The calcifuge and calcicole character of wild plants has been related to nutrient availability shortages, including iron (Fe)-deficiency. Surprisingly, just a few studies examined the relation between root Fe uptake and plant distribution in different soil types. We assessed the root Fe acquisition efficiency of two Ulmus species with calcareous (U. minor) and siliceous (U. laevis) soil distribution patterns in the Iberian Peninsula. Seedlings of both elm species were grown hydroponically with different Fe concentrations during 6 weeks. Plant physiological responses to Fe-limiting conditions were evaluated as were the ferric reductase activity and proton ($H^+$) extrusion capacity of the roots. Iron deprived elm seedlings of both species were stunted and suffered severe Fe-chlorosis symptoms. After Fe re-supply leaf chlorophyll concentrations rose according to species-dependent patterns. While U. minor leaves and seedlings re-greened evenly, U. laevis did so along the nerves of new growing leaves. U. minor had a higher root ferric reductase activity and $H^+$-extrusion capability than U. laevis and maintained a better nutrient balance when grown under Fe-limiting conditions. The two elm species were found to have different Fe acquisition efficiencies which may be related to their natural distribution in calcareous and siliceous soils of the Iberian Peninsula.

Keywords: calcifuge, iron chlorosis, iron reductase activity, nutrition, root proton extrusion, Ulmus

INTRODUCTION

Ulmus laevis is a Northern Hemisphere genus of importance in the ecological context of the Iberian Peninsula (García-Nieto et al., 2000). The indigenous elm species found in Spain are U. glabra Huds, U. laevis Pall, and U. minor Mill. The native character of U. laevis has been recently confirmed by DNA molecular markers (Fuentes-Utrilla et al., 2014). Despite U. minor being native to Spain (Richens and Jeffers, 1986; Gil and García-Nieto, 1990), its natural distribution is not clear since this species has been extensively planted for over 2,000 years, first for training vines and later for ornamental purposes (Gil et al., 2004). U. minor can be found in the whole Iberian Peninsula in azonal flood-plain forests, linked to shallow water-tables, since it tolerates floods as well as summer droughts. Nonetheless, it preferentially grows in Eastern Spain (Figure 1) were there is an abundance of calcareous, alkaline soils (Richens and Jeffers, 1986). In contrast, U. laevis is one of the few European tree species which thrives in damp soils that are seasonally flooded (Collin et al., 2000). In Spain its relic populations are scarce, small and fragmented (Figure 1, Venturas et al., 2013a; Fuentes-Utrilla et al., 2014). U. laevis chiefly grows in acid soils of the Western Iberian Peninsula as riparian forests, which are subjected to seasonal waterlogging and linked to aquifer discharge areas and/or endorheic basins.

The distribution of U. laevis and U. minor in the Iberian Peninsula may be related to calcifuge versus calcicole characteristics of each species. Whilst calcicole plants are able to take up nutrients when grown in calcareous soils (Zohlen, 2002), calcifuge species growth under high pH, alkaline soil conditions is limited by low nutrient availability especially phosphorus (P; Tyler, 1992; Zohlen and Tyler, 2004), iron (Fe; Zohlen and Tyler, 1997, 2000; Zohlen, 2002), and manganese (Mn; Messenger, 1986; Thomas et al., 1998; Kuster et al., 2013).

Iron is a vital element for plant growth, development and survival, since it is essential for the proper functioning of multiple metabolic and physiological processes (López-Millán et al., 2013; Sudre et al., 2013; Grillet et al., 2014). The detrimental effect of lime-induced chlorosis on wild plants grown in calcareous, high pH soils has been only analyzed in a few eco-physiological studies (Hutchinson, 1970; Anderson, 1984; Tyler, 1997; Zohlen and Tyler, 2004), iron (Fe; Zohlen and Tyler, 1997, 2000; Zohlen, 2002), and manganese (Mn; Messenger, 1986; Thomas et al., 1998; Kuster et al., 2013).

