Plant Soil (2010) 336:313–322
DOI 10.1007/s11104-010-0483-2

REGULAR ARTICLE

Rhizosphere humic acid interacts with root cell walls to reduce hydraulic conductivity and plant development

Sare Asli · Peter M. Neumann

Received: 2 May 2010 / Accepted: 24 June 2010 / Published online: 17 July 2010

Abstract Humic acids are ubiquitous, organic-end-products of the chemical and microbial degradation of dead biota in soils throughout the world. Humic acids can be transported in soil water as heterogeneous, supra-molecular, colloidal-agglomerates. Humic acid accumulation in the rhizosphere of transpiring plants may chemically stimulate development by increasing root availability of mineral nutrients and/or growth regulatory biomolecules. This report introduces novel, physical mechanisms by which humic acid can also reduce plant development. Effects of humic acid addition to the root media of intact maize plants (Zea mays L.) on their growth, transpiration and resistance to water deficits were assayed, as were the effects of external humic acid on the hydraulic conductivity of excised primary-roots. Humic acid reduced shoot growth, transpiration and resistance to water stress but not root growth. Root hydraulic conductivity was reduced by up to 44% via a time-, concentration- and size-dependent fouling mechanism resulting from humic acid accumulation at root cell-walls. Thus, humic acid is shown, apparently for the first time, to be able to exert novel physical effects in addition to its known chemical effects on plant development.

Keywords Rhizosphere · Humic acid · Cell-wall-fouling · Hydraulic-conductivity · Water-stress-resistance

Introduction

Humic acids are components of the ubiquitous, organic-end-products formed by the chemical and microbial degradation of dead biota in soils, waters and sediments throughout the world. They can represent a major part of total soil organic matter, act as sinks for atmospheric CO_2 and enable pollutant transport. In water, small units of humic acid with molecular masses in the 1,000 to 2,000 kDa range can form heterogeneous, micelle-like, supra-molecular colloidal-agglomerates with molecular masses >66,000 kDa and diameters reaching 800 nm. The agglomerates are held together by weak hydrophobic and electrostatic bonds and can be associated with mineral nutrients and small growth regulatory biomolecules which may affect plant, microbial and animal ecosystems (see comprehensive reviews and references cited by Chen and Aviad 1990; Piccolo 2001; Sutton and Sposito 2005; Steinberg et al. 2008).

Humic acid concentrations in fertile soils may reach hundreds of mg L^{-1} and external concentrations up to 300 mg L^{-1} can stimulate the growth of higher plants in hydroponic culture. The stimula-

Responsible Editor: Robert Reid.

S. Asli · P. M. Neumann (✉)
Department of Environmental, Water and Agricultural Engineering, Technion Israel Institute of Technology,
Haifa 32000, Israel
e-mail: agpetern@tx.technion.ac.il

tory mechanisms may involve increases in the availability to plant roots of essential mineral nutrients or growth regulatory substances. Soil solutions containing humic acids can be carried to the water absorbing root surfaces of transpiring plants by mass flow. There, partial disruption of humic acid agglomerates by root secreted organic acids may then release individual growth regulatory molecules that are small enough to penetrate through the cell wall matrix to the cell membrane and effect changes in gene expression or enzyme activities (Piccolo et al. 1996; Pinton et al. 1999; Canellas et al. 2002; Quaggiotti et al. 2004; Muscolo et al. 2007; Zandonadi et al. 2007; Schmidt et al. 2007; Canellas et al. 2008; Aguirre et al. 2009). In addition, indirect effects of humic acids on soil structure and soil microflora have also been suggested (Chen and Aviad 1990; Chen et al. 2000; Atiyeh et al. 2002: Arancon et al. 2006).

However, the stimulatory effects of humic acid on plants may dissipate with further increases in concentration and clear reductions in plant growth, as soil concentrations of humic acid increased, were shown by Vallini et al. (1993) and by Atiyeh et al. (2002). Effects of humic acid on plant development have generally been ascribed to the chemical effects of associated mineral nutrients or growth regulatory molecules. For example, a recent report by Muscolo and Sidari (2009) showed that known concentrations of a carboxylic fraction extracted from humic acid increased the growth of tissue cultured plant calli while a phenolic fraction at the same concentrations, reduced growth and even induced cell death. However, the concentrations and precise identities of any humic acid derived chemicals in the rhizosphere that may affect whole plant growth, have yet to be established (cf.Dobbss et al. 2007; Schmidt et al. 2007).

The involvement of humic acids in plant growth stimulation or reduction and the possible mechanisms involved, are of practical interest since natural soil levels of humic acid may be progressively increased by agricultural practices involving frequent soil additions of humate rich manures, composts, sewage sludge or processed sewage effluents (Rebhun and Manka 1971; Chen and Aviad 1990; Haynes and Naidu 1998). We report here on a novel, non-chemical mechanism by which humic acid can adversely affect plant development.

Asli and Neumann (2009) showed that inorganic, colloidal-suspensions of bentonite clay, or of nano-sized titanium dioxide particles, in the water supplied to plant roots could reduce root hydraulic conductivity by purely physical fouling mechanisms and thereby reduce transpiration and leaf growth. The possibility that organic colloids, such as the humic acids found in soil waters, may similarly interact with plants, does not appear to have been previously investigated. The specific aims of this laboratory study were therefore to determine:- 1). The effects of additions of humic acid (1 g L^{-1}) on physiological aspects such as growth, transpiration and ability to withstand soil water deficits, in whole maize plants (*Zea mays* L.). 2). The effects of humic acids, at 0.01, 0.3 or 1.0 g L^{-1}, on the hydraulic conductivity of maize seedling roots 3). The possible involvement of physical interactions between humic acid and root cell walls.

