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The alkaline tolerance in *Lotus japonicus* is associated with mechanisms of iron acquisition and modification of the architectural pattern of the root

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ABSTRACT

The response of fifty-four *Lotus japonicus* ecotypes, and of six selected ecotypes was investigated under alkaline conditions. Sensitive, but not tolerant ecotypes, showed interveinal chlorosis under all alkalinity conditions and high mortality under extreme alkalinity. Interveinal chlorosis was associated with Fe deficiency, as a reduced Fe^{2+} shoot content was observed in all sensitive ecotypes. In addition, some showed a decline in photosynthesis rate and PSII performance compared to the control. In contrast, some tolerant ecotypes did not change these parameters between treatments. Alkaline tolerance could be explained by a mechanism of Fe acquisition and a root structural modification. This conclusion was based on the fact that all tolerant, but not the sensitive ecotypes, presented high ferric reductase oxidase activity under alkaline stress compared to the control, and a *Herringbone* root pattern modification. On this basis, the analysis of these mechanisms of alkaline tolerance could be used in screening programs for the selection of new tolerant genotypes in the *Lotus* genus.

1. Introduction

Soil salinization is a serious ecological problem worldwide (Rengasamy, 2006; Zhang, 2011; Ladeiro, 2012). In particular, saline-alkaline soils cover 30% of the soil area over the world, with NaCl, Na_2SO_4, $NaHCO_3$, and Na_2CO_3 being the main harmful salts (Lin et al., 2012; Martínez-Cuenca et al., 2013). However, alkaline salts have a more severe effect on plant growth than neutral salts (Wang et al., 2011; Paz et al., 2012; Babuin et al., 2014; Lin et al., 2014). Saline soils generally cause osmotic stress and ion-induced injury to plant cells, but saline-alkaline soils present an additional negative effect derived from high pH (Zhang and Mu, 2009; Radi et al., 2012). The latter causes metal ions and phosphorus to precipitate, altering root physiological functions and cell structure, and affecting plant growth, development and survival (Marschner, 1995; Li et al., 2009; Wang et al., 2011). Particularly in calcareous soils, iron precipitates as ferric oxides and hydroxides, forming carbonates with CO_3^{2-}, which reduce its availability to plants (García-Mina et al., 2013). HCO_3^- has been regarded as the causal agent of interveinal chlorosis (IC), through its action as an inhibitor of root Fe uptake (Woolhouse, 1966; Abadía et al., 1999; Klein et al., 2012). Our own results have also shown alkalinity-induced available iron and intervenal chlorosis in the *Lotus japonicus* ecotypes Gifu B-129 and Miyakojima MG20 (Gifu and MG20, respectively; Babuin et al., 2014).

All plants (excepting grasses) that are exposed to low iron availability soils induce a set of morphological and physiological responses known as Strategy I (Jeong and Connolly, 2009; Jain et al., 2014). Briefly, the physiological processes of Strategy I consist in acidification, reduction and transport events. In this sense, the rhizosphere is acidified via H^+ extrusion by a root plasma membrane H^+-ATPase, in order to increase Fe^{3+} solubility. This ion is then reduced to extractable ferrous ion (Fe^{2+}) by a Ferric Reductase Oxidase (FRO), and subsequently taken up into root cells by a ferrous Fe transporter. In fact, we have determined by transcriptomic analysis that *L. japonicus* roots induced FRO-like genes under alkalinity (Babuin et al., 2014). Morphological processes of Strategy I

Abbreviations: FRO, ferric reductase oxidase; F_V/F_M, maximum quantum yield of primary PSII photochemistry; IC, interveinal chlorosis; SI, survival index; Pn, net photosynthesis rate; TT, topological trend.

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also include changes in root architecture, with an enhanced development of lateral roots and differentiation of specialized transfer cells (Guerinot and Yi, 1994; Campestre et al., 2016). Those species which are not able to adopt this strategy, or acquire it only partially, are called "Fe-inefficient" plants. These plants eventually develop an IC in their apical leaves, negatively affecting their survival (Von Wirén et al., 1993; Bacaicoa and García-Mina, 2009; Campestre et al., 2016).

For alkaline soil remediation, it is necessary to incorporate plants capable of withstanding alkaline unfavorable conditions in addition to the improvement of soil management practices. In this regard, the adaptive characteristics displayed by several *Lotus* species make them good candidates for restoration and phytoremediation of degraded environments (Escaray et al., 2012). Among them is *L. japonicus,* a model plant within the legume family and a valuable species for physiological and genetic studies focused on the tolerance mechanisms to biotic and abiotic stresses (Bordenave et al., 2013; Babuin et al., 2014; Campestre et al., 2016). The most well studied *L. japonicus* ecotypes are Gifu and MG20 (Klein et al., 2012; Bordenave et al., 2013; Striker et al., 2014). However, genetic resources collection and characterization of other *L. japonicus* ecotypes could provide useful information to identify germplasm adapted to alkaline environments. This information could be extrapolated to other economically and environmentally important *Lotus* species. Therefore, the aim of the present work was to understand and improve the knowledge of the physiological and biochemical mechanisms that form the basis of resistance to alkaline salts in *Lotus japonicus*.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds from 54 *L. japonicus* ecotypes, described by Hashiguchi et al. (2011), including the model laboratory ecotypes MG20 and Gifu were kindly provided by Dr. Ryo Akashi from the *Frontier Science Research Center* of Miyazaki University (Miyazaki, Japan). The seeds were scarified with sulfuric acid (98%), washed in distilled water and sown in Petri plates containing water-agar (0.8%). Plates were incubated until the emergence of cotyledons in a growth chamber with a 16/8 h photoperiod at 24/19 °C (day/night), 60/80 ± 5% relative humidity under fluorescent light bulbs providing 200 μmol photons m^{-2} s^{-1}.

