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ORIGINAL ARTICLE

Strictly NO_3- Nutrition Alleviates Iron Deficiency Chlorosis in *Arabidopsis thaliana* Plants.

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The effects of NO_3^- nutrition on iron deficiency responses were investigated in *Arabidopsis thaliana*. Plants were grown with or without 5 µM Fe, and with NO_3^- alone or a mixture of NO_3^- and NH_4^+. The results indicated that, NO_3^- nutrition induced higher dry matter production, regardless the Fe concentration. Fe deficiency reduced growth activity, photosynthetic pigment concentration and Fe content of plants, whatever the N forms. This decrease was more pronounced in plants grown with mixed N source; those plants presented the highest EL and MDA and anthocyanin contents compared to plants grown under Fe sufficient conditions. In iron free-solutions, with NO_3^- as the sole nitrogen source, enhanced FC-R activity in the roots was observed. However, in the presence of NH_4^+, plants displayed some decrease in in FC-R and PEPC activities. The presence of NH_4^+ modified typical Fe stress responses in *Arabidopsis thaliana* plants.

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Among abiotic stresses, iron deficiency is one of severe problems in worldwide agricultural production. Iron deficiency changed plant morphological traits (Briat 2007), decreased plant dry matter, leaf area and crop yield (Rabhi *et al.* 2007; Zocchi *et al.* 2007; Zaharieva *et al.* 2004). Around third of arable land on earth are affected by iron deficiency and the problems becoming an increasing threat to agriculture globally (Mtimet 2001). To overcome this constraint, plants have evolved specific adaptive mechanisms for iron acquisition, classified into two distinct “strategies”. The response to Fe deficiency of dicotyledonous and non-graminaceous monocotyledonous plants, involves a series of morpho-physiological and biochemical changes (Zaharieva *et al.* 2004) depicted as "strategy I". The main morphological changes are the increase of lateral root formation and the emergence of root hairs and transfer cells, increasing in this way the root surface and consequently Fe uptake (Schmidt *et al.* 2000). The strategy I response includes also a proton excretion

by roots, which lowers the rhizosphere pH, and an increase in the capacity to reduce Fe^{3+} (less soluble) to Fe^{2+} (more soluble) by a Fe(III)-chelate reductase (FCR) (Kim and Guerinot 2007). The reduction step, prior to Fe^{2+} uptake, has been shown to be critical for Fe uptake from Fe-deficient soil (Kim and Guerinot 2007).

Several metabolic changes have also been described in Fe-deficienct roots, including an increase in the activity of phosphoenolpyruvate carboxylase (PEPC) accompanied with an accumulation of organic acids in roots, mainly malate and citrate (Abadía *et al.* 2000). PEPC catalyses the carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate. The latter is reduced to malate via the cytosolic enzyme, malate dehydrogenase, then malate is transported to mitochondria through the malate-oxaloacetate shuttle and converted into citrate by citrate synthase (Andaluz *et al.* 2002). The role of organic acid accumulation in Fe deficiency response is mainly related to Fe transport to shoots as Fe citrate in xylem sap (Stephan 2002).

Nitrogen plays a pivotal role in the inorganic nutrition of plants and hence in determining growth. The form of N supply, to a great extent, controls the uptake ratio of cations and anions and thus, influences dry matter production and root rhizosphere and apoplastic pH (Mengel 1995; Marschner 1995). It has been reported that the uptake of NH_4^+ by plant roots leads to a further acidification of plant root zone (Marschner 1995; Von Wiren *et al.* 2001) and enhances Fe solubility. In contrast, in soils with high pH, where nitrate is the predominant N source, the uptake of NO_3^- anions results in an alkalinization of the rhizosphere (Marschner 1995; Touraine *et al.* 2001) limiting in this way Fe availability to roots (Lindsay 1984). Nitrate regime not only increases soil pH but also induces high leaf apoplastic pH (Kosegarten and Englisch 1994; Kosegarten *et al.* 2001) which reduces leaf FCR activity, resulting in Fe inactivation in rosette leaves (Mengel *et al.* 1994).

