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SHORT COMMUNICATION

Effects of humic substances on iron nutrition of lupin

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Abstract Poorly crystalline Fe oxides and organic matter are two important factors affecting Fe nutrition of plants. The main objective of this work was to study the contribution of humic substances to Fe nutrition of a typical Fe-chlorosis sensitive plant (white lupin, *Lupinus albus* L.). An experiment was performed involving two growing media (siliceous and calcareous) and different Fe sources: control without additional Fe added to the growing media, ferrihydrite (FH), FH + humic substances (HS, at two rates, 0.1 and 0.3 g C kg^{-1} growing media), Fe complexed to humic substances (Fe–HS), and Fe–EDDHA (as an effective Fe source in calcareous media). Chlorophyll meter readings and dry matter production (DM) were significantly greater with Fe–EDDHA and Fe–HS when compared with the other treatments in calcareous media. The positive effect of complexed Fe (to EDDHA or HS) on Fe nutrition can be, at least partially, related to an increase in Fe(III)-reducing capacity by roots, which seems to be improved by an enhanced root development. No positive effect on Fe nutrition was observed with the application of HS in a ferrihydrite enriched growing media (FH + HS) at 4 weeks, particularly with the application of HS at 0.3 g C kg^{-1} in calcareous media. Thus, the effect of HS on Fe availability was only positive if some Fe is complexed to HS. The efficiency of HS with Fe complexed (Fe–HS) in preventing Fe chlorosis was found to be similar to Fe–EDDHA. This is important not only for the knowledge of factors affecting Fe availability in soils, but also with a view of using Fe–HS complexes as effective products to correct this nutritional problem.

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Abbreviations

CA	citrate–ascorbate
DM	dry matter
DTPA	diethylenetriaminepentaacetic acid
EDDHA	ethylenediaminedihydroxyphenylacetic acid
FH	ferrihydrite
HS	humic substances

Introduction

Iron deficiency chlorosis is a major nutritional problem affecting cultivated plants in calcareous soils, which results in high economic losses, particularly in perennial crops (Tagliavini and Rombolà 2001; Gruber and Kosegarten 2002; Gil-Ortíz and Bautista-Carrascosa 2004). Besides carbonate content of soil, other properties such as the types of Fe oxide present and their content, the organic matter, water content, redox potential, carbonate mineralogy, and nutrient competition may also influence Fe availability to plants (Loeppert et al. 1984; Del Campillo and Torrent 1992a; Aly and Soliman 1998; Velázquez et al. 2004).

Most of the Fe present in calcareous soils is essentially in ferric oxide form not readily available to many plants (Miller et al. 1984; Mengel 1994; Lucena 2003). In the absence of complexing ligands, Fe acquisition by plants in calcareous soils is not only limited by the low solubility of Fe oxides, but also by their slow dissolution kinetics (Kraemer 2004). The mineralogy of these Fe oxides is particularly important in relation to the occurrence of this problem in various plant species in Mediterreanean areas (Yanguas et al. 1997;

Benítez et al. 2002). Among them, ferrihydrite, a poorly crystalline Fe oxide, has a significant influence on the supply of Fe to plants grown on calcareous soils (Loeppert and Hallmark 1985; Morris et al. 1990); this accounts for the frequently observed relationship between the chlorophyll content and oxalate- or citrate–ascorbate-extractable Fe in soil (Del Campillo and Torrent 1992b; Reyes et al. 2006; De Santiago and Delgado 2006).

