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Stomatal responses to humic substances and auxin are sensitive to inhibitors of phospholipase A_2

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Abstract

Two fractions of earthworm humic substances, differing in nominal molecular weight, containing a very low amount of free IAA, and exhibiting auxin-like properties, were prepared and characterised by infrared and ^{1}H-NMR spectroscopy. In this study we investigate their effects on stomatal opening, as influenced by phospholipase A_2, in leaf of the *Argenteum* mutant of pea (*Pisum sativum* L.) Both of the humic fractions caused stomatal opening in the epidermal peels. The response showed a broad biphasic dose dependence and the effective concentrations were similar for the two fractions. The maximal stomatal apertures in response to both humic substances were similar to that caused by IAA and somewhat less than the response to white light or fusicoccin. Two inhibitors of phospholipase A_2 selectively blocked the response of stomata to both IAA and humic substances, without affecting the response to light or fusicoccin. We conclude that stomatal opening in response to auxin and humic substances involves activation of a phospholipase A_2 that is not involved in signalling the response to light or fusicoccin.

Introduction

Humic substances (HS), the major components of soil organic matter, have multiple roles in plant growth (Tan, 1998) and are consequently the subject of study in various areas of agriculture, such as soil chemistry, fertility, plant physiology and environmental sciences. Humic matter has a very complex biological activity, depending on its origin, molecular size, chemical characteristics and concentration. Many of the most important functions of HS will remain obscure until the nature of these substances are elucidated. Considering that the genesis of HS involves combinations of several reaction pathways and a wide variety of chemical binding systems, it is very difficult to define a clear concept on their composition (Hayes, 1997). Considerable progress has been made in providing an awareness of some of the gross features of HS (Stevenson, 1994), by employing various spectroscopic procedures and Nuclear Magnetic Resonance spectroscopy. They are commonly believed to consist of high molecular weight and highly polydisperse heterogeneous molecules (Stevenson, 1994). Recent studies obtained by size exclusion chromatography (Conte and Piccolo, 1999a, b;

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Piccolo et al., 1996a, b) suggested an alternative model in which relatively small and heterogeneous humic molecules self-assemble in supramolecular conformations stabilised only by weak forces and hydrogen bonding. This novel interpretation implies that root-exuded organic acids present in soil solution may affect the stability of humic conformation and hence their effect on permeability of root membranes (Nardi et al., 1996).

In the rhizosphere, an interaction between the root system and humic matter is possible when humic molecules present in the soil solution are small enough to flow into the apoplast and reach the plasma membrane. A low molecular size humic fraction (Hef), extracted from earthworm faeces, has been shown to accumulate in the apoplast and at least some components are able to reach the plasma membrane (Muscolo and Nardi, 1999; Vaughan, 1986). This event occurs in the vicinity of the root surface, where the simultaneous release of protons and organic acids by both the root and microbes enables the dissociation of the humic macrostructures and the subsequent release of the otherwise unavailable bioactive fractions (Piccolo et al., 1992). These substances may enter the plant and affect plant metabolism by either inducing or repressing protein synthesis (Dell'Agnola et al., 1981; Vaughan and MacDonald, 1971), by enzyme activation or inhibition (Muscolo et al., 1993; 1996), and by inducing morpho-functional changes in root architecture (Canellas et al., 2002).

Low molecular size fractions of HS can selectively affect ion uptake (nitrate, sulphate and phosphate) in a manner that is related to the concentration of HS and the pH of the medium (Chen and Aviad, 1990; Clapp et al., 2001; Varanini and Pinton, 2001; Vaughan and Malcolm, 1985). Such effects are far from being fully understood and may be related to a syndrome of physiological effects on plant metabolism involving modulation of ion uptake, either directly or indirectly. Pinton et al. (1999) showed that a low molecular weight water-extractable humic fraction (WEHS) affected nitrate uptake and plasma membrane (PM) H^+-ATPase activity in maize roots. The authors demonstrated that maize seedlings exposed to WEHS have a higher capacity to absorb NO_3^- and a faster induction of nitrate uptake. Since the activity of the PM H^+-ATPase also increased, the authors proposed a possible role of WEHS in the modulation of nitrate uptake via an interaction with the PM H^+-ATPase. Quaggiotti et al. (2004) showed that Hef may exert direct effects on gene transcription in roots, as shown for the *Mha2* gene encoding an isoform of H^+-ATPase, as well as long-distance effects in shoots, as observed for the *ZmNrt2*.1 gene, encoding a nitrate transporter. More recently Canellas et al. (2002) showed that a high molecular weight fraction of humic acids, isolated from earthworm compost, also increased maize H^+-ATPase activity by enhancing the content of the enzyme.

Activation of the H^+-ATPase and induction of genes encoding this enzyme have also been reported in response to auxins. Indeed activation of this pump (together with other associated changes in membrane permeability) is one of the most rapid changes that can be measured following treatment with auxin, occurring within ten minutes (reviewed by Barbier-Brygoo, 1995). Longer term auxin treatment also induces genes encoding particular isoforms of the H^+ATPase (Frias et al., 1996). A variety of other auxin–like activities have been suggested for humic fractions. For example, Nardi et al. (1994) demonstrated that both IAA and Hef stimulated root growth on leaf explants of *Nicotiana plumbaginifolia* and that two inhibitors with anti-auxin activity (TIBA = 2,3,5-triiodobenzoic acid and PCIB = 4-chlorophenoxy-isobutyric acid) could block these effects. Muscolo et al. (1999) demonstrated auxin-like effects of HS on cell growth and nitrate metabolism.

