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Responses of two forage legumes to direct iron deficiency: a comparative study

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Iron deficiency-induced chlorosis is one of the most damaging and widespread nutritional disorders in plants grown in Mediterranean-type soils. The aim of the present paper was to compare the effects of direct iron deficiency on two *Medicago* species from Tunisia: *M. ciliaris* and *M. truncatula.* Seedlings of both species were grown on a nutrient solution containing 30 μM Fe (control) or 1 μM Fe (iron deficiency treatment) for 30 days. We found that growth of both species was severely affected by direct iron deprivation. Nevertheless, *M. truncatula* experienced more marked decrease in biomass production and more severe chlorosis symptoms than *M. ciliaris*. The latter showed a better K and Fe distribution within the plant and a much higher Fe use efficiency for chlorophyll synthesis than the former. In addition, a capacity of root acidification due to a noticeable proton release rate was observed in *M. ciliaris* aiming to remobilize and take up Fe traces from the medium. Therefore, *M. ciliaris* was more tolerant to the stress than *M. truncatula.* This could explain the abundance and the better vigor of the former as compared to the latter in their alkaline natural biotope, Soliman sabkha.

Key words: chlorophyll content, iron, *Medicago ciliaris*, *Medicago truncatula*, potassium, root acidification.

INTRODUCTION

Iron deficiency is a widespread nutritional disorder in plants particularly when alkaline or calcareous soils are concerned (Mengel, 1994). These types of soils are common in the Mediterranean area, including Tunisia. Their high bicarbonate level affects metabolic processes in roots and leaves, decreasing soil and plant Fe bioavailability (Mengel, 1994) and leading to lime-induced iron chlorosis. In calcareous soils, iron chlorosis represents a major problem for the majority of legumes (Nikolic & Kastori, 2000), where, both iron absorption and metabolism are controlled by high pH levels. In several Dicotyledonous species, roots respond to Fe deficiency by inducing physiological mechanisms to increase solubility and uptake of Fe from soils (Molassiotis *et al*., 2006; Zocchi *et al*., 2007). The most typical root responses of Dicotyledonous species are a decrease in rhizosphere pH, an enhancement in root Fe^{3+} reduction capacity, and an increase in root branching and hair formation (Zocchi & Cocucci, 1990; Donnini *et al*., 2009).

Medicago species are mainly used as pasture species in extensive farming systems, in particular to improve low quality of herbage in natural pastures. Indeed, they are able to fix enormous amounts of atmospheric nitrogen and therefore they do not need a nitrogen fertilizer supply (Papastylianou, 1990). Also, *Medicago* and other annual pasture legumes are characterized by high feeding quality determined by noticeable protein, mineral, and vitamin contents (Crespo, 1987). In Tunisia, *Medicago* plants are widely localized in the north and north-east regions, characterized by calcareous soils, even in salt-affected depressions where they grow inside perennial halophyte tufts (Rabhi *et al*., 2010). However, high pH values

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characterize these types of soils, which lead to iron precipitation and to a decrease in its bioavailability. It has been suggested that a reliable solution to solve this abiotic stress might be to select Fe-efficient genotypes (Jelali *et al*., 2011). In this context, the aim of the current research was to assess differences in Fe deficiency tolerance of two *Medicago* species (*M. ciliaris* and *M. truncatula*) that were found to grow on a relatively alkaline soil in their natural biotope, Soliman sabkha (NE Tunisia), by comparing growth parameters and physiological responses.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *M. ciliaris* and *M. truncatula* were collected from Soliman sabkha (NE Tunisia) and sown in Petri dishes on filter paper moistened with distilled water. After three days, seedlings were beforehand grown for three weeks in a growth chamber. The growth medium was a complete quarter-strength Hoagland's nutrient solution (Hoagland & Arnon, 1950). The growth chamber controlled environmental conditions were set to 14/10 hrs light/dark cycle, 22/18°C day/night temperature regime, light intensity of 200 $mmolm^{-2}\ s^{-1}$ PAR, and relative humidity of 60-80%. After 22 days, seedlings of each species were divided into two groups.

