

ARABIDOPSIS THALIANA TOLERATES IRON DEFICIENCY MORE THAN THELLUNGIELLA SALSUGINEA BY INDUCING METABOLIC CHANGES AT THE ROOT LEVEL

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Several studies have used *A. thaliana* as a model to identify the physiological and molecular mechanisms underlying iron deficiency tolerance in plants. Here, *Arabidopsis thaliana* and *Thellungiella salsuginea* were used to investigate the differential responses to iron deficiency of these two species. Plants were cultivated in hydroponic medium containing 5 or 0 μM Fe, for 10 days. Results showed that rosette biomass was more reduced in *T. salsuginea* than in *A. thaliana* when grown on Fe-deficient medium. As a marker for iron deficiency tolerance, the induction of ferric chelate reductase (FCR) and phosphoenolpyruvate carboxylase (PEPC) activities was observed only in *A. thaliana* roots. In addition, we found that the accumulation of phenolic acids in roots of N1438 ecotype of *A. thaliana* was stimulated by Fe deficiency. Furthermore, an increase of flavonoids content in the root and exudates was observed under Fe-deficiency in this ecotype. Unlike other abiotic stresses, it appears that iron deficiency effects were more pronounced in *Thellungiella* than in *Arabidopsis*. The higher tolerance of the *Arabidopsis* plant to iron deficiency may be due to the metabolic changes occurring in the roots.

Key words: *Arabidopsis thaliana*, enzymatic activities, iron deficiency, *Thellungiella salsuginea*, polyphenol production.

INTRODUCTION

Iron is a micronutrient required in low quantities by plants to achieve growth and development. Thus iron deficiency, like many other abiotic stresses, negatively affects the growth and development of plants and causes serious problems for farmers. Iron (Fe) deficiency is frequently encountered on calcareous soils. In these soils, the availability of iron to plants is reduced through the precipitation of iron at high pH (Rabhi et al., 2007). To overcome this constraint, plants showed several physiological and biochemical changes as adaptive responses to Fe limitation, including i) an induction of the activity of a ferric chelate reductase to convert Fe(III)-chelates to Fe(II) (Schmidt, 1999) and PEPC activity in root tissues, thus accumulating organic acids (mainly citrate) and accelerating the translocation of solubilised Fe to shoots (De Nisi and Zocchi, 2000; López-Millán et al., 2000); ii) the accumulation and exudation of phenolics compound reported to be the main components of root exudates in response to Fe

deficiency (Römheld and Marschner, 1986; Susin et al., 1996; Curie and Briat, 2003; Hell and Stephan, 2003).

Until now, *Arabidopsis thaliana* and *Thellungiella salsuginea* (also known as *T. halophila*), members of the Brassicaceae family (Al-Shehbaz et al., 1999), have been identified as potential model systems for the study of abiotic stress tolerance (Bressan et al., 2001; Inan et al., 2004; Volkov et al., 2004). These two species are closely related, both in their morphology and in their genome sequence (90–95% identity at the cDNA level; Bressan et al., 2001; Zhu, 2001). Furthermore, *A. thaliana* and *T. salsuginea* can be easily transformed and have several other characteristics such as a short life cycle and abundant seed production (Volkov and Amtmann, 2006). In response to abiotic stresses, *T. salsuginea* (an extremophile plant) appears to show resistance to stress caused by low temperature, drought, high salt and nitrogen deficiency as compared to *A. thaliana* (Volkov and Amtmann, 2006; Inan et al., 2004; Kant et al., 2008).

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To our knowledge, no studies have yet been carried out to compare the behaviour of *A. thaliana* and *T. salsuginea* in response to iron deficiency. In this study, physiological and biochemical experiments were conducted on both *A. thaliana* (N1438 and COL ecotypes) and *T. salsuginea* to study the physiological response to Fe deficiency in these species and to investigate the biochemical mechanisms that contribute to this variability. Based on these analyses, we exhibited that *Arabidopsis* has a higher tolerance than *Thellungiella* to a low external iron supply. This is accompanied by a variety of changes at the root level of the plant, mainly in FCR and PEPC activities and the accumulation and exudation of flavonoids in the external medium.