There is good evidence to suggest the involvement of a phospholipase A_2 (PLA_2) in auxin signalling leading to activation of the H^+-ATPase (reviewed by Macdonald, 1997; Scherer, 2002). Rapid activation of PLA_2 by auxin has been demonstrated in isolated microsomes and cultured cells (Paul et al., 1998; Scherer and André, 1989, 1993). In addition, inhibitors of animal PLA_2 blocked auxin-induced growth of Zucchini hypocotyls and maize coleoptiles (Scherer and Arnold, 1997; Yi et al., 1996). A PLA_2 enzyme is defined by its ability to catalyse the hydrolysis of the middle (*sn*-2) ester bond of substrate phospholipids to release lysophospholipids and free fatty acids. These lipid products are thought to be responsible for transmitting signals needed for the

induction of functional responses. Both fatty acids and lysophospholipids have been shown to produce auxin-like effects in maize coleoptiles (Yi et al., 1996) and to activate the H^+-ATPase *in vitro* (Palmgren et al., 1988). However, only fatty acids accumulated significantly after treatment of cultured plant cells with physiologically relevant concentrations of auxin (2 μM); lysophospholipids did not accumulate at auxin concentrations below 100 μM (Paul et al., 1998).

In animal systems, the PLA_2 superfamily includes a broad range of enzymes which have been extensively studied, classified and characterised (Six and Dennis, 2000). The 85 kDa cytosolic PLA_2 ($cPLA_2$) group has an important signalling role in animal cells, but no structural homologue of $cPLA_2$ has been found in the Arabidopsis genome (Holk et al., 2002). A group of calcium-independent, cytosolic phospholipases A_2 ($iPLA_2$) have been reported to have roles in phospholipid re-modelling and in signalling in animals (Winstead et al., 2000). Four cDNA sequences with homology to animal $iPLA_2$s have been isolated from Arabidopsis and two of them have been expressed in *Escherichia coli* and shown to have PLA activity *in vitro* (Holk et al., 2002; Rietz et al., 2004).

Activation of the plasma-membrane H^+-ATPase in guard cells causes proton extrusion and plasma-membrane hyperpolarization, driving uptake of K^+ via inward-rectifying potassium channels (K^+_{IN}) and uptake of Cl^- anions leading to stomatal opening. As well as auxins, red and blue light and the fungal toxin fusicoccin all activate H^+-ATPase and cause stomatal opening (reviewed by Schroeder et al., 2001). The mechanism is best understood for fusicoccin; it is thought that the toxin binds directly to a complex of the H^+-ATPase and a 14-3-3 protein, fixing the complex into a stable high activity state (Sze et al., 1999). The signalling pathways leading to stomatal opening in response to blue light have also been the subjects of much recent research. Stimulation of proton extrusion by phosphorylation of the C-terminus of the H^+-ATPase appears to be a key component of the response, but regulation of K^+_{IN} channels may also be important (reviewed by Assman and Shimizaki, 1999; Schroeder et al., 2001).

Stomatal opening can be observed in detached leaf epidermis, which consists of only two cell types, guard cells and epithelial cells in a single cell layer, which can be removed easily with a high percentage of both epidermal cells and guard cells remaining viable (Jewer et al., 1982). Because humic substances have been reported to affect the H^+-ATPase, and stomatal opening is an auxin response that involves activation of the H^+-ATPase, we hypothesised that HS might show auxin-like activity in this system. Since PLA_2 is a likely component of auxin signalling, and the single layer of cells in epidermal peels is readily accessible to lipid-soluble compounds, we went on to investigate the effect of inhibitors of PLA_2 on stomatal responses to auxin and HS. Finally, other stimuli that cause stomatal opening were used to discover whether the observed effects were specific. Our study demonstrates that two fractions of earthworm faeces humic substances, differing in nominal molecular weight and characterised by infrared and ^{1}H-NMR spectroscopy, exhibit auxin-like effects on stomatal opening as influenced by phospholipase A_2. It is relevant to validate the auxin-like action of humic matter extracted from earthworm faeces, because they produce castings with hormone-like activity and are the largest biomass of soil fauna, with a relevant ecological role in soil processes.

Materials and methods

Earthworm culture conditions

By grinding and mixing mineral constituents and organic matter with substances secreted by the gut, earthworms modify the composition of microbial communities and speed up the humification of organic matter, improving the quality of humus increasing the amount of organic carbon, humic carbon and C/N ratio (Dell'Agnola and Nardi 1987). The faeces of *Nicodrilus Allolobophora* (Eisen), *Aporrectodea* (Oerley) *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) (Minelli et al., 1995) were collected from the surface of the uncultivated couchgrass (*Agropyron repens*) fields, in the College of Agriculture Farm (University of Padua). Earthworm faeces were air-dried, cooled in a desiccator, and weighed.

The soils were classified as Calcaric Cambisol (CMc-FAO classification).

Preparation of humic fractions

The humic substances were extracted from the air-dried earthworm faeces with 0.1 *M* KOH (1:20 w/v) at room temperature for 16 h under a N_2 atmosphere and were freed from suspended material by centrifugation at 7000 *g* for 20 min. Purification and separation into fractions were as described by Nardi et al. (1991). Two fractions were obtained with low (<3500, Hef) and high (>3500, Hsp) nominal molecular weight.

Infrared spectroscopy

For each analysis, 2 mg of dried sample was mixed with 148 mg KBr (FT-IR grade, Aldrich Chemical Co. Milwaukee, WI), so that the mixture became homogeneous. After grinding, the sample mixture was heaped over the top of the micro sample cup. Any excess material was removed with a straight-edged tool. For the background, a micro sample cup of pure KBr was prepared. Diffuse reflectance infrared fourier transform (DRIFT) spectra were recorded with 200 scans collected at 4 cm^{-1} resolution using a Nicolet Impact 400 FT-IR Spectrophotometer (Madison, WI) and fitted with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). Spectra were collected and manipulated using the Omnic (3.1) software supplied from the manufacturer of the instrument.

^1H NMR spectroscopy

For each fraction, 20 mg were dissolved in 0.5 mL D_2O (Deuterium oxide). The spectra were recorded with a Bruker ACF 250 spectrometer using a 5-mm multinuclear probe. ^1H spectra were accumulated with 16 K data point, one pulse sequence, 40° pulse angle, 3 s relaxation delay and a sweep width of 2.5 kHz. To obtain a satisfactory signal to noise ratio 1000–2000 scans were needed. Gated irradiation was applied between acquisitions to pre saturate the residual water peak. Sodium 3-trimethylsilyl-propionate-2,2,3,3-d_4 (TSP) was added to the samples to provide a chemical shift standard.