Two treatments were applied: C = control (complete medium containing 30 μM Fe) and T = direct deficiency (complete medium containing 1 μM Fe). Nutrient solution (pH6.0) was weekly renewed. Iron solutions (Fe-EDTA) were prepared following Jacobson's method (Jacobson, 1951).

Plant growth and leaf chlorosis parameters

Eight plants of *M. ciliaris* and *M. truncatula* collected from the replicates of each treatment were harvested after 30 days of treatment. Plants were separated into leaves, stems, and roots. Plant material was rinsed with distilled water. The dry weight of each part was determined after drying at 60°C until constant weight.

Chlorotic status of young leaves was determined using two methods. The first was the non-destructive index (or score) of Gildersleeve & Ocumpaugh (1989) that measures visual chlorosis symptoms with values ranging from 0 (no apparent chlorosis) to 4 (severe chlorosis with necrosis). The index was followed up over 20 days (until its values were stabilized). The second method consisted of measuring chlorophyll concentration after harvesting according to Torrecillas *et al.* (1984).

Nutrient solution acidification

After 22 days of treatment (when iron chlorosis was obvious), medium pH was daily followed up with a Metrohm 633 pH-meter. On the harvest day, the capacity to extrude protons by roots was also determined. Roots were washed with 1% (v/v) HCl, then thoroughly rinsed several times with distilled water. Eight plants were incubated for three hours in a KCl (10 mM)/$CaCl_2$ (1 mM) solution initially adjusted to pH 6.3. Then proton extrusion fluxes were measured by a return titration using a NaOH solution (1 mM) (Rabhi *et al*., 2007).

Nutrient extraction and analysis

Mineral nutrients (potassium and iron) were extracted following the method described by Grusak (1995); samples from the ground plant material were wet-digested in borosilicate tubes by adding a mixture of nitric and perchloric acids (3/1, v/v), heating at 150°C for 1 hr, and then heating at 200°C until samples were near dryness. Digests were thereafter dissolved in 25 ml of 0.05% HNO_3. Ion concentrations were determined by atomic absorption spectrophotometry (VARIAN 220 FS).

Relative growth rate and Fe use efficiency parameters

Relative growth rate (RGR) was calculated according to Hunt's equation (Hunt, 1990):
$RGR = (\ln DW_2 - \ln DW_1)/(t_2 - t_1)$, with DW = total plant dry weight (mg), t = time (days) and the subscripts 1 and 2 indicate initial and final harvests, respectively. Fe use efficiency for biomass production was calculated as leaf, stem, and root biomass production over the treatment period divided by Fe content in each organ. Fe use efficiency for chlorophyll synthesis was calculated as leaf chlorophyll content divided by their Fe content.

Statistical analysis

Statistical analysis was performed according to Student's t test at $p \leq 0.05$.

RESULTS

Plant growth

Results presented in Table 1 showed an overall decrease in growth of all plant parts in the two *Medicago* species under iron deficiency conditions. Nevertheless, differences in growth were observed between species as well as between plant parts. Considering each organ apart, it can be obviously perceived that their growth was significantly more affected by the stress in *M. truncatula* than in *M. ciliaris*. In the former species, roots and stems were the most affected plant parts. Their biomass ranged from 45 to 49% of the control in *M. ciliaris* and from 31 to 32% in *M. truncatula*. Although clearly decreased, relative growth rate (RGR) was differently affected depending on the species (Table 1). Actually, *M. truncatula* showed a marked reduction in RGR (*ca* 47%), while this parameter was decreased by less than 25% in *M. ciliaris*. By contrast, root/shoot ratio displayed no significant variation under iron deficiency conditions in both species (Table 1). From these results, it seems that *M. ciliaris* tolerated iron deficiency significantly more successtully than *M. truncatula*.

