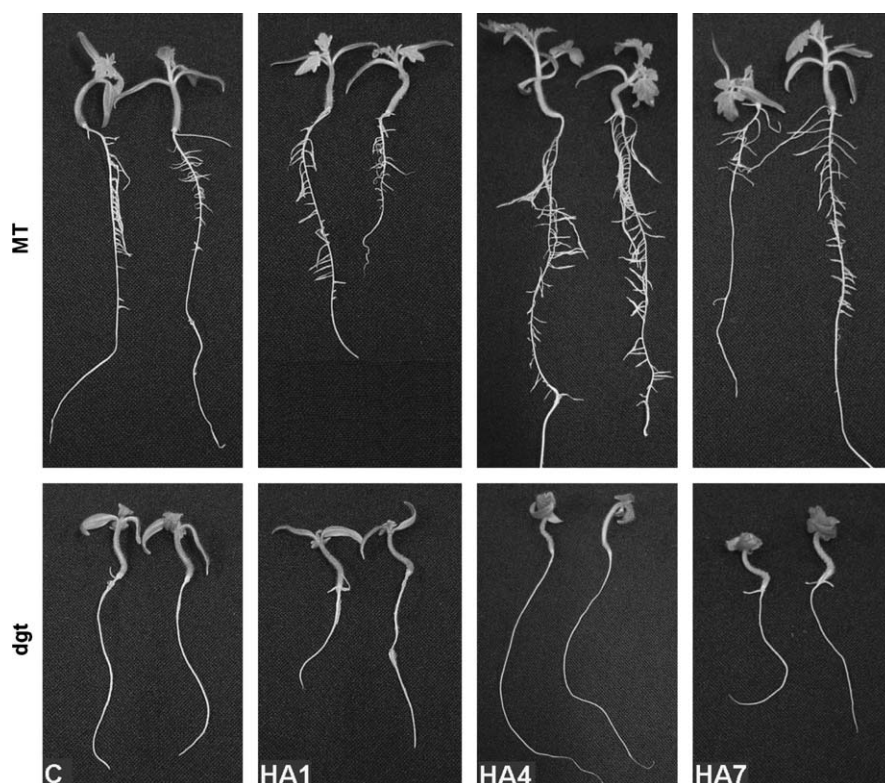
Figure 9 Effect of humic acids (HA; 40 mg L^{-1}) isolated from Xanthic Hapludox (P1), Sombrihumox (P4) and Rhodic Hapludox (P7) on the induction of lateral roots in micro-tom (MT; control) and dgt (an auxin-insensitive mutant) plants. Plants were cultivated *in vitro* for 30 days in Murashige–Skoog medium. No lateral root growth is noticed in dgt plants, suggesting that the various HA need the auxin-signalling transduction pathway to be active.

complex structures and it is very difficult to identify the relationship between the single compounds of HS and their biological activity. Recently, Sutton & Sposito (2005) reviewed the concept of structural features of HS and found no reasons to exclude biopolymers or any substances with well-defined biochemical structures as part of organic matter. Piccolo *et al.* (1996) provide evidence that the apparent size of humic fragments changes drastically with addition of simple organic acids. The effect of addition of carboxylic acid to humic fractions indicates that aggregate disruption is greatest when more hydrophobic humic materials are combined with organic molecules containing both hydrophobic and hydrophilic segments. This result, in turn, suggests that humic materials are held together by hydrophobic interactions, which are easily disrupted when simple organic molecules penetrate large hydrophobically bonded associations and separate them into smaller, higher energy H-bonded associations (Piccolo, 2002). Simpson (2002) examined HS solutions and observed both aggregation and disaggregation behaviour consistent with that described by Piccolo (2002). Changes brought about by organic acid exudation from plant roots or by H$^+$ pumping by plasma membrane ATPase may liberate substances

from the HS matrix, which promote clear changes in root development, as those observed in this study.

Acknowledgements

References

Aloni R., Aloni E., Langhans M., Ullrich C.I. (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Annals of Botany*, **97**, 883–893.

del Amor F.M. (2006) Growth, photosynthesis and chlorophyll fluorescence of sweet pepper plants as affected by the cultivation method. *Annals of Applied Biology*, **148**, 133–139.

Arancon N.Q., Edwards C.A., Bierman P., Metzger J.D., Lucht C. (2005) Effect of vermicomposts produced from cattle, food waste and paper waste on growth and yield of peppers in the field. *Pedobiologia*, **49**, 297–306.

Atiyeh R.M., Lee C.A., Edwards C.A., Arancon N.Q., Metzger J.D. (2002) The influence of humic acids derived from earthworm-processed organic wastes on plant growth. *Bioresource Technology*, **84**, 7–14.

Bottomley W.B. (1917) Some effects of organic-promotion substances auxinones on the growth of *Lemna minor* in mineral cultural solutions. *Proceedings of the Royal Society of London Biology*, **89**, 481–505.