MATERIALS AND METHODS

PLANT MATERIAL

Thellungiella salsuginea and *Arabidopsis thaliana* (COL and N1438 ecotypes) seeds were sown in pots containing a mixture of sand and peat (1V: 2V). They were irrigated with distilled water for one week, then with a complete nutrient solution containing 5 μM Fe-EDTA (Gay and Hauk, 1994). After three weeks, they were transferred into 300 ml plastic pots and acclimatized for one week. Then, plants were separated into two lots and irrigated with a nutritive solution containing 0 and 5 μM Fe. The medium was renewed weekly and the plants were harvested after ten days of treatment.

GROWTH MEASUREMENTS AND ANALYSIS OF INORGANIC IONS

Plants from each treatment were sampled to determine the leaf area and number. Leaves and roots were separated and their fresh weight (FW) was directly determined. Leaf surface area was measured using Optimas software. For dry weight (DW) determination, the leaves and roots were dried at 70°C for 48 h and weighed. The Fe^{2+} content was extracted with 1N HCl and determined by atomic absorption spectrophotometry.

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 80% (v/v) acetone. Absorbance of each of the acetone extracts was measured with a DU 640 Beckman spectrophotometer. Concentrations of chlorophyll (Chl) were calculated using the equations proposed by Lichtenthaler (1988).

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 root samples (200 mg) were ground in a mortar with 1 ml of extraction buffer (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 phenylmethylsulfonylfluoride (PMSF), and 5% polyvinylpyrrolidone (PVP) (w/v). The homogenate was centrifuged for 20 min at 14000 g and 4°C, the supernatant was collected and enzymatic activity was immediately measured.

The activity of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) was 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 was added to the reaction medium and then the activity was monitored at 340 nm for 15 min.

PHENOLIC COMPOUNDS ANALYSIS

Roots were dried at room temperature for two weeks. Organ extracts were obtained by magnetic stirring for 30 min of 2.5 g of dry organ powder with 25 ml of pure acetone. Then, extracts were kept at 4°C for 24 h, filtered through a Whatman No. 4 filter paper, and stored at 4°C until analysis.

To determine the total phenolics of the root exudates, roots (1 g) were collected from 12-day-old plants and homogenized in a mortar containing methanol and water (1:1) as the extraction medium. Then, the homogenate was centrifuged at 10 000×g for 10 min. (Jelali et al., 2010).

Total phenolics concentrations

Total phenolics were assayed using the Folin-Ciocalteu reagent, following Singleton and Rosi's (1965) method, based on the reduction of a phosphomolybdate-phosphomolybdate complex by phenolics to blue reaction products and slightly modified by Dewanto et al. (2002). An aliquot of diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin-Ciocalteu reagent. The

TABLE 1. The effect of iron concentration (0 or 5 μM) on growth parameters of *A. thaliana* and *T. salsuginea* plants treated over ten days.

	N1438		COL		THELL	
	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe
Rosette biomass (mg)	62.3 \pm 8.0b	46.1 \pm 8.5cd	88.9 \pm 9.6a	54.3 \pm 9.3bc	40.4 \pm 7.1d	14.5 \pm 2.6e
Root biomass (mg)	15.0 \pm 2.0a	11.5 \pm 2.5ab	15.5 \pm 2.7a	10.9 \pm 1.5b	10.7 \pm 1.2b	4.48 \pm 0.19c
Total leaf area (cm ²)	42.7 \pm 3.2a	26.3 \pm 2.2b	36.7 \pm 2.4a	20.0 \pm 3.7b	24.5 \pm 3.3b	12.1 \pm 1.2c
Leaf number	16 \pm 1.6a	15 \pm 0.8a	18.0 \pm 2.6a	15 \pm 1.7a	14 \pm 2.1a	9.0 \pm 0.6b
Single leaf area (cm ²)	2.7 \pm 0.3a	1.7 \pm 0.2b	2.0 \pm 0.3a	1.4 \pm 0.1b	1.5 \pm 0.2b	1.4 \pm 0.4b

Data are means of six replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

mixture was shaken and allowed to stand for 6 min, before the addition of 1.25 ml of 7% Na_2CO_3 . The solution was then adjusted with distilled water to a final volume of 3 ml and mixed thoroughly. After incubation in the dark, the absorbance at 760 nm was read versus the prepared blank. The total phenolic concentration in the roots was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE.g⁻¹ DW) through the calibration curve with gallic acid. All samples were analyzed in three replications.