TABLE 1. Plant growth and relative growth rate (RGR) in *M. ciliaris* and *M. truncatula* plants grown for 30 days on a nutrient solution containing 30 μM Fe (Control) or 1 μM Fe (Treated). Values are means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$). A column with the proportional differences between control and treatment (Xc-Xt)/Xc was provided for each species (Xc is the value of the control and Xt is the corresponding value of the treatment). (Xc-Xt)/Xc values were estimated by the control and treated means

Species	*M. ciliaris*			*M. truncatula*		
Treatment	Control	Treated	Dif.*	Control	Treated	Dif.*
Leaf DW (mg)	$386.8^{b} \pm 10$	$214.0^{a} \pm 22$	0.45	$82.1^{b} \pm 6$	$32.2^{a} \pm 6$	0.61
Stem DW (mg)	$280.7^{b} \pm 8$	$137.7^{a} \pm 11$	0.51	$50.4^{b} \pm 4$	$15.5^{a} \pm 3$	0.69
Root DW (mg)	$114.7^{b} \pm 5$	$52.2^{a} \pm 2$	0.54	$36.7^{b} \pm 5$	$11.6^{a} \pm 2$	0.68
Root/shoot	$0.16^{a} \pm 0.02$	$0.16^{a} \pm 0.01$	0.00	$0.27^{a} \pm 0.03$	$0.25^{a} \pm 0.01$	0.07
RGR (d^{-1})	$0.10^{b} \pm 0.01$	$0.08^{a} \pm 0.02$	0.20	$0.07^{b} \pm 0.01$	$0.04^{a} \pm 0.01$	0.43

* Dif. = (Control-Treated)/Control

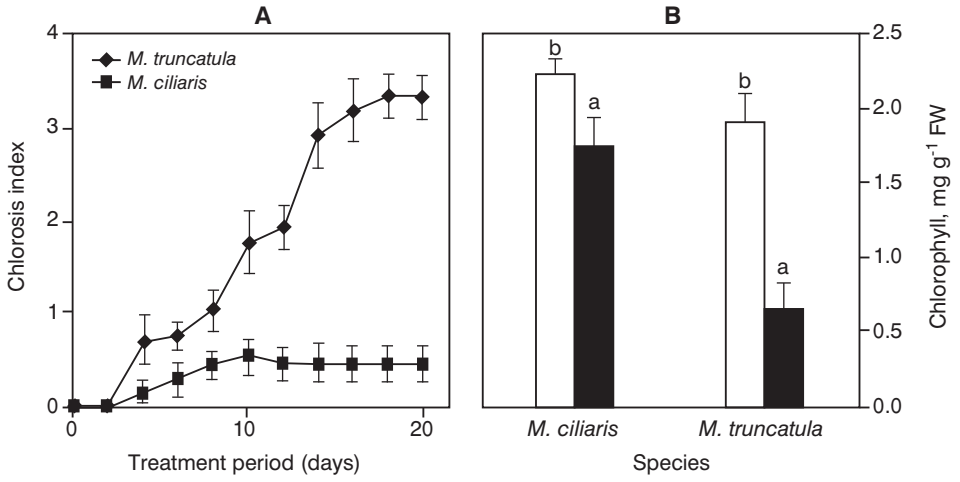

FIG. 1. A: Chlorosis index in *M. ciliaris* and *M. truncatula* plants grown during 20 days on a nutrient solution containing 1 μM Fe. Each point is a mean of 24 replicates ± SE; B: Chlorophyll concentration in young leaves of *M. ciliaris* and *M. truncatula* plants grown for 20 days on a nutrient solution containing 30 μM Fe (white bars) or 1 μM Fe (black bars). Bars are means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$).