Gas chromatography-mass spectroscopy (GC-MS) analysis

The sample (0.1–6 mg) was added to 1 mL of methanol and centrifuged at 10,000 rpm before the liquid phase (2 μL) was injected into the GC-MS system for IAA detection. All measurements were performed using an HP 6890 Gas Chromatograph coupled with an HP 5973 N Quadrupole. Chromatographic separation of analytes was obtained with an HP 5MS capillary column with the following dimensions: length 30 m; internal diameter 0.25 mm and film thickness 0.25 μm. The column had a constant helium flow of 0.7 mL min^{-1} and the temperature regime was 1 min at 150 °C followed by an increase of 10 °C/min to 250 °C, which was held for 15 min. The injector temperature was 250 °C. All injections were performed in splitless conditions for 0.5 minutes. The mass spectrometer was in SCAN acquisition mode to detect the ions produced by electron ionisation (70 eV) at the ion source temperature of 230 °C, in the mass range from 50 to 350 Daltons, with a scan rate of 4.72 scan sec^{-1}.

Plant material

Seeds of *Pisum sativum* L. *Argenteum* were sown in Levingtons F2S compost and grown under conditions of 16 h light, 22 °C and 8 h dark, 15 °C.

Tissue treatment and measurement of stomatal aperture

Epidermal peels were removed from the underside of fully expanded mature leaves of 3–5 week-old plants and floated cuticle side up in MES buffer solution (0.01 *M* 2-Morpholinoethanesulfonic acid-KOH, 0.05 *M* KCl, pH 6.15). Samples of peels were incubated in the dark for 1 h before treatment. The PLA_2 inhibitors 7,7-dimethyl-5,8-eicosadienoic acid (ETYA) (Sigma, Poole, UK) and Arachidonyltrifluoromethyl ketone ($AACOCF_3$) (Calbiochem, Nottingham, UK) were added at the same time as the treatment to a final concentration of 2.5 μM. For auxin-induced opening, 1 μM indole-3-acetic acid (IAA) was added to the buffer solution and peels were maintained in

the dark. Fusicoccin (Sigma, Poole, UK) was used at a concentration of 500 n*M* in the dark. Hsp and Hef were dissolved in water and used in the dark at concentrations between 0.01 and 100 mg C L^{-1}. For light-induced stomatal opening, peels were exposed to white light at 380 μmol m^{-2} s^{-1}. Measurements of stomatal aperture were made using a light microscope with bright-field illumination and an eyepiece graticule calibrated with a 100×10 μm slide micrometer scale. Experiments were performed three times, measuring between 25 and 30 stomatal apertures for each treatment. Where the percentages of open and closed stomata are recorded, stomata with apertures less than 2 μm were considered to be closed. IAA and inhibitor stock solutions were made up in ethanol and diluted to give a final solvent content of less than 0.02%. Ethanol at up to 0.5% has no effect on stomatal movement.

Viability tests

The viability of the epidermal tissue in the presence of inhibitors was tested by incubating peels in MES buffer solution containing fluorescein diacetate (0.01% w/v) for 5 min. The stain was visualised under UV light using a Nikon fluorescent microscope.

Results

Humic fraction characterization

^1H NMR spectroscopy

The spectra were divided into three main regions: aromatic hydrogens from 6.0 to 8.0 ppm; H attached to oxygen groups in carbon α (defined as sugar-like) from 4.2 to 3.0 ppm, and aliphatic H from 3.0 to 0.5 ppm. Distinct spectroscopic patterns were observed for the two humic fractions (Figure 1). Hsp showed a poorly resolved aromatic proton region and an intense and broad region attributed to sugar-like and polyether components (Wilson et al., 1983; Wershaw, 1985). Inspecting the aliphatic region revealed an intense doublet at 1.33 ppm that was identified as protons of the β CH_3 in lactate (Fan, 1996; Francioso et al., 2000; Wilson et al., 1988). Other broad and intense resonances at 0.83 ppm and

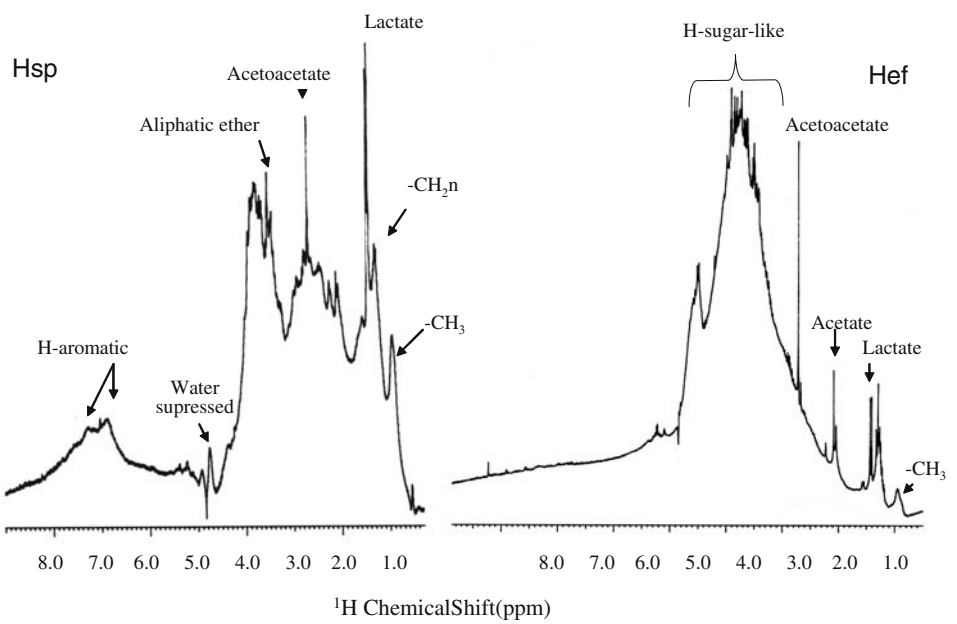

Figure 1. ^1H NMR spectrum of Hef and Hsp humic fractions extracted from earthworm faeces in D_2O. The spectra show an H_a region (6.0–8.0 ppm) poorly resolved, a H_{c-o} broad region (4.2–3.0 ppm) attributed to sugar-like components, polyether materials or methoxy groups, and an H_{al} region (3.0–0.5 ppm) with a finer proton structure which may correspond to low molecular weight organic acids.