Organic compounds have been demonstrated to play an important role in Fe nutrition of plants. Iron complexation by siderophores released by the roots is a basic acquisition strategy by plants (Römheld and Marschner 1986; Johnson et al. 2002; Ma et al. 2003). Microbial siderophores can also contribute to supply Fe to plants (Shenker et al. 1999; Kraemer 2004). Soil humic substances can contribute to enhance Fe availability to plants by preventing precipitation as Fe oxides (Schwertmann 1966), by formation of soluble complexes (Stevenson 1991; Mackowiak et al. 2001; Bocanegra et al. 2006), and by increasing Fe diffusion to roots (Pandeya et al. 1998; Cesco et al. 2000). Iron complexed to soluble humic substances has been demonstrated to be used as Fe source by Fe-deficient Strategy I plants grown in nutrient solution, at least in part, via reduction in complexed Fe(III) by the plasma membrane (Pinton et al. 1998; Mohamed et al. 1998; Pinton et al. 1999; Bocanegra et al. 2006); Strategy II plants seem to use this complexed Fe via ligand exchange between humic substances and phytosiderophores (Yehuda et al. 1996; Cesco et al. 2002).

All the evidences mentioned above reveal that poorly crystalline Fe oxides and natural organic matter (humic substances, HS) may account for important factors affecting Fe availability to plants in soils, Fe complexed to HS (Fe–HS) being a possible Fe source contributing to Fe nutrition. However, most of the evidences obtained for the assumption of Fe–HS complexes being Fe sources for plants have been obtained growing plants in a nutrient solution. The main objective of this work was to study in a system closer to soil than hydroculture (calcareous and siliceous growing media): (1) the contribution of Fe–HS to Fe supply to plants, and (2) how the interaction of HS with poorly crystalline Fe oxides affects Fe availability in soil.

Materials and methods

Experimental design

An experiment was performed following a completely randomized design with four replications (pots) and two factors:

(a) Growing media: (1) siliceous growing media, and (2) calcareous growing media. Siliceous sand (>99% quartz) and calcareous sand (>99.5% $CaCO_3$) were sieved between 0.2 and 0.5 mm to obtain a good aeration and hydraulic conductivity in the media. Part of the quartz sand was washed several times with diluted Na_2CO_3 to disperse and eliminate Fe oxides bound to sand particles. Calcareous growing media were prepared carefully mixing nonwashed siliceous sand with calcareous sand at the same rate. Siliceous growing media were prepared mixing one part of nonwashed quartz sand (50%) with another part of Na_2CO_3 washed quartz sand (50%). Final citrate–ascorbate-extractable Fe (measure of Fe associated to poorly crystalline Fe oxides) in both growing media was 20 mg kg^{-1} because the contribution of calcareous sand to Fe content in the calcareous media was negligible.

(b) Iron sources: (1) control without additional Fe supply to the growing media, (2) ferrihydrite enriched media (FH), (3) ferrihydrite enriched media + humic substances (HS) at 0.1 g organic C per kg of growing media (FH + $HS_{0.1}$), (4) ferrihydrite + HS at 0.3 g organic C per kg of growing media (FH + $HS_{0.3}$), (5) iron-humic substances complex (Fe–HS), and (6) Fe–EDDHA. All the treatments involving the use of ferrihydrite were prepared mixing washed siliceous sand coated with ferrihydrite following the procedure of Rahmatullah and Torrent (2000) with nonwashed siliceous sand (siliceous media) or calcareous sand (calcareous media) at the same rate. With this mixture, a citrate–ascorbate-extractable Fe content in the media of 150 mg Fe kg^{-1} was achieved. HS were prepared using a commercial liquid mixture of humic and fulvic acids (Solfer húmicos®, Valencia, Spain), which was dialyzed into 15 KDa cut-off Visking tubing (Sigma, Barcelona, Spain) against deionized water until the electrical conductivity of the solution was <1 dS m^{-1}. After that, the pH was lowered until 8 using HCl. The dialyzed fraction contains compounds with a greater molecular mass than that used by Pinton et al. (1999) but in the range of that used by Bocanegra et al. (2006). The resulting extract was purified of complexed metals by elution through a cation exchange resin Amberlite IR-120 (Sigma) in H^+ form (Nardi et al. 2005), the remaining Fe (1.4 mmol l^{-1}) being ascribed mainly to microcrystalline Fe oxides (Loeppert and Inskeep 1996). Main properties of dialyzed humic substances are shown in Table 1. Iron-humic substances complex (Fe–HS) was prepared according to Pinton et al. (1999) by treating the HS extract with Amberlite IR-120 saturated with $FeCl_3$. Iron content in HS and Fe–HS was determined by atomic absorption spectrometry following digestion with HNO_3 in a microwave oven (Milestone, provided by Gomensoro, Madrid, Spain). Organic C in HS and Fe–HS was determined using a LECO CNS analyzer