The aim of this work was to study the effect of nitric regime under Fe-deficient conditions on growth characteristics and physiological responses in *Arabidopsis thaliana* (isolate Col).

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *A. thaliana,* Col, were sown in pots containing a mixture of sand and peat (1V: 2V). After germination, seedlings were grown in a growth chamber under controlled conditions (Gibeaut *et al.* 1997): 12 h daily light, 150 µmol m^{-2} s^{-1} photon flux density; 22/18°C day/night temperature regime, and 60/80% day/night relative humidity regime. They were irrigated with distilled water for 8 days then with a complete nutrient solution containing 5 µM Fe-EDTA (Gay and Hauk 1994). After 3 weeks, they were transferred into 300 ml plastic pots and acclimated over one week. Then, four treatments were started. Plants were separated in 4 lots, each one placed on one of the following nutrient solutions: T1: 100% NO_3^- N + 5 µM Fe; T2: 100% NO_3^-N + 0 µM Fe; T3: 50% NO_3^- N:50% NH_4^+N + 5 µM Fe; T4: 50% NO_3^-N:50% NH_4^+N + 0 µM Fe. The medium was weekly-renewed and the plants were harvested after 10 days of treatment.

Pigment concentrations

For each treatment, six plants were used, and individually treated. Fresh leaves of each plant were separately incubated in the dark for 72 h at 4°C in acetone 80% (v/v). Absorbance of each of the acetone extracts was measured with a DU 640

Beckman spectrophotometer. Concentrations of chlorophyll and total carotenoids were calculated using the equations proposed by Lichtenthaler (1988). Anthocyanin levels were estimated in methanolic extracts form A_{530} and A_{653} as indicated by Murray and Hackett (1991).

Ferrous iron content

Bivalent iron (Fe^{2+}) was extracted following the method of Liorente *et al.* (1976). Plant materials were washed with distilled water, dried at 70 ºC for 72 h, weighed then ground with a mortar. The obtained powder samples were digested using 1N HCl solution. Fe^{2+} content was determined by atomic absorption spectrophotometry.

Electrolyte leakage

Electrolyte leakage was determined as described by Dionisio-Sese and Tobita (1998). Leaf samples (approximately 200 mg FW) were submerged into 10 ml distilled water and kept at 32ºC over 2 h. Then, the initial electrical conductivity of the medium (EC1) was measured. After the conductivity measurement, the leaf tissues were killed by autoclaving at 121ºC for 20 min to release all electrolytes, cooled to 25ºC, and then the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was calculated as EL = 100 EC1/EC2.

The roots were immersed 5 min in a solution of $CaCl_2$ (0.5 mM), then incubated in 25 ml of distilled water for one hour in the dark and the EC1 values were measured. Subsequently, they were transferred to 25 ml of boiling distilled water for 5 minutes and the EC2 values were measured. The EL was calculated from EL= EC1/ (EC1+EC2).

Lipid peroxidation

The level of lipid peroxidation was determined by a procedure based on the method of Heath and Packer (1968). 0.5 g fresh leaves were ground in 5 mL of ice-cold phosphate buffer solution (0.05 mM, pH 7.8) containing 1% PVP. The homogenate was centrifuged at 10,000 g for 30 min. 2 mL of supernatant was mixed with 2 mL of thiobarbituric acid (TBA) (0.5% TBA, 20% TCA). The mixture was heated at 100°C for 30 min, chilled on ice, and then centrifuged at 1000 g for 10 min. Absorbance of the supernatant was measured at 532 nm and adjusted for non-specific absorbance at 510 nm and 560 nm.