Total flavonoid concentration

Total flavonoids were measured using a colorimetric assay developed by Dewanto et al. (2002). An aliquot of diluted sample or standard solution of (+)-catechin was added to 75 μl of NaNO_2 solution (7%), and mixed for 6 min, before adding 0.15 ml AlCl_3 (10%). After 5 min, 0.5 ml of 1 M NaOH solution was added. The final volume was adjusted to 2.5 ml, thoroughly mixed, and the absorbance of the mixture was determined at 510 nm. Total flavonoids were expressed as mg (+)-catechin equivalent g⁻¹ DW (mg CE g⁻¹ DW), through the calibration curve of (+)-catechin (0-400 $\mu\text{g}\cdot\text{ml}^{-1}$ range). All samples were analyzed in three replications.

STATISTICS

Statistical analysis was performed with StatisticaTM software, using one-way analysis of variance (ANOVA). Statistical assessments of differences between mean values were performed by Duncan's multiple range test at $P \leq 0.05$.

RESULTS

PLANT GROWTH

Fe-deficiency resulted in a restriction of *A. thaliana* leaf development. At 0 μM Fe, rosette biomass was reduced to 74% and 61% that of control plants for

N1438 and COL ecotypes, respectively (Table 1). This restriction concerned mainly the leaf expansion (individual leaf surface area: reduction by 38% and 30% for N1438 and COL ecotypes, respectively), with the leaf number much less affected by iron deficiency. For *T. salsuginea* rosette, the growth inhibition was stronger than for *A. thaliana* (36% of control at 0 μM Fe). It was associated with an inhibition of both leaf initiation (64% of control) and leaf expansion (leaf area reduced by 51%) (Table 1).

In both species root growth was less affected by iron deficiency. In *A. thaliana* plants, root biomass was not significantly reduced. However, a reduction by 48% was observed in *T. salsuginea* under Fe-deficient conditions (Table 1).

CHLOROPHYLL CONCENTRATION

Under control conditions, *T. salsuginea* showed a higher pigment concentration than *A. thaliana*. Differences were also observed under Fe limiting conditions; iron deficiency had a more marked influence on chlorophyll a, and chlorophyll b concentrations in *T. salsuginea* than in *A. thaliana*. In fact, the reductions of total chlorophyll concentrations observed in *T. salsuginea* and *A. thaliana* was 50% and 38%, respectively (Fig. 1). A greater decrease of Chl a concentration caused a decrease in Chl a/Chl b ratio, in *T. salsuginea* plants (Fig. 1).

FE CONTENT

Fe^{2+} concentrations in the tissues decreased with iron concentration in the medium, but differently in the two species (Fig. 2). The larger diminution was observed in *T. salsuginea* and reached 72% and 51% in shoots and roots, respectively.

FCR AND PEPC ACTIVITY

Fe deficiency increased significantly the Fe-reducing activity in root segments of both accessions of *A. thaliana*. However, Fe^{3+} reduction activity did not

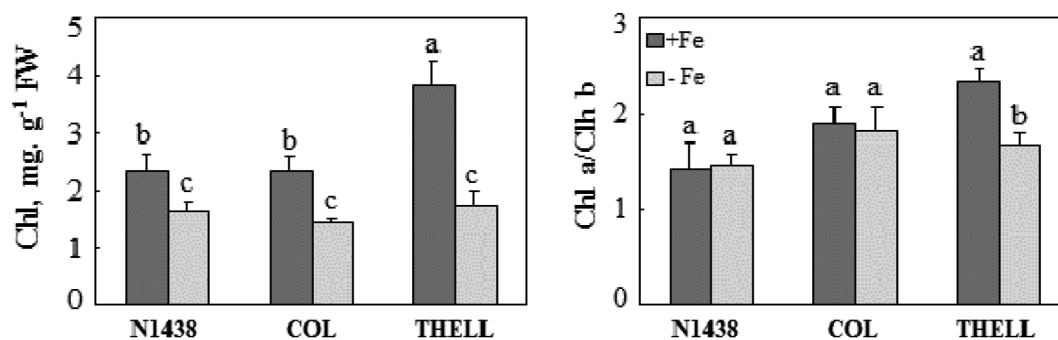


Fig. 1. The effects of iron concentration (0 or 5 μ M) on chlorophyll content and Chl a/Chl b ratio in the rosette leaves of *Arabidopsis thaliana* and *T. salsuginea* plants treated over ten days. Bars are means of six replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