1.3 ppm are due to the protons of terminal methyl groups of methylene chains, respectively (Malcolm, 1990). Two further intense resonances appeared at 2.9 ppm and 3.40 ppm and were assigned to protons in α CH_3 of acetoacetate and ether aliphatics, respectively (Aldrich, 1993; Fan, 1996). In contrast, Hef did not show resonances due to aromatic protons and was characterised by a broad unresolved region assigned to sugar-like components. However, the spectrum also contains some resonances due to the presence of low molecular weight organic substances such as lactate (1.33 ppm) and acetoacetate (2.9 ppm) and a singlet at 1.47 ppm assigned to proton in βCH_3 of alanine.

FT-IR spectroscopy

The spectra are shown in Figure 2. There were few significant differences between the spectra for the two humic fractions. The major bands were assigned as follows: a broad band around at 3200 cm^{-1} is attributed to O–H stretching of carboxylic and alcoholic groups in different electrostatic environments (Bellamy, 1975); around at 2940 cm^{-1} the band is assigned to asymmetric C–H stretching of aliphatic groups. The band appearing at 1719 cm^{-1} is characteristic of undissociated carboxyl groups (C = O) vibrations (Niemeyer et al., 1992; Rao, 1963). The bands at 1660 and 1514 cm^{-1} likely correspond to carbonyl C = O stretching and both N–H deformation and C–N stretching vibrations. Moreover, these bands are due to C = C and C–C vibrations in aromatic rings, respectively. The region around at 1400–1300 cm^{-1} correspond to CH_2 asymmetric bending and carboxylate symmetric stretching motions (Bellamy, 1975; Niemeyer et al., 1992; Stevenson, 1994) the band appearing around 1230 cm^{-1} can be attributed to C–O stretch vibrations in alcohols, phenols and carboxyl groups. The strong band appearing around at 1043 cm^{-1} is attributed to C–O stretching of carbohydrates and alcohols (Bellamy, 1975; Stevenson, 1994), as well as to C–C stretching motions of aliphatic groups, and in-plane C–H bending of aromatic rings. The broad band at 2626 cm^{-1} in Hef is consistent with the formation of intermolecular hydrogen bonding between OH groups in oxygenated compounds (Rao, 1963). In addition the intense bands in Hef at 1718 and 1223 cm^{-1} confirmed the presence of COOH groups in acid dimers (Rao, 1963). These data were supported by NMR spectra that showed intense chemical shift of the β-CH_3 protons assigned to lactate.

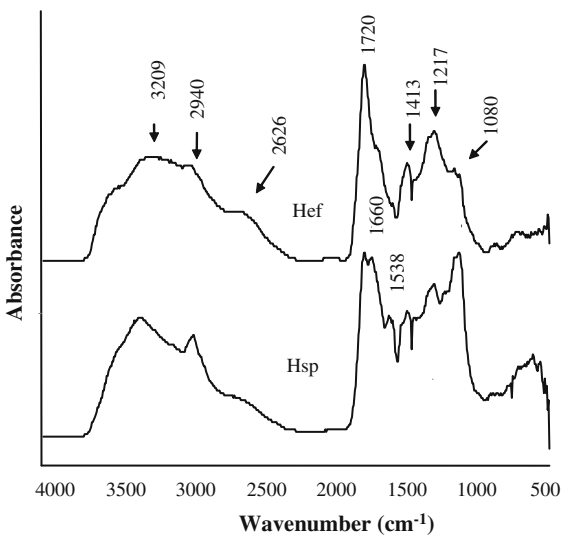

Figure 2. DRIFT spectra of Hef and Hsp humic fractions extracted from earthworm faeces. The bands correspond to ν (O–H) vibration of carboxylic and alcoholic groups (3200 cm^{-1}); asymmetric ν(C–H) motions of aliphatic groups (2960 cm^{-1}); undissociated carboxyl groups ν(C = O) vibrations (1719 cm^{-1}); ν(O–H) vibration of carboxyl groups (2626 cm^{-1}); ν(C = O) vibration in amide I and both δ(N–H) and ν(C–N) vibrations in amide II (1612 and 1514 cm^{-1}); ν(C–O) vibrations of alcohols, phenols and carboxyl groups (1220 cm^{-1}); C–O stretching of carbohydrates and alcohols, as well as C–C stretching motions of aliphatic groups (1083 cm^{-1}).

GC-MS analysis

The IAA standard showed a GC retention time of 14.56 min in the GC/MS conditions and the mass spectrum had a base peak at 130 m/z, consistent with the ion formed by elimination of the -CO_2Me radical from methylated IAA. This signal was not detectable in the spectrum for methylated Hef (data not shown).

Stomatal opening experiment

Optimisation of auxin-induced stomatal opening

To determine the optimum concentration of IAA for induction of stomatal opening in the *Argenteum* mutant of *P. sativum*, epidermal peels were incubated in buffer in the dark for one hour and then exposed to different concentrations of IAA or to a buffer control for a further hour. Peels