Despite the ubiquitous presence of Fe in the earth’s crust, the low solubility of Fe(III) compounds in many soils prevents plant Fe uptake and induces the development of Fe deficiency symptoms (Lindsay, 1984; Lucena, 2006). Iron chlorosis is principally observed in calcifuge plants growing in calcareous soils, where CaCO$_3$ buffers soil solution pH in the range of 7.5–8.5 (Lindsay and Schwab, 1982), and in the presence of high bicarbonate concentrations (Lucena, 2000). According to the specific root uptake mechanisms for the acquisition of Fe, plants have been...
classified as (Marschner and Römheld, 1994): Strategy I (dicotyle-
doneous and non-graminaceous monocotyledonous species) or
Strategy II (restricted to graminaceous species), which are addi-
tionally capable of producing phyto-siderophores. Iron uptake
mechanisms in Strategy I plants may involve metabolic and mor-
phological changes (Rombolà et al., 2002; Abadía et al., 2011;
López-Millán et al., 2013), but the major components are: (i) the
occurrence of a Fe-reductase enzyme of the FRO (membrane-
bound ferric reductase) family, (ii) the induction of an IRT (iron
regulated transporter) Fe(II) transporter that belongs to the ZIP
family (ZRT, IRT-like protein), (iii) acidification of the rhizo-
sphere via the excretion protons by a H^+ -ATPase, and (iv) the
excretion of organic compounds, such as carboxylates, pheno-
lcs, and flavonoids, which can affect Fe-availability directly or
indirectly. The relative importance and efficacy of the differ-
ent root Fe uptake mechanisms may vary among plant species,
varieties and populations, which may be associated with the tol-
erance or susceptibility when grown in high pH, calcareous soils
(Dell’Orto et al., 2013).

Information on the root response mechanisms of trees to lime-
induced chlorosis is mostly limited to some fruit species such as
peach (Gogorcena et al., 2000; Jiménez et al., 2011), Amnona
(Ojeda et al., 2003), olive, pear, quince (De la Guardia and Alcántara,
2002; Donnini et al., 2011), and to a few forest or woody species
like cork-oak (Gogorcena et al., 2001), poplar (Fodor et al., 2005),
carob and trifoliate orange (Pestana et al., 2012). On the other hand,
the response of some calcicole and calcifuge plants to lime-
induced chlorosis (e.g., Zohlen and Tyler, 1997, 2000; Zohlen,
2002; Donnini et al., 2012) has been principally evaluated on the
basis of tissue Fe determinations.

Therefore, given the markedly different distribution of U. lae-
vis and U. minor in siliceous and calcareous soils of Spain and
as a first approach, a greenhouse study was undertaken to assess
the effect of growing these species under hydropionic, Fe-limiting
conditions. With the aim of comparing the performance of both
elm species under Fe-limiting conditions, we tested the following
hypotheses: (i) Do U. laevis and U. minor differ in their degree
of tolerance to lime-induced chlorosis in relation to growth, defi-
ciency symptom development and nutrient homeostasis?, and (ii)
is U. minor more efficient than U. laevis in acquiring Fe via the
root system as measured by ferric reductase activities and proton
extrusion capacities?

MATERIALS AND METHODS

PLANT CULTURE

Ulmus minor P-VV1 genotype seeds (the code refers to the Spanish
Elm Breeding Program) were collected at Puerta de Hierro nurs-
ery (Madrid, 40°27’N, 3°45’W) conservation plot, and U. laevis
M-VD.R29 genotype seeds (referring to the Spanish Elm Breeding
Program) at Valdelatas forest (Madrid, 40°32’N, 3°40’W). In the
first experimental week, seeds were germinated in 4 L pots filled
with perlite. All the materials used for plant growth (perlite and
pots) were previously washed (two washes in 0.1 M HCl, followed
by a wash in 100 μM ethylenediaminetetraacetic acid disodium
(Na2EDTA) and several rinses in pure water) to limit the poten-
tial occurrence of Fe contamination. Seeds were initially irrigated
with distilled water (type II analytical grade, obtained with an Elix
5 apparatus, Millipore, USA) until the development of the first
true leaf (c.a. 3 weeks after seed sowing). Seedlings were subse-
dually grown in 1/4 strength nutrient solution (T0, as described
below) for 1 week, 1/2 strength for another 2 weeks, and finally
in full-strength solution until the end of the experimental period.
The base nutrient solution without Fe (T0) was changed once per
week and had a composition of: (i) macronutrients (mM): 1.0
Ca(NO3)2, 0.9 KNO3, 0.3 MgSO4, and 0.1 KH2PO4; (ii) EDTA-
buffered metal micronutrients (μM): 2.5 MnSO4, 1.0 CuSO4, 10.0
ZnSO4, 1.0 NiCl2, 1.0 CoSO4, and 115.5 Na2EDTA; and (iii) other
micronutrients (μM): 35 NaCl, 10 H3BO3, and 0.05 Na2MoO4
(Nadal et al., 2012). The pH of the solution was buffered with
4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) at
0.1 mM and adjusted to 7.5 with 1.0 M KOH (Nadal et al.,
2012). Seedlings were grown in a greenhouse under a 16/8 h
day/night regime and temperatures ranging from 10 to 25°C. A
total of 72 homogeneous seedlings from the two species were
selected and transferred to hydroponic culture 1 month after sow-
ing. Twelve plants from the same species were placed together
in continuously aerated (approximately 100 L of air per hour
and container) 6 L containers filled with full-strength nutrient
solution.

