

## ORIGINAL PAPER

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## Water- and pyrophosphate-extractable humic substances fractions as a source of iron for Fe-deficient cucumber plants

Received: 6 November 1996

**Abstract** The capacity of Fe-deficient cucumber plants to utilise water-extractable and pyrophosphate-extractable humic substances as a source of Fe was investigated. Plants were grown for 13 days in nutrient solution in the presence or absence of Fe and during the last 7 days water-extractable and pyrophosphate-extractable humic substances were added to the solution at a final concentration of  $5 \mu\text{g organic C ml}^{-1}$ . The water-extractable humic fraction did not significantly modify leaf area and dry matter accumulation, leaf total Fe or chlorophyll content of cucumber plants adequately supplied with Fe. In contrast, pyrophosphate-extractable humic substances caused a slight but significant decrease of all the leaf parameters considered, with the exception of the chlorophyll content. Root Fe content of Fe-sufficient plants was decreased by more than 50% in the presence of each humified fraction. Addition of each humic fraction to Fe-deficient plants led to a partial disappearance of leaf chlorosis symptoms with a significant increase in chlorophyll and leaf Fe content. Fe content of roots was also significantly increased in Fe-deficient plants by the addition of humic substances to the nutrient solution. These results show that Fe-deficient cucumber plants can utilise Fe contained in the two fractions of humified organic matter. However, by calculating the amount of total Fe accumulated per plant in the presence of water-extractable or pyrophosphate-extractable humic substances, it could be seen that Fe contained in the water-extractable humic fraction was almost totally used by Fe-deficient cucumber plants, while that present in the pyrophosphate-extractable fraction could only be partially absorbed. The results strongly support a role of humified organic matter in Fe nutrition of plants and are discussed in terms of a possible interaction between soil humic substances and the biochemical mechanisms involved in the plant response to Fe deficiency.

**Key words** Humic substances · Cucumber · Iron nutrition · Iron deficiency · Root acidification

### Introduction

Soil humic substances have been widely regarded as playing a beneficial role in Fe acquisition by plants (Chen and Aviad 1990). This effect has been mainly attributed to the complexing properties of humic and fulvic acids increasing the availability of the micronutrient from sparingly soluble hydroxides (Stevenson 1991). Difficulties in obtaining humic and fulvic acids free of Fe with the procedure usually employed for the fractionation of humic matter were taken as evidence for the formation of stable complexes (Kodama et al. 1988; Stevenson 1991). However, the direct contribution of such complexes to Fe nutrition is not easily defined, especially in solution culture, and many studies have produced conflicting results (Linehan 1985). This could be due to the use of supra-optimal Fe concentration in the nutrient solution and to the presence of other added chelating agents. Furthermore, marked differences have been observed due to the type (fulvic or humic acids) and amount of humic molecules employed (Ernst et al. 1987; Linehan and Shepherd 1979) or to the origin of the humic fractions (Dormaar 1975). It has been emphasised that a direct role of humic substances on Fe nutrition, and more generally on plant nutrition, could be most pronounced in conditions of limited nutrient availability (David et al. 1994; Kuiters and Mulder 1993).

In this study the capacity of Fe-deficient cucumber plants to utilise two different fractions of humic substances as a source of Fe was investigated. The effect of the same fractions on Fe nutrition of cucumber plants adequately supplied with Fe(III)-EDTA was also evaluated. In order to minimise structural modifications, humified organic fractions were obtained using mild extraction procedures, namely water and  $\text{Na}_4\text{P}_2\text{O}_7$ , respectively.

## Materials and methods

### Humic substances and the experimental protocol

Pyrophosphate-extractable humic substances were obtained from 100 g of air-dried soil, collected at a depth of 5–15 cm from the Ao horizon of a mollic leptosol (FAO) in the Carnia Alps (Sauris, Italy), with 1 l of 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$  at pH 7.1, after shaking for 1 h under  $\text{N}_2$  at room temperature. The suspension was centrifuged at 2500 g for 30 min and the supernatant filtered on a Whatman WCN 0.45  $\mu\text{m}$  membrane filter. Ultrafiltration was carried out through an Amicon YM5 membrane in an Amicon apparatus continuously supplied with 0.1 M  $\text{Na}_4\text{P}_2\text{O}_7$ , pH 7.1 at a flux of 100  $\text{ml h}^{-1}$  and under  $\text{N}_2$  atmosphere at a pressure of 4 atm. The pyrophosphate-extractable fraction of nominal molecular weight larger than 5KDa was continuously diafiltered with distilled water, brought to pH 8 with NaOH, in order to eliminate pyrophosphate (final theoretical concentration less than 0.2  $\mu\text{M}$ ), then with 0.1 M EDTA, in order to remove chelated metals, then again with distilled water.