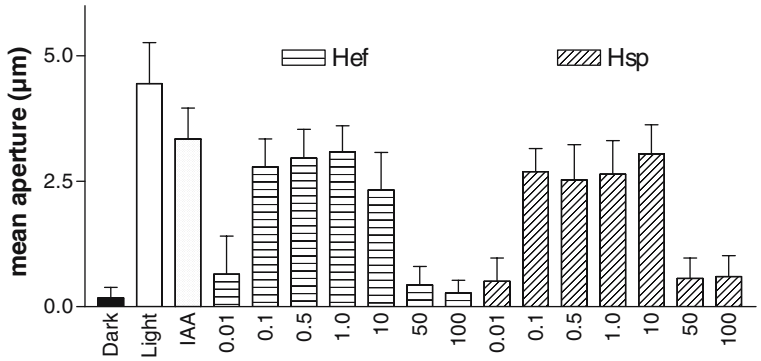

Figure 3. The response of pea stomata to IAA, white light and humic substances. Epidermal peels were pre-incubated in the dark for one hour, when mean stomatal apertures were 0.12 μm $\pm$0.19, and then treated as follows for 1 h: dark, no treatment; white light (380 μM m^{-2} s^{-1}), 1 μM IAA in the dark, Hef or Hsp in the dark at the concentrations shown (mg C L^{-1}). Bars represent the mean stomatal aperture $\pm$ SE ($n=75$).

exposed to 1 μM IAA had 68% of stomata open in comparison to only 15% in controls. Peels exposed to 0.1, 10, and 100 μM IAA all showed less than 20% of open stomata after the same incubation period (data not shown). IAA was used at 1 μM in all subsequent experiments. Benzoic acid (a weak acid which has structural similarity to IAA, but no auxin activity) had no effect on stomatal aperture at these concentrations (data not shown).

Stomatal opening in response to humic substances

The humic substances Hef and Hsp were tested for their effect on stomatal aperture at concentrations between 0.01 mg C L^{-1} and 100 mg C L^{-1} (Figure 3). Epidermal peels treated with IAA, white light or maintained in the dark were included as controls. Both humic fractions caused stomatal opening at concentrations between 0.1 mg C L^{-1} and 10 mg C L^{-1}. Concentrations below (0.01 mg C L^{-1}) and above (50 and 100 mg C L^{-1}) this range were ineffective. The mean stomatal apertures obtained with Hef or Hsp at 1 mg C L^{-1} were not significantly different from that obtained with 1 μM IAA, but somewhat lower than that obtained in white light.

Effect of animal PLA_2 inhibitors on stomatal opening

Inhibitors of animal PLA_2 activity were used to investigate the involvement of PLA_2 in stomatal opening in response to auxin, light, HS and the fungal toxin fusicoccin. Epidermal peels were pre-incubated in the dark for one hour to ensure closure of stomata, then inhibitors were added and the peels were subjected to the appropriate stimulus. Figure 4a shows the mean stomatal aperture in peels treated with each of the stimuli, with or without the inhibitor $AACOCF_3$, a preferential inhibitor of $cPLA_2$ and $iPLA_2$ in animals. Before treatment, stomata were closed (aperture less than 1 μm). After treatment with IAA, Hef and Hsp in the absence of inhibitor, stomata opened to a mean aperture of between 2.7 and 2.9 microns. However, when $AACOCF_3$ was included, stomata remained closed with a mean aperture equivalent to that of the dark control. In contrast, light and fusicoccin caused stomata to open to somewhat larger apertures and this response was not affected by the presence of $AACOCF_3$. Similar results were obtained using another inhibitor, ETYA (Figure 4b). The mean stomatal apertures in peels treated with IAA, Hef or Hsp were significantly reduced when the inhibitor was present, but the responses to light or fusicoccin were not affected.

The inhibitors were tested for their effects on the viability of guard cells and epithelial cells as judged by staining of treated epidermis with fluorescein diacetate. No effects were seen at concentrations up to 50 μM (data not shown).

Discussion

Activation of the PM H^+-ATPase is a key step leading to stomatal opening in response to a variety of stimuli including auxin (reviewed by

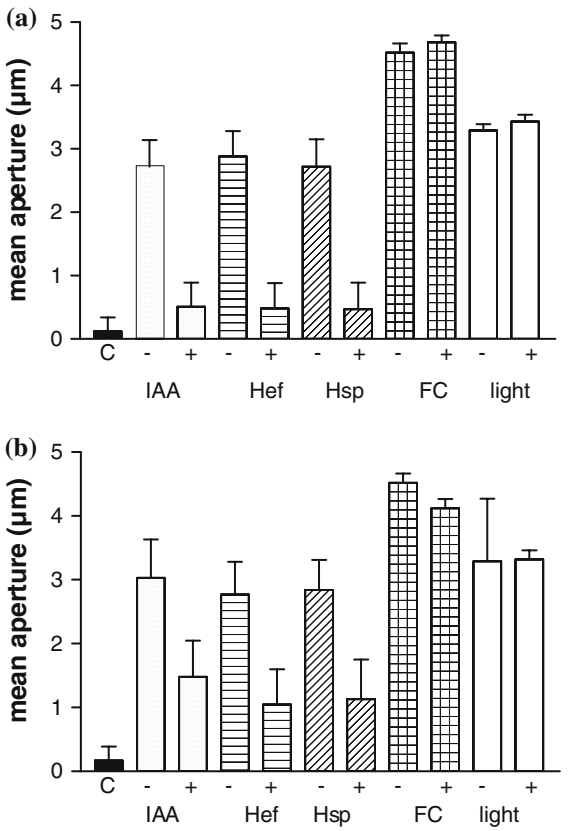

Figure 4. The effect of PLA_2 inhibitors on IAA-, Hef, Hsp, light- and fusicoccin- induced stomatal opening. Epidermal peels were pre-incubated in the dark for one hour and then treated as follows in the dark: no treatment (C), 1 μM IAA for 1h, 1 mg C L^{-1} Hef for 1 h, 1 mg C L^{-1} Hsp for 1 h, or 0.5 μM FC for 30 min (all in the dark) or placed under white light (380 μM m^{-2} s^{-1}) for 1 h. (a) Where indicated (+), $AACOCF_3$ (2.5 μM) was added at the same time as the other treatments. Mean stomatal apertures before treatment were 0.23 ± 0.37. (b) Where indicated (+), ETYA (2.5 μM) was added at the same time as the other treatments. Mean stomatal apertures before treatment were 0.15 ± 0.21. Bars represent the mean stomatal aperture ± SE ($n = 75$).