7-day-old seedlings were transferred from plates to plastic pots containing autoclaved sand. Pots were irrigated with an irrigation system according to Paz et al. (2012), with half-strength Hoagland's nutrient solution composed of 3 mM KNO_3, 2 mM, $Ca(NO_3)_2{\cdot}4H_2O$, 1 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $NH_4H_2PO_4$, 50 μM $NaFeO_8EDTA{\cdot}2H_2O$, 4.5 μM $MnCl_2{\cdot}4H_2O$, 23 μM H_3BO_3, 0.16 μM $CuSO_4{\cdot}5H_2O$, 17.5 μM $ZnSO_4{\cdot}7H_2O$, and 12 μM $Na_2MoO_4{\cdot}2H_2O$ (Campestre et al., 2016) for 7 d. Then, the alkalinity treatment was initiated by gradually adding $NaHCO_3$ to the nutrient solution, until a final concentration of 10 mM (pH = 8.3, 1.9 mmhos/cm) or 30 mM (pH = 10 and 2.51 mmhos/cm) for the survival assay. The $NaHCO_3$ concentration was maintained until the end of each experiment. For the control treatment, plants were cultivated in sand and irrigated with half-strength Hoagland's nutrient solution as described before.

Alternatively, 7-day-old plants cultivated in sand and irrigated as indicated above, were grown hydroponically with the same nutrient solution for 7 more days. Then, 10 mM $NaHCO_3$ was added in half of the plants (alkaline treatment), while the others were kept under the same conditions (control treatment). The nutrient solutions were renewed every three days until the end of each experiment.

2.2. Determination of survival index for the selection of L. japonicus ecotypes

The survival index (SI) was calculated as the percentage of plants that were alive at the end of the survival assay. 54 ecotypes were used in the experiment. Only plants with complete defoliation and extreme dehydration were considered as dead. On this basis, MG63, MG4, MG57, MG65, MG88 and MG68 were selected for further studies.

2.3. Determination of growth

Plant fresh weight was determined directly after harvest. The determination was made on roots and shoots of the six selected contrasting ecotypes.

2.4. Determination of extractable ferrous ion

Fe^{2+} was determined according to Babuin et al. (2014). Shoots and roots were harvested, carefully washed with deionized water, frozen in liquid N_2 and stored at −80 °C until analysis. Plant organs were cut into small pieces with clean scissors. Tissue samples (100 mg) of each organ were shaken in 1.2 mL of 80 mM 2.2′-dipyridyl-HCl (pH 3.0) in 10% methanol in the dark (24 h). Extracts were passed through a 0.45 μm syringe filter and 1 mL of the filtrate volume was assayed at 522 nm using a spectrophotometer (Zeltec ZL-5000 UV/VIS Spectrometer, Argentina). Fe^{2+} concentrations were calculated from a standard curve using a Fe atomic absorption standard solution.

2.5. Total chlorophyll content determination

Leaves were harvested and stored at −80 °C until use. For pigments extraction, 40 mg of plant material grounded in liquid nitrogen were shaken in 100% acetone (4 °C, overnight). The extract was cold centrifuged and the supernatant removed. Measurements were made at wavelength 663 nm (chlorophyll a) and 647 nm (chlorophyll b) in a spectrophotometer (Zeltec ZL-5000 UV/VIS Spectrometer, Argentina), and pigments concentration calculated according to Babuin et al. (2014).

2.6. Determination of net photosynthesis rate and chlorophyll fluorescence fast-transient test

Net photosynthesis rate (Pn) was measured on blades of the third expanded leaf at light saturation (1200 μmol photons m^{-2} s^{-1} illumination, LED light) using a portable photosynthesis system (TPS-2 Portable Photosynthesis System, MA, USA).

Non-invasive chlorophyll fluorescence fast-transient test (JIP test) was performed according to Gazquez et al. (2015) on blades of the third expanded leaf with a portable chlorophyll fluorometer (Pocket PEA, Hansatech Instrument, UK). The JIP parameter maximum quantum yield of primary PSII photochemistry (F_V/F_M) was analyzed. For this purpose, leaves were covered with leaf clips to adapt them to darkness for 20 min. Then, leaf clips were opened, and samples were exposed during 3 s to 3500 μmol photons m^{-2} s^{-1} (637 nm peak wavelength). The pocket PEA software (PEA plus v1.1, Hansatech Instrument Ltd., UK) was used to analyze PSII properties according to Strasser et al. (2000).

2.7. Determination of H^+ extrusion by roots

H^+ extrusion was determined according to Campestre et al. (2016) with some modifications. Root H^+ extrusion was determined during 5 d in the hydroponic solutions of plant cultures with or

Table 1
Survival index of plants ecotypes under 30 mM $NaHCO_3$. To determine the survival index (SI), plants were cultivated 44 d under 30 mM $NaHCO_3$. The SI was calculated as the percentage of plants alive at the end of experiment. Asterisks indicate the six representative selected ecotypes (n = 4).

Survival index (%)				
100	75	50	25	0
MG52	MG71	MG4 *	MG5	MG3
MG58	MG72	MG75	MG95	MG1
MG57 *	MG63 *	MG51	MG94	MG68 *
MG20	MG67	MG79	MG93	MG35
MG90	MG49	MG39	MG88 *	MG84
	MG122	MG91	MG83	MG98
		MG91	MG77	MG23
		MG46	MG76	MG65 *
		MG101	MG74	MG86
			MG73	MG44
			MG71	MG119
			MG62	
			MG40	
			MG34	
			MG17	
			MG16	
			MG14	
			MG127	
			MG125	
			MG100	
			Gifu	

without 10 mM $NaHCO_3$, using a digital pHmeter. For each treatment, a control beaker without plant was performed. The pH of the hydroponic solutions, pH 6 and 8.3 for control and alkaline plants, respectively, was only adjusted at the initial time without changing the volume of the solution during all assay. pH gradient value (ΔpH) was calculated as:

$$\Delta\text{pH} = \text{pHbeakerwithplant} - \text{pHbeakerwithoutplant}$$

2.8. Root ferric reductase activity measurement

The root of each plant was rinsed with deionized water and their root tips were obtained and weighted for reductase activity measurement (Zouari et al., 2001). Root tip segments were transferred to a full-strength Hoagland's solution (see above) in the presence of 0.1 mM Fe^{3+}-EDTA and 0.4 mM 2,2′-bipyridyl for one hour, keeping the same growing conditions as those utilized for seedlings growth. The activity was determined in a spectrophotometer (Zeltec ZL-5000 UV/VIS spectrometer, Argentina) at 520 nm by measuring the complex Fe^{2+}-dipyridyl formed.