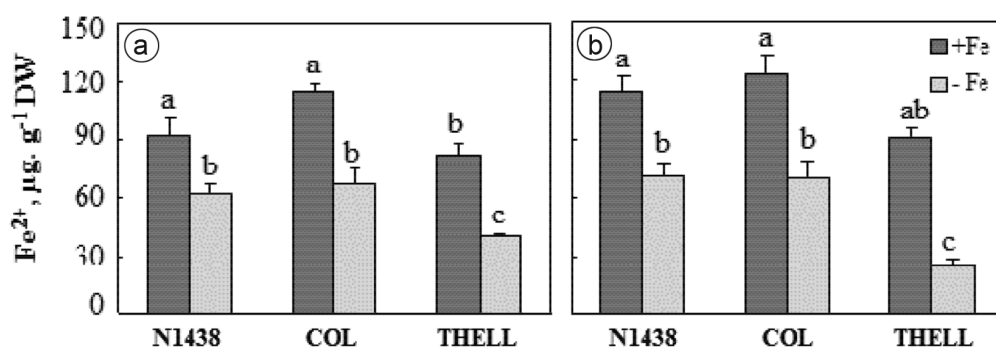


Fig. 2. The effects of iron concentration (0 or 5 μ M) on iron content in roots (a) and rosette leaves (b) of *Arabidopsis thaliana* and *T. salsuginea* plants treated over ten days. Bars are means of six replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

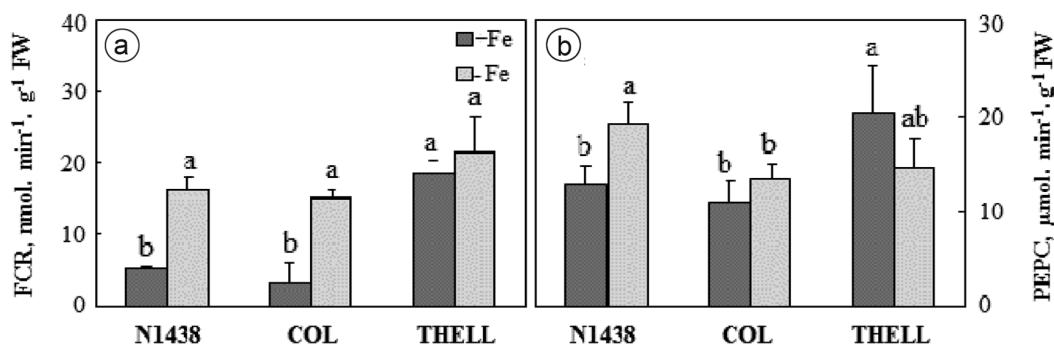


Fig. 3. The effects of iron concentration (0 or 5 μ M) on FCR (a) and PEPC (b) activities in roots of *Arabidopsis thaliana* and *T. salsuginea* plants treated over ten days. Bars are means of six replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

change significantly in *T. salsuginea* with iron deficiency (Fig. 3a).

PEPC activity was also assayed in root extracts from the two species, grown under Fe-deficient conditions. Fe deficiency resulted in a significant

increase of PEPC activity in root extracts of *A. thaliana* plants. However, the extent of stimulation compared to the control was stronger in N1438 than in COL, whereas PEPC activity was diminished by 29% in *T. salsuginea* (Fig. 3b).

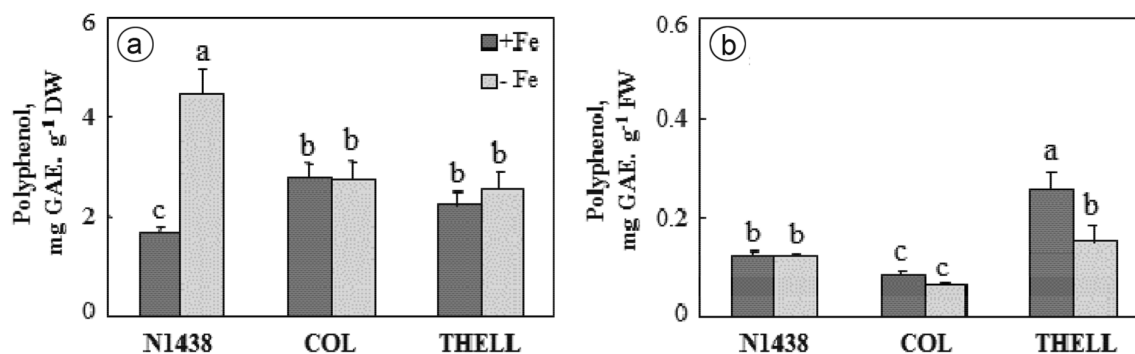


Fig. 4. The effects of iron concentration (0 or 5 μ M) on the accumulation (a) and exudation (b) of polyphenols in roots of *Arabidopsis thaliana* and *T. salsuginea* plants treated over ten days. Bars are means of four replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