Leaf chlorosis parameters

During the two first days of treatment, no chlorosis symptom was observed in young leaves of -Fe plants (Fig. 1A). On the 4th day, a typical leaf yellowing was noticed in the two *Medicago* species and the chlorosis index was significantly higher in *M. truncatula* than in *M. ciliaris*. In the latter, chlorosis index increased slowly with the treatment duration and was stabilized at 0.6 on the 10th day. In *M. truncatula* however, it continued to increase at a much higher rate to reach 3.4 on the 18th day. As for the chlorosis index, a significant difference was noticed between the two species in terms of chlorophyll concentrations in young leaves. Indeed, although both species experienced a de-

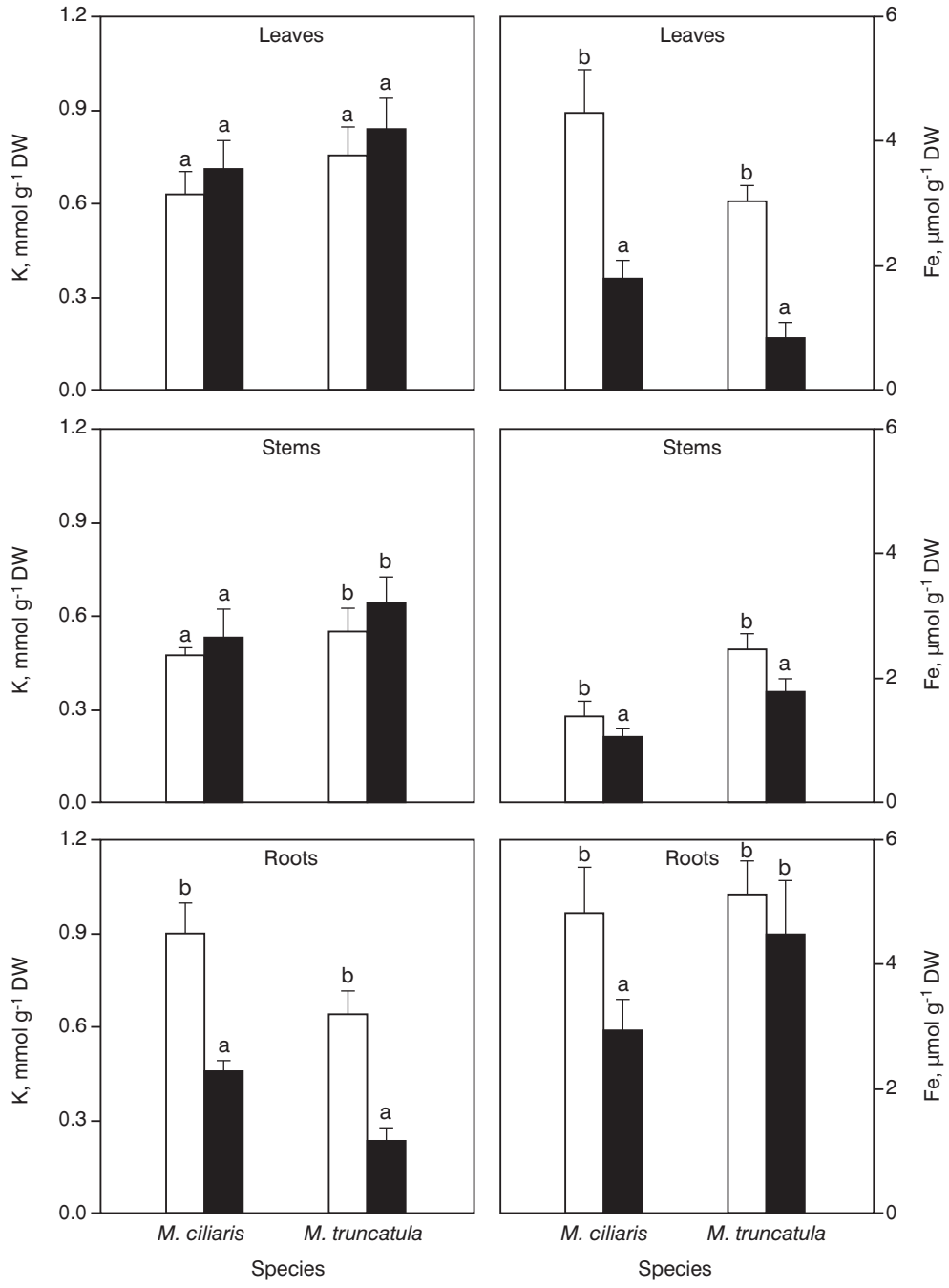

FIG. 2. Potassium and iron concentrations in leaves, stems, and roots of *M. ciliaris* and *M. truncatula* plants grown for 30 days on a nutrient solution containing 30 µM Fe (white bars) or 1 µM Fe (black bars). Values are means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$).

crease in this parameter, *M. ciliaris* maintained a better chlorophyll status under iron deficiency conditions (70% of the control) than *M. truncatula* (32% of the control) (Fig. 1B).