TREATMENT APPLICATION

When seedlings were 2 months old the nutrient solution was
changed for the onset of the different root Fe treatments. When
supplied to the plants, iron was applied as Fe(III)-HBED pre-
viously synthesized in the laboratory by complexing free Fe(III)
supplied as Fe(NO3)3 9H2O with HBED (ADOB PPC, Poznan,
Poland) at 1:1 (Fe:ligand) ratios.

Three different Fe treatments per species were initially added to
the base nutrient solution, namely: 0 μM Fe (i.e., T0 containing no

FIGURE 1 | Ulmus laevis populations (Venturas et al., 2013a)
represented as white dots over an Iberian Peninsula soil pH map
(Rodriguez et al., 2009). Ulmus minor is thought to be native east of the
broken line (Richens and Jeffers, 1988), although it grows all over Spain
because this species has been extensively used by humans (Gil et al.,
2004).
Fe, as described above), 5 μM Fe (T5) and 20 μM Fe (T20). Four containers per species received 0 μM Fe (T0). Nutrient solutions were changed once per week until the end of the experimental period (after 6 weeks). To achieve a Fe-deficiency state similar to what may occur to Fe-chlorotic field trees and an intermediate chlorosis level, an additional 1 μM Fe treatment (T1) was given for 2 weeks to one T0 container per species (i.e., to 12 plants).

PLANT PHYSIOLOGICAL RESPONSES
After Fe-resupply, plant heights and SPAD values (measured with a SPAD 502, Minolta, Osaka, Japan) were determined on a weekly basis for 6 weeks on 12 plants per treatment and species. For SPAD index determinations, which give an estimation of leaf chlorophyll (Chl) concentrations (Nadal et al., 2012), two sub-apical leaves per plant were measured, with two measurements per leaf. Total Chl contents were determined by the method of McKinney (1941) and correlations with SPAD values were established for both species. In brief, Chl concentrations were measured from fresh tissue after extraction in 80% acetone and spectrophotometric reading at 664 nm for Chl a and 647 nm for Chl b (7315 Spectrophotometer Jenway, Bibby Scientific Limited, UK).

Plant tissues were analyzed following standard laboratory procedures and limiting the risk of Fe contamination by rising the pH to 7.0 (measurements carried out with a Micro-pH 2002 pH-meter, Crison Instruments, Spain). Since solutions were continuously aerated, the contribution of root respiration to solution acidification was negligible. Shoot and root fresh weights (FWs) were recorded at the end of the experimental period. The daily H⁺ extrusion per plant (μmol g⁻¹ root FW day⁻¹) was calculated as the H⁺ increment since the previous day.

RESULTS

Plant Physiological Responses
For the two species, Fe-supply led to a steep Chl concentration increment during the first 2 weeks (Figure 2), which was...
maintained until the end of the experimental period. In general and for a similar root Fe supply level *U. laevis* had higher SPAD values than *U. minor* leaves, and there were no significant differences between the 20 and 5 μM Fe treatments at the end of the trial. The supply of 1 μM Fe to Fe-deficient seedlings (indicated with an arrow) in week 3 led to a steep Chl increase (Figure 2).

Both elm species fully supplied with iron (20 μM Fe treatment) were green, had a healthy appearance and a large size (especially *U. laevis*) during the whole experimental period (Table 1, Figure 3). For this treatment, *U. laevis* leaves had 37% more total Chl concentrations than *U. minor* (Table 1). Iron-sufficient, *U. laevis* leaves also had 34% higher tissue Fe concentrations than *U. minor* leaves (Table 1).

There were significant differences in SPAD values between the species and Fe treatments after 6 weeks (P < 0.0001; Table 1, Figure 2). Both elm species developed strong Fe-chlorosis symptoms in the lack of Fe. The seedlings subjected to the 0 μM Fe treatment were stunted and leaves were severely chlorotic with necrotic spots, chiefly in the case of *U. laevis* (Figures 3 and 4A,C). Once supplied Fe, the re-greening patterns of seedlings and leaves were different in both species. The re-greening process in *U. laevis* leaves began along the veins of apical leaves and basal leaves took longer to re-green and never reached a homogeneous green coloration (Figure 4B). Meanwhile, *U. minor* re-greened evenly all over the plant and leaf surface (Figure 4D). Seedlings of the 5 and 20 μM Fe treatments reached their maximum SPAD values in week 3 (Figure 2). Regarding tree height and for both species, no significant differences were found between the 5 and 20 μM Fe treatments, while plants receiving 1 and 0 μM Fe in the nutrient solution had a stunted growth (Figure 3).