Materials and methods

Plant material Seeds of *Zea mays* L (Cv 32p75, Milchan Brothers, Ashdod, Israel) were germinated and unless otherwise stated, were grown in aerated hydroponic media in a light and temperature regulated growth chamber under 12 h photoperiods. Further details concerning growth conditions and some of the assay techniques briefly described below are reported in Asli and Neumann (2009).

Humic acids Humic acid derived from an unidentified source of coal was purchased from Sigma Aldrich. In addition, certified sources of Pahokee peat derived humic acid and of Leonardite derived humic acid were purchased from the International Humic Substances Society (St. Paul MN, USA). The humic acids were prepared according to the protocol in Hong and Elimelech (1997) and aqueous suspensions of ≤ 1 g L^{-1} at pH 6.8 were used for the assays.

Leaf and root growth rates Leaf elongation was assayed at 5–10 h intervals for 48 h by following length increases in emerging primary leaves of uniform maize seedlings. Leaf growth rates were based on up to 20 h of linear growth before the end of the logarithmic phase of elongation. Primary root elongation rates were based on the increase in length of marked roots over 3 d.

Transpiration Transpiration rates were assayed gravimetrically using whole seedlings grown in hydroponics. The seedlings which were selected for uniform size, had open first and second leaves and an emerging third leaf. The seedling roots were loosely sealed in light proof plastic vials which were 3/4 filled with 0.1 mM $CaCl_2$ with or without humic acid at 1 gL^{-1}. Plants with their foliage removed and the remaining stump capped with Parafilm, were used as controls. Transpiration was assayed by following weight loss for 3 h with an electronic balance, subtracting control values and dividing by leaf surface area. A factor relating 1 g of leaf fresh weight to 65.1 cm^2 of open leaf area was determined for 30 plants and used to convert leaf fresh weight of assayed plants to leaf area (circa 13 cm^2 per plant). Oxygen levels in the root media at the beginning and end of 3 h transpiration assays were measured using a dissolved O2 probe (Cyberscan, DO 300; Eutech Instruments Europe BV, Nijkerk, the Netherlands) and decreases of circa 20% were measured with or without humic acid.

Root cell wall pore size The effects of prior humic acid perfusion on mean diameters of the nano-sized pores traversing the cell wall matrices of living maize roots were determined by observation of cytorrhysis i.e. root collapse (Carpita et al. 1979; Asli and Neumann 2009). This was induced by sequential exposure to hypertonic solutions of PEG molecules with hydrodynamic diameters ranging from 0.9 nm to 7 nm (Kuga 1981). The concentrations of PEG 200, 1,500, 4,000, 6,000 and 10,000 (57.84, 180.71, 204.17, 201.62 and 210.54 g PEG per 1,000 g water, respectively) were fixed using the calibration of Money (1989) to give the same solution water potential of −0.7 MPa for all PEG treatments. Batches of 10 control or colloid treated roots were gently rinsed and transferred to Petri dishes containing 50 ml of PEG 200 solution and then transferred at 50 min intervals to PEG solutions with higher molecular weights and hydrodynamic diameters. When the external PEG molecules were too large to penetrate the pores in the cell walls, an osmotically induced efflux of cell water resulted in inward collapse and root cytorrhysis. Flattened, ribbon-like, roots could then be observed through a binocular microscope and average pore diameters calculated.

Leaf curl determination Uniform seedlings were planted in well drained pots containing 500 g of local clay soil with one maize seedling per pot and five pots per treatment. The pots were flood irrigated with 0.1 strength nutrient solution at 48 h intervals for 7 days. The treatment was continued with or without addition of 1 g L^{-1} humic acid for a further 7 days. The plants then had 4 open leaves and drought was initiated by cessation of irrigation. Leaf curling, an early symptom of plant water stress (e.g. Cochard 2002) was initiated circa 30 h after cessation of irrigation and plants routinely attained 100% leaf curl circa 3 hours later. The total percentage of leaves in each treatment showing leaf curl was determined at the indicated times.

Root hydraulic conductivity One cm lengths at the cut ends of excised primary roots (6 cm long) were fitted into marked glass capillary tubes. Silicon grease and Parafilm wrapping were used to carefully seal the junction between capillary tube and root. The protruding 5 cm of up to 11 roots with intact tips were immersed vertically in 1 liter light proof containers with a 1 cm space between each root. The containers were filled with continuously stirred solutions of 0.1 mM $CaCl_2$ without or with humic acid. Water-flow through the roots was followed by measuring the rise of menisci in the protruding capillaries induced by pressurizing the root container to 10 KPa. Hydraulic conductivity was linear for at least 5 h and assays were based on flow rates measured at 10 min intervals from 30 to 40 min after commencement of pressurization. Axial hydraulic conductivity of the xylem increased with xylem maturation from 2 cm behind the tip (not shown). Thus, 3 cm of root starting 2 cm behind the tip was taken to represent the region of effective water uptake in the calculation of hydraulic conductivity. Roots which gave pressurized flow rates >0.3 $\mu L\ min^{-1}$ in the first 30 min also allowed aberrant transport of high mol wt dextran blue (0.4 g L^{-1}) through to the glass capillary; these roots were considered leaky and discarded.

