



## Review

**Physiological effects of humic substances on higher plants**Serenella Nardi<sup>a,\*</sup>, Diego Pizzeghello<sup>a</sup>, Adele Muscolo<sup>b</sup>, Angelo Vianello<sup>c</sup><sup>a</sup>*Dipartimento di Biotecnologie Agrarie, Agripolis, Università di Padova, Strada Romea 16, 35020 Legnaro, Padova, Italy*<sup>b</sup>*Dipartimento di Agrochimica ed Agrobiologia, Università di Reggio Calabria, Piazza S. Francesco 4, 89061 Gallina di Reggio Calabria, Italy*<sup>c</sup>*Dipartimento di Biologia ed Economia Agro-industriale, Sezione di Biologia Vegetale, Università di Udine, Via Cotonificio 108, 33100 Udine, Italy*

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**Abstract**

The physiological effects of humic substances (HS) on some aspects of plant growth and metabolism are examined. Evidence has been presented on that the effect of HS on plant growth depends on the source, concentration and molecular weight humic fraction. While a low molecular size (LMS < 3500 Da) fraction easily reaches the plasmalemma of higher plant cells and, in part, is taken up into them, a high molecular size fraction (HMS > 3500 Da) is not absorbed and can interact only with the cell wall. Therefore, a LMS fraction is the major candidate for determining the positive effects of HS on plant growth. The latter effects are in part exerted at the level of the plasma membrane by positively influencing the uptake of some nutrients, and in particular that of nitrate. The effects on the intermediary metabolism are less understood, albeit it seems that HS may influence both respiration and photosynthesis. Humic matter appears also to display an hormone-like activity. It is not clear if this activity is strictly linked to the chemical structure of HS or whether it depends on hormones of microbial origin entrapped into them. In any case, HS exhibit stimulatory effects on plant cell growth and development. © 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Humic matter; Chemical characteristics; Hormone-like activity; Intermediary metabolism; Ion uptake; Plant cells

**1. Introduction**

Humic substances (HS), the major component of soil organic matter, are the subject of study in various areas of agriculture, such as soil chemistry, fertility, plant physiology as well as environmental sciences, because of the multiple roles played by these materials that can greatly benefit plant growth (Tan, 1998).

The beneficial effects of HS on plant growth may be related to their indirect (increase of fertilizer efficiency or reducing soil compaction), or direct (improvement of the overall plant biomass) effects. In particular, the increase of root growth is generally more apparent than that of the shoot (Vaughan and Malcom, 1985). The stimulatory effects of HS have been correlated to the maintenance of Fe and Zn in solution at effective concentrations (Clapp et al., 2001). In this context, HS have been widely regarded as playing a beneficial role in Fe acquisition by plants (Chen and Aviad, 1990; Pinton et al., 1999b). This effect has been mainly attributed to the complexing properties of HS, which

increase the availability of micronutrients from sparingly soluble hydroxides (Stevenson, 1991). Although only in part, HS, in particular those with a low molecular mass, are taken up by plants and, therefore, may also actively modify the plant metabolism (Vaughan and Malcom, 1985; Muscolo and Nardi, 1999). Their effects appear to be mainly exerted on cell membrane functions, promoting nutrient uptake (Visser, 1986; Varanini and Pinton, 1995), or plant growth and development, by acting as hormone-like substances (Vaughan and Malcom, 1985; Nardi et al., 1996).

This issue has been examined in recent reviews (Clapp et al., 2001; Varanini and Pinton, 2001). Therefore, this paper aims to reassess some physiological effects of HS on higher plant cells.

**2. Chemical characterization of humic matter**

Many of the most important functions of HS will remain obscure, until the nature of these substances will not be elucidated. It is known that the chemical composition of humic matter includes many aromatic rings that interact

\* Corresponding author. Tel.: +39-49-8272905; fax: +39-49-8272929.  
E-mail address: serenella.nardi@unipd.it (S. Nardi).

with each other and with aliphatic chains, giving rise to macromolecules with different masses. 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). Many of the original classical methods to understand the nature of HS were based on elemental composition, but the results obtained represent averages for agglomerations of molecules and it is impossible to derive precise empirical formulae from these data (Hayes et al., 1989).

Later, valuable information was gained from chemical degradation techniques (acid/base catalyzed hydrolysis, oxidative and reductive processes and thermal procedures), involving possible chemical constituents and building blocks of HS (Hayes, 1997). However, because the major linkages in the 'core' of HS are not hydrolysable, the energy inputs needed to cleave the links between the component molecules give rise to products that can be vastly different from the molecules that compose the macromolecules.

