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Differential response of pea (*Pisum sativum* L.) genotypes to iron deficiency in relation to the growth, rhizosphere acidification and ferric chelate reductase activities

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Abstract

Calcareous soils are known problematic lands for agricultural systems because of the low availability of nutrients, particularly iron (Fe). The so-called strategy I plant (e. g. Pea, *Pisum sativum* L.) which groups dicotyledons and monocots other than grasses, developed root membrane activities that contribute to the improvement of Fe availability. Among the functions considered to be a critical phase in iron absorption is rhizosphere acidification by H-ATPase and Fe(III) reduced by Fe(III) chelate reducctase (FeCR). In order to experimentally investigate the importance of root FeCR in Fe nutrition, its relationship with rhizosphere acidification and the genotypic differences in response to iron deficiency in pea (*Pisum sativum* L.), a glasshouse experiment was conducted hydroponically on four genotypes Merveille de Kelvedon (MK); Lincoln (Lin); Douce de Provence (DP) and Alexandra (Alex). Plants of each genotype were distributed into two plots, the first one received full nutrient solution (+ Fe), the second one received nutrient solution devoid of iron (- Fe). Plant growth, Fe distribution, SPAD index and root acidification and ferric chelate reductase activities were evaluated. Fe deficiency decreased plant growth and SPAD index along with the significant increase of H-ATPase and FeCR activities. Some genotypic differences were observed as follows; Alex showed high tolerance to Fe deprivation as compared to other genotypes. Important H-ATPase and FeCR activities, high Fe use efficiency and adequate membrane efficiency are the main reasons for this tolerance. These physiological parameters could be used as tools of tolerance for further breeding programs.

Keywords: Fe-chelate reductase, Fe use efficiency, iron deficiency, membrane efficiency, rhizosphere acidification.
Abbreviations: DW_dry weight, FeCR_ferric chelate reductase , FeUEFeCR_Fe use efficiency for ferric chelate reductase activity. FW_fresh weight, ME_membrane efficiency

Introduction

Plant growth in the soil is a complex process. Roots play vital roles in anchoring and mechanical support of plants, absorption of water and nutrients, biosynthesis and storage of chemical compounds, and interactions with abiotic and biotic factors in the soil environment (Yu et al., 2019). Calcareous soils are one of these problematic areas, which constitute more than 30% of the planet's cultivated land (Wallace and Lunt, 1960). Mortvedt (1991) demonstrated that the soil solution does not provide more than 10% of Fe's plant requirements, leading to the so-called lime-induced chlorosis, affecting many annual crops and perennial plants.

Iron is an essential element for almost all organisms due to its role in various metabolic processes. In plants, iron plays inevitable roles in intracellular metabolic pathways, such as electron transfer in photosynthesis and respiration (Briat et al., 2015; Nozoye et al., 2016), and is required for many enzymes in most living organisms to complete biological functions (Thomine and Vert, 2013). However, these achievements haven't helped to eliminate iron deficiency in the agricultural system. Iron deficiency annually affects numerous crop plants, which can cause a heavy reduction in fruit yield and quality (Zhao et al., 2020) pH is considered to be the main factor that affects the solubility of metals in the soil. The solubility of metals decreases at high pH and increases at low pH values (Sheoran et al., 2016). Multiple studies reported a range of adaptive mechanisms that evolve to increase plant tolerance to Fe deficiency. Plant tolerance to Fe deficiency is strongly correlated with the root ability to reduce rhizosphere pH by increasing proton extrusion and improving the ferric chelate reductase (FeCR) activity (Krouma et al., 2003). Reduction of Fe (III) to the more soluble form Fe (II) by FeCR enzyme located in the root cell plasma membrane seems to be a mandatory step in Fe uptake in Strategy I plant (Curie and Briat, 2003). This phenomenon which enhances Fe acquisition from the rhizosphere is one of the major physiological responses of plants to Fe deficiency (González-Vallejo et al., 2000; Krouma et al., 2003). Kosegarten et al. (2004) demonstrated that FeCR is highly sensitive to pH and is inhibited in alkaline soils. High soil pH, often accompanied by an elevated bicarbonate concentration in calcareous soils is proposed to reduce root iron acquisition and transportation in plants (Karimi and Tari, 2017; Ding et al., 2019).