Root Fe(III)-reductase activity

Fe(III)-chelate reductase activity was estimated as in *vivo* reduction of Fe(III)-EDTA by intact plant roots. The formation of the red Fe(II)-bathophenanthrolinedisulphonate (BPDS) complex was followed by measuring its absorbance at 535 nm (Chaney *et al.* 1972). The reaction was performed for 30 min with BPDS (0.3 mM) and Fe(III)-Na-EDTA (0.1 mM) in full-strength nutrient solution, buffered with 10 mM MES-KOH (pH 5.5).

Enzyme extraction and assay

Fresh leaf or root samples were ground in a mortar with 100 mM Tris-bicine (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol (v/v), 5 mM $MgCl_2$, 1% mercaptoethanol (v/v), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5% polyvinylpyrrolidone (PVP) (w/v of sample FW). After centrifugation at 12000 X *g* for 20 min at 4°C, the supernatant was collected and enzyme activities were immediately measured.

The activities of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and Rubisco (Rubisco E.C.4.1.1.39) were assayed according to Ouerghi *et al.* (2000). PEPC reaction mixture contained 100 mM Tris-bicine (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 5 mM $NaHCO_3$, 0.2 mM NADH, 4 mM phosphoenolpyruvate, 5 enzyme units of

malate dehydrogenase (MDH). The crude extract (100 µl) was added to the reaction medium then the activity was monitored at 340 nm for 15 min.

Statistics

Statistical analysis was performed with Statistica™ software, using two-way ANOVA and Newman–Keuls test for post-hoc mean comparison. The ANOVA was performed over the whole set of data

RESULTS

Plant aspect and growth

Chlorosis symptoms appeared first on the fifth day after the beginning of the treatments in the young leaves of plants grown without Fe (0 µM Fe) with whatever the N form. After 10 days of treatment, chlorosis intensity of plants grown without Fe and N source as 50% NO_3^-:50% NH_4^+ N was higher than those of plant grown without Fe and N as 100% NO_3^-.

At the end of treatment, plants grown with 5 µM Fe and 100% NO_3^- showed significantly higher total dry weight compared to plants grown with 5 µM Fe and 50% NO_3^-:50% NH_4^+ (Fig. 1). Iron deficiency reduced dry weight of plants whatever the N source. This reduction was more pronounced for plants grown with 50% NO_3^-:50% NH_4^+ (Fig. 1).

Fe content

Root and shoot Fe concentration of *Arabidopsis* plants grown without Fe (0 µM Fe) was found to be significantly lower than in plants with 5 µM Fe, regardless of the N form (Fig. 2) Whereas, regardless of the Fe level, Fe concentration in plants grown with 100% NO_3^- was significantly higher than that of plants gown with both forms of N.

Pigments contents

A significant reduction in chlorophyll content was found in Fe-deficient plants as compared to Fe-sufficient plants. This effect was more pronounced in NH_4^+ fed plants (Fig. 3). Fe deficiency did not affect carotenoids content whatever the N form (Fig. 3). Anthocyanins content was significantly induced by iron deficiency when plants are grown with % NO_3^-:50% NH_4^+. However, an opposite effect was observed in NO_3^- treated (Fig. 4).

Lipid peroxidation and electrolyte leakage

The extent of the Fe-induced oxidative damage was assessed by measuring the MDA formation and electrolyte leakage (Fig. 5). Amount of electrolyte leakage increased under iron deficiency conditions only for plants grown with both forms of N. However, in plant grown in nitric medium, electrolyte leakage was not affected by iron deficiency (Fig. 5A). Similarly, Fe deficiency increased MDA contents in plants grown with 50% NO_3^-:50% NH_4^+, whereas MDA content was not significantly affected by iron deficiency if NO_3^- was used as the sole nitrogen source (Fig. 5B).

Nutrient solution acidification

The changes in pH depended primarily on nitrogen source. In the fact, NO_3^- uptake increases the pH of the outer solution, whereas NH_4^+ uptake doesn't affects pH of the medium. When plants are grown without Fe, the pH of the nutrient solution remained stable (Fig. 6A).