Considerable progress has been made in the last few years in providing an awareness of some of the gross features of HS (Hayes et al., 1989; Stevenson, 1994), by employing various spectroscopic procedures (Preston, 1996). Infrared spectroscopy has been the most widely used for studies of HS (MacCarthy and Rice, 1985), but the overlapping and the uncertainty of the assignments remain. Other approaches, which include electron spin resonance (Senesi and Steelink, 1989), Raman, ultraviolet–visible, fluorescence, X-ray photoelectron spectroscopy, have also been used.

The surface enhanced Raman spectroscopy technique has recently been employed (Francioso et al., 1996). Since its discovery, surface-enhanced Raman spectroscopy and surface-enhanced resonance Raman spectroscopy had been largely applied to the study of humic materials, demonstrating that it is possible to gather valuable information about the aromatic groups and the special conformation of these macromolecules in aqueous solution. The combined use of these techniques has shown an increase of oxygenated groups in HS with low molecular masses (Francioso et al., 1996).

In recent years, with the aim of studying HS, considerable effort has been focused on applications of solution- and solid-state  $^{13}\text{C}$  NMR (nuclear magnetic resonance) spectroscopy to studies of HS composition. This technique has demonstrated that aliphatic compositions in HS are often as important or, occasionally, more important than aromatic structures. Aromaticity of HS, extracted from soils of differing pedological origins, ranges from 30 to 60%, with many in the 47–60% range. A substantial portion of aliphatic C in HS consists of paraffinic C. One of the advantages of  $^{13}\text{C}$  NMR is that it indicates the presence in HS of a variety of structures whose determinations by others methods would either be laborious and time-consuming, or not possible at all. Even more valuable information on

the chemical nature HS can be obtained in an integrated approach by combining  $^{13}\text{C}$  NMR with chemical and mass spectrometric methods. Of considerable interest is a comparison of solid-state  $^{13}\text{C}$  NMR data of humic acids (HA) and fulvic acids (FA), extracted, respectively, from a Mollisol Ah horizon and from a Haplaquod Bh horizon (Table 1) (Schnitzer and Schulten, 1998). The spectra are divided into the following regions: 0–40 ppm (C in the straight-chain, branched, and cyclic aliphatics); 41–60 ppm (C in branched aliphatic, amino acids, and  $\text{OCH}_3$  groups); 61–105 ppm (C in carbohydrates, and in aliphatic containing C bounded to OH, ether oxygens, or occurring in five- or six-membered rings bonded to O); 105–150 ppm (aromatic C), 151–170 ppm (phenolic C), and 171–190 ppm (C in  $\text{CO}_2\text{H}$  groups). The main differences between HA and FA concern the following aspects: C distribution in the two humic fractions; HA are slightly more aromatic than FA, but the FA are considerably richer in  $\text{CO}_2\text{H}$  groups; HA are richer in paraffinic C, but poorer in the carbohydrate-C than FA. Nevertheless, on the whole, the main features, such as aromaticity and aliphaticity are similar.

### 3. Uptake of humic matter

The assertion that HS can have a direct effect on plant metabolism, implies that these substances are taken up into plant tissues (Vaughan and Malcom, 1985). Earlier work relied on color changes in plant organs as an indication of uptake (Prat, 1963). Later, isotopes of carbon were used, particularly  $^{14}\text{C}$ -labeled HS (Vaughan and Ord, 1981; Vaughan, 1986). Vaughan (1986), using excised (25–35 mm long) roots from 2 d-old peas (*Pisum sativum*) found that the amount of radioactivity associated with roots increased with the concentration of HA and FA (Table 2). At all the concentrations used in the incubation media, FA were absorbed more than HA. When pea roots were

Table 1  
Distribution of C (%) in a Mollisol humic acid (HA) and a Haplaquod fulvic acid (FA), determined by  $^{13}\text{C}$  NMR (modified from Schnitzer and Schulten (1998))

Chemical shift range (ppm)	% of C	
	HA	FA
0–40	24.0	15.6
41–60	12.5	12.8
61–105	13.5	19.3
106–150	35.0	30.3
151–170	4.5	3.7
171–190	10.5	18.3
Aliphatic C (0–105 ppm)	50.0	47.7
Aromatic C (106–150 ppm)	39.5	34.0
Phenolic C (151–170)	4.5	3.7
Aromaticity <sup>a</sup>	44.1	41.6

<sup>a</sup> ((aromatic C + phenolic C)/(aromatic C + phenolic C + aliphatic C)) × 100.

Table 2  
Incorporation of radioactivity by pea roots treated with increasing of concentrations of  $^{14}\text{C}$  humic (HA) and  $^{14}\text{C}$  fulvic acids (FA) (modified from Vaughan (1986))

Humic concentration (mg l <sup>-1</sup> )	HA (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	FA (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)
10	43	58
25	73	117
50	124	197
100	193	290
150	233	352
200	252	398
250	298	413

incubated in labeled humus at different temperatures and in different experimental conditions (Tables 3 and 4), two uptake components were operating, the first was an initial and rapid passive process, while the second was a slower, but continuous, active uptake dependent on metabolism. Other data indicate that the initial uptake of HS is mainly confined to the cell wall (Vaughan, 1986). In agreement with the latter results, the different treatments (chelation with EDTA, pronase treatment or NaOH wash), used to remove the bound activity, had little effect. This indicates that almost all the labeled HS were tightly bound to the cell wall (Vaughan, 1986).