For killed "root-ghost" assays the protruding 5 cm of roots fitted to capillaries as above were contacted with hot (80°C) ethanol for 1 min and gently rinsed in water prior to the flow assay. Killed roots which gave pressurized flow rates >0.55 $\mu L\ min^{-1}$ were considered leaky and discarded. Hydraulic conductivity of live and killed roots was calculated as m^3 solution transported per m^2 effective root area per second per MPa applied pressure.

Statistics All experiments were replicated one or more times with similar results. Differences between treatments were estimated by paired two-sample t tests or one way ANOVA, using the analysis tool box in GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

Results

Adverse whole plant responses to root addition of humic acid

Soil grown plants in pots were repeatedly irrigated for 6 weeks with nutrient solution containing 1 g L^{-1} humic acid. This treatment resulted in reductions in shoot fresh and dry weights as compared to control plants irrigated with nutrient solution only (Table 1). Similarly, reductions in leaf growth were observed when humic acid at 1 g L^{-1} was added to the nutrient solution of hydroponic maize plants for 4 weeks although humic acid at 0.1 g L^{-1} and 0.3 g L^{-1} appeared to stimulate growth (not shown). Finally, rapid reductions in leaf growth rate were induced within days of adding 1 g L^{-1} humic acid to the root media of hydroponic seedlings. These were not accompanied by reductions the elongation rates or the healthy appearance of the primary roots (Table 2).

Typical kinetics of the rapid reductions in transpiration also induced by humic acid addition to the roots of hydroponic plants are shown in Fig. 1 and the pooled rates of transpiration for three replicate experiments are shown in Table 2. These findings suggested that the presence of humic acid somehow reduced essential water transport from the root medium to the shoot thereby reducing transpiration.

Table 1 Inhibition of shoot growth of potted maize plants irrigated with humic acid. Plants were grown in a local clay soil in 1 L pots with drainage holes at base and sides and the soil flooded by sub irrigation with 0.1 strength modified Hoagland solution with or without 1 g L^{-1} of humic acid, at 48 h intervals. Treatment and controls each consisted of three replicate pots with six plants per pot. After 6 weeks the shoots were excised at soil level and weighed. Data are means ± SE for individual shoots, $n=18$. Different letters in columns indicate significant weight differences at $P<0.05$ using paired *t*-test

	Shoot fresh weight (g)	Shoot dry weight (g)
Control	16.08±0.30 a	2.94±0.70 A
Humic acid	12.90±0.40 b	2.20±0.06 B

Table 2 Humic acid in the root media inhibits transpiration and leaf growth but not root growth. Values are pooled means ± SE for three similar experiments comprised of five replicate plants each for transpiration assay, 18–23 replicate plants each for leaf growth assay and 16 plants each for root growth assay. Different letters in rows indicate significant differences between each treatment mean and the control, $P<0.05$. (Paired *t*-test)

	+ Humic acid	Control
Transpiration rate (mg H_2O cm^{-2} h^{-1})	6.80±0.60b	10.90±1.70a
Leaf growth rate (mm h^{-1})	0.90±0.03b	1.04±0.02a
Root growth rate (mm h^{-1})	2.97±0.13a	2.87±0.14a

In order to determine whether similar levels of humic acid in soil media might also reduce water availability to the shoots, potted maize plants were flood-irrigated with nutrient solution with or without 1 g L^{-1} humic acid at 48 h intervals for 7 d, and irrigation was then stopped. As the soil dried, the plants previously irrigated with humic acid showed accelerated onset of drought induced leaf curl by comparison with control plants (Fig. 2). Leaf curl is a

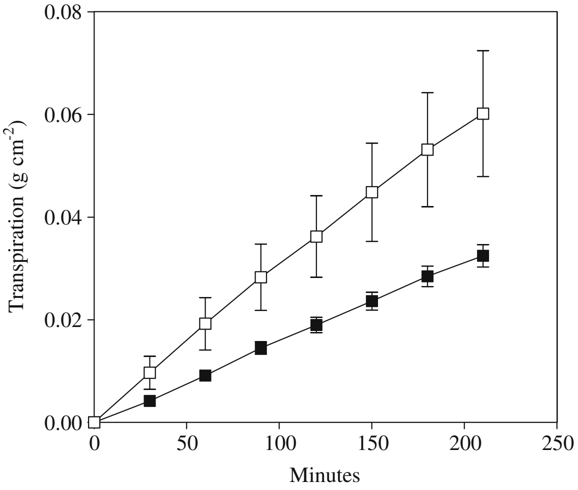

Fig. 1 Kinetics of humic acid induced reduction in cumulative transpiration. Humic acid at 1 g L^{-1} was added to the root media of intact hydroponic maize seedlings at the 3 leaf stage at time zero. Control and humic acid treatments are represented by *open* and *filled squares* respectively. Means ± SE, $n=5$