Root ferric chelate reducing activity

The highest level of FCR activity was recorded in plant grown in presence of Fe and with 50% NO_3^-:50% NH_4^+. Whereas, elimination of Fe from the growth medium significantly decreased FCR activity, in these conditions. In plants grown with 100% NO_3^-, iron deficiency had a significant positive effect on FCR activity (Fig. 6B).

PEPC activity in roots extracts

The activity of PEPC, enzyme involved in organic

acid metabolism was measured in the root tips of the plants from all treatments after 10 days of Fe starvation. Fe deficiency significantly decreased PEPC activity in NH_4^+ fed plants, whereas no significant effect in NO_3^- treated plants was observed (Fig. 7).

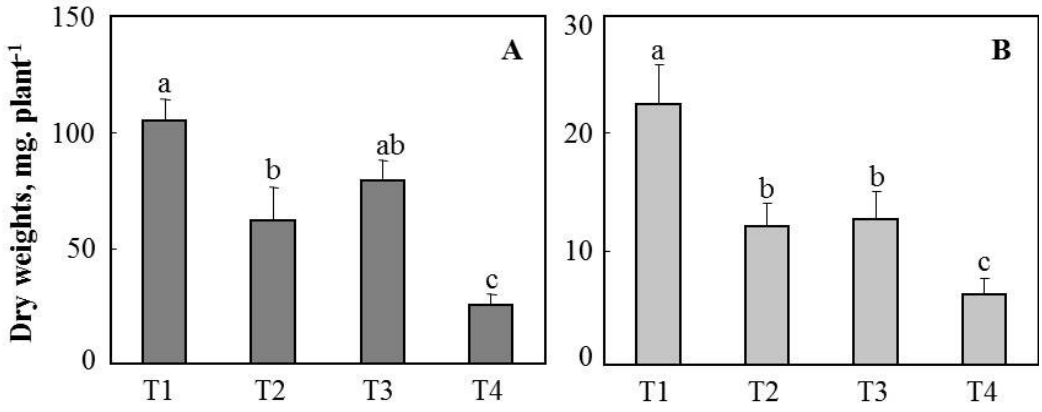

Figure 1 : Shoot (A) and Root (B) dry weights of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/NO_3^- supply. Bars are means of 8 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

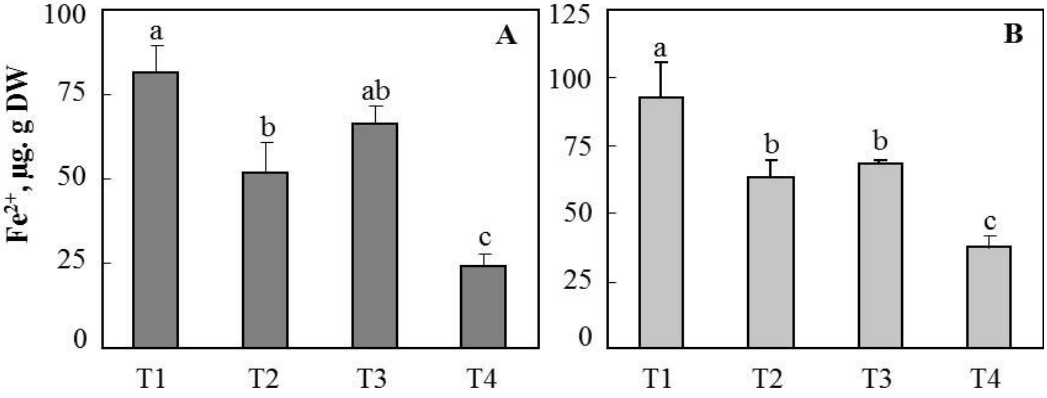

Figure 2. Shoot (A) and Root (B) Fe content of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