Nutrient status

Fe deficiency did not statistically modify potassium concentration in leaves and stems of both species (Fig. 2), whereas root K^+ concentration was noticeably decreased, mainly in *M. truncatula*. Decreases were 50 and 71% in *M. ciliaris* and *M. truncatula*, respectively. Thus, iron deficiency resulted in a redistribution of this nutrient within plant. In both species, the percentage of potassium accumulated in stems was not modified (30-31% in *M. truncatula* and 38-39% in *M. ciliaris*) by the stress, whereas those of roots and leaves were drastically modified (Fig. 3). Leaf-accumulated K^+ was enhanced from 43% in +Fe plants of both species to 51 and 61%, in *M. ciliaris* and *M. truncatula*, respectively. On the contrary, root-contained K^+ was decreased from 19 to 10% in *M. ciliaris* and from 26 to 9% in *M. truncatula.*

In both species, a significant decrease in total iron concentration was observed in Fe-deficient leaves as compared to that of Fe-sufficient ones (Fig. 2). The effect was much more pronounced in *M. truncatula* (–76%) than in *M. ciliaris* (–57%). Similarly to leaves, chlorosis conditions affected iron concentration in stems of the two species and this detrimental effect was more obvious in *M. truncatula* (–22 %) (Fig. 2). The most important differences were found in the variations of root concentrations. Indeed, iron deficiency resulted in a decline (40%) in root-accumulated iron of *M. ciliaris*, whereas it showed no significant effect on that of *M. truncatula*. Fe distribution within *M. ciliaris* plant (Fig. 3) did not change under Fe deficiency conditions. Conversely, *M. truncatula* plants showed Fe accumulation in roots (49%) on the expenses of leaves (24%), the percentage of stem iron quantity being treatment-independant.

Fe use efficiency (FeUE)

The two species experienced opposite behaviours as regards FeUE for chlorophyll synthesis in young leaves under Fe deficiency conditions (Table 2). Indeed, while *M. ciliaris* enhanced this parameter by 54%, *M. truncatula* decreased it by 60%. Fe-deficient plants of both species showed a marked increase in FeUE for biomass production in all plant parts (Table 2).