Further investigations on the uptake by plant roots of humic fractions, with different molecular masses, have supplied new insights. When pea roots were cultured at metabolic temperatures, in the presence of radioactive LMS fractions, the humic matter was taken up to a greater extent. In addition, it was found that 70% of the radioactivity was present in the supernatant fraction of pea roots. This was in contrast with the value of 25% recovered in the supernatant for the radioactive HMS humic fraction. When pea roots were cultured at low temperatures and in the presence of the two-labeled humic fractions, only the HMS fraction was absorbed by pea roots. These results support the interpretation that HS of all molecular weights can be absorbed and

Table 3  
Uptake of  $^{14}\text{C}$  humic acid (HA) by pea roots after 3, 6, 12, 18 h in different experimental conditions (modified from Vaughan (1986))

Root treatment	3 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	6 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	12 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	18 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)
Living (25 °C)	69	122	160	179
Living (4 °C)	41	49	56	65
Dead (25 °C)	35	42	37	43
Living (25 °C) plus 2.5 μg ml <sup>-1</sup> CHM	48	87	195	117

Table 4  
Uptake of  $^{14}\text{C}$  humic acid (HA) and fulvic acid (FA) by pea roots after 1, 4, 18 h (modified from Vaughan, (1986))

Humic fraction	1 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	4 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)	18 h (μg <sup>14</sup> C 100 mg l <sup>-1</sup> tissue)
HA	54	83	134
HA water insoluble	49	81	122
HA water soluble	34	94	223
FA	31	89	231

show evidence that the uptake of LMS is dependent on the active component of transport (Vaughan, 1986). The LMS fraction absorbed by roots was then transferred to the shoots, but, even in these cases, the amount transferred was not higher than 10–12% (Vaughan, 1986). This pattern has been confirmed by Muscolo and Nardi (1999), utilizing LMS and HMS fractions conjugated with fluorescein isothiocyanate (FITC). They showed that only the LMS humic fraction was able to interact with the plasma membrane of cultured carrot cells.

#### 4. Role of the humic matter in the ion absorption

The influence of soil humus on ion uptake, and more in general on plant growth, has been examined by Vaughan and Malcom (1985), Chen and Aviad (1990), Varanini and Pinton (2001) and Clapp et al. (2001). The effects of HS on ion uptake appear to be more or less selective and variable, in relation to their concentration and to the pH of the medium. In beetroot disks, HA stimulated the development of an uptake capacity for Na<sup>+</sup>, Ba<sup>2+</sup>, while that of Ca<sup>2+</sup> and Zn<sup>2+</sup> were unaffected (Vaughan and MacDonald, 1976). This fraction also enhanced the development of phosphate uptake capacity, but retarded that of chloride (Vaughan and MacDonald, 1971). The development of the Na uptake capacity was related to protein synthesis, because cycloheximide and D-threo-chloramphenicol (two protein synthesis inhibitors) inhibited it. However, HA were not able to overcome this inhibitory effect and did not affect the incorporation and distribution of  $^{14}\text{C}$ -labeled amino acids into proteins (Vaughan and MacDonald, 1976). In addition, it has been reported that HMS and LMS fractions (Albuzio et al., 1986) and HA and FA may affect NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and K<sup>+</sup> uptake by barley and oat seedlings (Maggioni et al., 1987). The most prominent stimulatory effect concerns NO<sub>3</sub><sup>-</sup> uptake in oat roots, although this was evident only after several hours of exposure (Nardi et al., 1991).

The effects of HS on ion absorption by plant roots are not easily explainable, owing to the complex and still unknown nature of these substances. Furthermore, the effects described in these papers are difficult to compare, because HS with different features (due to the origin of the soil and