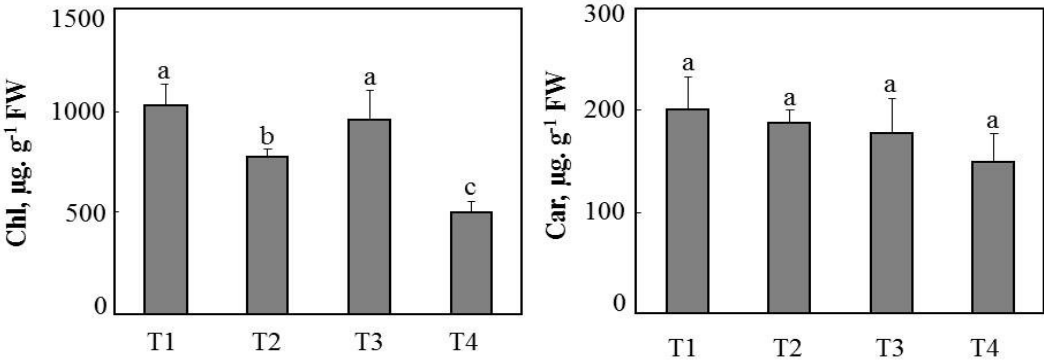

Figure 3. Chlorophyll and carotenoid contents in shoot of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

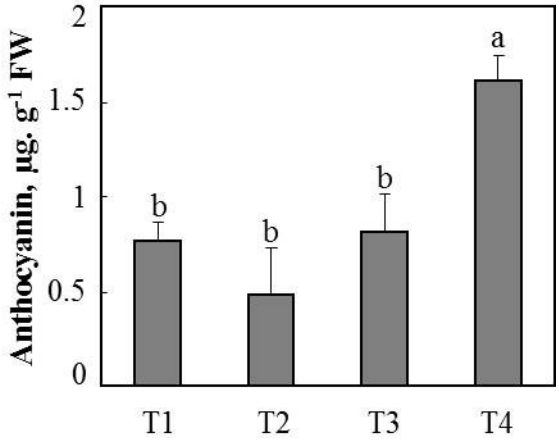

Figure 4. Anthocyanins content in shoot of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/ NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

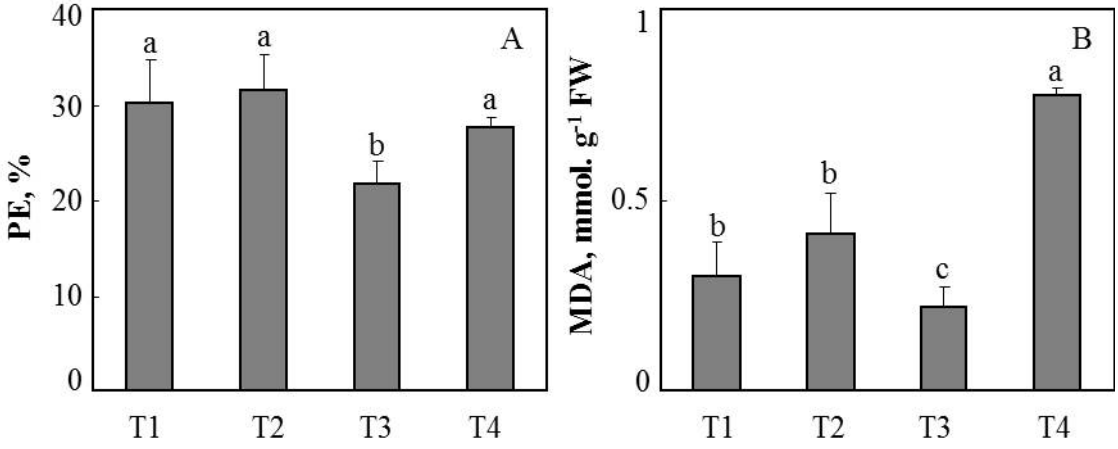

Figure 5. Electrolyte leakage (A) and MDA content (B) in shoot of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/ NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