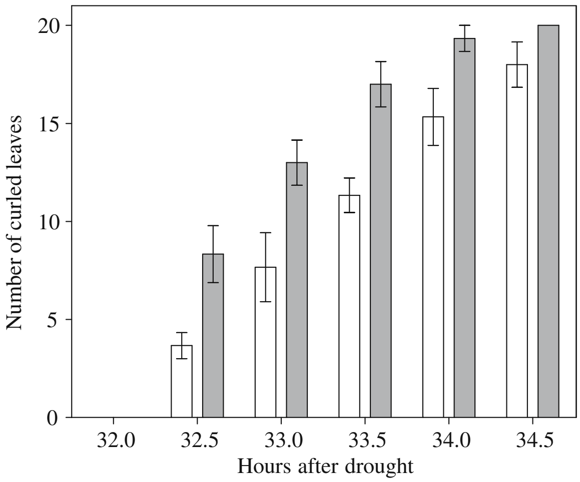

Fig. 2 Soil humic acid accelerates drought induced leaf curl. Seedlings were grown in 400 ml pots with 1 seedling per 0.5 kg of soil. Every 48 h for the first 7 d after shoot emergence, the five replicate pots in each treatment were flooded by sub irrigation for 1 h with 0.1 X nutrient solution and then allowed to drain. Nutrient solution with the addition of humic acid at 1 g L^{-1} was then used to irrigate treated plants for a further 7 d while control plants continued to receive nutrient solution alone After14 d, when there were 4 open leaves per plant, irrigation was stopped. The critical time for the first appearance of leaf curl symptoms was circa 32 h after cessation of irrigation and the number of leaves showing onset of drought-induced curling was then assayed at indicated times. The data are pooled means ± SE for 3 replicate experiments. Control and humic acid treatments are indicated by *open* and *filled columns* respectively

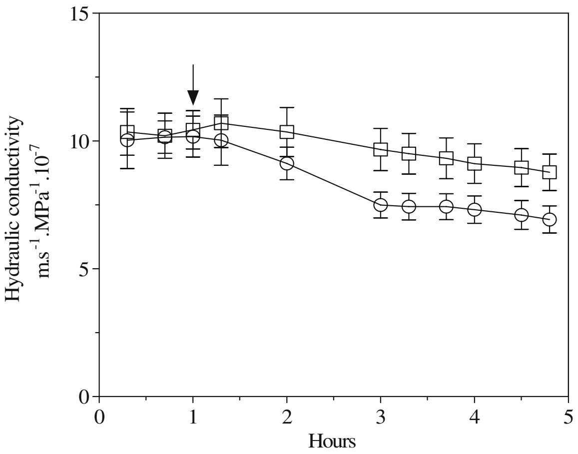

Fig. 3 Kinetics of progressive decreases in root hydraulic conductivity induced by increases in external concentration and exposure time to humic acid. A 1 cm length at the cut end of excised primary roots of maize seedlings was sealed into capillary tubes and 5 cm of protruding root with intact tip was immersed in 1 L of 0.1 mM $CaCl_2$ with or without humic acid. Flow through batches of roots sealed separately into 1 L containers was initiated by applying hydrostatic pressure of 10 KPa. Flow from the cut ends of the roots into the marked capillaries protruding from the root containers was then followed at intervals and used to calculate hydraulic conductivities, as in Materials and methods. *Upper* and *lower lines* represent humic acid concentrations of 0.3 g L^{-1} and 1 g L^{-1} respectively in root media. *Arrow* indicates time of addition of humic acid. Means ± SE, *n*=9

known symptom of plant water stress. Thus, root addition of humic acid could reduce transpiration of plants in hydroponics and accelerate the onset of water-stress symptoms in plants in a drying soil. The possibility that humic acid reduced water supply to the shoot by directly reducing root hydraulic conductivity was next investigated.

External additions of humic acid reduce the hydraulic conductivity of excised roots

The assay method described in Materials and methods allowed simultaneous assays of hydraulic conductivity in groups of up to 11 excised primary roots with intact tips. Unless otherwise stated, measurements were completed within 5 h, while flow through the control roots remained linear (Asli and Neumann 2009). The kinetics in Fig. 3 show that humic acid perfusion at both 0.3 g L^{-1} and 1.0 g L^{-1} caused gradual, time and concentration dependent reductions in the hydraulic conductivity of excised maize primary roots, during a 4 h perfusion. Root perfusion with much lower concentrations of humic acid (10 mg L^{-1}) for 10 h also produced small but significant reductions (P=0.05, Student's t test) in root hydraulic conductivity, from 13.3±0.5 m s^{-1} MPa^{-1}. 10^{-7} for control roots to 11.9±0.7 m s^{-1} MPa^{-1}. 10^{-7} for the roots with humic acid (pooled means ± SE for 3 batches of 9 to 11 roots).

Sigma humic acid derived from an unspecified coal was routinely used in most of these experiments because large amounts were involved and sources of certified humic acid were relatively expensive. Nevertheless, certified humic acids extracted from Leonardite or Pahokee peat, that were purchased from the International Humic Substances Society (IHSS), induced reductions in root hydraulic conductivity that were similar to those induced by the Sigma humic acid (Table 3). Thus, a 70 min perfusion with the Sigma humic acid at 1 g L^{-1} caused a 44% reduction in root hydraulic conductivity while both the humic acids from IHSS caused 33% reductions. The inhibitory effect on root hydraulic conductivity of Sigma

Table 3 Humic acids from different sources similarly reduce root hydraulic conductivity. Humic acids were obtained from Sigma Aldrich or as certified sources from the International Humic Substances Society. Hydraulic conductivities were based on 40 min of control perfusion with 0.1 mM $CaCl_2$ followed by 70 min perfusion with humic acids at 1 g L^{-1} in 0.1 mM $CaCl_2$, and then, 30 min with 0.1 mM $CaCl_2$ alone after humic acid removal. Data are pooled means ± SE, from 3 replicate experiments with a total of 23 to 25 roots. Different letters in columns indicate that treatment means differed from the appropriate control values at $P<0.05$ using one way ANOVA