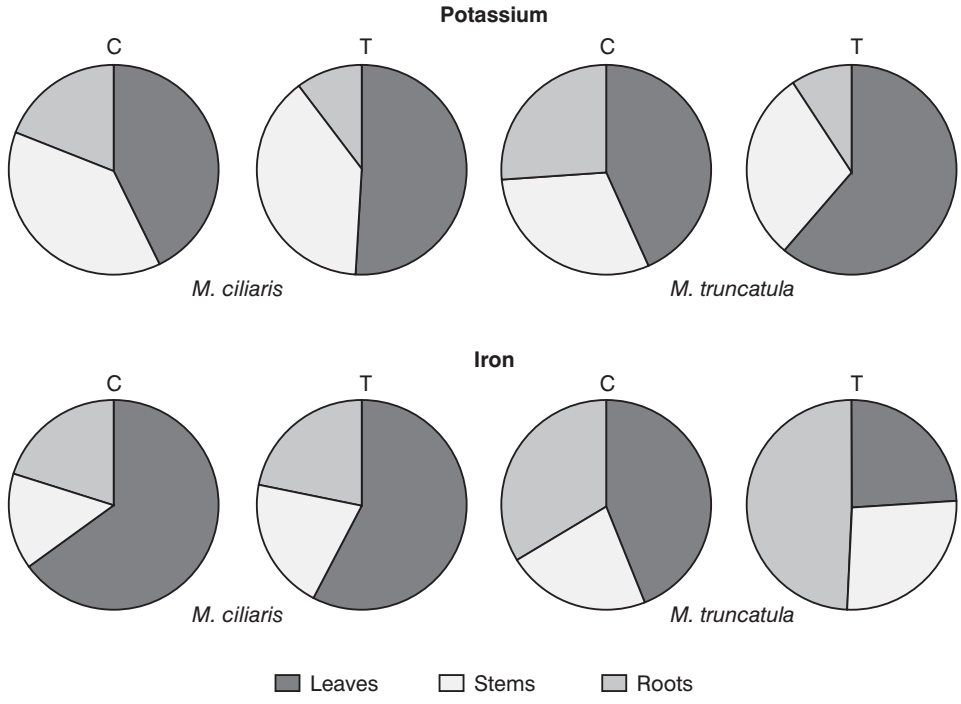

FIG. 3. Potassium and iron distribution within *M. ciliaris* and *M. truncatula* plants grown for 30 days on a nutrient solution containing 30 μM Fe (C) or 1 μM Fe (T).

TABLE 2. Fe use efficiency (FeUE) for biomass production and chlorophyll synthesis in *M. ciliaris* and *M. truncatula* plants grown for 30 days on a nutrient solution containing 30 μM Fe (control) or 1 μM Fe (treated). Values are means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$). A column with the proportional differences between control and treatment (Xc-Xt)/Xc was provided for each species (Xc is the value of the control and Xt is the corresponding value of the treatment). (Xc-Xt)/Xc values were estimated by the control and treated means

Species	*M. ciliaris*			*M. truncatula*		
Treatment	Control	Treated	Dif.*	Control	Treated	Dif.*
FeUE for chl (mg chl μg^{-1} Fe)						
Young leaves	$59.7^{a} \pm 5$	$91.8^{b} \pm 11$	–0.54	$60.7^{b} \pm 3$	$23.2^{a} \pm 2$	0.62
FeUE for DW (mg DW μg^{-1} Fe)						
Leaves	$4.0^{a} \pm 0.33$	$9.9^{b} \pm 1.5$	–1.48	$5.4^{a} \pm 1$	$22.3^{b} \pm 1.7$	–3.13
Stems	$12.8^{a} \pm 2$	$17.9^{b} \pm 2$	–0.40	$6.5^{a} \pm 0.9$	$9.9^{b} \pm 0.7$	–0.52
Roots	$3.8^{a} \pm 0.5$	$6.4^{b} \pm 1.4$	–0.68	$3.1^{a} \pm 0.25$	$4.0^{a} \pm 0.33$	–0.29