Schroeder et al., 2001). Since this enzyme has been shown to be activated by HS (Canellas et al., 2002; Pinton et al., 1999; Quaggiotti et al., 2004) and HS have been suggested to have auxin-like activities in a number of systems, it seemed likely that HS would stimulate stomatal opening. Both of the humic fractions tested here were indeed able to cause stomatal opening in epidermal peels of pea. The maximal extent of stomatal opening was similar for the two humic fractions and for IAA, somewhat lower than the maximal stomatal aperture obtained when either white light or fusicoccin was the stimulus. The two humic fractions were effective in promoting stomatal opening at similar concentrations, with a broad biphasic dose response. Concentrations over a 100-fold range between 0.1 mg C L^{-1} and 10 mg C L^{-1} were effective for both Hef and Hsp, while higher and lower concentrations were ineffective. A biphasic dose response curve is typical for many auxin responses and stomatal opening in response to IAA is no exception. However, the range of effective concentrations is narrower than for the humic substances – concentrations 10-fold lower or higher than the optimum of 1 μM are significantly less effective.

There is good evidence that a PLA_2 is involved in transmitting the auxin signal leading to activation of the PM H^+-ATPase (reviewed by Macdonald, 1997; Scherer, 2002). We tested two inhibitors of PLA_2 (ETYA and $AACOCF_3$) at low concentrations and showed that they were able to block the response of stomata to both HS and IAA. However, they did not block opening by light or fusicoccin at these concentrations, showing that they are not inhibiting any process required for stomatal opening *per se*. This also rules out a more general toxic effect on the cells, in agreement with our observation that the inhibitors had no effects on cell viability at concentrations up to 20-fold higher than those used here. These observations suggest that the signalling pathway(s) activated by IAA and humic acids and leading to stomatal opening involve a PLA_2 that is sensitive to ETYA and $AACOCF_3$. In animals, $AACOCF_3$ is often reported to be selective for $cPLA_2$, but also inhibits $iPLA_2$. Holk et al. (2002) showed that activity of an $iPLA_2$ homologue from A*rabidopsis thaliana* is sensitive to both $AACOCF_3$ and ETYA, thus these enzymes are candidates for involvement in auxin and HS signalling. However, the inhibitors do not affect the response to light and fusicoccin, suggesting that these stimuli do not signal through the same pathway as auxin. In agreement with these data, Scherer and Arnold (1997) demonstrated that ETYA was able to block elongation of zucchini hypocotyls in response to auxin, but not in response to FC.

Our data show that Hef and Hsp have similar effects on stomata, whereas previous studies of the effect of HS on plants have suggested rather different activities for Hef and Hsp. Indeed auxin-like effects have been mainly attributed to

the low molecular weight components of HS, such as Hef, in part because of the ability of Hef to reach the plasma membrane of cells (for review, see Nardi et al., 2002), whereas Hsp has been reported to be tightly bound to the cell wall (Vaughan, 1986). There are also differences in the structure of the two humic fractions. Carbon-13 NMR spectroscopy has been successfully applied to humic substances and represents a powerful technique for structural investigations of humic substances (Piccolo and Conte, 1998; Preston, 1996). Quantitative ^{13}C NMR spectra (Muscolo et al. 1996) showed clear differences between the two humic fractions Hef and Hsp. The Hef fraction (low molecular weight) was rich in aliphatic- and carboxylic-C groups, whereas the Hsp fraction had a higher content of peptidic-, carbohydratic- and aromatic-C groups. There were no significant differences in the amount of phenolic-C groups, between the Hef and Hsp humic fractions. Here we have compared the FT-IR DRIFT and ^{1}H NMR spectra of the two fractions. The DRIFT spectra showed few significant differences between the two fractions, but there were clear differences in the ^{1}H NMR spectra, in particular the higher content of aromatic groups in Hsp was confirmed. The ^{1}H NMR spectra also suggested the presence of low-molecular weight substances, such as lactate and acetoacetate, in both Hsp and Hef. In Hsp (fraction retained after dialysis), these substances are presumed to be bound to the macrostructure.

We were unable to detect IAA in Hef using GM-MS with samples of up to 6 mg. Callenas et al. (2002) previously reported detection of IAA in a 30 mg sample of humic acids, although the signal was only about twice the noise level. The limit of detection of the method would be expected to be of the order of a few ng, thus both sets of data are consistent with an IAA content of less than 0.001% w/w in HS. Previously, Muscolo et al. (1998) detected IAA at about 0.5% in both Hef and Hsp by radioimmune assay (RIA). The different IAA contents estimated by RIA and GC-MS may reflect the ability of the RIA to detect IAA bound to the HS macro structure, whereas GC-MS would only detect free IAA in the solvent. In any case, even the higher estimate of IAA content (by RIA) is too low to account for the auxin-like activity of HS on stomata at most of the concentrations used in this study. The highest concentration of Hef and Hsp that caused stomatal opening (10 mg C L^{-1}) would contain 0.45 μM IAA. This would be expected to cause some stomatal opening, since the optimal concentration of IAA is 1 μM with a greatly reduced effect at 0.1 μM. However, Hef and Hsp still caused stomata to open when used at 0.1 mg C L^{-1}, corresponding to an IAA concentration of only 4.5 nM. Even if the IAA content of the HS has been underestimated by the RIA, the results cannot be simply explained by free IAA in the samples, since the dose response to HS is much broader than for IAA. Similar apertures are produced over a 100-fold concentration range with HS, whereas the response to IAA decreases significantly at concentrations 10-fold lower or higher than the optimum. Thus, the two humic fractions exhibit auxin-like activity that cannot be fully explained by their content of IAA. It is possible that the HS also contain other auxins, such as phenylacetic acid and indole butyric acid in addition to IAA. The spectroscopic analysis confirmed the presence of low molecular weight organic acids in both fractions. Alternatively, there may be some other unknown component of HS that has auxin-like activity. All known auxins contain a carboxyl group in addition to a hydrophobic ring and the DRIFT analysis indicated a high content of free carboxyls in both fractions. Finally, it is possible that IAA may be produced, either chemically or enzymatically, from HS in contact with the plant cell wall.