Root hydraulic conductivity ms^{-1} MPa^{-1}. 10^{-7}			
	Pahokee Peat	Leonardite	Sigma
Control	14.9±0.7 A	13.4±0.7 a	12.25±1.20 a′
Humic acid	10.0±0.6 B	9.0±0.6 b	6.88±0.65 b′
Humic acid removed	10.8±0.6 B	10.3±0.5 b	12.93±1.28 a′

humic acid was reversed during a 30 min perfusion with humic acid-free solution whereas reversal of the inhibitory effects of the other two humic acids was less complete, presumably because of a greater degree of humic acid attachment to the roots. However, the flow inhibitory effects of the Sigma humic acid also became less reversible after a longer (4 h) perfusion (not shown). It was concluded that root perfusion with both Sigma humic acid and certified humic acid from other sources, could similarly reduce root hydraulic conductivity.

Mechanisms by which humic acid reduces root hydraulic conductivity

The hypothesis investigated was that large supra-molecular colloidal agglomerates of humic acid in external water sources are carried by mass flow to the root surfaces of transpiring plants where they reduce water flow through the tortuous nano-sized pores that traverse the polysaccharide fiber matrices of the epidermal cell walls. Several experimental approaches were used to specifically determine whether aqueous organic colloids could be retained by root cell walls and then restrict water passage.

Solutions of polyethylene glycols were first used to investigate possible relationships between the sizes of organic molecules in the water supplied to roots and induced reductions in root hydraulic conductivity. Polyethylene glycols (PEG) are water soluble, non-ionic, organic polymers that are produced in a range of molecular sizes. PEG 1,000 and PEG 6,000 with mean molecular weights of 1,000 Da and 6,000 Da respectively, were each supplied externally to the roots at 1 g L^{-1}, the highest concentration of humic acid used in equivalent root conductivity assays. At this relatively low concentration both types of PEG had negligible osmotic potential.

PEG 1,000 with a reported molecular diameter of 1.8 nm (Kuga 1981) was expected to readily traverse root cell walls pores with diameters ranging from 4 to 8 nm (Asli and Neumann 2009 and references therein) and had no apparent effects on the hydraulic conductivity of live roots during 70 min perfusions (Fig. 4 inset). Thus, the PEG 1,000 molecules did not have cell wall-fouling, osmotic or viscous effects large enough to reduce water flow through the roots. In contrast, root perfusion with equivalent solutions of PEG 6,000 (5.4 nm molecular diameter) resulted in progressive reductions in root hydraulic conductivity (Fig. 4). The progressive nature of the reductions indicates that the bulk viscosity of PEG 6,000 was not limiting flow since high viscosity would have immediate effects. Instead, these findings suggested that PEG 6,000 and by inference, humic acids, were too large to readily penetrate the root cell wall matrices; thus, accumulation could occur at the root surfaces, thereby reducing water flow.

Perfusion of live roots for 4 h with 1 g L^{-1} humic acid caused reductions in estimated cell wall pore diameters (Table 4). Perfused roots had a brown coloration which was retained after rinsing. This indicated that some humic acid had accumulated on or in the cell wall pores. Moreover, perfusion with humic acid of root cell wall 'ghosts' obtained by hot alcohol extraction of live roots (in order to disrupt cell plasma membranes) also decreased root hydraulic conductivity. The hydraulic conductivity values for root ghosts perfused without or with 1 gL^{-1} humic acid were 23.7±1.5 m s^{-1} MPa^{-1}.10^{-7} and 20.3± 1.6 m s^{-1} MPa^{-1}.10^{-7} respectively (pooled means ± SE, for 20 to 25 roots from 3 replicate experiments) and differed significantly ($P<0.05$ Student's *t*-test). The effective disruption of membrane and cytoplasmic barriers by the hot alcohol treatment was reflected in relatively higher root hydraulic conductivities than

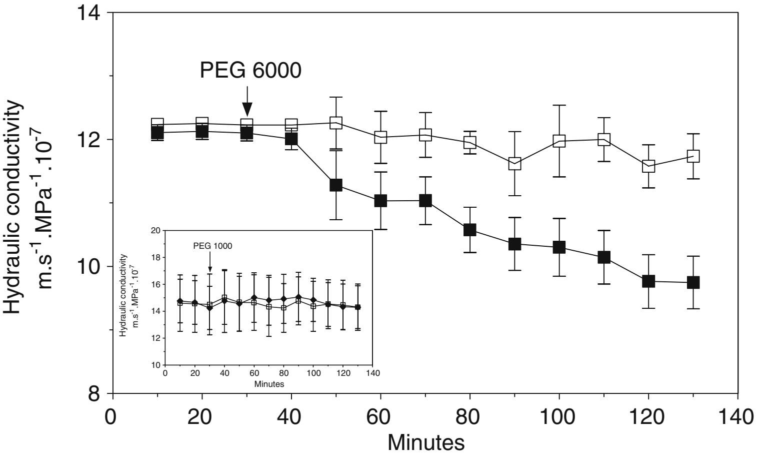

Fig. 4 Polyethyleneglycol induced decreases in root hydraulic conductivity are dependent on molecular size. Batches of excised roots were perfused with PEG 6,000 at 1 g L^{-1} in 0.1 mM $CaCl_2$ as in Materials and methods. Inset shows lack of effect of smaller PEG 1,000 molecules at the same concentration. Means ± SE, n=7 to 11

in live roots. Humic acid therefore, appeared to restrict flow via an interaction with the root cell walls alone.