Water-soluble humic substances were extracted from finely ground sphagnum peat (2.5 g) by adding 50 ml of distilled water and shaking for 15 h at room temperature. Thereafter, the suspension was centrifuged at 8000 rpm for 30 min and the supernatant filtered on a Whatman WCN 0.2  $\mu\text{m}$  membrane filter. The resulting solution was acidified to pH 2 with  $\text{H}_2\text{SO}_4$  and loaded onto a Amberlite XAD-8 column (100 $\times$ 20 mm). Adsorbed humic substances were washed with 100 ml of distilled water according to Aiken et al. (1979) and eluted from the column with 0.1 N NaOH. The solution was treated with Amberlite IR-120 ( $\text{H}^+$  form) to bring its pH to neutrality. Both fractions were freeze-dried before storage and immediately dissolved before use at a concentration of 1 mg organic C  $\text{ml}^{-1}$ .

Before elemental analysis both fractions were solubilised and treated with a cation exchange resin to bring the pH to between 2 and 3.

Elementary composition of the humic substances was determined by an automated Dumas procedure using a CHN analyser (Carlo Erba, Milano), while element contents were determined by inductively coupled argon plasma emission spectroscopy (ICP-OES).

High performance size exclusion chromatography (HPLC-SEC) was performed on a porous silica column (Bio Sil SEC 250, Bio-Rad) connected to a Waters 590 HPLC pump and a Waters 490 UV-VIS detector set at 400 nm, using 0.025 Tris-phosphate buffer (pH 7) at a flow rate of 1  $\text{ml min}^{-1}$ . The column was calibrated with an appropriate set of humic substance fractions of reduced molecular weight polydispersity obtained by ultrafiltration and extracted from an Haplumbrept, USDA (De Nobili and Fornasier 1996).

Cucumber seeds (*Cucumis sativus* L., cv. Serpente cinese, Dotto Sementi S.p.A., Italy) were germinated on filter paper moistened with 1 mM  $\text{CaSO}_4$ . After 6 days 18 seedlings were transferred to 2.3 l plastic vessels containing aerated nutrient solution either with or without Fe supply, and grown for up to 13 days under a 16/8 h light/dark regime at 25°C, relative humidity 65–75%, light intensity 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The nutrient solution had the following composition (mM):  $\text{K}_2\text{SO}_4$  0.7, KCl 0.1,  $\text{Ca}(\text{NO}_3)_2$  2,  $\text{MgSO}_4$  0.5,  $\text{KH}_2\text{PO}_4$  0.1; ( $\mu\text{M}$ ),  $\text{H}_3\text{BO}_3$  10,  $\text{MnSO}_4$  0.5,  $\text{ZnSO}_4$  0.5,  $\text{CuSO}_4$  0.2,  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$  0.01 (Fe contamination not higher than 10 ppb). Fe, when added, was supplied as Na-Fe(III)-EDTA at a final concentration of 80  $\mu\text{M}$ . During the last 7 days in water culture, water- or pyrophosphate-extractable humic substances were added daily to the nutrient solution at a final concentration of 5  $\mu\text{g organic C ml}^{-1}$  per day apportioning 2 and 20  $\mu\text{g}$  of Fe per plant during the whole 7 days period, respectively. The nutrient solutions were changed every 3 days and adjusted to pH 6.3 with 1N NaOH.

### Analyses

The changes in the pH of the nutrient solutions were recorded daily. After 13 days in nutrient solution plants were harvested; roots and leaves were separated from stems and cotyledons and used for analytical determinations. Chlorophylls were extracted from leaf tissues by acetone and quantitatively measured as reported by Lichtenthaler (1987). The Fe content of plant tissues was determined by ICP-OES