Table 1 Some characteristics of humic substances used in the study

C	H	O	Ash	Total acidity[a] (eq H^+ kg^{-1})	A_{254}[b] (l g^{-1} cm^{-1})	E_4/E_6[b]	Molecular weight distribution [(KDa)[c]; (percent of total organic C)]			
37.94	3.04	35.8	22.6	3.6	75.71	4.16	15–30 (65.8)	30–50 (nd)	50–100 (13.1)	>100 (21.1)

nd, not detectable

[a] Determined according to Sierra et al. (2004)

[b] A_{254}, specific spectral absorbances at 254 nm per unit of dissolved C in 0.1 *N* NaOH; E_4/E_6, ratio of measured spectral absorbances at 465 nm (E_4) and 665 nm (E_6), determined according to Shirshova et al. (2006)

[c] Molecular weight distribution determined by ultrafiltration according to Xu et al. (2006)

(LECO Spain, Madrid, Spain). Iron complexed to humic substances in Fe–HS was 84 μmol g^{-1} organic C.

Humic substances were applied diluted in deionized water before planting. Fe–HS and Fe–EDDHA were applied with irrigation: 10 μM complexed Fe in the nutrient solution, a similar concentration to a Hoagland type nutrient solution, the total amount of applied Fe being 1.1 mg kg^{-1} growing media, and the total amount of organic C applied as Fe–HS 0.25 g kg^{-1}.

Plant material and growth conditions

White lupin (*Lupinus albus* L.) was used for the experiment as typical Fe-chlorosis sensitive plant (White and Robson 1989). This plant usually shows significant yield reductions when it is grown in soil of pH above 7.2 (Kerley and Huyghe 2001). One plant was grown in each pot (350-ml, 5.5 cm diameter, 15 cm-high polystyrene cylinders) containing 350 g of each growing media. Previously, seeds were sown in perlite irrigated with deionized water, and after 14–15 days (four true leaves stage), the lupin plants were planted in the growing media and grown for 28 additional days. No chlorosis symptoms were observed at planting. The experiment was conducted in a growing chamber with a photoperiod of 14 h, a 24/20°C day/night temperature, 60% RH, and a 22 W m^{-2} light intensity. Pots were irrigated daily with 25 ml of a Hoagland-type nutrient solution having the following composition (all concentrations in mmol l^{-1}): $MgSO_4$ (4), $Ca(NO_3)_2$ (5), KNO_3 (5), KH_2PO_4 (2), H_3BO_3 (0.092), $MnCl_2$ (0.018), $CuSO_4$ (0.0016), $ZnSO_4$ (0.0025), and H_2MoO_4 (0.0023). Iron was applied with the nutrient solution only in the treatments involving the application of Fe-chelates (Fe–HS and Fe–EDDHA in the first experiment).

Plant analysis

At 21 and 28 days after planting, chlorophyll was measured with a Minolta SPAD-502 chlorophyll meter (Minolta Camera, Osaka, Japan). Previously, accurate correlation between SPAD units and the chlorophyll content in *L. albus* leaves was checked (Chlorophyll = 0.2 exp(0.04 SPAD), R^2=0.88, P<0.001, n=30). Chlorophyll measurements were done in triplicate in the youngest completely expanded leaf.

At the end of the experiments, Fe(III)-reducing capacity of the roots was determined with intact plants as described by Johnson et al. (2002). After that, shoots and roots of each plant were separated and dry weight determined after drying plant material at 65°C for 48 h in a forced-air oven. For Fe analysis, dried plant material (aerial part) was ground to pass a 1-mm sieve. Then, an aliquot of 0.25 g was digested with HNO_3 in teflon containers in a microwave oven (Milestone), according to the manufacturer instructions. Iron contents in diluted digests were determined by atomic absorption spectrometry.