It is well established that the application of chemical Fe fertilizers effectively controls Fe chlorosis (Pestana et al., 2003). However, these fertilizers are costly and polluting the

soil and environment. An environmentally friendly alternative strategy to improve crop production under Fe-deficiency is organic, economical and sustainable methods to screen tolerant genotypes. The exploration of the genotypic differences in response to Fe deficiency to screen tolerant genotypes can represent the appropriate alternative. The present work consists of the exploration of the genotypic differences in the response of pea plants to Fe deficiency based on:

* Their capacity of rhizosphere acidification (H-ATPase),
* Fe chelate reductase (FeCR) activity,
* Fe use efficiency and,
* Membrane efficiency

Four pea (*Pisum sativum* L.) genotypes from the Ministry of Agriculture, Water resources and Fishing are used in a glasshouse experiment. A deep dissection of the relationship between H-ATPase, FeCR, Fe availability, chlorophyll and plant growth was made. Some new parameters such as Fe use efficiency and membrane efficiency were calculated to express the genotypic differences and highlight useful traits for further screening programs.

Results

Plant growth and Fe nutrition

Under iron deficiency, pea plants expressed Fe chlorosis symptoms on young leaves one week after the onset of the treatment. These visual symptoms were more severe in MK, Lin and DP than in Alex. The SPAD index measurement, which reflects the leaves' chlorophyll status, confirmed this result (Table 1). Table 1 shows that iron deficiency decreased significantly the SPAD index in MK, Lin and DP (respectively by 31 %, 28 % and 28 %), with a less pronounced effect in Alex (13%).

Plant growth also decreased under iron starvation in all genotypes. Some genotypic differences were observed (Table 1). Total fresh weight decreased by 25% in MK and DP, by 11% in Lin and no clear effect on Alex's growth. Alex's FW was 1.3 times more important than that of MK and DP cultivated under iron deficiency.

Measurements made on Fe repartition in the plant organs demonstrated that Fe concentrations decreased significantly in shoots and roots under deficient conditions. Nevertheless, plant organs are differently affected, Fe concentration in leaves decreased by less than 50% in all genotypes (Figure 1a in roots, Figure 1b). We calculated extractable Fe quantities in the plants to deeply analyze the Fe nutrition and repartition under problematic conditions (Table 1). Obtained results demonstrated that under Fe starvation, the accumulation of Fe were decreased significantly in plants with some genotypic differences. The quantities of Fe decreased by 65 %, 51 %, 58 % and 43 %, respectively, in MK, Lin, DP and Alex when subjected to iron deficiency. Under these iron deficiency conditions, Alex accumulates 60 %, 30 % and 60 % more iron in its organs than MK, Lin and DP, respectively. This genotype remains the least affected, considering Fe concentration as well as Fe quantities.

Root H-ATPase and FeCR activities

The acidification capacity of roots was measured directly in the nutrient solution during 8 days of treatment. Figure 4 shows no clear modification of pH in control plants. Nevertheless, plants subjected to iron deficiency starts to acidify their nutrient solution one day after the onset of the treatment and continue during 5 days in MK and Lin (reaching the values of 5.3 and 5.21, respectively), 6 days in DP (reaching the value 5.27) and 7 days in Alex reaching the lowest value of pH (4.92) (Figure 2).

FeCR in roots was stimulated in stressed plants as compared to control ones. This stimulation was estimated to 15% in MK, 25% in Lin, 41% in DP and 80% in Alex (Figure 3). The genotypic differences that previously observed were maintained. FeCR was 1.4, 1.2 and 1.6 more important in Alex than MK, Lin and DP, respectively, under iron deficiency.