2.9. Determination of root morphological patterns

After harvest, roots were washed with water to remove the remaining sand and were carefully extended on a flat-glass and scanned. Images were analyzed with the Image-ProPlus v4.1 software (Media Cybernetics, Bethesda, MD, USA). Root topology was calculated according to Paz et al. (2012). Two extreme root patterns, the *Herringbone* (one single main root with one order of laterals) and the *Dichotomous* (all external links form new branches with equal probability) were studied. To estimate the tendency of the root topology, the equation of Topological Trend (TT) was used which ranges from 1 to 0. The *Herringbone* pattern ranges from 0.5 up to 1, while the *Dichotomous* pattern ranges from 0 up to 0.5 (Fitter and Stickland, 1991; Kiswara et al., 2009). The mathematical expression was:

$$\text{TT} = [P_e0 - P_e(\text{min})]/[P_e(\text{max})2P_e(\text{min})],$$

where P_e0 is the external total path length observed and, P_e(min) and P_e(max) are the possible minimum and maximum P_e values, respectively.

2.10. Statistical analyses

Data were subjected to *t*-tests using the INFOSTAT statistical software package (InfoStat version 2010. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, http://www.infostat.com.ar).

3. Results

3.1. Characterization of L. japonicus ecotypes under alkaline stress

In order to select contrasting ecotypes to alkaline stress, 54 *L. japonicus* ecotypes were grown in sand with 30 mM $NaHCO_3$ during 44 d. The $NaHCO_3$ concentration was achieved after gradual alkalinization during 10 d. The MG20 and Gifu ecotypes, already classified as tolerant and sensitive to high alkalinity (Babuin et al., 2014), respectively, were also included as reference. The number of surviving plants was recorded at the end of the experiment in order to calculate a SI (Table 1). The results revealed variations among

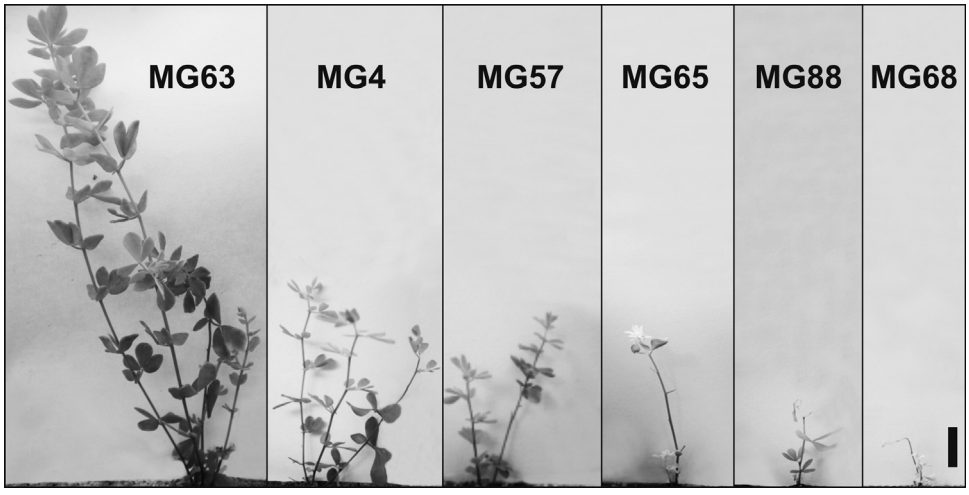

Fig. 1. *Characterization of L. japonicus ecotypes under extreme alkaline stress.* Images of a survival plant of each selected ecotypes under 30 mM $NaHCO_3$ at day 30. The black bar represents 1 cm.

ecotypes regarding the response to alkaline stress. In most of the sensitive ecotypes characterized by a low SI, the youngest leaves developed IC at 10 mM $NaHCO_3$ (pH 8.3), and subsequent bleaching at 20 mM of the salt. However, several ecotypes, regardless their SI, developed at least one or more events of plant death, preceded by desiccation of apical leaves, partial defoliation and total defoliation, at 30 mM $NaHCO_3$ (pH 10). Five ecotypes, MG52, MG58, MG57, MG20 and MG90 showed the maximum SI after 44 d at extreme alkalinity conditions. Among them, only MG57 and MG20 did not showed IC or other apparent alkaline stress symptoms. Interestingly, some of these five ecotypes, and others with high SI were also able to complete their life cycle generating pods and viable seeds. The highly tolerant ecotype MG57 and others with intermediate SI values – MG4 and MG63 – were selected as representative tolerant ecotypes (Table 1). A common trait among them was that they presented no IC throughout the test, even at extreme alkaline conditions. In parallel, The MG65, MG88 and MG68 were selected as sensitive ecotypes (Table 1). Both tolerant and sensitive ecotypes were phenotypically contrasting, and a marked IC was observed in the last ones (Fig. 1). However, a reduced shoot fresh weight accumulation under alkaline conditions was a common trait in the six selected ecotypes (Fig. 2A). Root fresh weight reduction under the stress condition was also recorded for the sensitive ecotypes, although a similar tendency was also found for the tolerant ones (Fig. 2B). Some authors relate IC with a low concentration of photosynthetic pigments under alkalinity (Abadía et al., 1999; Klein et al., 2012). However, in our alkalinity condition, the total chlorophyll content was decreased in the six studied ecotypes (Fig. 3). Moreover, IC is a symptom closely related with an Fe deficiency (Jelali et al., 2011; Rodríguez-Celma et al., 2013). Therefore, the representative tolerant and sensitive ecotypes were used in an assay of Fe content determination.