Canellas L.P., Façanha A.R. (2004) Relationship between nature of soil humified fractions and their bioactivity. *Pesquisa Agropecuária Brasileira*, **39**, 233–240.

Canellas L.P., Olivares F.L., Façanha A.L.O., Façanha A.R. (2002) Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H$^+$-ATPase activity in maize roots. *Plant Physiology*, **130**, 1951–1957.

Chen Y., Aviad T. (1990) Effects of humic substances on plant growth. In *Humic Substances and Crop Science: Selected Readings*, pp. 161–186. Eds P. MacCarthy, C.E. Clapp, R.L. Malcolm and P.R. Bloom. Madison, WI: Soil Science Society of America.

Chen J., Gu B., Royer R.A., Burgos W.D. (2003) The roles of natural organic matter in chemical and microbial reduction of ferric ion. *Science of the Total Environment*, **307**, 167–178.

Dharmasiri N., Dharmasiri S., Estelle M. (2005) The F-box protein TIR1 is an auxin receptor. *Nature*, **435**, 441–445.

Dong L., Wang L., Zhang Y., Zhang Y., Deng X., Xue Y. (2006) An auxin-inducible F-box protein CEGENDUO negatively regulates auxin-mediated lateral root formation in *Arabidopsis*. *Plant Molecular Biology*, **60**, 599–615.

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária (1979) *Manual de métodos de análise de solo*. Rio de Janeiro, Brazil: Serviço Nacional de Levantamento e Conservação de Solos.

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária (1980) *Boletim Técnico n° 62*. Rio de Janeiro, Brazil: Serviço Nacional de Levantamento e Conservação de Solos.

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária (1999) *Sistema Brasileiro de Classificação de Solos*. Rio de Janeiro, Brazil: Centro Nacional de Pesquisa de Solos.

Estelle M.A., Somerville C.R. (1986) The mutants of *Arabidopsis*. *Trends in Genetics*, **2**, 89–93.

Façanha A.R., Façanha A.L.O., Olivares F.L., Guridi F., Santos G.A., Velloso A.C.X., Rumjanek V.M., Brasil F., Schripsema J., Braz-filho R., Oliveira M.A., Canellas L.P. (2002) Bioatividade de ácidos húmicos: efeito sobre o desenvolvimento radicular e sobre a bomba de prótons da membrana plasmática. *Pesquisa Agropecuária Brasileira*, **37**, 1301–1310.

Garcia-Mina J.M., Antolin M.C., Sanchez-Diaz M. (2004) Metal-humic complexes and plant micronutrient uptake: a study based on different plant species cultivated in diverse soil types. *Plant and Soil*, **258**, 57–68.

Gray W.M., Kenpiski S., Rouse D., Leyser O., Estelle M. (2001) Auxin regulates SCFTIR1-dependent degradation of AUX/AIA proteins. *Nature*, **414**, 271–276.

Hoagland D.R., Arnon D.I. (1950) *The Water Culture Method for Growing Plants Without Soil*. California Agricultural Experiment Station. Circular 347.

Hodge A. (2004) The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist*, **162**, 9–24.

Kelly M.O., Bradford K.J. (1986) Insensitivity of the *diageotropica* tomato mutant to auxin. *Plant Physiology*, **82**, 713–717.

Kepinski S., Leyser O. (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature*, **435**, 446–451.

Lea P.J., Azevedo R.A. (2006) Nitrogen use efficiency. 1. Uptake from the soil. *Annals of Applied Biology*, **149**, 243–247.

López-Bucio J., Cruz-Ramírez A., Herrera-Estrella L. (2003) The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology*, **6**, 280–287.

Medici L.O., Azevedo R.A., Smith R.J., Lea P.J. (2004) The influence of nitrogen supply on antioxidant enzymes in plant roots. *Functional Plant Biology*, **31**, 1–9.

Moniz A.C. (1972) Mineralogia do Solo. In *Elementos de Pedologia*, pp. 391–408. Ed. A.C. Moniz. São Paulo, Brazil: Editora da Universidade de São Paulo.

Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, **15**, 437–497.

Muscolo A., Cutrupi S., Nardi S. (1998) IAA detection in humic substances. *Soil Biology & Biochemistry*, **30**, 1199–1201.

Muscolo A., Sidari M., Attinà E., Francioso O., Tugnoli V., Nardi S. (2006) Biological activity of humic substances is related to their chemical structure. *Soil Chemistry*, **71**, 75–85.

Nannipieri P., Grego S., Dell'Agnola G., Nardi S. (1993) Proprietà biochimiche e fisiologiche della sostanza organica. In *Ciclo della sostanza organica nel suolo: aspetti agronomici, chimici, ecologici y selvicolturali*, pp. 67–78. Ed. P. Nannipieri. Bologna, Italy: Patron.

Nardi S., Arnoldi G., Dell'Agnola G. (1988) Release of the hormone-like activities from *Allophobora rosea* (Sav.) and *Allophobora caliginosa* (Sav.) feces. *Canadian Journal of Soil Science*, **68**, 563–567.