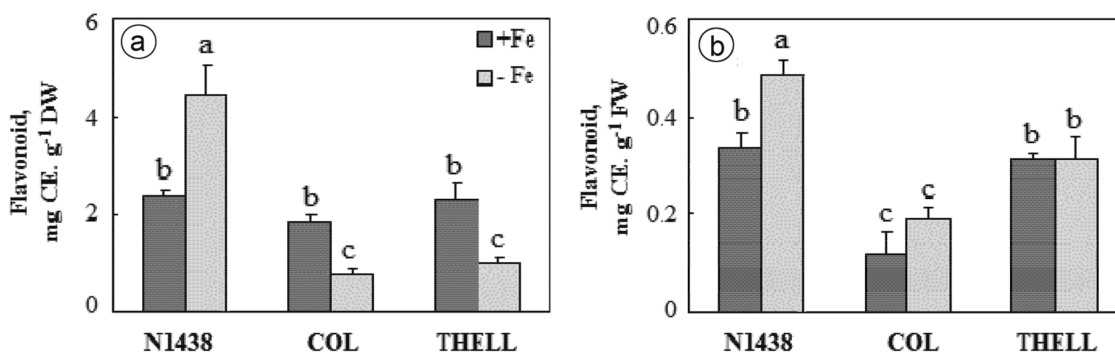


Fig. 5. The effects of iron concentration (0 or 5 μ M) on the accumulation (a) and exudation (b) of flavonoids in roots of *Arabidopsis thaliana* and *T. salsuginea* plants treated over ten days. Bars are means of four replicates \pm SE. For each parameter, different letters indicate significant differences at $P \leq 0.05$ as determined by Duncan's multiple range tests.

TOTAL PHENOL CONCENTRATIONS IN ROOTS AND EXUDATES

Higher concentrations of phenols were observed in Fe-deficient roots of N1438 accessions, compared to the control plants. In COL and *T. salsuginea* plants, this concentration remained stable after iron deficiency (Fig. 4a). In root exudates, the concentration of phenols remained close to the control value in *A. thaliana*, whereas it was diminished by 40% in *T. salsuginea* (Fig. 4b).

FLAVONOID CONCENTRATIONS IN ROOTS AND EXUDATES

Under Fe deficiency, the root concentration of flavonoids underwent a significant increase in N1438 accession. However, it diminished by 57% in COL and *T. salsuginea* (Fig. 5a). The exudation of flavonoids was significantly stimulated in *A. thaliana*, but not in *T. salsuginea* plants (Fig. 5b).

DISCUSSION

In an attempt to understand the variability of responses to iron deficiency between *A. thaliana* and *T. salsuginea* we paid attention to the metabolic changes that occurred under such conditions. Before discussing these in detail it should be noted that *T. salsuginea* had a slower growth rate and a lower leaf area than *A. thaliana* under iron deficient-conditions. The reduction in leaf development that accompanied iron deficiency was observed for *A. thaliana* as for *T. salsuginea*. However, it affected *T. salsuginea* more severely than *A. thaliana*. The difference between the two species could be attributed to (i) an inhibition of leaf induction in *T. salsuginea*, practically absent in *A. thaliana*, and (ii) a restriction of leaf expansion, present in both species but more important in *T. salsuginea*. In addition, the biomass production of shoots was more affected in *T. salsuginea* as compared to *A. thaliana*. Roots, however, were clearly less sensitive to the stress than shoots, showing no significant

variation between control and treated plants in *A. thaliana* plants.