In conclusion, the results give strong evidence that both high and low molecular weight fractions of HS promote stomatal opening in pea with a broad biphasic concentration dependence in spite of their low content of free IAA. The extent of opening is similar to that produced by auxin, and a component sensitive to inhibitors of $iPLA_2$ is involved in signalling the response to auxin and HS, but not the response to white light or fusicoccin.

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References

Aldrich Library 1993 Aldrich library of 13C and 1H FT NMR spectra, Vol. III. Eds. C J Pouchert and J Behnke.

Assmann S M and Shimizaki K 1999 The multisensory guard cell. Stomatal responses to blue light and abscisic acid. Plant Physiol. 119, 809–815.

Barbier-Brygoo H 1995 Tracking auxin receptors using functional approaches. Critical Reviews in Plant Sci. 14, 1–25.

Bellamy J 1975 The Infrared Spectra of Complex Molecules. Chapman and Hall, London.

Canellas L P, Olivares F L, Okorokova-Facanha A L and Facanha 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 Physiol. 130, 334–340.

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

Clapp C, Chen Y, Hayes M H B and Cheng H H 2001 Plant growth promoting activity of humic substances. *In* Understanding and Managing Organic Matter in Soils, Sediments and Waters. Eds. R S Swift and K M Sparks. pp. 243–255. International Humic Science Society, Madison, WI.

Conte P and Piccolo A 1999a High pressure size exclusion chromatography (HPSEC) of humic substances: molecular sizes, analytical parameters and column performance. Chemosphere. 38, 517–528.

Conte P and Piccolo A 1999b Conformational arrangement of dissolved humic substances. Influence of solution composition on association of humic molecules. Environ. Sci. Techn. 33, 1682–1690.

Dell'Agnola G, Ferrari G and Nardi S 1981 Antidote action of humic substances on atrazine inhibition of sulphate uptake in barley roots. Pesticide Biochem. Physiol. 15, 101–104.

Dell'Agnola G and Nardi S 1987 An overview of earthworm activity in the soil. *In* On Earthworms. Eds. A M Bonvicini Pagliai and P. Omodeo. pp. 103–112. Mucchi, Modena.

Fan T W M 1996 Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog. Nucl. Magn. Reson. Spectrosc. 28, 161–219.

Francioso O, Ciavatta C, Sánchez-Cortés S, Tugnoli V, Sitti L and Gessa C 2000 Spectroscopic characterization of soil organic matter in long-term amendment trials. Soil Sci. 165, 495–504.

Frías I, Caldeira M T, PérezCastiñeria J R, NavarroAviñó J P, Culianez-Maciá F A, Kuppinger O, Stransky H, Pagés M, Hager A and Serrano R 1996 A major isoform of the maize plasma membrane H^+-ATPase: Characterization and induction by auxin in coleoptiles. Plant Cell 8, 1533–1544.

Hayes M H B 1997 Emerging concepts of the compositions and structure of humic substances. *In* Humic Substances in Soils, Peats and Waters-Health and Environmental Aspects. Eds. M H B Hayes and W S Wilson. pp. 3–30. The Royal Society of Chemistry, Cambridge.

Holk A, Reitz S, Zahn M, Quader H and Scherer G F E 2002 Molecular identification of cytosolic, patatin-related phospholipase A from *Arabidopsis* with potential functions in plant signal transduction. Plant Physiol. 130, 90–101.

Jewer P C, Incoll L D and Shaw J 1982 Stomatal responses of Argenteum - a mutant of *Pisum sativum* L. with readily detachable leaf epidermis. Planta 155, 146–153.

Macdonald H 1997 Auxin perception and signal transduction. Physiol. Plantarum 100, 423–430.

Malcolm R L 1990 The uniqueness of humic substances in each of soil, stream and marine environments. Anal. Chim. Acta 232, 19–30.

Minelli A, Omodeo P, Rota E and Sambugar B 1995 Anellida Clitellata, Aphanomeura. *In* Checklist delle Specie della Fauna Italiana. Eds. A Minelli, S Rufo and S La Posta. pp. 12–13. Calderoni, Bologna.

Muscolo A, Bovalo F, Gionfriddo F and Nardi S 1999 Earthworm humic matter produces auxin-like effects on *Daucus carota* cell growth and nitrate metabolism. Soil Biol. Biochem. 31, 1303–1311.

Muscolo A, Cutrupi S and Nardi S 1998 IAA detection in humic matter. Soil Biol. Biochem. 30, 1199–1201.

Muscolo A, Felici M, Cancheri G and Nardi S 1993 Effect of earthworm humic substances on peroxidase and esterase activity during growth of leaf explants of *Nicotiana plumbaginifolia*. Biol. Fertil. Soils 15, 127–131.

Muscolo A and Nardi S 1999 Effetti di due frazioni umiche sul metabolismo azotato di cellule di *Daucus carota*. In Le Ricerche di Base e le Applicazioni delle Sostanze Umiche alle Soglie del 2000. pp. 103–106. IV Congresso Nazionale IHSS. Alghero.

Muscolo A, Panuccio M R, Abenavoli M R, Concheri G and Nardi S 1996 Effect of molecular complexity and acidity of earthworm faeces humic fractions on glutamate dehydrogenase, glutamine synthetase, and phosphoenolpyruvate carboxylase in *Daucus Carota* a II cells. Biol. Fertil. Soils 22, 83–88.

Nardi S, Pizzighello D, Muscolo A and Vianello A 2002 Physiological effects of humic substances in higher plants. Soil Biol. Biochem. 34, 1527–1537.

Nardi S, Concheri G and Dell'Agnola G 1996 Biological activity of humic substances. *In* Humic Substances in Terrestrial Ecosystems. Ed. A. Piccolo. pp. 361–406. Elsevier, Amsterdam.

Nardi S, Concheri G, Dell'Agnola G and Scrimin P 1991 Nitrate uptake and ATPase activity in oat seedlings in the presence of two humic fractions. Soil Biol. Biochem. 23, 833–836.

Nardi S, Panuccio M R, Abenavoli M R and Muscolo A 1994 Auxin-like effect of humic substances extracted from faeces of *Allolobophora Caliginosa* and *Allolobophora Rosea*. Soil Biol. Biochem. 26, 1341–1346.