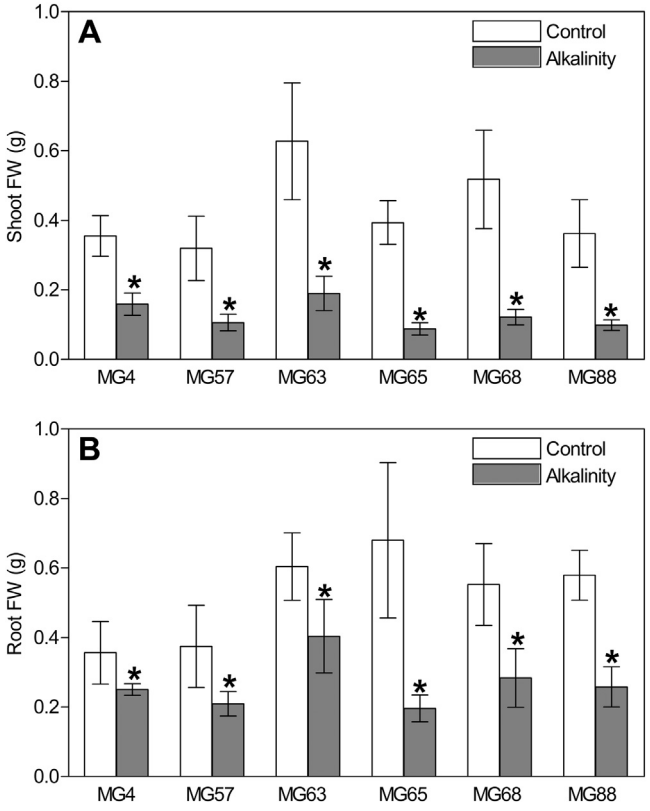

Fig. 2. *Determination of shoot and root growth in plants of selected ecotypes under 10 mM $NaHCO_3$.* Determination of fresh weight in shoots (A) and roots (B) in plants under control and alkalinity conditions at day 21. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

3.2. *Chlorosis symptoms were related with decrease of Fe^{2+}, net photosynthesis rate and PSII performance in sensitive ecotypes*

It is worth noting that the determination of total Fe does not always translate into the amount of Fe actually available for the physiological processes in plants (Abadía, 1992; Morales et al., 1998; Römheld, 2000), including *L. japonicus* (Klein et al., 2012). Several of our experiences showed that chlorotic leaves of *L. japonicus* plants under alkalinity may contain the same (Babuin et al., 2014), or higher (Campestre et al., 2016) total Fe concentrations than green leaves of control plants. However, in all of these cases, chlorotic leaves were always accompanied by a decrease in Fe^{2+} content. For this reason, only Fe^{2+} was determined on the selected ecotypes grown in hydroponic medium, with the addition of 10 mM $NaHCO_3$ during two weeks. IC was observed since the first week of alkaline treatment in MG68 and MG88, but not in MG4 and MG63 apical leaves (Fig. 4A). Curiously, MG57, but not MG65, also presented IC at day 15. The Fe^{2+} determination indicated a drop in shoot Fe^{2+} levels with respect to the control in all ecotypes that developed IC under alkalinity (Fig. 4B). In contrast, the ecotypes without IC in the apical leaves (excepting MG65) showed

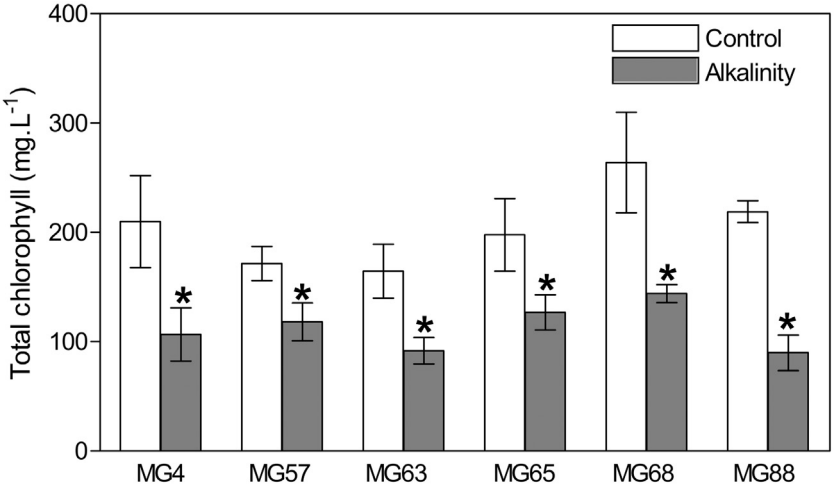

Fig. 3. *Total chlorophyll contents in apical leaves of selected ecotypes grown under 10 mM $NaHCO_3$.* Total chlorophyll (mg/L) was performed on apical leaves at day 15 and determined by spectrophotometry. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