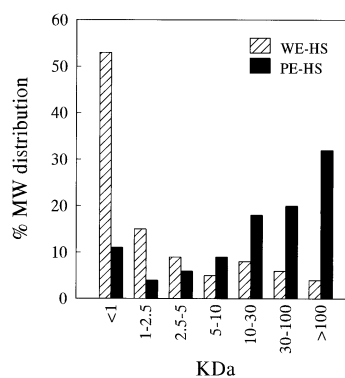
after digestion of tissues with concentrated  $\text{HNO}_3$ . Leaf area was determined by using a leaf area meter LI-3000 (LI-COR, Nebraska). Data on whole plants reported in Table 5 include those referring to stems and cotyledons. Fe(III)-reducing capacity was determined by using the BPDS (bathophenanthrolinedisulfonate) reagent (Chaney et al. 1972). The roots of intact cucumber plants were incubated for 1 h in 5 ml of a solution containing  $\text{CaSO}_4$  0.5 mM, NaFe(III)-EDTA 0.25 mM, BPDS 0.6 mM, Mes-NaOH 10 mM (pH 5.5) in the dark at 25°C. Thereafter, the absorbance at 535 nm of the solution was read with a UV-VIS spectrophotometer. BPDS forms a stable, water-soluble, red complex with Fe(II); the amount of Fe(III) reduced was calculated by the concentration of the Fe(II)-BPDS complex formed using an extinction coefficient of 22.1  $\text{mM}^{-1}\text{cm}^{-1}$ .

## Results and discussion

The two humic substance fractions examined in this work differed widely in their chemical and physico-chemical properties, not only because of their different origin but also because of the different way in which they were prepared. As was expected, the water-soluble fraction extracted from sphagnum peat contained mostly humic substances of low molecular weight (Fig. 1), whereas in the pyrophosphate-extractable fraction most of the humic molecules of nominal molecular weight lower than 5 KDa were eliminated by ultrafiltration. Although both fractions were prepared using mild conditions during both extraction and purification to avoid hydrolysis and alterations of humic molecules, the water-soluble fraction was extracted from a peat already very low in metals and treatment with a cation exchange resin (which can remove exchangeable Fe only) was sufficient to bring its Fe content down to about 3  $\mu\text{moles g}^{-1}$  (Table 1).

In the case of pyrophosphate-extractable humic substances, on the other hand, the fraction was exhaustively diafiltered with 0.1 M EDTA to avoid treatment with strong acids. Even this treatment failed to reduce the Fe content to a level comparable to that of the other fraction. The fact that this fraction was diafiltered with a solution containing such a strong chelating agent means that Fe was held in particularly stable chelated complexes.

In a first set of experiments the effect of two humic fractions on root and leaf dry matter accumulation and Fe



**Fig. 1** Percent molecular weight distribution of water-extractable (WE-HS) and pyrophosphate-extractable (PE-HS) humic fractions as determined by HPLC-SEC in Tris-phosphate buffer at pH 7

**Table 1** Elemental composition of water-extractable (WE-HS) and pyrophosphate-extractable (PE-HS) humic substances (*DW* dry weight)

	Humic Substances	
	WE-HS	PE-HS
	g·100 g <sup>-1</sup> DW	
C	43.4	47.0
H	3.8	4.1
O (by difference)	49.2	41.2
N	0.4	2.6
Ash	3.2	5.1
	mg·100 g <sup>-1</sup> DW	
Na	36.13	129.68
Al	9.37	67.26
B	1.88	2.03
Ca	161.81	18.84
K	30.47	38.50
Mg	8.44	10.28
Fe	19.37	209.81
Mn	0.05	1.29
Zn	2.84	0.06
Cu	0.45	0.73

**Table 2** Effect of water-extractable (WE-HS) or pyrophosphate-extractable (PE-HS) humic substances on dry matter (mg per plant), chlorophyll (mg per plant), Fe content ( $\mu\text{g}$  per plant) and area ( $\text{cm}^2$  per plant) of leaves of cucumber plants grown for 13 days in a nutrient solution with (+Fe) or without (-Fe) 80  $\mu\text{M}$  Na-Fe(III)-EDTA. Humic substances were added to the solution at a final concentration of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  during the last 7 days of water culture. Data with the same *letter* within each row are not significantly different by the Duncan's multiple range test ( $P=0.05$ ). Values in parentheses represent percent of control

	+Fe			-Fe		
	Control	WE-HS	PE-HS	Control	WE-HS	PE-HS
Dry matter	129 a (100)	124 a (96)	114 b (79)	39 b (100)	40 b (102)	51 a (131)
Chlorophyll	13.4 a (100)	12.5 a (93)	12.5 a (93)	1.7 c (100)	2.2 b (129)	3.7 a (217)
Fe content	55 a (100)	50 a (91)	39 b (71)	3.6 c (100)	4.2 b (117)	6.0 a (167)
Leaf area	67 a (100)	65 a (97)	57 b (85)	15 b (100)	17 b (113)	21 a (140)