Discussion

Mechanisms by which humic acid affects plant growth

Humic acid is best known for its ability to stimulate plant growth (e.g. Chen and Aviad 1990; Nardi et al. 2002; Canellas et al. 2002; Arancon et al. 2003, 2006; Dobbss et al. 2007). However, humic acid at 1 g L^{-1} consistently inhibited the shoot growth of both hydroponic and soil-grown plants (Tables 1 and 2). It has been previously suggested that plant growth reductions by supra-optimal levels of humic acid may be related to the excessive chemical activity of small growth regulatory biomolecules released from humic acid agglomerates after interactions with organic acids in the rhizosphere (Piccolo et al. 1996; Canellas et al. 2002; Canellas et al. 2008; Muscolo and Sidari 2009). In our experiments, humic acid at 1 g L^{-1} caused reductions in maize leaf growth but did not reduce root growth rate, or the healthy appearance of the roots, even though the roots were more directly exposed to humic acid than the leaves. It seems unlikely that any growth inhibitory levels of chemicals released by humic acid into the rhizosphere, or possible humic acid interference with mineral nutrient availability caused specific reductions in leaf growth without similarly affecting the roots. The fact that root water deficits tend to inhibit leaf growth to a greater extent than root growth is however, well established (e.g. Sharp and Davies 1979; Lu and Neumann 1999; Hsiao and Xu 2000). The involvement of an additional, physical-mechanism that restricted root water availability for shoot growth without inhibiting root growth can therefore be hypothesized.

Table 4 Root treatment with humic acid reduces cell-wall pore diameters. Following perfusion with humic acid in 0.1 mM $CaCl_2$ for 4 h, roots were rinsed briefly in 0.1 mM $CaCl_2$ alone and assayed for cell-wall pore diameter as in Materials and methods. Mean ± SE for 3 replicate experiments each with 7–8 roots. Different letters indicate treatment means were significantly different from control values at $P<0.05$ using a paired t-test

	Cell wall pore diameter (nm)
Control	6.6±0.2 a
Humic acid	2.8±0.3 b

Four lines of evidence support the hypothesis that the physical accumulation of supramolecular agglomerates of humic acid on or in water conducting root cell walls limited root water transport and thereby restricted shoot development:-

1. Root perfusion with waters containing humic acids from 3 different sources consistently decreased the hydraulic conductivity of live roots in a concentration and time dependent manner. In addition, humic acid also reduced the hydraulic conductivities of hot alcohol extracted root ghosts. This showed clearly that

humic acid interactions with cell walls alone could cause significant decreases in root hydraulic conductivity.

2. Colloidal suspensions of humic acid can accumulate via electrostatic and Van der Waal interactions, at the surfaces of synthetic micro or nano filtration membranes used in water purification. Formation of cake layers and other types of pore blockage can then progressively decrease membrane hydraulic conductivity (Hong and Elimelech 1997; Yuan and Zydney 2000; Goosen et al. 2004; Lee et al. 2005; Contreras et al. 2009). These reports illuminate probable mechanisms by which humic acid could similarly foul the micro-porous root cell walls.
3. Humic acid perfusion of excised roots was shown to cause brown colouration and reductions in the diameters of root cell wall pores, indicating that some of the humic acid had became attached to the cell wall pores. Moreover, reductions in root hydraulic conductivity induced by humic acid were not completely reversible, especially after pronged perfusion, again indicating wall-attachment of the humic acid.
4. The perfusion of excised roots with solutions of the water soluble organic polymer PEG 6,000 at 1 g L^{-1}, but not the smaller PEG 1,000, reduced root hydraulic conductivity, as did the equivalent concentration of humic acid. PEG 6,000 has a relatively large molecular diameter (of 5.4 nm) and its passage across tortuous plant cell wall pores is restricted (Carpita et al. 1979; Kuga 1981; Chazen et al. 1995). The molecular weights and diameters of aqueous supramolecular agglomerates of humic acid can vary with the source of the humic acid, the assay technique used and the ionic status or pH of the environment. Low values for molecular diameters can be in the 4 nm to 14 nm range and high values can reach hundreds of nanometers (cf. Kawahigashi et al. 2005; Sutton and Sposito 2005). Published estimates of plant cell wall pore diameters range between 4 nm and 8 nm and the mean value for maize root cell-wall pore diameters was 6.6 nm. Thus, humic acid in water taken up by roots should be largely excluded by root cell walls.

It therefore appears that a physical accumulation of humic acid at root cell wall surfaces during water uptake can lead to the formation of cake layers and pore blockages that cause physical reductions in root hydraulic conductivity. In whole plants, induced reductions in root to shoot water transport have often been shown to have restrictive effects on shoot growth (cf. Sharp and Davies 1979; Matyssek et al. 1991; Chazen and Neumann 1994; Lu and Neumann 1999; Hsiao and Xu 2000; Neumann et al. 2010). The observed reductions in shoot growth, transpiration, and resistance to soil water deficits that accompanied root additions of humic acid could therefore result from the physical reduction of root hydraulic conductivity. Inorganic colloids had similar effects (Asli and Neumann 2009; Asli and Neumann unpublished data) and we suggest the term “colloid stress” to describe such interactions.

Does colloid stress occur under field conditions?