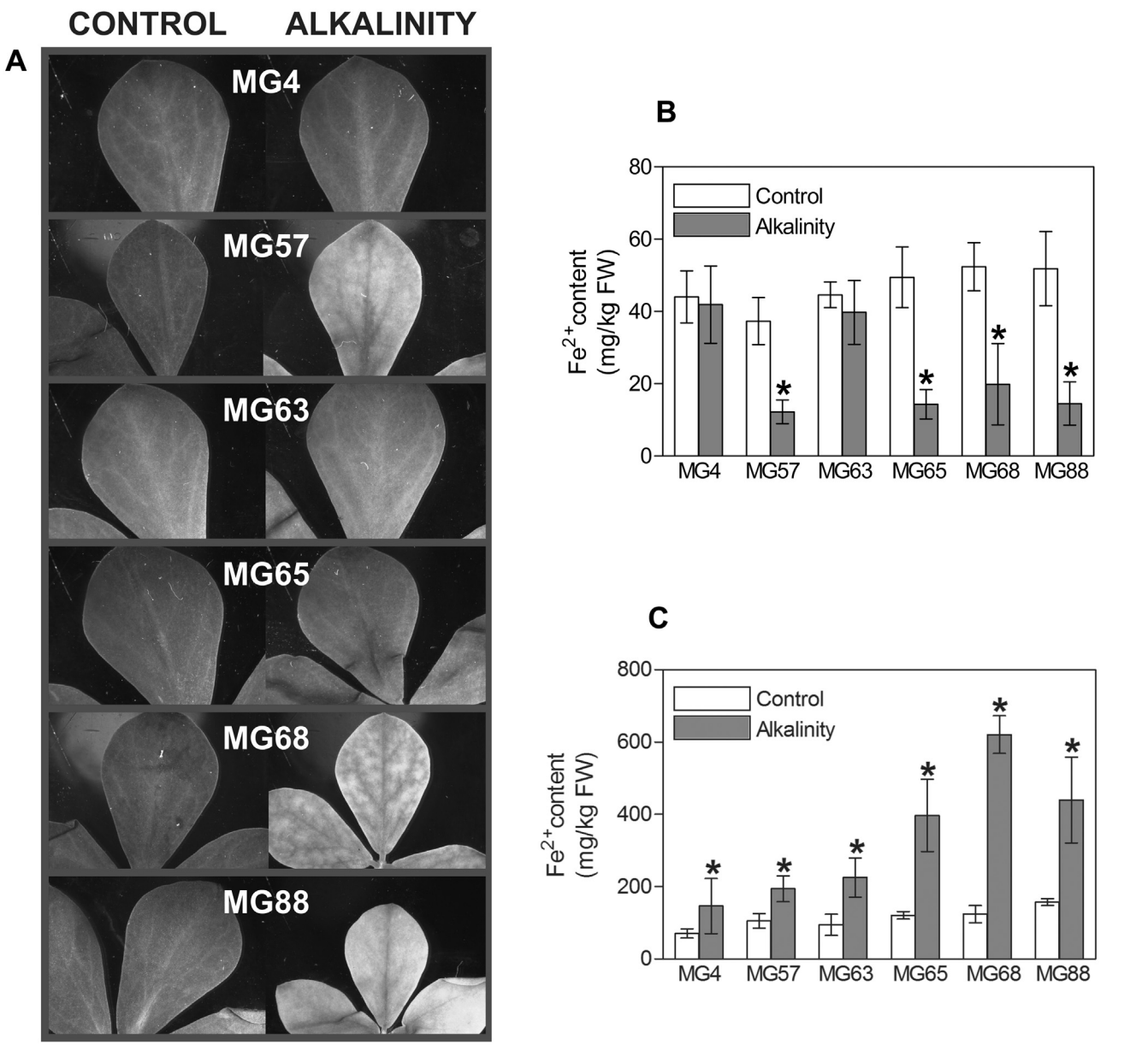

Fig. 4. *Interveinal chlorosis and Fe^{+2} content in plants of selected ecotypes under 10 mM $NaHCO_3$.* A. Observation of the presence or absence of IC in the third expanded leaf under control and alkaline conditions at day 15. Fe^{+2} content (mg/kg FW) in shoots (B) and roots (C) determined by spectrophotometry. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

no changes in Fe^{2+} shoot contents under alkalinity, compared to control plants. In addition, all ecotypes increased their roots Fe^{2+} contents under alkalinity, in comparison with controls (Fig. 4C). However, the Fe^{2+} accumulation was (4–6 fold) higher for the sensitive ecotypes than for the tolerant ones (2-fold).

Because Fe^{2+} also participates as a cofactor of many important proteins related to photosynthetic processes (Eichert et al., 2010; Ivanov et al., 2012), determinations of Pn and PSII performances were performed. Pn was reduced only in the sensitive ecotypes MG68 and MG88 under alkalinity, dropping to a level 50% lower than control plants (Fig. 5A). PSII performance was estimated through the JIP parameter F_V/F_M. The F_V/F_M values were only reduced in the sensitive ecotypes MG68 and MG88 (more obviously in the last) under alkaline conditions, with respect to controls (Fig. 5B).

Because differences in Fe^{2+} contents were recorded between the tolerant and sensitive ecotypes, we decided to further investigate whether the Fe acquisition mechanisms associated with Strategy I were contrasting between these two ecotype groups.

3.3. Tolerant ecotypes kept H^+ extrusion and a high ferric reductase activity under alkaline stress

Root H^+ extrusion was determined during 5 d by pH measurements of the plant culture hydroponic solutions. Under control treatment, all ecotypes decreased the pH of the solution, releasing H^+ throughout the experiment (Fig. 6). The same behavior was found under alkalinity, but only during the first 4 d. However, at 5 d, the sensitive but not the tolerant ecotypes increased medium alkalinity, reaching zero ΔpH values (MG65 and MG88), or even positive (MG68).

In addition, increasing Fe availability was tested by determination of FRO activity (Fig. 7). Results revealed that FRO activity was significantly higher in all the tolerant ecotypes under alkalinity,