Growing media analysis

After each crop, two Fe extractions were performed: DTPA according to Lindsay and Norvell (1978), which has been usually considered as an Fe availability index (Sims 2000), and citrate–ascorbate-extractable Fe according to Reyes and Torrent (1997), which can be considered an estimate the amount of Fe associated with poorly crystalline Fe oxides. These extractions were not performed in treatments involving the application of complexed Fe (Fe–EDDHA and Fe–HS) since the small amount of total applied Fe does not allow to appreciate differences. All Fe extractions were carried out in triplicate using polypropylene flasks. Iron in the extracts was determined by atomic absorption spectrometry.

Statistical analysis

A two-way analysis of variance on the effects of growing media and Fe source on chlorophyll meter readings, dry matter (DM) production (shoots and roots), number of leaves, Fe content in aerial part, and Fe(III)-reducing capacity by roots was performed using the General Linear Model procedure of Statgraphics Plus 5.1 (StatPoint 2000).

Mean comparison was performed according to the Least Significant Difference (LSD) test. To study the effect of different Fe sources on variables mentioned above in calcareous media, a one-way analysis of variance was performed and means for each Fe source treatment compared using LSD test.

Results and discussion

The growing media were found to have a significant effect on chlorophyll meter readings, dry matter production, number of leaves, and Fe in aerial part; the siliceous one being, as it was expected, that exhibiting the greatest values (Table 2). Furthermore, the Fe source showed a significant effect on most of these variables (Tables 2 and 3). A significant interaction between the two factors in chlorophyll meter readings was observed ($P<0.001$), which can be explained by the different effect of the Fe source depending on the growing media: while control showed the greatest SPAD values at 4 weeks, but only significantly greater than FH + HS treatments, in siliceous media (Table 2), SPAD readings were significantly greater with Fe–EDDHA and Fe–HS when compared with the other treatments in the calcareous one (Table 3). Dry matter production exhibited a different behavior; total DM was significantly smaller in ferrihydrite combined with HS than in control or Fe–HS in siliceous media, and nonsignificant differences between control and treatments with complexed Fe (Fe–HS or Fe–EDDHA) were observed in DM production in these growing media (Table 2 shows DM in shoots and roots). In calcareous media, total DM production was greater with Fe–EDDHA when compared with Control and FH treatments (alone or with HS), but not significantly different than with Fe–HS (Table 3 shows DM in shoots and roots).

In general terms, when the calcareous media were only taken into account, the best results were achieved by the two treatments with complexed Fe (Fe–EDDHA and Fe–HS, Table 3), the Fe–HS being the only treatment that resulted in significantly increased Fe in aerial part relative to control, and Fe–EDDHA the only one that significantly increased the root reducing capacity when compared with control (Table 3). Differences in total DM production were mainly ascribed to different root development, Fe–EDDHA and Fe–HS being the treatments promoting the highest root DM production in calcareous media (Table 3). Nonsignificant differences in SPAD readings and DM production (total and per organs) were observed between Fe–HS and Fe–EDDHA, the last one being supposed an efficient Fe source for Fe-chlorosis sensitive plants grown in calcareous media. The fact that the positive effect of Fe–HS on SPAD readings, root DM production, and Fe content (Table 3) was only observed in calcareous media is in agreement with previous findings, which revealed that the role of HS on Fe nutrition was more evident in conditions of low Fe availability (David et al. 1994; Kuiters and Mulder 1993). However, SPAD readings at 3 and 4 weeks were smaller in FH+HS treatments when compared with other Fe sources (ferrihydrite alone, Fe–HS, and Fe–EDDHA) in calcareous media (Table 3). Furthermore, DM production (total and root) in FH + HS (0.3 g C kg^{-1}) was significantly smaller than in Fe–HS or Fe–EDDHA. Thus, no positive effect on Fe nutrition was observed with the application of HS at 4 weeks of growth, particularly with the application of HS at 0.3 g C kg^{-1} in calcareous media, this amount of C being similar to that applied with Fe–HS, a treatment that enhanced Fe nutrition in these media as described above.