the methods of extraction) were assayed. It is possible that HS may exert several effects on plant functions and that some of these may result, directly or indirectly, in a modulation of ion uptake. In this scenario, a first line of evidence is based on experiments carried out by utilizing both transcription (6-methylpurine) and translation (cycloheximide) inhibitors. It has been shown that HA stimulated carrier-protein synthesis in barley roots at a post-transcriptional level (Dell'Agnola and Ferrari, 1971; Dell'Agnola et al., 1981). This conclusion has been recently confirmed by determining the messenger RNA amount, after treatment of maize seedlings with LMS fraction (Nardi et al., 2000b). The analysis of the synthesized polypeptides revealed a post-transcriptional effect of humic matter on protein synthesis and, consequently, on the overall plant nutrition machinery. However, doubts remain concerning the exact step in which these substances could exert their effects. In any case this mechanism is in line with both relatively old and recent findings, showing that HS (HMS and LMS fractions) stimulated  $\text{NO}_3^-$  by promoting the expression of nitrate carrier proteins (Vaughan et al., 1985), and which also resulted in the modification of some kinetic parameters (Cacco et al., 2000). But the effects of HMS and LMS fractions on  $\text{NO}_3^-$  uptake could also be explained by considering that these substances may act as hormone-like substances (Cacco and Dell'Agnola, 1984; Dell'Agnola and Nardi, 1987; Nardi et al., 1988), or that they induce genome modifications (Attinà et al., 1992). In particular, it has been shown by Nardi et al. (2000b) that only LMS fraction, endowed with gibberellin-like activity, could increase  $\text{NO}_3^-$  uptake, while they strongly inhibited microsomal ATPase activity and  $\text{H}^+$  extrusion by roots, in the same way as when gibberellic acid was used. In any case, the mechanisms suggested can explain why the stimulatory effects of LMS fraction on  $\text{NO}_3^-$  uptake required long periods of incubation through a regulation of the 'coarse' type.

As it is known, primary active transport by plant cells depends on the presence of a vanadate-sensitive proton-pumping ATPase ( $\text{H}^+$ -ATPase) that builds up an electrochemical proton gradient across the plasma membrane (Morsomme and Boutry, 2000). The latter energizes secondary active transport, accomplished by carrier proteins via symport or antiport. In this context,  $\text{NO}_3^-$  is taken up by an inducible  $\text{H}^+/\text{NO}_3^-$  symport with a stoichiometry of 2:1 (Miller and Smith, 1996). Another line of evidence supports the hypothesis that LMS fraction could interact with these transport proteins ('fine' regulation), leading to modulation of  $\text{NO}_3^-$  uptake. This contention is reinforced by the observation that LMS fraction can reach the apoplast and interact with the plasma membrane of roots (Vaughan, 1986) and cultured carrot cells (Muscolo and Nardi, 1999). The first evidence for an effect of HS on transport proteins concerns the stimulation caused by HMS and LMS fractions on the activity of the  $\text{K}^+$ -stimulated ATPase (believed to be coincident with the  $\text{H}^+$ -ATPase of plasma membranes) of microsomal fractions (Maggioni et al., 1987; Nardi et al.,

1991; Pinton et al., 1992). Similar results were also obtained by showing that HA stimulated proton extrusion by roots in a vanadate-sensitive manner, although this increase was apparent only after 2–4 h of incubation (Pinton et al., 1997). Nevertheless, this effect has been interpreted as a consequence of a direct stimulation of HA on the proton pump ( $\text{H}^+$ -ATPase). LMS fraction could also stimulate the  $\text{H}^+$ -ATPase activity of isolated plasma membranes (Varanini et al., 1993), thus determining an increase of the electrochemical proton gradient which might be, at least in part, responsible for the stimulation of  $\text{NO}_3^-$  uptake (Pinton et al., 1999a).

Finally, owing to their polyanionic (acid) nature, HS could simply act as surface-active molecules (Visser, 1986; Nardi et al., 1991). By decreasing the pH at the surface of the plasma membranes of root cells, HS may counteract the alkalization which occurs when  $\text{NO}_3^-$  is used as a N source and that is responsible for inhibiting the  $\text{H}^+/\text{NO}_3^-$  symport (Raven and Smith, 1976). Concordantly, when there was a decrease of  $\text{NO}_3^-$  uptake, at the same time, an increase in the  $\text{NH}_4^+$  uptake takes place (Barber, 1984).

The plasma membranes of plant cells possess several redox activities that can be related to both plant nutrition and cell wall formation and lignification (Lüthje et al., 1997; Bérczi and Møller, 2000). In this context, it has been shown that, in oat roots, HMS humic fractions inhibited NADH oxidation in either the presence or absence of an artificial electron acceptor (ferricyanide), whereas LMS fractions inhibited this oxidase only if the electron donor (NADH) and acceptor (ferricyanide) were contemporarily added (Pinton et al., 1995). While the first effect could be related to the activity of surface peroxidases that can be involved in cell wall formation and thickening (Vianello and Macrì, 1991), the second seems to be exerted on a different redox system with an unknown function.

It is well known that Fe absorption by roots of dicotyledonous plants requires a preliminary reduction of Fe(III) to Fe(II) by a Fe(III)-chelate reductase of plasma membranes (Moog and Brüggemann, 1994). It has been shown that Fe-deficient cucumber plants, at least in part, could use Fe complexed with HS to reduce Fe(III) before being absorbed by the roots (Pinton et al., 1998; Pinton et al., 1999b).