We analyzed the between these physiological parameters modulating the genotypic differences in response to iron deficiency in pea. For the first time, we connected plant growth to their total Fe content (Figure 4). Fig 4a shows no relationship between biomass production and total Fe content in plants under sufficient Fe nutrition. Nevertheless, a close relationship occurs between plant growth and Fe content, when subjected to iron deficiency (Figure 4b). The genotype Alex is characterized by its high plant growth capacity and Fe^{2+} accumulation compared to the other ones.

We represented the FeCR activity with root Fe concentration (Figure 5a, b). The first connection, a close relationship between Fe chelate reductase and root Fe concentration, is established under iron deficiency. As previously, Alex induces higher FeCR activity in its root membrane and remobilizes more iron.

Succeeding this in-depth analysis strategy of these physiological responses, we connected H-ATPase and FeCR activities in pea roots (Figure 6a, b). Fig 6a shows no relationship between H-ATPase and FeCR activities in control plants. When subjected to iron deficiency, a close and strict correlation between these two root membrane activities occurs. Alex also develops a higher capacity of root FeCR and H-ATPase activities than the other genotypes.

Discussion

Our results show that iron deficiency-induced iron chlorosis in pea plants reduces plant growth and chlorophyll concentration with a clear distinction of Alex as the least affected than other genotypes. Previous studies demonstrated that iron is involved in chlorophyll and carotenoid biosynthesis (Morales et al., 1990; Thoiron et al., 1997), photosynthesis (Rutherford, 1985; Sandmann, 1985) as well as the metabolism of plastidial proteins (Spence et al., 1991). Therefore, the induced iron chlorosis and the decrease of chlorophyll concentration observed in this study can be explained by the drastic decrease of iron availability for these functions leading to growth fall. These results are those of Ksouri et al. (2007) on the grapevine, who demonstrated that lime-induced Fe shortage is genotype-dependent. The growth parameters and leaf chlorophyll content were differently affected, depending on both genotype and bicarbonate dose. In Prunus rootstocks, Athanassios et al. (2005) demonstrated that Fe deficiency decreases plant growth and chlorophyll concentration and stimulates Fe- reductase activity. Valentinuzzi et al. (2020) demonstrated that Fe application to Fe deficient plants, even though at low levels (e.g. 1µM vs. 80–100 µM in normal conditions), is sufficient to promote the reactivation of the primary metabolism in plants (e.g. photosynthesis, nitrogen acquisition and assimilation) with a consequent increase of the biomass accumulation. Other studies showed that iron deprivation decreased chlorophyll content in leaves and inhibited the Fe translocation to growing plant tissue (Bisht et al., 2002).

Table 1. Different physiological parameters were measured in four Pea genotypes. + Fe: plants cultivated on a full nutrient solution, - Fe: plants cultivated on nutrient solution deprived of iron during 22 days. Means with the same letter are not significantly different at p≤ 0.05 according to Fisher's Least Significant Difference. Standard errors of means of 10 replicates.

	MK		Lin		DP		Alex	
	+ Fe	- Fe	+Fe	-Fe	+Fe	-Fe	+Fe	-Fe
SPAD index	38.6^{b} ± 3.5	26.5^{d} ± 2.9	39.8^{b} ± 3.1	28.7^{c} ± 2.2	42.4^{a} ± 2.1	30.5^{c} ± 1.9	44.3^{a} ± 1.7	38.6^{b} ± 2.3
FW	9.3^{b} ± 1.23	6.96^{c} ± 1.42	11.68^{a} ± 1.35	8.57^{bc} ± 1.04	78.99^{b} ± 0.65	6.34^{c} ± 1.14	11.98^{b} ± 1.52	10.04^{b} ± 1.02
Fe	86.13^{a} ± 8.2	30.45^{f} ± 4.5	78.34^{b} ± 9.2	38.17^{e} ± 4.4	74.16^{c} ± 6.8	30.86^{f} ± 3.5	86.66^{a} ± 6.8	48.97^{d} ± 5.2
Δ pH	-0.23^{d} ± 0.05		-0.40^{c} ± 0.06		-0.59^{b} ± 0.09		-1.09^{a} ± 0.09	
ΔFeCR	74.2^{d} ± 8.2		104.7^{c} ± 12.4		176^{b} ± 20.1		372.3^{a} ± 28.3	
FeUE FeCR	14.15^{d} ± 1.61	44.26^{ab} ± 2.83	12.39^{d} ± 1.19	31.68^{c} ± 2.72	13.73^{d} ± 1.7	41.43^{b} ± 23	12.44^{d} ± 16	47.31^{a} ± 35
ME	82.4^{cd} ± 5.8	98.5^{bc} ± 6.3	70^{d} ± 7.1	93.4^{c} ± 8.3	70.8^{d} ± 7.2	110.7^{b} ± 13.2	77.1^{cd} ± 6.1	169.8^{a} ±20.1