Niemeyer J, Chen Y and Bollag J M 1992 Characterization of humic acids, composts, and peat by diffuse reflectance Fourier-transform infrared spectroscopy. Soil Sci. Soc. Am. J. 56, 135–140.

Palmgren M G, Sommarin M, Ulvskov P and Jorgensen P L 1988 Modulation of plasma-membrane H^+-ATPase from oat roots by lysophosphatidylcholine, free fatty-acids and phospholipase A_2. Physiol. Plantarum 74, 11–19.

Paul R U, Holk A and Scherer G 1998 Fatty acids and lysophospholipids as potential second messengers in auxin

action. Rapid activation of phospholipase A_2 activity by auxin in suspension-cultured parsley and soybean cells. Plant J. 16, 601–611.

Piccolo A, Nardi S and Concheri G 1996a Macromolecular changes of humic substances induced by interaction with organic. Eur. J. Soil Sci. 47, 319–328.

Piccolo A, Nardi S and Concheri G 1996b Micelle-like conformation of humic substances as revealed by size exclusion chromatography. Chemosphere 33, 595–602.

Piccolo A and Conte P 1998 Advances in nuclear magnetic resonances and infrared spectroscopies of soil organic particles. *In* Structure and Surface Reactions of Soil Particles. Eds. P M Huang, N Senesi and J Buffle. pp. 183–250. Wiley & Sons Ltd, Sussex.

Piccolo A, Nardi S and Concheri G 1992 Structural characteristics of humic substances as related to nitrate uptake and growth regulation in plant systems. Soil Biol. Biochem. 24, 373–380.

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

Preston C M 1996 Applications of NMR to soil organic matter analysis: history and prospects. Soil Sci. 161, 44–166.

Quagiotti S, Ruperti B, Pizzighello D, Francioso O, Tugnoli V and 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, 1–11.

Rao C N R 1963 Chemical Applications of Infrared Spectroscopy. Academic Press, London 132 pp.

Rietz S, Holk A and Scherer G F E 2004 Expression of the patatin-related phospholipase A gene AtPLA IIA in *Arabidopsis thaliana* is up-regulated by salicylic acid, wouding, ethylene, and iron and phosphate deficiency. Planta 219, 743–753.

Scherer G F E 2002 Second messengers and phospholipase A_2 in auxin signal transduction. Plant Mol. Biol. 49, 357–372.

Scherer G F E and André B 1989 A rapid response to a plant hormone: auxin stimulates phospholipase A_2 in vivo and in vitro. Biochem. Biophys. Res. Commun. 163, 111–117.

Scherer G F E and André B 1993 Stimulation of phospholipase A_2 by auxin in microsomes from suspension-cultured soybean cells is receptor-mediated and influenced by nucleotides. Planta 191, 515–523.

Scherer G F E and Arnold B 1997 Inhibitors of animal phospholipase A_2 enzymes are selective inhibitors of auxin-dependent growth. Implications for auxin-induced signal transduction. Planta 202, 462–469.

Schroeder J I, Allen G J, Hugouvieux V, Kwak J M and Waner D 2001 Guard cell signal transduction. Ann. Rev. Plant Physiol. Plant Mol. Biol. 52, 627–658.

Six D A and Dennis E A 2000 The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta 1488, 1–19.

Stevenson F J 1994 Humus Chemistry: Genesis, Composition, Reactions. 2Wiley & Sons, New York 496 pp.

Sze H, Li X and Palmgren M G 1999 Energization of plant cell membranes by H+-pumping ATPases: regulation and biosynthesis. Plant Cell 11, 677–689.

Tan K H 1998 Colloidal chemistry of organic soil constituents. *In* Principles of Soil Chemistry. Ed. K H Tan. pp. 177–258. Marcell Dekker, New York.

Varanini Z and Pinton R 2001 Direct versus in direct effects of soil humic substances on plant growth and nutrition. *In* . Eds. D Basel, R Pinton, Z Varanini and P Nannipieri. pp. 141–158. The Rhizosphere. Marcel Dekker, Switzerland.

Vaughan D 1986 Effetto delle sostanze umiche sui processi metabolici delle piante. *In* Sostanze umiche effetti sul terreno e sulle piante. Eds. R G Burns, G Dell'Agnola, S Miele, S Nardi, G Savoini, M Schnitzer, P Sequi, D Vaughan and S A Visser. pp. 59–81. Ramo Editoriale degli Agricoltori, Roma.

Vaughan D and Macdonald I R 1971 Effects of humic acid on protein synthesis and ion uptake in beet discs. J. Exp. Bot. 22, 400–410.

Vaughan D and Malcolm R E 1985 Influence of humic substances on growth and physiological processes. *In* Soil Organic Matter and Biological Activity. Eds. D Vaughan and R E Malcolm. pp. 37–75. Martinus-Nijhoff, Boston.

Wershaw R L 1985 Application of Nuclear Magnetic Resonance Spectroscopy for determining functionality in humic substances. *In* Humic Substances in Soil, Water and Sediment: Geochemistry, Isolation and Characterization. Eds. G R Aiken, D M McKnight, R L Wershaw and P MacCarthey. pp. 561–570. Wiley International, New York.

Wilson M A, Collin P J, Malcolm R L, Perdue E M and Cresswell P 1988 Low molecular weight species in humic and fulvic fractions. Org. Geochem. 12, 7–12.

Wilson M A, Collin P J and Tate K R 1983 ^{1}H NMR nuclear magnetic resonance spectroscopy of soil humic acid. J. Soil Sci. 34, 297–304.

Winstead M V, Balsinde J and Dennis E A 2000 Calcium-independent phospholipase A_2: structure and function. Biochim. Biophys. Acta 1488, 28–39.

Yi H, Park D and Lee Y 1996 In vivo evidence for the involvement of phospholipase A and protein kinase in the signal transduction pathway for auxin-induced corn coleoptile elongation. Physiol. Plantarum 96, 359–368.

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