Table 2 Effect of the different Fe sources applied on the siliceous growing media on chlorophyll meter readings at 3 and 4 weeks of growth (SPAD 3 and SPAD 4, respectively), dry matter production (DM) in aerial part and shoots, number of leaves, Fe content in aerial part, Fe(III) reduction capacity by roots, and Fe in the media (DTPA and citrate–ascorbate extractable) at the end of the experiment (after 4 weeks of growth)

Treatment	SPAD 3	SPAD 4	DM in aerial part (g $plant^{-1}$)	DM in roots (g $plant^{-1}$)	Number of leaves	Fe in aerial part (mg kg^{-1} DM)	Fe(III) reduction capacity (μmol Fe g^{-1} FW)	DTPA–Fe[1] (mg kg^{-1})	CA–Fe[1] (mg kg^{-1})
0	66a	66a	1.89a	1.73a	18a	117a	0.42a	0.9b	23c
Ferrihydrite (FH)	55b	61ab	1.12bc	1.39ab	19a	73a	0.25ab	3.11a	146b
FH + HS (0.1 g C kg^{-1})	66a	58b	0.56c	0.73b	16a	64a	0.07b	2.74a	164a
FH + HS (0.3 g C kg^{-1})	63a	56b	1.14bc	1.19ab	17a	112a	0.31ab	2.50a	142b
HS–Fe	63a	62ab	1.89a	1.48ab	18a	110a	0.22ab		
EDDHA–Fe	65a	65ab	1.69ab	1.53a	17a	84a	0.29ab		

Means followed by the same letter in each column are not significantly different according to the LSD test at a probability level of 0.05
DM, dry matter; *FH*, ferrihydrite; *HS*, humic substances; *HS–Fe*, Fe–humic substances complex; *FW*, fresh weight
[1] DTPA and citrate–ascorbate (*CA*) extractable Fe only in control without Fe application and in ferrihydrite-enriched media

Table 3 Effect of the different Fe sources applied on the calcareous growing media on chlorophyll meter readings at 3 and 4 weeks of growth (SPAD 3 and SPAD 4, respectively), dry matter production (DM) in aerial part and shoots, number of leaves, Fe content in aerial part, Fe(III) reduction capacity by roots, and Fe in the media (DTPA and citrate–ascorbate extractable) at the end of the experiment (after 4 weeks of growth)

Treatment	SPAD 3	SPAD 4	DM in aerial part (g plant^{-1})	DM in roots (g plant^{-1})	Number of leaves	Fe in aerial part (mg kg^{-1} DM)	Fe(III) reduction capacity (μmol Fe g^{-1} FW)	DTPA–Fe[1] (mg kg^{-1})	CA–Fe[1] (mg kg^{-1})
0	26bc	25c	0.68a	0.56c	10b	28b	0.14b	0.34c	10d
Ferrihydrite (FH)	46a	40b	0.65a	0.93bc	19a	46b	0.22b	0.91b	25c
FH + HS (0.1 g C kg^{-1})	33b	25c	0.69a	0.63bc	15a	49b	0.06b	1.15ab	33b
FH + HS (0.3 g C kg^{-1})	24c	21c	0.68a	0.55c	15a	35b	0.08b	1.30a	44a
HS–Fe	49a	49a	1.07a	1.61ab	19a	83a	0.55ab		
EDDHA–Fe	52a	51a	1.13a	2.62a	19a	50b	1.62a		

Means followed by the same letter in each column are not significantly different according to the LSD test at a probability level of 0.05.
DM, dry matter; *FH*, ferrihydrite; *HS*, humic substances; *HS–Fe*, Fe–humic substances complex; *FW*, fresh weight
[1] DTPA and citrate–ascorbate (*CA*) extractable Fe only in control without Fe source and in ferrihydrite enriched media