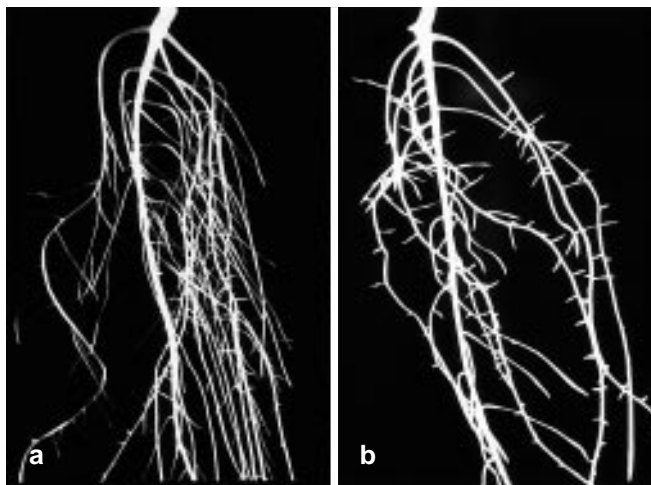
content was evaluated in cucumber plants grown in nutrient solution adequately supplied with Fe. Addition of water-extractable, low molecular weight humic substances at a final concentration of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  to the nutrient solution containing 80  $\mu\text{M}$  Na-Fe(III)-EDTA did not significantly affected dry matter, total Fe and chlorophyll content, or area of leaves of cucumber plants (Table 2). In contrast, when pyrophosphate-extractable humic substances (5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$ ) were present in the nutrient solution, dry matter and Fe content showed lower values than the untreated plants, while chlorophyll content was not significantly affected. This behaviour confirms that

**Table 3** Effect of water-extractable (WE-HS) or pyrophosphate-extractable (PE-HS) humic substances on dry matter (mg per plant) and Fe content ( $\mu\text{g}$  per plant) of roots of cucumber plants grown for 13 day in a nutrient solution with (+Fe) or without (-Fe) 80  $\mu\text{M}$  Na-Fe(III)-EDTA. Humic substances were added to the solution at a final concentration of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  during the last 7 days of water culture. Data with the same *letter* within each row are not significantly different by the Duncan's multiple range test ( $P=0.05$ ). Values in parentheses represent percent of control

	+Fe			-Fe		
	Control	WE-HS	PE-HS	Control	WE-HS	PE-HS
Dry matter	29 a (100)	26 a (90)	24 a (83)	10 a (100)	11 a (110)	12 a (120)
Fe content	45 a (100)	19 b (42)	19 b (42)	0.6 c (100)	0.9 b (150)	1.7 a (283)

this fraction was able to form Fe-humic complexes even more stable than Fe-EDTA, in contrast with numerous reports showing a stimulation of plant growth by fulvic and humic acids (Rauthan and Schnitzer 1981; Tan and Nopamornbodi 1979). However, a great variability in response to humic molecules has been recorded between different plant species (Vaughan and Malcolm 1985); in addition, the effect on plant growth has commonly been investigated using concentrations of humic substances much higher than that employed in the present work. Dry matter of Fe-sufficient cucumber roots was almost unaffected by the addition of both humic fractions (Table 3); however, total root Fe content was decreased by about 60% after 7 days of growth in the presence of each humic fraction. This latter effect is consistent with the results reported by Ernst et al. (1987) and Guminski et al. (1983) and suggests a lower Fe uptake by humus-treated cucumber roots. However, this behaviour might be ascribed to the decrease in the activity of free  $\text{Fe}^{3+}$  at the root surface and in the apoplast, due to the presence of humic ligands (Linehan and Shepherd 1979). Since determination of total Fe content of cucumber roots included adsorbed Fe, it is possible that the formation of negatively charged complexes of Fe with humic molecules would have decreased the amount of Fe adsorbed onto the root surface and in the apoplast (Dunemann et al. 1991). Despite the strong effect on root Fe content, accumulation of the micronutrient in the shoot was, as shown above, significantly affected only by the pyrophosphate-extractable humic fraction. In this respect it is noteworthy that it has been shown that the decrease in root Fe content does not limit Fe accumulation in the leaves of plants treated with fulvic fractions (Aso and Takenaga 1975; Guminski et al. 1983; Linehan and Shepherd 1979).