When plants were grown in a control medium, the chlorophyll concentration in the leaves of *T. salsuginea* was much higher than in *A. thaliana*. In Fe-deficient conditions, the chlorophyll concentration was diminished in both species. However, diminution was more pronounced in *T. salsuginea* than in *A. thaliana*. This loss of chlorophyll is often considered as a marker of iron deficiency in plants (Briat and Vert, 2004). Thus, these results support that *T. salsuginea*, was more stressed than *A. thaliana* when plants were grown in a Fe-deficient medium. Moreover, the decrease in the Chl *a/b* ratio, due to a greater loss of Chl *a* in Fe-deficient plants of *T. salsuginea* suggests a considerable lowering of the rate of protochlorophyllide re-synthesis, and thus Chl *a* biosynthesis, (Argyroudi-Akoyunoglou and Akoyunoglou, 1970). The plants' Fe content was severely decreased by Fe deficiency in *A. thaliana* as well as in *T. salsuginea*. It seems that the Fe status cannot be used to discriminate between the two species.

These different behaviours observed in *A. thaliana* and *T. salsuginea* plants may be attributed to the adaptive biochemical mechanisms developed at the root cell plasma membrane. Since Fe deficiency stimulated FCR activity in roots of both ecotypes of *A. thaliana*, but COL showed a higher induction of this enzyme, contrarily to the Fe deficient roots of N1438, in which this activity was lower (Fig. 2a). In contrast, the FCR activity was not affected by iron deficiency in the *T. salsuginea* plant. The induction of root FCR activity was correlated to the tolerance to Fe deficiency in several species, as it was reported for lettuce (Msilini et al., 2012), chickpea (Mahmoudi et al., 2005), grapevine (Ksouri et al., 2006) and medicago (M'sehli et al., 2009). Several lines of evidence support the role of PEP-carboxylase (PEPC) in the adaptation of plants to environmental changes. The activation of PEPC has been associated to organic acid synthesis and to the need for cytoplasmic pH homeostasis in Fe-deficient roots (López-Millán et al., 2000). Here, we showed that direct Fe deficiency elicited an increase in PEPC activity for both *A. thaliana* ecotypes. This increase would poise the root cells for the efficient acquisition of Fe. However, in *T. salsuginea* a significant decrease in PEPC activity was observed in plants grown with 0 μM Fe as compared to control ones. This decrease pointed towards the severe effect of iron deficiency on the acquisition of iron in *T. salsuginea* plants.

In addition to the activation of FCR and PEPC enzymes (Zocchi and Cocucci, 1990; Dell'Orto et al., 2000), several other biochemical mechanisms such as root exudation and/or the accumulation of organic compounds (phenolics and flavonoids) remain of major importance (Jelali et al., 2010). It has been suggested that phenolic compounds enhance the Fe availability in rhizosphere soil, through chelating and

reducing insoluble Fe in the apparent free space of the roots and/or in the external solution (Dakora and Phillips, 2002). In addition, Jin et al. (2007) demonstrated that secreted phenolics play a critical role in facilitating the reutilization of the apoplastic Fe in roots. Here, we found that the accumulation of phenolic acids in the roots of N1438 ecotype was stimulated by Fe deficiency. Similar results were found in other species such as pea (Jelali et al., 2010) and red clover (Jin et al., 2007). Furthermore, an increase of flavonoids content in the root and exudates was observed under Fe-deficiency in these plants. The accumulation of flavonoids in the *A. thaliana* root was also observed when plants were exposed to high light intensity (Hemm et al., 2004). In addition, Keilig and Müller (2009) suggest that flavonoids could be an additional factor in heavy metal tolerance in *Arabidopsis thaliana*. Flavonoids accumulation among diverse plant species suggests that these compounds may serve important roles in root biology, plant defence and as precursors to root exudates. Nonetheless, further investigation is needed to understand the role of flavonoids in plant responses to iron deficiency.

CONCLUSION

In conclusion, *Arabidopsis* appears to be more tolerant to Fe deficiency as compared to *Thellungiella*. The capacity of *Arabidopsis* to prevent the effects of iron deficiency was mainly due to the higher accumulation and exudation of polyphenol and increased FCR and PEPC activities, especially in N1438 ecotype. These changes reveal a modest but real variability in the capacity of *Arabidopsis* and *Thellungiella* to induce metabolic changes in response to Fe deficiency.

AUTHORS' CONTRIBUTIONS

NM and JF performed experiments, analyzed data and wrote the manuscript; MCh contributed to the biochemical assays; ML contributed to the reagents, materials and analysis tools; ZO supervised the whole project.

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