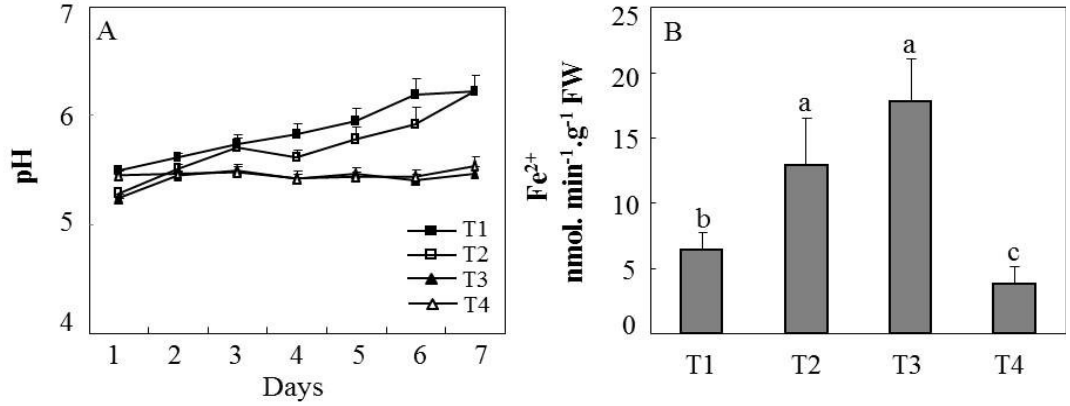

Figure 6. pH values of the culture media (A) and Fe-reductase activity in root (B) of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/ NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

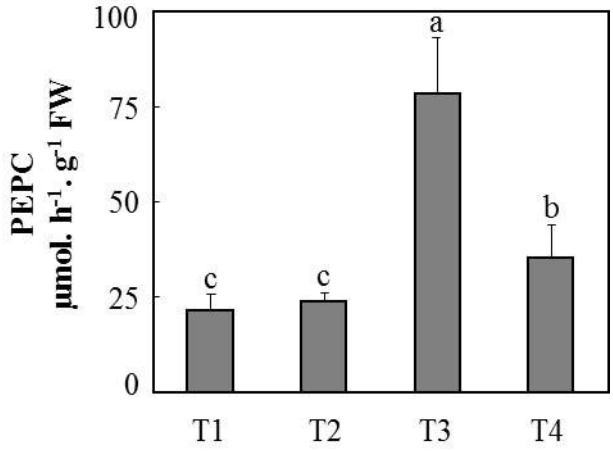

Figure 7. PEPC activity in root of *Arabidopsis thaliana* plants grown during 10 days with 0 µM Fe(III)-EDTA or 5 µM Fe(III)-EDTA, and with NO_3^- as the only source of nitrogen or with a mixed NH_4^+/NO_3^- supply. Bars are means of 6 replicates ± SE. Bars labelled by the same letter are not statistically different according to the ANOVA test at $P \leq 0.05$.

DISCUSSION

The results from this study showed that the interaction between different Fe levels and N forms in the nutrient solution had a significant effect on vegetative growth, nutrient concentration and biochemical response in *Arabidopsis* plants. Concerning the total growth, plants supplied with N as 100% NO_3^- produce significantly higher dry matter than plants with mixed N nutrition. A similar result has been recorded in tomato plants (Claussen 2002) who have noted that tomato plants cultivated on a nitric medium produced higher dry weight than plants cultivated in a mixed medium. Moreover, Fe deprivation reduced plants growth whatever the N form, this reduction was more pronounced for plants grown in the presence of NH_4^+ in the medium culture.