* SPAD index, * FW, Fresh weight expressed as g. plant^{-1}, * Δ pH, calculated as the difference between pH in stressed plants and pH in control ones,
*** Δ** FeCR, calculated as the difference between FeCR in stressed plants and FeCR in control ones, * Fe, quantities of Fe measured in plants (Fe, µg. plant^{-1}),
* FeUE FeCR, Fe Use Efficiency for FeCR, calculated as the ratio of FeCR (nmol Fe^{2+} .g^{-1} FW roots) to root Fe (µg.plant^{-1}), * ME, Membrane Efficiency, expressed as the ratio of FeCR to H-ATPase.

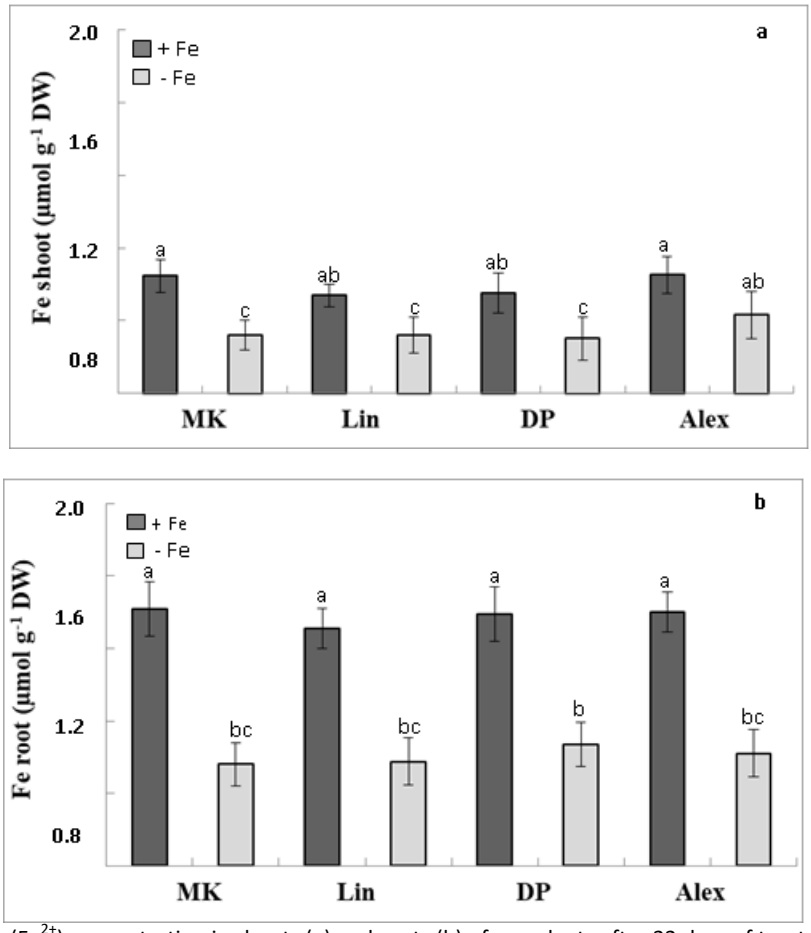

Figure 1. Extractible Fe (Fe^{2+}) concentration in shoots (a) and roots (b) of pea plants after 22 days of treatments. + Fe: control plants cultivated on standard nutrient solution, -Fe: plants cultivated on nutrient solution deprived from Fe. Within columns, means with the same letter are not significantly different at p≤ 0.05 according to Fisher's Least Significant Difference. Standard errors of means of 10 replicates.