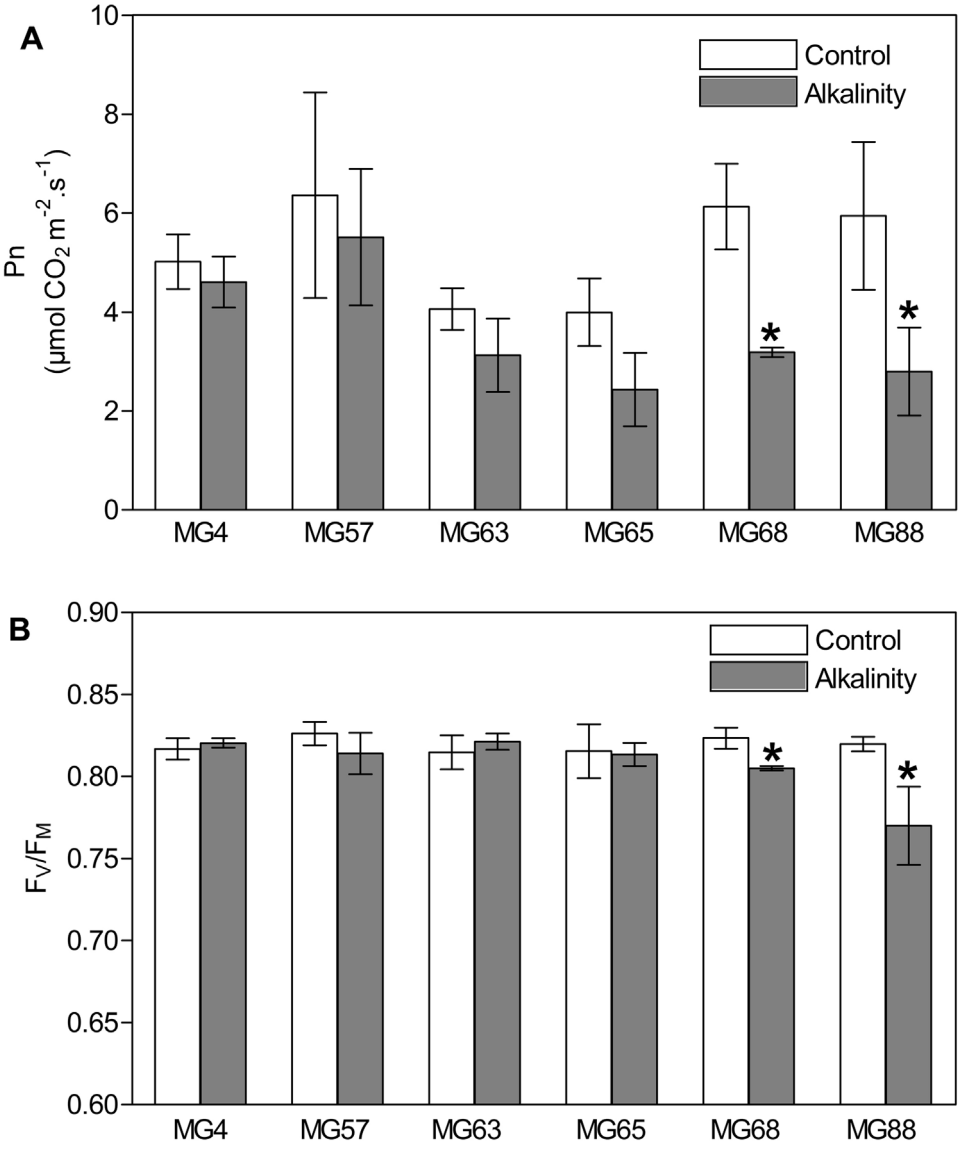

Fig. 5. *Net photosynthesis rate and chlorophyll fluorescence fast-transient test of selected ecotypes grown under alkalinity.* A) Pn and B) JIP test were performed at day 15 on the third expanded leaf. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

compared to control, throughout the test. In addition, all sensitive ecotypes showed a higher FRO activity under the alkaline treatment, compared to control, only at 5 d. As a whole, the tolerant and sensitive ecotypes increased, in average, 10 and 2 times their FRO activity in the fifth day, respectively.

3.4. *Evaluation of the root topology of* L. japonicus *ecotypes exposed to alkaline conditions*

The proliferation of lateral roots, which leads to the modification of the global root architecture, proved to be another mechanism acquired by higher plants to achieve the Fe uptake in the soil (López-Bucio et al., 2003; Hodge et al., 2009). Root architecture studies are characterized by the use of a topological model based on the arrangement of links in the root system (Paz et al., 2012). All ecotypes cultivated under control conditions developed a *Dichotomous* pattern with TT values between 0.1 and 0.2 (Fig. 8). However, under alkaline growth conditions, the tolerant ecotypes modified their root topology, reaching values near or above 0.5, which indicates a *Herringbone* pattern. In the case of the sensitive ecotypes, MG65 did not change the TT parameter between treatments. Meanwhile, MG68 and MG88 showed higher TT values under alkalinity than in controls. However, the root of both ecotypes conserved a *Dichotomous* pattern, their values not higher than 0.3.

4. Discussion

One of the aims of the present work was to characterize *L. japonicus* ecotypes with contrasting performance under alkalinity. Our results revealed variations of SI among different ecotypes with respect to their responses to alkaline stress, a trait used to select 3 tolerant and 3 sensitive ecotypes for further studies. We selected the highly tolerant ecotype MG57, with an equal SI than the MG20 ecotype, and the tolerant ecotypes MG63 and MG4 with a slightly lower SI. In addition, the highly sensitive ecotype, MG65, with a SI value below the Gifu SI, and two other sensitive ecotypes, MG68 and MG88, were selected. The observation of the alkaline stress symptoms and other biochemical and physiological determinations, supported their grouping according to their SI determination.

Alkaline tolerance could be explained by a mechanism related to high FRO activity in root, which is associated with Strategy I. In this sense, all tolerant ecotypes showed a higher FRO activity