## 5. Effect of humic matter on intermediary metabolism

A first aspect concerns the effect of HS on respiration, although it is poorly understood. Our knowledge is mainly based on results that have already been critically examined in some reviews (Vaughan and Malcom, 1985; Chen and Aviad, 1990; Varanini and Pinton, 1995; Nardi et al., 1996). Surprisingly, the effect of HS on respiration of plant cells, despite its relevance, has received little attention in recent

years. Therefore, in this section we only reassess this issue in the light of our more recent progresses on this aspect of plant cell physiology (Affouret et al., 2001).

There are many reports showing that HS, extracted from a wide range of soils, were able to enhance respiration of higher plants (Vaughan and Malcom, 1985), with the effects of FA more pronounced than that of HA. These results have been interpreted in varying ways. The possibility that HS-induced stimulation can depend on the property of these substances to act as substrates or respiratory chain catalysts is no longer acceptable. In addition, the stimulation of O<sub>2</sub> consumption is only in the order of 25–30% and obtained with intact plants, such as tomato (Sladky, 1959) or beet slices (Vaughan, 1967a). But this increase could also be linked to a stimulation of peroxidase activity by HS (Muscolo et al., 1993). Indeed the latter activity implies an O<sub>2</sub> uptake, which is not distinguishable, at the tissue level, from that linked to respiration, being both cyanide-sensitive.

This aspect has also been addressed by using isolated rat liver mitochondria. It has been shown that HS partially uncoupled oxidative phosphorylation after a short exposure (Visser, 1987). This effect can explain previous results showing that synthetic HS also uncoupled oxidative phosphorylation, thus decreasing ATP concentration. These partial uncoupling has been confirmed by using isolated higher plant mitochondria (Flaig, 1968), albeit this effect was also accompanied by an increase of dry matter and sometimes, as in cereals, of yield grain. This apparent contradictory results have been explained suggesting that the partial uncoupling renders some inorganic phosphate available, without depleting cellular ATP, which is then used in some phosphorylating reactions linked to biosynthetic pathways. However, it has been demonstrated that incubation of mitochondria with HS for a long period resulted in a positive influence on oxidative phosphorylation (Visser, 1987), a result that could explain the finding that HS caused an increase of ATP production (Khristeva et al., 1980). The latter observations are, however, difficult to reconcile with the former. In addition, more recent results show that HS determined a decrease (30–40%) of cellular ATP, without affecting O<sub>2</sub> consumption (Nardi et al., 1991).

From the above findings and considerations, it is not clear whether HS influence respiration by directly or indirectly interfering with mitochondria, thereby making new experimental work necessary prior to drawing a firmer conclusion.

A second aspect that has been examined concerns photosynthesis. Even in this case, our information is fragmentary and not very recent. Although indirect, the most prominent effect of HS application to growing plants was an increase of chlorophyll content which, in turn, could affect photosynthesis (Sladky, 1959). However, the increase of chlorophyll alone did not necessarily result in higher yields. HS, applied to the growth solution, stimulated enzyme activities related to the photosynthetic sulphate

reduction pathway (Ferretti et al., 1991). This positive effect of HS has also been observed on the main photosynthetic metabolism in maize leaves, where a decrease in starch content was accompanied by an increase of soluble sugars (Merlo et al., 1991). This change appeared to be mediated by variations of the activity of the main enzymes involved in carbohydrate metabolism.

## 6. Hormone-like activity of HS

In a series of papers published between 1914 and 1920 (Bottomley, 1914a,b; 1917, 1920), Bottomley showed that HS enhanced plant growth by providing substances called ‘auximones’, a conclusion that has been independently and successively reached also by Hillitzer (1932) and Chaminate and Boucher (1940). Later, using isolated root tips from peas, O'Donnell (1973) concluded that HS exhibited an auxin-like activity, confirming a previous result obtained by Paszewski et al. (1957). These findings have been further supported and extended by showing that humic fractions have a high hormonal activity (Cacco and Dell'Agnola, 1984; Dell'Agnola and Nardi, 1987; Nardi et al., 1988; Piccolo et al., 1992).

In this context, new information arising from more recent papers has further supported this hypothesis. In particular, it has been shown that only LMS fractions induced morphological changes similar to those caused by indole-3-acetic acid (IAA) (Muscolo et al., 1993). In addition, the LMS fraction increased both peroxidase and IAA oxidase activity, albeit IAA increased IAA oxidase, but inhibited peroxidase activity. Again, Nardi et al. (1994), utilizing two inhibitors of auxin (TIBA, 2,3,5-triiodobenzoic acid and PCIB, 4-chlorophenoxy-isobutyric acid), demonstrated that the IAA and LMS fractions induced root growth of *Nicotiana plumbaginifolia*, while TIBA or PCIB alone inhibited it. The presence of TIBA plus LMS fractions or TIBA plus IAA inhibited root growth, while explants, grown in the presence of IAA plus PCIB or LMS fractions plus PCIB, showed roots similar to those obtained with IAA or LMS fractions, respectively. These results thus confirm that the LMS component of humic matter is the fraction endowed with auxin-like activity, although the pathways followed by the IAA and the LMS fraction in inducing their effects may be somewhat different (Fig. 1).