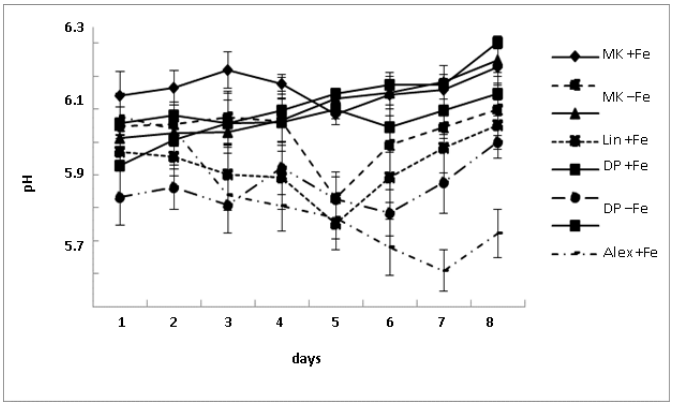

Figure 2. Root H-ATPase activity expressed as acidification capacity during 8 days. Measurements were made on plants subjected (-Fe) or no (+Fe) to iron deficiency during 2 weeks. Vertical bars represent ± standard errors of means of 10 replicates, p≤ 0.05.

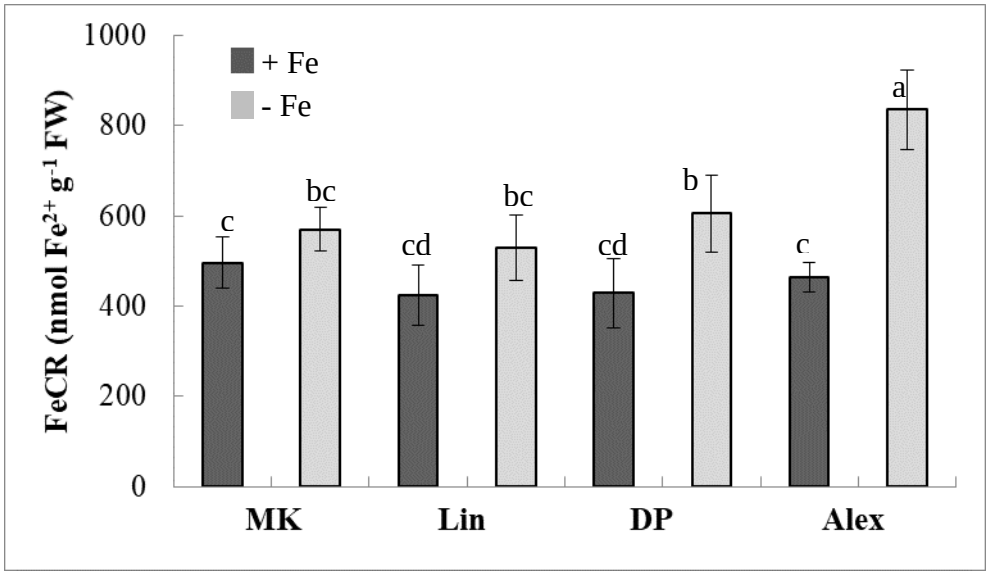

Figure 3. Fe(III)-chelate reductase (FeCR) activity in roots of Pea plants after 22 days of treatments. + Fe: control plants cultivated on standard nutrient solution, -Fe: plants cultivated on nutrient solution deprived from Fe. Within columns, means with the same letter are not significantly different at p≤ 0.05 according to Fisher's Least Significant Difference. Standard errors of means of 10 replicates.