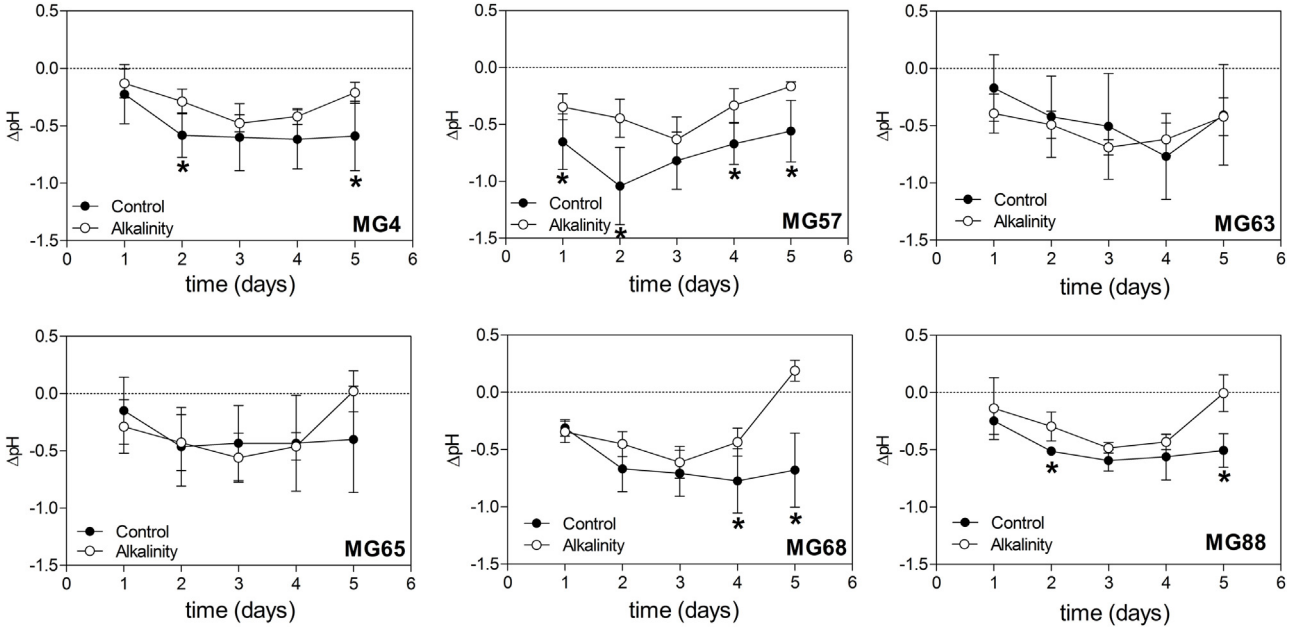

Fig. 6. *H⁺ extrusion by roots of plants grown under alkalinity.* The pH of the nutrient solution was measured during 5 d, and for each treatment, a control beaker without plant was performed. ΔpH was calculated as: ΔpH = pH beaker with plant − pH beaker without plant. Points (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

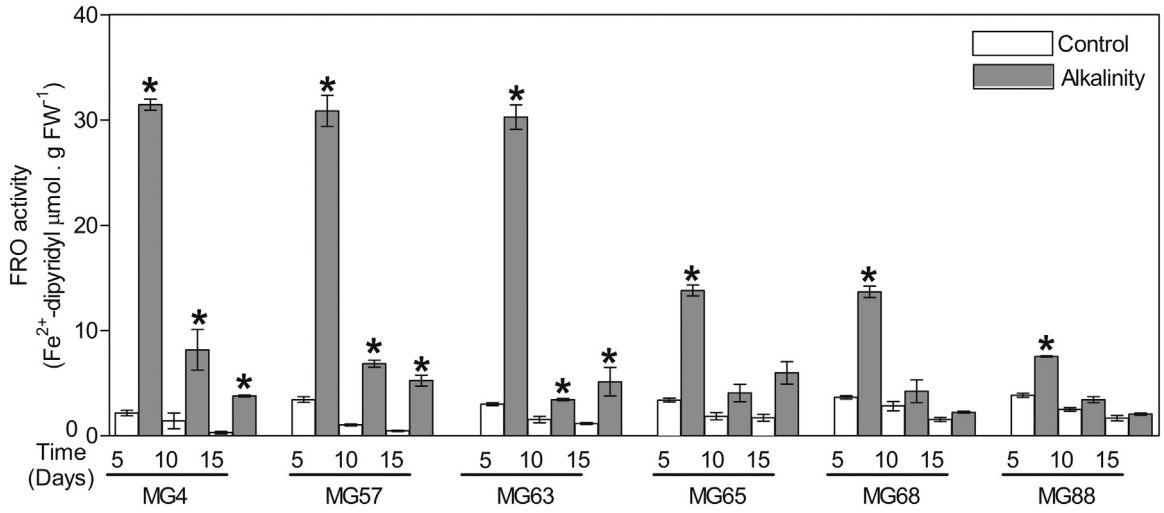

Fig. 7. *Ferric reductase oxidase activity in roots of plants grown under alkalinity.* The FRO activity was determined at 5, 10 and 15 d by spectrophotometry at 520 nm by measuring of the complex Fe^{2+}-dipyridyl formed. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

under alkaline stress relative to their controls during the whole assay. On the contrary, higher FRO activity under alkalinity was only observed in the sensitive ecotypes, compared to controls, at 5 d. This may explain, at least partially, why the sensitive ecotypes developed evident symptoms of Fe deficiency. Kabir et al. (2012) reported that two *Pisum sativum* L. genotypes (one Fe-efficient and one Fe-inefficient) increased FRO root activity when exposed to alkaline stress since days 3–5 of the beginning of the experiment. However, a decrease was observed between days 6–7, implying that all of the genotypes studied suffered Fe deficiency.

With respect to root H^+ extrusion, the sensitive ecotypes, but not the tolerant ones, increased medium alkalinity on the fifth day of alkaline treatment. Although these results could be the consequence of secondary effects associated to prolonged iron deficiency, they could also be related with a Strategy I mechanism. In this sense, Kabir et al. (2012) also reported that the *Pisum sativum* L. Fe-efficient genotype showed H^+ extrusion between 2 and 7 days under alkalinity, with a maximum observed at day 5. Instead, the *P. sativum* Fe-inefficient ecotype showed a ceased in their H^+ extrusion at the fifth day.

Additionally, the alkaline tolerance could also be explained through a change in root structure. All of the tolerant, but not the sensitive ecotypes modified their root architecture under alkalinity and acquired a *Herringbone* root pattern. This pattern, characterized by a proliferation of lateral roots, could prove to be another mechanism to achieve increased Fe uptake in the soil (López-Bucio et al., 2003). In line, Li et al. (2016) reported that Fe^{2+} is highly toxic and detrimental to plant metabolism when it is in high concentrations, because of its influence on redox chemistry. These authors also described the inhibition of primary root growth as a consequence of its accumulation. In this regard, all sensitive ecotypes showed a higher accumulation of Fe^{2+} in their alkalized roots,