These results suggested that the HS used in this study could enhance Fe nutrition if some Fe was complexed to these organic compounds previously to its application to the growing media. The initial Fe content remaining in HS did not contribute significantly to Fe supply to plants. After cation exchange resin treatment, most of this Fe might be associated to oxides, dispersed in the alkaline extraction of humic compounds. In soils and other environmental samples, poorly crystalline Fe oxides are present in part as coatings on humic substances (Violante et al. 2003). A poor contribution to available Fe to lupins in calcareous media could be expected from this initial Fe content in HS because the amount applied as Fe oxide was very small to improve Fe nutrition (less than 4 mg Fe kg^{-1} growing media).

Contrasting with the observed results in siliceous media, chlorophyll meter readings were significantly greater with Ferrihydrite than with Control in calcareous media (Table 3). This revealed a positive effect of increasing the content in poorly crystalline Fe oxides in the media on plant response to Fe-chlorosis, in agreement with previous works which revealed that carbonate and poorly crystalline Fe oxides contents were the main factors explaining Fe availability to lupins in calcareous soils (De Santiago and Delgado 2006). On the other side, in siliceous media, very small contents in poorly crystalline Fe oxides were sufficient to ensure an accurate Fe supply to lupin plants because nonsignificant differences were found between the control and ferrihydrite after 4 weeks of growth (Table 2). Even more, the most efficient Fe sources found in calcareous media (Fe–HS and Fe–EDDHA) did not significantly increase SPAD, DM, or Fe content in aerial part (Table 2) when compared with control in the siliceous media.

Total DM production was significantly correlated with SPAD readings at 4 weeks in plants grown in calcareous media (r=0.7, P=0.001, n=24). However, root DM was more significantly related to SPAD at 4 weeks (r=0.67, P<0.001) than shoot DM (r=0.47, P<0.05), thus revealing that the decreased root development in lupins plants grown in calcareous media was a symptom related to Fe deficiency (Peiter et al. 2001). This has been also observed in other Fe-chlorosis sensitive plants (Tsipouridis et al. 2006). In these growing media, Fe content was slightly correlated with SPAD readings at 4 weeks (r=0.4, P<0.05, n=24), and not correlated with SPAD readings at 3 weeks and total DM production. This poor relationship between Fe content and chlorosis symptoms has been observed in previous studies growing lupins on calcareous soils (Kerley 2000; De Santiago and Delgado 2006). In calcareous media, total DM production and SPAD readings at 4 weeks were related to Fe(III)-reducing capacity by roots (linear regression, R^2=0.69, P<0.001 for DM, and logarithmic regression, R^2=0.47, P<0.001, for SPAD). These relationships were in agreement with previous evidences of Fe-reducing capacity being a mechanism involved in Fe supply to strategy I plants grown under Fe-stress growing conditions (De la Guardia et al. 1995; Agnolon et al. 2002; De la Guardia and Alcántara 2002). However, when DM in different organs was taken into account, reducing capacity by roots was significantly related to root DM (exponential regression, R^2=0.75; P<0.001), but not to shoot DM, perhaps indicating that factors affecting root development were also affecting root reducing capacity in calcareous media.

In calcareous media, Fe(III)-reducing capacity was greater in Fe–EDDHA treatment compared with control or ferrihydrite, and nonsignificantly different from that observed in Fe–HS. Thus, the positive effect of complexed Fe (to EDDHA or HS) might be, at least partially, ascribed to an increase in root reducing capacity. This effect of HS on reducing capacity has been described as one of the main reasons for the positive effect of these substances on Fe