In an attempt to evaluate the possible interaction of the LMS fraction with plasma membranes (target of IAA) of carrot cells, Muscolo and Nardi (1999) labeled with fluorescein isothiocyanate (FITC) IAA, HMS and LMS. The results showed that fluorescent plasma membrane staining was only observed in IAA- and LMS fraction-treated cell cultures. Prior treatment of carrot cells with unconjugated IAA or LMS humic fractions blocked the fluorescein staining of both the FITC-IAA and FITC-LMS humic fraction, giving indirect evidence of the possible binding site of LMS humic

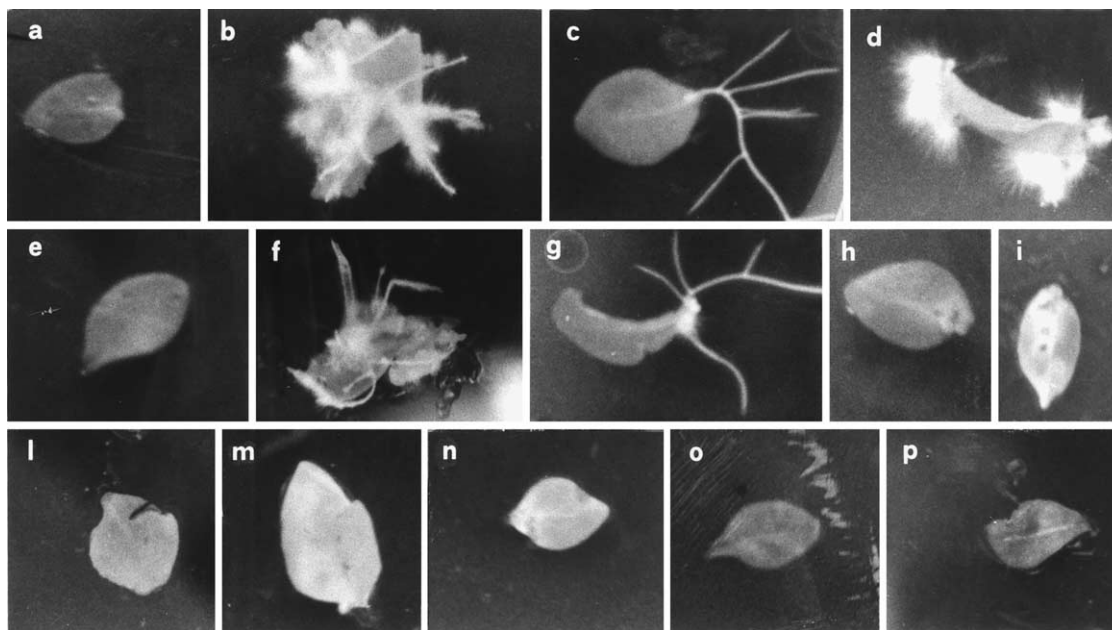


Fig. 1. Photographs of leaf explants of *Nicotiana plumbaginifolia* treated with low molecular size humic fraction (LMS), indole-3-acetic acid (IAA), inhibitors of IAA (TIBA, 2,3,5-triiodobenzoic acid and PCIB, 4-chlorophenoxy-isobutyric acid) and cycloheximide (a, control; b, IAA; c, LMS; d, IAA + LMS; e, PCIB; f, PCIB + IAA; g, PCIB + LMS; h, TIBA; i, TIBA + IAA; l, TIBA + LMS; m, PCIB + TIBA; n, cycloheximide; o, cycloheximide + IAA; p, cycloheximide + LMS).

fraction to the IAA cell membrane receptors (Fig. 2). It is important to emphasize that the interaction of HS with cellular membrane is not due to the possible presence of auxin components in this preparation. In fact, using different approaches, the IAA content was identified in the 0.5% (w/v) to 3.7% range in the LMS humic fraction, according to the different sensitivities of the assays and the methodology used (Muscolo et al., 1998). A recent result seems to corroborate the above findings (H. MacDonald, pers comm). She has shown that IAA and LMS fractions had the same effect on the stomatal opening in pea leaves. This inducing effect appears to be mediated by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and protein kinase C (PKC), both enzymes involved in the signal transduction pathway leading to the response of plants to IAA (Scherer and Andre, 1989; Nemeth et al., 1998).

Very recently the hormone-like activity of humic matter has been questioned in favor of a major effect of these substances on the plasma membrane H<sup>+</sup>-ATPase (Varanini and Pinton, 2001), or on an increased availability of micro-elements (Fe and Zn) (Clapp et al., 2001). These conclusions appear, however, somewhat simplistic and do not consider the very complex nature of HS.