Nardi S., Reniero F., Concheri G. (1997) Soil organic matter mobilization by root exudates of three maize hybrids. *Chemosphere*, **35**, 2237–2244.

Nardi S., Pizzeghello D., Muscolo A., Vianello A. (2002*a*) Physiological effects of humic substances on higher plants. *Soil Biology & Biochemistry*, **34**, 1527–1536.

Nardi S., Sessi E., Pizzeghello D., Stuarto A., Rella R., Parvoli G. (2002*b*) Biological activity of soil organic matter mobilized by root exudates. *Chemosphere*, **46**, 1075–1081.

Nardi S., Morari F., Berti A., Tosoni M., Giardini L. (2004) Soil organic matter properties after 40 years of different

use of organic and mineral fertilizers. *European Journal of Agronomy*, **21**, 357–367.

O'Donnell R.W. (1973) The auxin-like effects of humic preparations from leonardite. *Soil Science*, **116**, 106–112.

Parry G., Estelle M. (2006) Auxin receptors: a new role for F-box proteins. *Current Opinion in Cell Biology*, **18**, 152–156.

Piccolo A. (2002) The supramolecular structure of humic substances: a novel understanding of humus chemistry and implications in soil science. *Advanced Agronomy*, **75**, 57–134.

Piccolo A., Nardi S., Cancheri G. (1996) Macromolecular changes of soil humic substances induced by interactions with organic acids. *European Journal of Soil Science*, **47**, 319–328.

Pinton R., Cesco S., Santi S., Agnolon F., Varanini Z. (1999) Water extractable humic substances enhance iron deficiency responses by Fe deficient cucumber plants. *Plant and Soil*, **210**, 145–157.

Pizzeghello O., Nicolini G., Nardi S. (2001) Hormone-like activity of humic substances in *Fagus sylvatica* forests. *New Phytologist*, **151**, 647–657.

Pratt L.H., Codonnier-Pratt M.M., Kelmenson P.M., Lazarova G.I., Alba R.M. (1997) The phytochrome gene family in tomato (*Solanum lycopersicum*). *Plant, Cell and Environment*, **20**, 672–677.

Quaggiotti S., Ruperti B., Pizzeghello D., Francioso O., Tugnoli V., Nardi S. (2004) Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). *Journal of Experimental Botany*, **55**, 803–813.

Ramírez-Chavez E., Lopez-Bucio J., Herrera-Estrella L., Molina-Torres J. (2004) Alkamides isolated from plants promote growth and alter root development in *Arabidopsis*. *Plant Physiology*, **134**, 1058–1068.

Simpson A.J. (2002) Determining the molecular weight, aggregation, structures and interactions of natural organic matter using diffusion ordered spectroscopy. *Magnetic Resonance in Chemistry*, **40**, 572–582.

Singh R.B. (2000) Environmental consequences of agricultural development: a case study from the green revolution state of Haryana, India. *Agriculture, Ecosystems and Environment*, **82**, 97–103.

Sorin C., Bussell J.D., Camus I., Ljung K., Kowalczyk M., Geiss G. (2005) Auxin and light control of adventitious rooting in *Arabidopsis* require Argonaute1. *Plant Cell*, **17**, 1343–1359.

Stevenson J.F. (1994) *Humus Chemistry: Genesis, Composition, Reactions*. 2nd edn. New York, NY: John Wiley & Sons Inc., 496 pp.

Sutton R., Sposito G. (2005) Molecular structure in soil humic substances: the new view. *Environmental Science and Technology*, **39**, 9009–9015.

Tanksley S.D., Ganal M.W., Prince J.P., Vicente M.C., Bonierbale M.W., Broun P., Fulton T.M., Giovannoni J.J., Grandillo S., Martin G.B., Messeguer R., Miller J.C., Miller L., Paterson A.H., Pineda O., Roder M.S., Wing R.A., Wu W., Young N.D. (1992) High density molecular linkage maps of the tomato and potato genomes. *Genetics*, **132**, 1141–1160.

Torrey J.G. (1986) Endogenous and exogenous influences on the regulation of lateral root formation. In *New Root Formation in Plants and Cuttings*, pp. 31–66. Ed M.B. Jackson. Dordrecht, the Netherlands: Martinus Nijhoff.

Vaughan D., Malcolm R.E. (1985) Influence of humic substances on growth and physiological processes. In *Soil Organic Matter and Biological Activity*, pp. 37–75. Eds D. Vaughan and R.E. Malcolm. Dordrecht, the Netherlands: Martinus Nijhoff/Dr W. Junk Publisher.

Whitehead D.C. (1963) Some aspects of the influence of organic matter on soil fertility. *Soil Fertility*, **26**, 217–223.

Zandonadi D.B., Canellas L.P., Façanha A.R. (2007) Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H$^+$ pumps activation. *Planta*, **225**, 1583–1595.

Zhang H., Jennings A., Barlow P.W., Forde B.G. (1999) Dual pathways for regulation of root branching by nitrate. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 6529–6534.