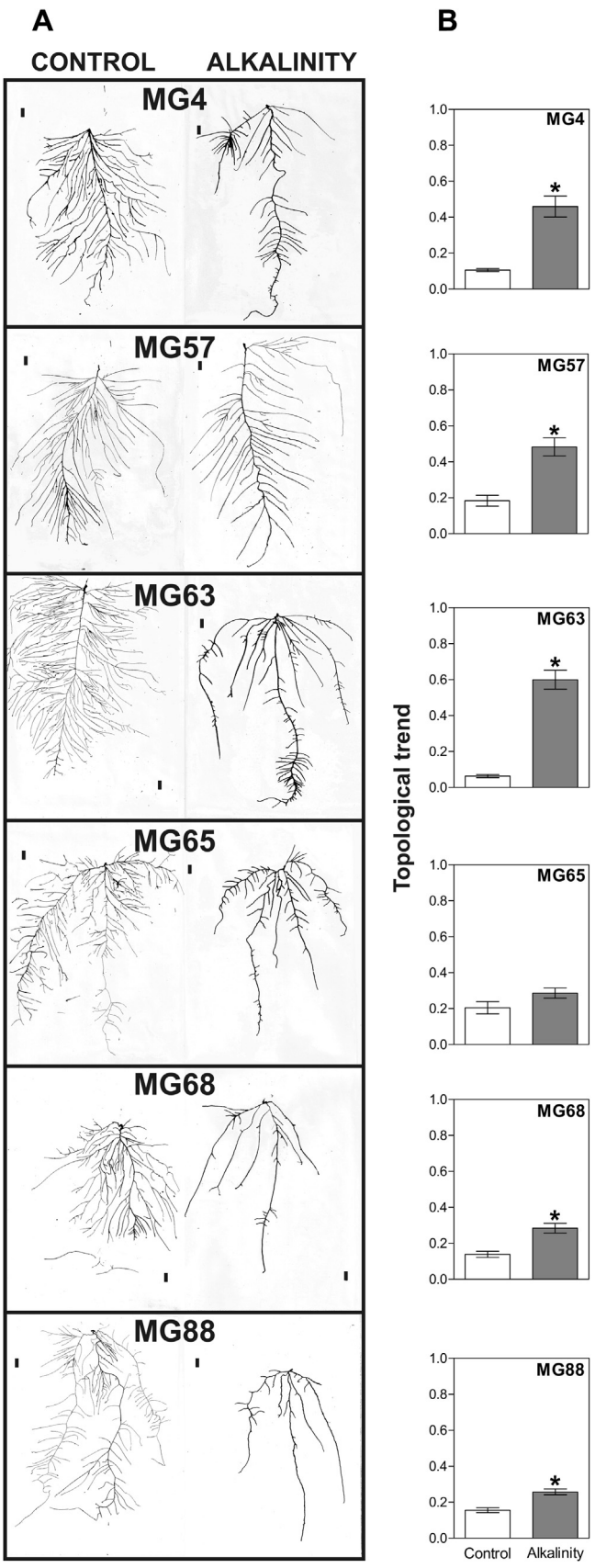

Fig. 8. *Analysis of root topology pattern acquired under control and alkalinity conditions.* A. Images of root architecture in plants under control and alkalinity conditions at day 21. The black line into each image represents 1 cm B. To estimate the tendency of the root topology the equation of TT was used which ranges from 1 to 0. The *Herringbone* pattern ranges from 0.5 up to 1 and the *Dichotomous* pattern ranges from 0 up to 0.5. Bars (mean ± SD; n = 5) with asterisks represent significant differences between treatments (T test; $P < 0.05$).

when compared to the tolerant ecotypes. Therefore, the root topology developed by the sensitive cultivars under alkalinity could be caused by this ion accumulation.

In addition, the root architecture could explain the presence or abscence of IC observed in MG57 and MG65, respectively, under alkalinity. In this sense, the IC observed in MG57 plants grown in hydroponic medium, but not in the sand substrate, could have been the result of an impairment in the formation of a *Herringbone* root pattern. In this regard, the three tolerant ecotypes, cultivated in sand, managed to change their root morphology to a root pattern efficient for this nutrient uptake (Paz et al., 2012). In contrast, when plants were grown in a hydroponic system, roots did not have this type of resistance by the lack of solid support. This observation agrees with Graves (1992) who indicated that sand, and not hydroponic medium cultures, caused root hair development *in Gleditsia triacanthos*. Moreover, we have recently studied the effect of inoculating the Fe-inefficient Gifu ecotype of *L. japonicus* with a growth promoting bacteria *Pantoea eucalypti* M91 (Campestre et al., 2016). In this work we reported that the inoculation of Gifu plants induced a *Herringbone* root pattern when plants were grown in alkalinity, improving their Fe availability.

Despite of MG57 lower Fe^{2+} shoot concentration under alkalinity, compared to control, its PSII activity and its Pn level were not altered. There is experimental evidence showing that the optimization of the metabolic Fe use in the shoots could include, or be linked with the re-mobilization of the inactive Fe stored in reservoirs within the plant (García-Mina, 2013). This process could involve the biosynthesis of specific components which solubilizes the precipitated or immobilized Fe in the plant (García-Mina, 2013). As a consequence, MG57 could present another mechanism of re-utilization or re-mobilization of inactive Fe.

In contrast, IC was observed in MG65 plants grown in sand substrate but not in hydroponic medium. The root architecture could be again the cause of this event noted in the highly sensitive ecotype. The IC observed in MG65 plants grown in sand substrate could be associated with a root architecture that affects the Fe^{2+} translocation from roots to shoots.

Alkaline soils reaching pH values between 8 and 10 generally have deficiencies in some nutritional elements that become insoluble, as Fe, Zn, Cu and Mn (Imas, 2000), and several elements deficiency can occur simultaneously (Vince and Zoltán, 2011). However, in this study we limit ourselves to analyzing only the Fe deficiency. In light of the presented results, our conclusion is that the survival of tolerant ecotypes to alkaline stress could partly be explained by the efficient Fe^{2+} acquisition of their roots and their architectural pattern. This efficiency could be associated not only with high FRO activity or the medium acidification, but also with a necessary modification of the root morphology to a *Herringbone* pattern. The identification of these tolerant traits could be used in screening programs for the selection of new alkaline-tolerant genotypes in the *Lotus* genus.

Authors' contributions

Conceived and designed the experiments: OR, AAR, SJM, MPC. Performed the experiments: MPC. Analyzed the data: MPC, AAR, CA, PIC. Contributed reagents/materials/analysis tools: OR. Wrote the paper: MPC, AAR.

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