It is known that different soils vary in their native auxin content (Hamence, 1946) and fertile soils contain greater amounts of auxins than those that are less fertile (Stewart and Anderson, 1942; Dahm et al., 1977). Auxin and gibberellin concentrations are usually higher in the rhizosphere than in the bulk soil, probably as a consequence of increased microbial populations or of an accelerated

metabolism owing to the presence of root exudates. Although numerous soil and rhizosphere micro-organisms, as well as the root systems of higher plants have been reported to produce auxins (Lebuhn and Hartmann, 1993) and gibberellins (Rademacher, 1992), there is little information about their stability. Therefore, only indirect conclusions have been drawn about their presence in amounts high enough to be biologically-active (Frankenberger and Arshad, 1995). Perhaps, HS could be considered as a sort of memory of microbial population and plant cover. Frankenberger and Arshad (1995) have found that the active ingredients in humus were not mineral nutrients, but were organic substances and biologically-active metabolites of various microbes. Indeed, mineral substances applied in equal amounts to soil had little effects on plant growth. The favorable effects of organic substances were observed primarily after decomposition and processing of humus, compost, and peat. Biological and biochemical transformations are most likely to occur upon degradation of these materials. This implies that the biologically active substances of humus are not the original parent compounds, but are products of microbial metabolism. The starting organic materials may comprise compounds that serve as precursors or as substrates for the synthesis of biologically active substances, including hormone-like substances, by the heterotrophic activity of the soil microbiota. These plant growth regulators, kept within HS, are of ecological importance because they do not leach and, at the same time, become available for plants (Nardi et al., 2000a; Pizzeghello et al., 2001).