Soil additions of endogenous humic acid at 1 g L^{-1}were found by us to reduce shoot growth and water stress resistance in pot experiments. However, concentrations of humic acid in soil waters in the field only range from tens to a few hundred mg L^{-1} (Chen and Aviad 1990). Thus, the humic acid concentrations used by us to characterize flow inhibitory effects on excised roots (300 to 1,000 mg L^{-1}) were at the high end of the natural spectrum. Albeit, a relatively long (10 h) perfusion of roots with humic acid at the much lower concentration of 10 mg L^{-1} also caused statistically significant reductions in root hydraulic conductivity, as compared with humic acid free controls. Moreover, under natural conditions the colloidal clay particles, bacteria and other organic polymers in soil waters might also contribute to reductions in root hydraulic conductivities (cf. Goosens et al. 2004; Lee et al 2005; Asli and Neumann 2009). We therefore suggest that in the field, the extended periods of uptake by transpiring plants of soil waters containing suspended humic acid and other colloids are likely to contribute to root hydraulic limitations.

Plant adaptation or acclimation to colloid stress

Since the water uptake capacity of entire plant root systems is a function of the total root area available for water uptake, plants could in theory act to reduce colloid stress responses via preferential allocation of carbon resources to additional root development. For example, a doubling of root surface area available for water uptake could compensate for a colloid-induced halving of the specific hydraulic conductance of individual roots. Interestingly, humic acids have been widely reported to stimulate root elongation, root hair and lateral root production (cf. Canellas et al. 2002; Zandonadi et al. 2007; Schmidt et al. 2007; Canellas et al. 2009). In an eco-physiological context, the diversion of carbon resources towards increased root production in response to the colloid stress induced by humates or other colloids could represent a constitutive plant adaptation or a short term acclimation. However, even if accelerated root production can minimize reductions in water availability, accompanying limitations in carbon resource availability in the shoots might still limit shoot growth. Thus, the interactions between plants and humates involve complex, time-related chemical, physical and physiological mechanisms that may reduce plant resistance to water deficits and be associated with either increases or decreases in shoot growth.

Colloid stress induced by interactions between plants and humates could have environmental implications of practical importance. For example, colloid induced fouling of the roots may limit the benefits associated with repeatedly applying composts, manures or sewage sludge that are all rich in humates and other colloids, as organic soil amendments (Chen and Aviad 1990; Haynes and Naidu 1998). Moreover, decreased rainfall as a result of climate change and population driven increases in urban and industrial freshwater requirements can reduce the availability of freshwater for irrigation-use in agriculture, particularly in arid and semi arid regions of the world. Irrigation with the alternative water resources provided by recycled domestic sewage effluents that are also rich in humates and other colloids, may similarly lead to eventual root fouling. This could however, be avoided by further (tertiary) water-treatment to remove the colloids (cf. Rebhun and Manka 1971; Tal 2006).

In summary, several lines of evidence indicate that humic acid in water taken up by plant roots will accumulate at epidermal cell wall surfaces. Resultant formation of surface cake layers and partial fouling of the cell wall pores can then cause reductions in root hydraulic conductivity, leaf growth, transpiration, and plant resistance to water deficits. Thus, humic acid is shown, apparently for the first time, to be able to exert novel physical effects, in addition to its known chemical effects on plant development.

Acknowledgements Funding by FMW chair (PMN) and Technion graduate school (SA) is gratefully acknowledged.

References

Aguirre E, Lemenager D, Bacaicoa E, Fuentes M, Baigorri R, Angel Z, Jose GM (2009) The root application of a purified leonardite humic acid modifies the transcriptional regulation of the main physiological root responses to Fe deficiency in Fe-sufficient cucumber plants. Plant Physiol Biochem 47:215–223

Asli S, Neumann PM (2009) Colloidal suspension of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. Plant Cell Environ 32:577–584

Atiyeh RM, Lee S, Edwards CA, Arancon NQ, Metzger JD (2002) The influence of humic acids derived from earthworm-processed organic wastes on plant growth. Bioresour Technol 84:7–14

Arancon NQ, Clive A, Edwards CA, Lee S, Byrne R (2006) Effects of humic acid from vermicompost on plant growth. Eur J Soil Biol 42:565–569

Arancon NQ, Lee S, Edwards CA, Atiyeh RM (2003). Effects of humic acid derived from cattle, food and paper-waste vermicomposts on growth of greenhouse plants. Pedobiologia 47:741–744

Canellas LP, Olivares FL, Facanha AL, Facanha AR (2002) Humic acid isolated from earthworm compost enhance root elongation, lateral root emergence and plasma membrane H^+-ATPase activity in maize roots. Plant Physiol 130:1951–1957

Canellas LP, Junior LRLT, Dobbss LB, Silva CA, Medici LO, Zandonadi DB, Facanha AR (2008) Humic acids cross-interactions with root and organic acids. Ann Appl Biol 153:157–166

Canellas LP, Spaccini R, Piccolo A, Dobbss LB, Okorokova-Facanha AL, Santos GD, Olivares FL, Facanha AR (2009) Relationships between chemical characteristics and root growth promotion of humic acids isolated from Brazilian oxisols. Soil Sci 174:611–620

Carpita N, Sabularse D, Montezinos D, Delmer D (1979) Determination of the pore size of cell walls of living plant cells. Science 205:1144–1147

Chazen O, Neumann PM (1994) Hydraulic signals from the roots and rapid cell-wall hardening in growing maize (Zea mays L.) leaves are primary responses to polyethylene glycol-induced water deficits. Plant Physiol 104:1385–1392