Valipour et al. (2020) showed that both Fe deficiency (direct or lime induced) decreased leaf chlorophyll, carotenoid and Fe concentrations in quince seedlings. Lopez-Millan et al. (2001) concluded that chlorophyll biosynthesis is considered as an indicator of net physiologically available iron to the plant.

The genotypic differences previously observed in plant growth and chlorophyll accumulation are also maintained for Fe nutrition. Alex shows the least affected leaves and the most affected Fe concentration roots than the other genotypes. Nevertheless, it maintains the higher capacity of iron accumulation (Table 1). This result lets us think that the performance of Alex (represented by its capacity of plant growth and fewer chlorosis symptoms) is linked to its ability of iron uptake by roots and translocation to the shoots to support photosynthetic and other metabolic ways. This micronutrient calculation demonstrated that Alex accumulated up to 59%, 28% and 61% more Fe than DP, Lin and MK, respectively (Table 1) under Fe starvation. At the same time, this genotype stimulated its FeCR more significantly (+ 80% as compared to control plants) than MK (+ 15% as compared to control plants), Lin (+ 25% as compared to control plants) and DP (+ 41% as compared to control plants). Thus, we can explain Fe's critical improvement in Alex subjected to iron deficiency by its high FeCR induction capacity under such conditions. The **Δ**FeCR calculated as the difference of FeCR between stressed and control plants (Table 1) is 5.1, 3.56 and 2.11 times more important in Alex than MK, Lin and DP, respectively. Similarly, our results show an important induction of H-ATPase activity in all genotypes under Fe deficiency, but Alex is discriminated against by the other ones. The **Δ**pH calculated as the pH difference between stressed and control plants (Table 1) is 4.7, 2.7 and 1.8 times more important in Alex than that of MK, Lin and DP, respectively. In light of these results, we can conclude that the rhizosphere acidification improvement constitutes a good environment for FeCR and confirms these two membrane activities' interdependence. Previously, Kosegarten et al. (2004) demonstrated that FeCR is highly sensitive to pH and is inhibited in alkaline soils. Karimi and Tari (2017) and Ding et al. (2019) demonstrated that high soil pH, often accompanied with an elevated bicarbonate concentration in calcareous soils, are proposed to reduce root iron acquisition and transportation in plants. Bicarbonate in the rhizosphere can also inhibit the iron reductase activity of roots by neutralizing protons released by H^+-ATPase (Rabotti and Zocchi, 1994; Romera et al., 1992). Under iron-deficient conditions, pear, as a Strategy I plant, releases H^+ and phenolic compounds to the rhizosphere and up-regulates root ferric-chelate reductase activity to enhance Fe^{3+} reduction, and then the reductive Fe^{2+} was uptaken by IRT transporter proteins located at the cell membrane (Garnica et al., 2018; En-Jung and Waters, 2016). In this study, the relative tolerance to iron deficiency observed in Alex, as compared to MK, Lin and DP genotypes, seems to be linked to its high capacity of rhizosphere acidification which constitutes a good environment for the induction of FeCR enzyme leading to the improvement of FeII availability for the plant.

Nevertheless, the various connections established in this study demonstrated the close relationship between plant growth and its Fe accumulation (Figure 4), between FeCR and Fe concentration in roots (Figure 5) and between FeCR and H-ATPase (rhizosphere pH). Thus, we can conclude the presence of a common thread between H-ATPase, FeCR, Fe content and plant growth. The tolerant genotype (Alex in this study) can acidify his rhizosphere, stimulating his FeCR activity to conduct a critical Fe remobilization and adequate plant growth. In order to explore more the variability of response to iron deficiency in pea genotypes and to identify other traits of tolerance, we calculated the Fe use efficiency for FeCR activity (FeUEFeCR). This parameter is calculated as the ratio of FeCR (nmol Fe^{2+} g^{-1} FW roots) to root Fe (µmol g^{-1} DW roots) (Table 1). All genotypes increased their FeUEFeCR when subjected to iron deficiency, and the genotypic differences previously observed were maintained. Alex expressed higher efficiency of Fe use for FeCR activity (+ 20% as compared to control plants; + 3% in MK and Lin and + 10% in DP)