Cucumber plants grown for 13 days in nutrient solution without added Fe showed visual deficiency symptoms (leaf chlorosis). As a consequence, leaf area and dry matter, and Fe and chlorophyll contents were significantly decreased as compared to plants adequately supplied with Fe(III)-EDTA (Table 2). Lobartini and Orioli (1988) reported that in *Spirodella intermedia*, sunflower and maize plants that were Fe-deficient, the chlorosis symptoms disappeared upon addition of a crude humic acid to the nutri-



**Fig. 2** Roots of 6-day-old cucumber plants grown in nutrient solution with (a) or without (b) 80  $\mu\text{M}$  Na-Fe(III)-EDTA

ent solution. Addition of water-extractable or pyrophosphate-extractable humic substances (5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$ ) for 7 days to Fe-deficient cucumber plants led to a partial recovery of the leaf parameters considered and to a gradual re-greening of the leaf tissue. In particular, chlorophyll and Fe contents were significantly increased in humus-treated plants (Table 2). Since the sole source of Fe was that represented by the endogenous content of the humic fractions (see Table 1), it is clear that plants could use both forms of chelated Fe.

Root apparatus of Fe-deficient cucumber plants showed morphological changes (proliferation of lateral roots and sub-apical root hairs) characteristic of the response to Fe-deficiency (Fig. 2), which were accompanied by an increase in the rate of net proton release and by the development of a higher Fe(III)-reducing activity (Table 4). The Fe content of roots was also significantly increased in Fe-deficient plants grown for 7 days in the presence of the humified fractions (Table 3). By calculating the amount of total Fe accumulated by the whole plant in the presence of either humic fractions, it can be seen (cf. Tables 1, 5) that Fe contained in the water-extractable fraction was almost totally used by the Fe-deficient cucumber plants, while that present in pyrophosphate-extractable humic substances could be only partially absorbed. In fact, the daily addition of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  of the water-soluble or pyrophosphate-extractable humic fractions to the nutrient solution resulted in an input of 2 and 20  $\mu\text{g}$  Fe per plant in 7 days, respectively. As can be seen in Table 5, the difference in Fe content between humus-treated and control Fe-deficient plants was 1.9 (9.5 vs 7.6) and 3.8 (11.4 vs 7.6)  $\mu\text{g}$  Fe per plant due to the presence of water- and pyrophosphate-extractable humic substances, respectively. This behaviour can be due to a difference in the Fe-binding capacity and/or in the solubility of the two humic fractions at changing pH values (Stevenson 1991). Fe-deficient cucumber roots acidify the nutrient solution in response to Fe-deficiency (Fig. 2), even in the presence of a  $\text{N-NO}_3^-$  source (Röm-

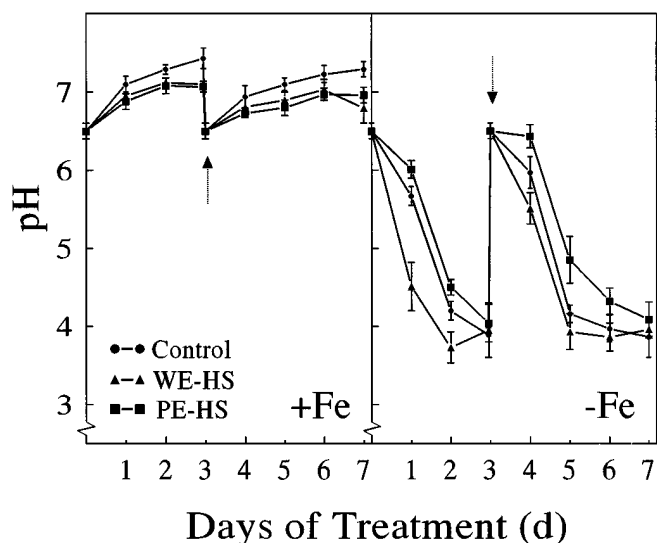
**Table 4** Iron(III)-reducing capacity [ $\text{nmol Fe(II)} \times \text{g}^{-1}$  fresh weight  $\times \text{h}^{-1}$ ] and acidification of the nutrient solution (pH) by roots of 6-day-old cucumber plants grown in the presence (+Fe) or absence (-Fe) of 80  $\mu\text{M}$  Na-Fe(III)-EDTA. Data represent means  $\pm$  SD of five independent experiments