uptake by plants (Mohamed et al. 1998; Sánchez-Sánchez et al. 2006), Fe–HS being a natural substrate for the plasma membrane Fe(III)-chelate reductase (Pinton et al. 1999). Furthermore, an improved Fe supply to plants overcoming chlorosis must increase root development (Table 3). According to Chen et al. (2004), an enhanced Fe nutrition is a major mechanism of plant growth stimulation by HS. The increased root growth has been also ascribed to the presence of auxin groups in the macrostructure of HS> 14 KDa (Canellas et al. 2002). This increased root growth may account for an increased Fe(III)-reducing capacity since this property seemed to be related to the development of the organ, and thus for an improved Fe supply by Fe–HS in calcareous media. In siliceous media, with an accurate Fe supply, root reducing capacity was also related to root DM (linear regression, R^2=0.67, P<0.001). Contrasting to that observed in calcareous media, in the siliceous one Fe–HS and Fe–EDDHA did not promote increased root DM and root reducing capacity when compared with other treatments (Table 2). Thus, it can be supposed that root development was a key factor explaining Fe(III)-reducing capacity in roots, and thus affecting Fe nutrition in lupins.

Humic substances applied without complexed Fe in a ferrihydrite enriched calcareous media increased DTPA and CA-extractable Fe (Table 3), the last accounting for readily reductable Fe mainly associated with poorly crystalline Fe oxides (Reyes and Torrent 1997; De Santiago and Delgado 2006). This last extractant mimics the reductant effect of lupin roots (related to citrate excretion, Dinkelaker et al. 1989). The increased amount of extractable Fe may be ascribed in part to Fe complexation by HS because some ligand exchange between HS and DTPA likely occurs (enhancing DTPA extraction) and also some HS with complexed Fe must be extracted by citrate (De Santiago and Delgado 2006). In addition, the reductant effect of HS on ferrihydrite described by Cesco et al. (2000) could contribute to an increased amount of Fe extracted by CA. However, this increased amount of extractable Fe did not account for an increased Fe content in plants, SPAD readings, or DM production. The decreased SPAD observed with HS alone in calcareous media was probably related to a decreased root development (significant at HS rates of 0.3 g C kg^{-1}, Table 3), which accounted for a depressed Fe nutrition. This was in agreement with previous findings by Brunner et al. (1996) who observed that root growth was inhibited in the presence of HS of large molecular size (>10 KDa) due to the presence of phytotoxic aromatic compounds.

In addition, in siliceous media, HS application in a FH-enriched media accounted for depressed SPAD readings and total DM when compared with control. This was at least in part related to a depressed root development by HS because significantly smaller root DM and Fe(III) reduction capacity was observed at HS rates of 0.1 g C kg^{-1} (Table 3, not significantly different than with 0.3 g C kg^{-1}). In these media, however, treatments with FH showed a smaller shoot DM than other treatments (included control). Ferrihydrite treatment accounted for six times more CA–Fe than control (Table 2), the reductant effect of citrate and ascorbate being much greater than in calcareous media (Table 3). However, Fe content in plants revealed that an excessive Fe supply is not the reason for the decreased development in FH (Table 3). Ferrihydrite significantly increased the sorption capacity for some nutrients, such as P or Zn, in the growing media through surface complexation (Lindsay 1991; Rahmatullah and Torrent 2000; Uygur and Rimmer 2000). Phosphorus sorption capacity in the growing media, considering a specific surface in the ferrihydrite around 150 m^2 g^{-1} (Montilla et al. 2003), was around 30 times greater than the total amount of P applied in nutrient solution according to Torrent et al. (1994). Thus, a decreased concentration of some nutrients, particularly P, can be expected in the growing media solution, which could explain, at least in part, a depressed growth in FH-enriched media. This effect was more evident in siliceous media because, in calcareous, Fe was a more limiting growing factor than P.

Conclusions

Ferrihydrite was found to be an iron source, which significantly contributes to Fe nutrition of lupins grown in calcareous sand. Furthermore, humic substances (HS) significantly affected Fe nutrition of lupins grown in calcareous media. However, this effect was only positive if some Fe was complexed to HS. The efficiency of HS with Fe complexed (Fe–HS) in preventing Fe chlorosis was found to be similar to Fe–EDDHA at the same rate of complexed Fe applied, thus revealing that Fe–HS could be an effective product to correct this nutritional problem.

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