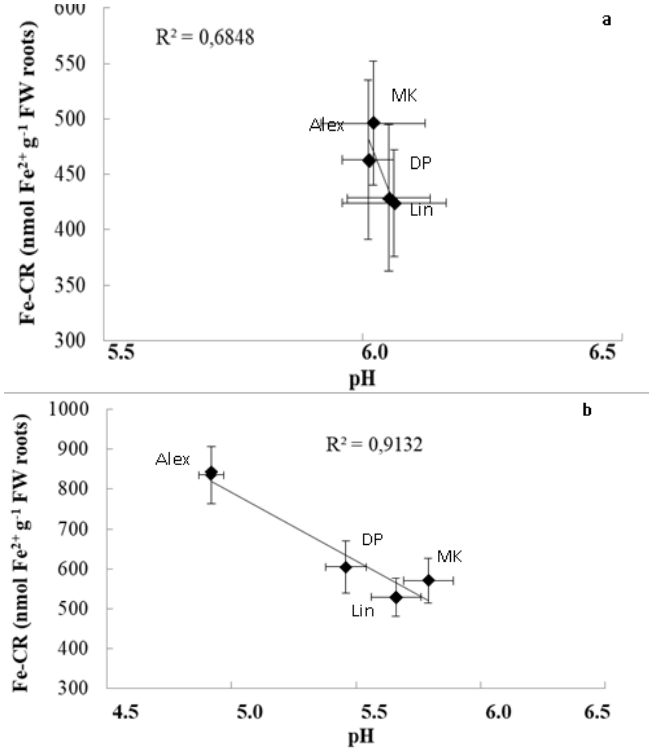

Figure 5. Relationship between ferric chelate reductase (nmol Fe^{2+} g^{-1} FW) and root Fe concentration (µmol g^{-1} DW) in Pea plants cultivated on standard nutrient solution (a) or nutrient solution devoid of iron (b). Vertical and horizontal bars represent ± standard errors of means of 10 replicates, p≤ 0.05.

Following this strategy of deep dissection of pea response to iron deficiency, we calculated the membrane efficiency (ME) expressed as the ratio of FeCR to H-ATPase (Table 1). All genotypes increased their ME when subjected to iron starvation. This stimulation was estimated to 20%, 33%, 56% and 120%, respectively, in MK, Lin, DP and Alex. At this level, Alex was also distinguished by its higher root membrane efficiency. Even under Fe deficiency, ME was 1.7, 1.8 and 1.5 more important in Alex than MK, Lin and DP, respectively.

In this study, we emphasise the relative tolerance of Alex compared to MK, Lin and DP. Such a genotypic variation in response of pea to Fe deficiency confirms previous intraspecific variations reported in green bean (Hemantaranjan and Garg, 1986), chickpea (Rai et al., 1982), lentil (Rai et al., 1984), peanut (O'Hara et al., 1988), lupine (Tang et al., 1990) and quince seedlings (Valipour et al., 2020). We suggest that iron deficiency-induced, firstly, roots H-ATPase, then root FeCR activity due to the new adequate condition of rhizosphere pH, and finally produced Fe^{2+} is uptaked and allocated to shoots to support chlorophyll biosynthesis, photosynthesis and plant growth. In accordance with our results, Yi and Guerinot (1996) showed that root FeCR is necessary for Fe uptake under Fe deficiency. Santi et al. (2005) suggested that two plasma membrane H-ATPase genes are deferentially expressed in Fe-deficient plants.