Chazen O, Hartung W, Neumann PM (1995) The different effects of PEG 6000 and NaCl on leaf development are associated with differential inhibition of root water transport. Plant Cell Environ 18:727–735

Chen Y, Aviad T (1990) Effects of humic substances on plant growth. In: MacCarthy P, Clapp CE, Malcolm RL, Bloom

PR (eds) Humic substances in soil and crop sciences: selected readings. American Society of Agronomy and Soil Science Society of America, Madison, pp 161–186

Chen Y, Katan G, Gamliel A, Aviad T, Schnitzer M (2000) Involvement of soluble organic matter in increased plant growth in solarized soils. Biol Fertil Soils 32:28–34

Cochard H (2002) Xylem embolism and drought-induced stomatal closure in maize. Planta 215:466–471

Contreras AE, Kim A, Li Q (2009) Combined fouling of nanofiltration membranes: Mechanisms and effect of organic matter. J Membr Sci 327:87–95

Dobbss LB, Medici LO, Peres LEP, Pino-Nunes LE, Rumjanek VM, Facanha AR, Canellas LP (2007) Changes in root development of *Arabidopsis* promoted by organic matter from oxisols. Ann Appl Biol 151:1–13

Goosen MFA, Sablani SS, Ai-Hinai H, Ai-Obeidani S, Al-Belushi R, Jackson D (2004) Fouling of reverse osmosis and ultrafiltration membranes: a critical review. Sep Sci Technol 39:2261–2297

Haynes RJ, Naidu R (1998) Influence of time fertilizer and manure application on soil organic content and soil physical conditions:a review. Nutr Cycle Agroecosyst 51:123–137

Hong S, Elimelech M (1997) Chemical and physical aspects of natural organic matter (NOM) fouling of nanofiltration membranes. J Membr Sci 132:159–181

Hsiao TC, Xu LK (2000) Sensitivity of growth of roots versus leaves to water stress:biophysical analysis and relation to water transport. J Exp Bot 51:1595–1616

Kawahigashi M, Sumida H, Yamamoto K (2005) Size and shape of soil humic acids estimated by viscosity and molecular weight. J Colloid Interface Sci 284:463–469

Kuga S (1981) Pore size distribution analysis of gel substances by size exclusion chromatography. J Chromatogr A 206:449–461

Lee S, Cho J, Elimelech M (2005) Combined influence of natural organic matter (NOM) and colloidal particles on nanofiltration membrane fouling. J Membr Sci 262:27–41

Lu Z, Neumann PM (1999) Water stress inhibits hydraulic conductance and leaf growth in rice seedling but not the transport of water via mercury-sensitive water channels in the root. Plant Physiol 120:143–151

Matyssek R, Tang AC, Boyer JS (1991) Plants can grow on internal water. Plant Cell Environ 14:925–930

Money NP (1989) Osmotic pressure of aqueous polyethylene glycols: relationship between molecular weight and vapor pressure deficit. Plant Physiol 91:766–769

Muscolo A, Sidari M (2009) Carboxyl and phenolic humic fractions affect Pinus Nigra callus growth and metabolism. Soil Sci Soc Am J 73:1119–1129

Muscolo A, Sidari M, Attina E, Francioso O, Tugnoli V, Nardi S (2007). Biological activity of humic substances is related to their chemical structure. Soil Science Society of America Journal 71:75–85

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

Neumann PM, Weissman R, Stefano G, Mancuso S (2010) Accumulation of xylem transported protein at pit membranes and associated reductions in hydraulic conductance. J Exp Bot 61:1711–1717

Piccolo A (2001) The supramolecular structure of humic substances. Soil Sci 166:810–832

Piccolo A, Nardi S, Concheri G (1996) Macromolecular changes of soil humic substances induced by interactions with organic acids. Eur J Soil Sci 47:319–328

Pinton R, Cesco S, Iacolettig G, Astolfi S, Varanini Z (1999) Modulation of NO_3^- uptake by water-extractable humic substances: involvement of root plasma membrane H^+ATPase. Plant Soil 215:155–161

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.). J Exp Bot 55:803–813

Rebhun M, Manka J (1971) Classification of organics in secondary effluents. Environ Sci Technol 5:606–609

Schmidt W, Santi S, Pinton R, Varanini Z (2007) Water-extractable humic substances alter root development and epidermal cell pattern in *Arabidopsis*. Plant Soil 300:259–267

Sharp RE, Davies WJ (1979) Solute regulation and growth by roots and shoots of water stressed maize plants. Planta 147:43–49

Steinberg CEW, Meinelt T, Timofeyev MA, Bittner M, Menzel R (2008) Humic substances (review series). Part 2: interactions with organisms. Environ Sci Pollut Res 15:128–135

Sutton R, Sposito G (2005) Molecular structure in soil humic substances: the new view. Environ Sci Technol 39:9009–9015

Tal A (2006) Seeking sustainability: Israel's evolving water management strategy. Science 313:1081–1084

Vallini G, Pera A, Avio L, Valdrighi M, Giovannetti M (1993) Influence of humic acids on laurel growth, associated rhizospheric microorganisms, and mycorrhizal fungi. Biol Fertil Soils 16:1–4

Yuan W, Zydney AL (2000) Humic acid fouling during ultrafiltration. Environ Sci Technol 34:5043–5050

Zandonadi DB, Canellas LP, Facanha AR (2007) Indole acetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H^+ pumps activation. Planta 225:1583–1595