On the other hand, Fe deficiency caused iron chlorosis in *Arabidopsis* plants of both N forms. Chlorosis symptom was accompanied by a significantly decrease of chlorophyll content in the shoots. The impact of iron deficiency, on chlorophyll formation has been reported in several studies. For example, bicarbonate presence in the medium significantly reduced chlorophyll concentration in sunflower leaves (Gharsalli and Hajji 1992) as well as in two peach rootstocks (Molassiotis *et al.* 2006). These findings are explained by the primordial role of Fe^{2+} on the formation of the chlorophyll precursors ð-aminolevulinic acid and protochlorophyllide (Marschner 1995).

The shoot and root Fe content was significantly higher in plants grown with sufficient Fe and 100% NO_3^- compared either to plants with sufficient Fe and both form of N or without Fe and whatever N form. Similar findings were showed by Zornoza and Gonzalez (1998) who found that the presence of 20% N as NH_4^+ reduced the uptake of Fe, by spinach plants. Contrary to our findings, (Assimakopoulou, 2006) demonstrated that Fe concentration increased with the addition of mixed N nutrition. Regardless of the N form, the Fe deprivation from the nutrient solution significantly decreased shoot and root Fe concentration.

It is well known that, Fe deficiency induces oxidative stress in dicotyledonous species which causes disturbance of expression and activity of diverse antioxidant enzymes (Ranieri *et al.* 2001). In

this work we assessed oxidative stress by measuring the anthocyanin and MDA content and electrolyte leakage. Under Fe deficiency conditions, MDA content and electrolyte leakage increased markedly only for plant grown with mixed N nutrition, suggesting that cell membrane stability was affected under theses conditions. Lipid peroxidation of membranes was accompanied by an increase of anthocyanin content, in the same conditions. According to (Sgherri *et al.* 2004) anthocyanin production, known as a hallmark of plant stress, allows the plant to develop resistance to a number of environmental stresses, especially oxidative damages.

Acidification of the rhizosphere is known to be part of the mechanism by which some dicotyledonous plants respond to Fe starvation. This acidification has been attributed to activation of the root plasma membrane H^+-ATPase (Dell'Orto *et al.* 2000b). When grown without Fe, regardless of the nitrogen source, pH of the medium was not affected. Whereas, a slight increase in pH was observed when plants were supplied with NO_3^-. It has been shown that medium acidification and root FeIII reducing capacities are related to plants adaptation to Fe deficiency (Brancadoro *et al.* 1995; Schmidt 2006; Zaharieva *et al.* 2004). Under our growth conditions, we did not observe root acidification under Fe-deficient conditions, however, Fe-chelate reductase activity was significantly increased in Fe-deficient plants fed with NO_3^-. On the opposite when supplied with NH_4^+ under Fe-deficient conditions, activity of FCR was significantly reduced. These results are in agreement with those of Rivero *et al.* (2003) who found higher FCR activity in the roots of both tomato and watermelon plants treated with NO_3^- in comparison with those treated with NH_4^+. Kosegarten *et al.* (2004) and Nikolic and Romheld (2003) demonstrated close relationships between the form of nitrogen in the nutrient solution, root apoplast pH and FC-R activity in *Helianthus annuus* L.. Based on these relationships, the stimulation of FC-R activity may be linked to the decrease in pH associated with NH_4^+ uptake. Nevertheless, our results are not in agreement with the expected effect of various nitrogen forms on Fe^{3+} reduction. In addition, the application of nitrate in the nutrient solution did not affect PEPC activity. In contrast, a significant decrease of this activity was observed under ammoniacal regime.

Our results showed that nitrogen uptake and metabolism interfere with Fe metabolism in *Arabidopsis* plants. In a mixed medium (50% NO_3^-:50% NH_4^+), growth activity and membrane integrity of *Arabidopsis* was greatly affected by iron deficiency. In these conditions, some typical biochemical responses to Fe deficiency (medium acidification, root FC-R and PEPC activity) are not induced in *Arabidopsis* plants. However, plants fed with 100% NO_3^- showed a better behavior towards iron deificiency. In the fact, growth activity was less affected and an induction of root FC-R activity in the roots when subjected to Fe deficiency in these conditions.

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