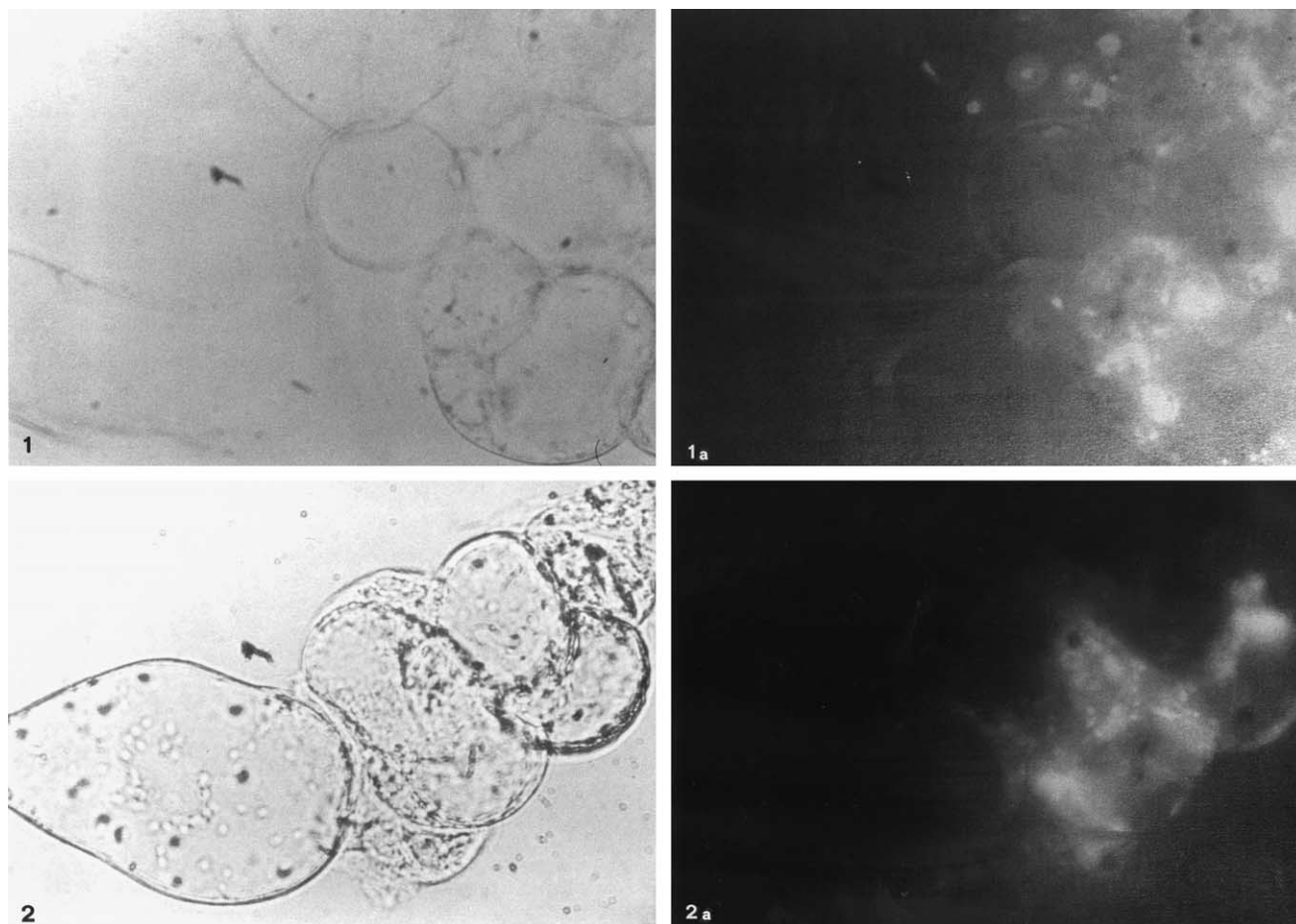


Fig. 2. Light (1 and 2) and fluorescence (1a and 2a) microscopy of carrot cells incubated with fluorescein isothiocyanate FITC-HMS (high molecular size) (1 and 1a) and FITC-LMS (2 and 2a) humic fractions.

## 7. Structure–activity relationships of HS

The lack of detailed knowledge on the composition of HS renders it very difficult to identify the relationships between the structure and the activity of these substances. The study of these relationships is complicated, as seen above, by the presence of other molecules, such as hormones of microbial origin. Thus, attempts to relate these two aspects have produced conflicting results. Nevertheless, it has been suggested that functional carboxylic and hydroxylic groups of HS could play a major role in determining their activity (Mato et al., 1972; Malcom and Vaughan, 1978; Pflug and Ziechmann, 1981), but the manner by which they exert their effects remains to be elucidated (Vaughan and Malcom, 1985). Low molecular weight components of HS were shown to be particularly active (Vaughan, 1967a,b, Mato et al., 1972; Vaughan et al., 1974), although high molecular mass components had a residual activity (Ladd and Butler, 1971; Malcom and Vaughan, 1979). The effectiveness of LMS fractions was due to a combination of the low molecular weight and the high content of aromatic, carboxylic and phenolic groups (Piccolo et al., 1992; Nardi et al., 1998,

2000a,b). This aspect has already been described by Visser (1986), who noticed that LMS fractions and FA possessed a higher metal binding capability with respect to HMS fractions, because of the larger number of functional groups (in particular carboxylic and phenolic OH groups). This could explain how they improve nutrient assimilation and plant metabolism. Moreover, LMS humus complexes entered cells more easily than their HMS counterparts. High molecular mass humic fractions could have an opposite effect in plants, promoting the plant growth, but decreasing enzyme activity (Visser, 1986; Nardi et al., 1988). In any case, HMS substances have been reported to be irreversibly fixed on the external cell surface (Vaughan and Ord, 1981) and, as previously described, the majority of HMS fraction was tightly bound to the cell wall (Vaughan, 1986). These results are in line with those of Nardi et al. (1996, 2000a), who showed that HMS humus treatment induced a higher rate of root differentiation and the stimulation of enzyme activities in metabolic processes related to plant growth and differentiation. Again, Sessi et al. (2002) showed that LMS fractions greatly stimulated  $\text{NO}_3^-$  uptake, while HMS substances required a long period of exposure in  $\text{NO}_3^-$

medium in order to exhibit a low stimulation. This result agrees with that Vaughan (1986) obtained, who demonstrated that only 25% of the radioactivity of HMS substances remained in the supernatant of pea root seedlings which had been incubated in HMS humus radioactive solution.

Such observations indicate that the LMS fraction, which is endowed with a high aromatic, carboxylic and phenolic C and with a low molecular weight, acts at the symplast and directly influences plant metabolism. One idea is that HMS fraction operates mainly on the cell wall influencing the differentiation and growth process at the apoplast. Clearly, more work is required in this important area of plant nutrition.

## 8. Conclusions

It is clear from the above that HS may positively influence higher plant metabolism. This function seems to be carried out more readily by LMS humic fractions, because they are able to reach the plasma membrane of root cells and then to be translocated. Unfortunately, the as yet unknown nature of HS prevents us from drawing more conclusive results concerning the effects of HS on plant growth. We can only affirm that HS appear to influence the metabolism of plant cells at different levels. Their effects may, therefore, be different and be additive, overlapping, or, in some cases, mechanistic related. This apparently puzzling situation can be however, rationalized by hypothesizing that HS have several targets that can be explained partly by their chelating capacity and partly by their hormone-like activity. This is not surprising, considering the complex and differentiated nature of HS. Therefore, more research is necessary to explain the positive effects of HS on higher plants. In particular these studies have to be, primarily, focused on the following topics: (1) the availability of humus in the soil solution and in the rhizosphere; (2) the link between humus activity and the presence in the soil solution of active metabolites of various microbes; and (3) the use of more characterized HS in experiments on plant metabolism.

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