Materials and methods

Plant materials and growth conditions

Four pea genotypes, Merveille de Kelvedon (MK), Lincoln (Lin), Douce de Provence (DP) and Alexandra (Alex), sourced from the Ministry of Agriculture, water resources and fishing, are used. Healthy seeds of uniform size were disinfected with 2 % Calcium hypochlorite solution and were germinated in Petri dishes. 6-day-old plantlets were transferred in a glasshouse, at the Faculty of Sciences and Techniques of Sidi Bouzid (35° 2' 7.58" N 9° 29' 2.18" E) under natural light with 16 hours photoperiod and a temperature of 25 ºC/ 17 ºC (± 2 ºC, day / night), relative humidity about 75 % and using the following nutrient solution (Hewitt 1996): Ca(NO3)2 (1.25mM), KNO3 (1.25mM), KH2PO4 (1.60 mM), $MgSO_4$ (0.50 mM), K_2SO_4 (1.50 mM), $CaSO_4$ (3.50 mM), H_3BO_3 (4 µM), $MnSO_4$ (4 µM), $ZnSO_4$ (1 µM), $CuSO_4$ (1 µM), $CoCl_2$ (0.12 µM), $(Na)_6(Mo)_7O_{24}$ (0.12 µM).

Experimental design

Cultures were conducted hydroponically and individually in 1 L pots, and plants were separated into two plots; the first one (10 pots) received the above nutrient solution added with 30 µM Fe as K-Fe-EDTA (control plants, +Fe), the second plot (10 pots) received the same nutrient solution without iron (stressed plants, - Fe). The nutrient solution was aerated with a flow of 400 ml min^{-1} of filtered air via a compressor and "spaghetti tube" distribution system and changed every 3 days during two weeks of treatment. After that (apparition of visual symptoms of chlorosis), nutrient solution pH was adjusted to 6 and measured daily for over 8 days. Finally, Fe-chelate reductase activity and SPAD index were performed, and plants were harvested for biomass and extractable Fe (Fe^{2+}) quantification. All measurements and expressed parameters are means of 10 replicates.

SPAD Index

Relative leaf chlorophyll concentrations were estimated in vivo on the youngest fully expanded leaf using a SPAD-502 (Konica-Minolta, Japan). Measurements were made on 10 plants of each treatment. Presented results are means of 10 replicates per genotype and treatment. Values are expressed as SPAD units.

Acidification capacity

The acidification capacity was measured daily in the continuously aerated nutrient solution, adjusted to pH 6.0 with 0.1 N NaOH (Vizzotto et al. 1999).

Fe(III) Chelate reductase

the Fe-chelate reductase activity was measured in plant roots subjected to iron starvation for 8 days according to the method of Krouma et al. (2003) in 100 mL of a solution containing 0.5 mM CaSO4, 0.1 mM Fe(III)EDTA, 0.3 mM bathophenanthrolinedisulfonic acid (BPDS) and 10 mM 2-(N-morpholin)ethane sulfonic acid (MES) adjusted to pH 5.5 with 0.1 N NaOH. The Fe(II)BPDS3 complex's absorbance was measured at 535 nm based on the extinction coefficient of 22 mM cm^{-1}.

Iron determination

Fe^{2+} extraction was made in powdered digested samples using the HCl method. 20 mg of fine powder was digested in 5 ml of 1 N HCl for 4 hours, then 5 ml of deionized water were added and the extract was filtered. Fe content was determined by the atomic absorption spectrophotometry method (Köseoglu and Açikgöz, 1995).

Statistical analysis

Data and statistical analyses were performed using the software StatPlus Pro. All data are presented as mean ± standard error. Analysis of variance (ANOVA) was performed to determine whether the effects of treatments (+ Fe, – Fe) on the respective factor were significant. The significance of differences among treatment means was determined by Fisher's least significant difference test (LSD) at 5 %. Treatment means were declared significantly when the difference between any two treatments was more significant than the LSD value generated from the ANOVA. They are marked by different letters in the figures.

Conclusion

The present study shows that plant growth, SPAD index, Fe nutrition, root H-ATPase and root FeCR are interdependent. Our results confirm the relative tolerance of the genotype Alex to iron deficiency as compared to the other ones. The primordial reasons for this tolerance are its higher root membrane efficiency represented by the important capacity of rhizosphere acidification and Fe chelate reductase activity and its remarkable Fe use efficiency. FeUECR and ME discriminated against the studied genotypes and can be used as trait of tolerance for further screening programs.

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