* Dif. = (Control-Treated)/Control

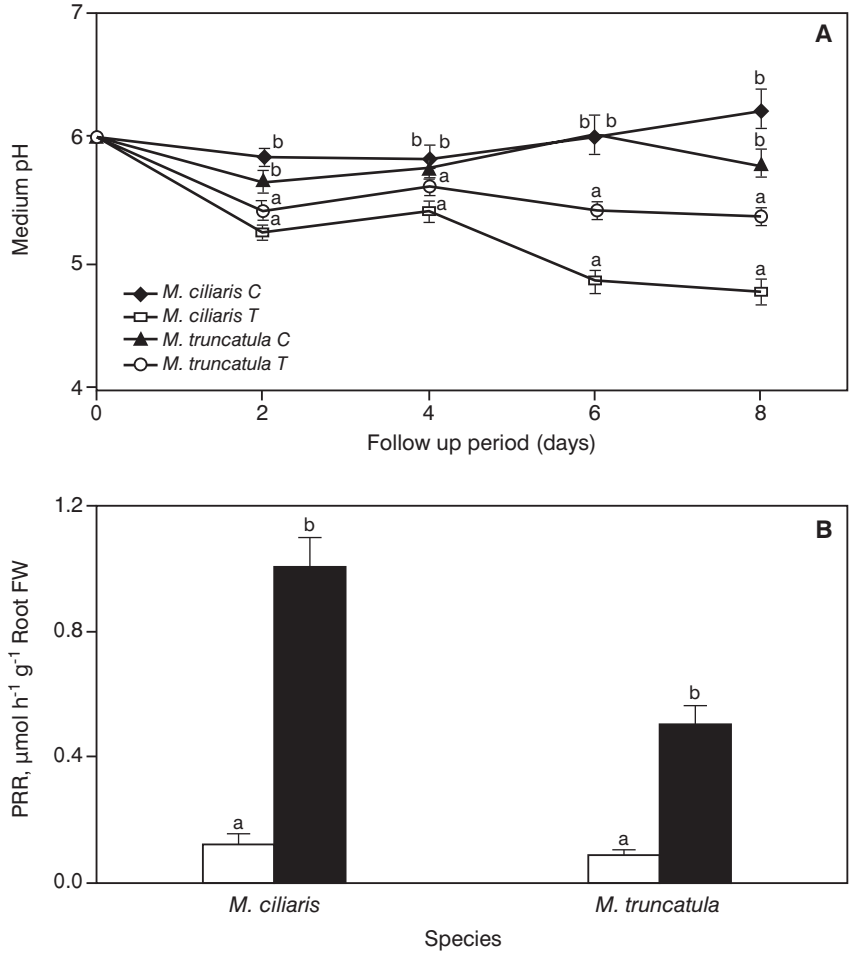

FIG. 4. A: pH values of the nutrient media during the last 8 days of treatment in *M. ciliaris* and *M. truncatula* plants grown for 30 days on a nutrient solution containing 30 μM Fe (C) or 1 μM Fe (T). Points are means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$); B: Proton release rate (PRR) as measured after a 3-hour incubation in a KCl (10 mM)/$CaCl_2$ (1 mM) solution just before the harvest in *M. ciliaris* and *M. truncatula* plants grown over 30 days on a nutrient solution containing 30 μM Fe (white bars) or 1 μM Fe (black bars). Means of 8 replicates ± SE. For each species, means denoted by the same letter did not significantly differ (Student's t test, $p \leq 0.05$).

Medium acidification

Nutrient medium acidification was regularly assessed by pH measurements during the last 8 days of the experiment. The two *Medicago* species maintained their medium pH at about 6.0 upon physiological non-stress conditions. Under Fe-deficiency conditions, *M. ciliaris* exhibited a decline in the medium pH by 1.4 units, whereas *M. truncatula* roots decreased their medium pH by 0.8 units (Fig. 4A). Proton extrusion by roots was also measured on the harvest day. It showed that Fe-deficient plants released more protons than Fe-sufficient plants in both species. In addition, under iron deficiency conditions, *M. ciliaris* plants showed much higher levels of proton release (1 μmol h^{-1} g^{-1} Root FW) than *M. truncatula* (0.5 μmol h^{-1} g^{-1} Root FW) (Fig. 4B).

DISCUSSION

Many species of the legume genus *Medicago* are native to the Mediterranean basin. Compared to *M. truncatula*, the model chosen for studies in nitrogen fixation, *M. ciliaris* is important as agricultural crop. In the present work, we showed that, regardless of the treatment, *M. ciliaris* plants exhibited higher weight compared to *M. truncatula* plants (Table 1). The observed difference was due to intra-genus variability in the genus *Medicago*. Iron deficiency decreased plant growth in the two studied species. However, *M. ciliaris* showed a relative higher capacity to respond to Fe stress than *M. truncatula*. Indeed, our findings showed that *M. ciliaris* growth was less affected by Fe deficiency in comparison with that of *M. truncatula* (Table 1), suggesting a higher tolerance of the former to the stress than the latter. These results are in agreement with previous reports in chickpea varieties (Mahmoudi *et al*., 2007), in citrus (Pestana *et al*., 2005), and in grapevine (Bavaresco & Poni, 2003). Chlorophyll status in young leaves may also give an idea not only about chlorosis degree, but also about the differential behaviour of genotypes to Fe deprivation (Sudahono & Rouse, 1994). In this context, the obtained data (chlorosis index and chlorophyll content) indicated that *M. ciliaris* maintained a significantly higher chlorophyll concentration as compared to *M. truncatula* (Fig. 1A and B), suggesting that the latter is more sensitive to Fe chlorosis. Previously, Chouliaras *et al.* (2004) and Ksouri *et al.* (2004) found, that Fe deficiency tolerance is related to the maintenance of a high chlorophyll concentration in citrus and grapevine, respectively. These authors used such parameters to select Fe-efficient genotypes. Our results support this point of view. Actually, based on plant growth and chlorophyll status, it seems clear that *M. ciliaris* is more tolerant to Fe chlorosis than *M. truncatula*.