	+Fe	-Fe
pH	7.1 $\pm$ 0.1	4.2 $\pm$ 0.2
Fe(III)-reduction	30 $\pm$ 3	700 $\pm$ 20

**Table 5** Effect of water-extractable (WE-HS) or pyrophosphate-extractable (PE-HS) humic substances on dry matter (mg per plant) and Fe content ( $\mu\text{g}$  per plant) of whole cucumber plants grown for 13 days in nutrient solution with (+Fe) or without (-Fe) 80  $\mu\text{M}$  Na-Fe(III)-EDTA. Humic substances were added to the solution at a final concentration of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  during the last 7 days in water culture. Data with the same letter within each row are not significantly different by the Duncan's multiple range test ( $P=0.05$ ). Values in parentheses represent percent of control

	+Fe			-Fe		
	Control	WE-HS	PE-HS	Control	WE-HS	PE-HS
Dry matter	226 a (100)	216 a (96)	187 b (83)	97 a (100)	104 a (107)	111 a (114)
Fe content	120 a (100)	88 b (73)	77 b (64)	7.6 c (100)	9.5 b (125)	11.4 a (150)

held et al. 1984); thus, a decrease in the pH of the root external medium may have partially precipitated the high molecular weight pyrophosphate-extractable fraction (Stevenson 1994). As shown in Fig. 3, an increase in the pH of the nutrient solution by Fe-sufficient  $\text{NO}_3^-$ -fed cucumber roots was observed. The presence of water-extractable or pyrophosphate-extractable humic substances induced a lower increase in the pH value of the solution, probably as the result of the effect of humic molecules on ion transport (Maggioni et al. 1987) and membrane-associated  $\text{H}^+$ -translocating enzymes (Pinton et al. 1992; Varanini et al. 1993). Roots of Fe-deficient plants treated with water-soluble or pyrophosphate-extractable humic fractions still show a high capacity to acidify the nutrient solution, suggesting that Fe supplied by the two fractions was not enough to switch off the mechanisms responsible for the increase in the net proton release. In the presence of the water-soluble fraction the pH decrease was even steeper. Acidification of the rhizosphere by roots of Fe-deficient dicotyledon is most probably the result of the activation of the plasma membrane  $\text{H}^+$ -ATPase (Rabotti and Zocchi 1994). Varanini et al. (1993) reported that low molecular weight humic molecules, essentially represented by fulvic acids, stimulated  $\text{H}^+$ -ATPase activity of isolated plasma membrane vesicles. Thus, it is possible that, besides supplying Fe, the water-soluble humic fraction might specifically stimulate proton release from the root cells into the apoplast and in the rizosphere.



**Fig. 3** Effect of water-extractable (WE-HS) or pyrophosphate-extractable (PE-HS) humic substances on the pH of the nutrient solution. Cucumber plants were grown for 13 days in the presence (+Fe) or absence (-Fe) of 80  $\mu\text{M}$  Na-Fe(III)-EDTA; during the last 7 days humic fractions were added daily at a final concentration of 5  $\mu\text{g}$  organic C  $\text{ml}^{-1}$  per day and the pH of the nutrient solution was recorded. Arrows indicate changes of the nutrient solution. Data are means  $\pm$  SD from three independent experiments run in triplicate

## Conclusion

Data reported in the present work show that Fe-deficient cucumber plants can utilise complexed Fe present in two fractions of humified organic matter. Available data do not allow conclusions to be drawn about the different contribution of the two fractions to plant Fe-nutrition. However, of particular interest is the behaviour of the water-extractable humic fraction. Due to its high solubility and low molecular weight, this fraction is likely to act as a source of Fe in the soil solution. Furthermore, an interaction with the biochemical mechanisms involved in the response to Fe deficiency might be hypothesised. In addition to a stimulatory action on the proton-extruding root plasma membrane ATPase, soluble complexes between Fe and humic molecules could represent a natural substrate for the Fe-stress-inducible Fe(III)chelate reductase.

**Acknowledgements** Research was supported by grants from Italian M.I.R.A.A.F., special project "Plant Biotechnologies". The authors thank Prof. P. Nannipieri, University of Firenze for helpful discussions and Dr. Claudio Mondini, Istituto Sperimentale per la Nutrizione delle Piante of Gorizia, Italy, for the determination of element content of humic fractions.

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