Fe deficiency treatment induced noticeable changes in K^+ distribution within plant (Figs 2 and 3). López-Millán *et al*. (2001) also found that iron deprivation induced an enhancement in potassium content from 15 to 20 mM in pear. According to Tagliavini & Rombolà (2001), this cation plays a major role in Fe assimilation under iron deficiency conditions, by increasing root plasma membrane H^+-ATPase activity. Although some authors suppose that chlorotic plant parts frequently exhibit high K^+ contents, iron starvation is often associated with potassium starvation (Loué, 1993). In our case, we observed that potassium redistribution within plant was more pronounced in the less tolerant species. This suggests that K^+ remobilization from roots and its transport to shoots depends on chlorosis severity. In addition, in recent works (Jelali *et al*., 2011; Houmani *et al*., 2012) we showed that Fe deficiency resulted in a greater accumulation of potassium in young leaves of two tolerant *Pisum sativum* cultivars and those of the halophyte *Suaeda fruticosa*, suggesting the likely implication of potassium distribution in the tolerance of this species to iron deficiency. Fe concentration was decreased by Fe deficiency treatments in all plant parts of *M. truncatula* as well as in *M. ciliaris* (Fig. 2), which is in agreement with previous results found by Tagliavini *et al.* (2000). Interestingly, the detrimental effect of iron shortage was more pronounced in the less tolerant species (*M. truncatula*) characterized by an iron accumulation in roots. It seems that the ability of *M. ciliaris* plants to remobilise iron from their roots and transport it to leaves is a key physiological response to Fe deficiency. These results are similar to those found by Mahmoudi *et al.* (2007) in chickpea. Fe use efficiency for biomass production did not clearly discriminate the two *Medicago* species; both ameliorated iron management for growth mainly in stems and leaves (Table 2). By contrast, a very obvious discrimination between the two species was obtained with Fe use efficiency for chlorophyll synthesis; while this parameter was noticeably increased in the more tolerant species, it was drastically decreased in the less tolerant one.

In our work, an obvious decrease in the nutrient solution pH (Fig. 4A) and a marked increase in proton extrusion rate (Fig. 4B) were experienced by

roots of the two *Medicago* species under Fe deficiency. This observation suggests that both genotypes are relatively Fe-efficient. However, large differences were observed between them, which support again the idea that *M. ciliaris* is more tolerant to iron deficiency than *M. truncatula*. Similarly, Dell'Orto *et al.* (2000) and Mahmoudi *et al.* (2005) showed the existence of a noticeable relationship between rhizosphere acidification and iron deficiency tolerance. This is an accessory mechanism that enhances Fe dissolution and facilitate Fe(III)-chelate reductase activity by neutralizing apoplastic negative charges (Rabotti & Zocchi, 1994; Vizzotto *et al*., 1999).

In summary, it appears that the higher iron deficiency tolerance found in *M. ciliaris* as compared to *M. truncatula* is due to several mechanisms such as the preservation of adequate chlorophyll concentration, a better iron remobilization from roots to shoots, as well as a higher ability to acidify its rhizosphere. This could explain the abundance and the better vigor of *M. ciliaris* as compared to *M. truncatula* in their alkaline natural biotope, Soliman sabkha.

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