### Table 1 | SPAD values, leaf chlorophyll (Chl) and iron leaf and stem concentrations of *U. laevis* and *U. minor* seedlings 6 weeks after Fe-resupply.

| Species | Treatment | Mean aerial part DW (g) | Δ SPAD | [Fe] (mg Kg⁻¹) | Total [Chl] (mg cm⁻²) | [Fe] (mg Kg⁻¹) |
|---------|-----------|-------------------------|--------|----------------|----------------------|---------------|
| *U. laevis* | 0 μM Fe | 0.10 ± 0.02 a | −6.4 ab | 21.0 ± 0.4 a | 0.08 | 6.2 ± 0.1 b |
| | 1 μM Fe | 0.47 ± 0.13 b | 14.4 c | 23.8 ± 5.8 ab | 1.98 | 8.1 ± 0.2 bc |
| | 5 μM Fe | 1.95 ± 0.30 c | 29.6 d | 37.0 ± 3.6 bc | 2.73 | 10.8 ± 1.3 c |
| | 20 μM Fe | 2.07 ± 0.51 c | 28.4 d | 60.3 ± 2.7 d | 3.01 | 13.7 ± 1.0 cd |
| *U. minor* | 0 μM Fe | 0.23 ± 0.15 a | −10.5 a | 10.8 ± 0.2 a | 0.13 | 4.3 ± 0.1 a |
| | 1 μM Fe | 0.34 ± 0.18 a,b | −2.6 b | 11.9 ± 0.1 a | 0.70 | 9.0 ± 0.2 c |
| | 5 μM Fe | 2.81 ± 1.57 c,d | 16.3 c | 38.8 ± 2.3 bc | 2.12 | 9.9 ± 0.8 c |
| | 20 μM Fe | 1.23 ± 0.35 c | 16.2 c | 44.9 ± 1.5 c | 2.19 | 15.0 ± 0.9 d |

The headings correspond to: mean aerial part dry weight (DW; g), Δ SPAD (SPAD increment of Week 6 – Week 0; n = 12), total leaf [Chl], and leaf and stem [Fe] (mg Kg⁻¹ DW; means ± SE; n = 7). Letters correspond to Tukey’s HSD 95% homogeneous groups.
ROOT PROTON EXCRETION

The proton extrusion per day was significantly different between the species (P < 0.0001), but was not found to be significant for treatments nested within species (P = 0.57). The highest rate of proton extrusion was measured on the first day of the trial when 280 and 97 μmol H+ g⁻¹ day⁻¹ were recorded for U. minor and U. laevis, respectively. During the following 8 days, root extrusion values decreased to reach a steady average of 17 (U. minor) and 9 (U. laevis) μmol H⁺ g⁻¹ day⁻¹.

ROOT FERRIC REDUCTASE ACTIVITY

The root ferric reductase activity was significantly different between species (P = 0.0004) and treatments nested within species (P < 0.0001; Figure 5). For the 1 μM Fe and 20 μM Fe treatments, U. laevis had a much higher reductase activity than U. laevis. However during 2 h of the assay, Fe-deficient (0 μM Fe) plants of both species had a limited root ferric reductase activation capacity in the presence of Fe(III) in the nutrient solution (Figure 5).

MINERAL ELEMENT STATUS

The macro- and micro-nutrient concentrations of leaves and stems in relation to the different treatments are shown in Tables 1–3. Leaf and stem Fe concentrations decreased under Fe-limiting conditions (Table 1). At the end of the experimental period and as derived from Figure 3, the aerial part dry weight (DW) gradually increased with higher root Fe levels for U. laevis. While a similar trend was observed for U. minor seedlings, the highest mean aerial plant DW was recorded for the 5 μM Fe treatment, which however, had a high SD (Table 1). This implies that the highest Fe absorption rates were achieved by the 20 μM Fe U. laevis and 5 μM Fe U. minor treated seedlings, suggesting that the later species possibly reached an optimum when supplied 5 μM Fe via the root system.

For all elements excepting K, Fe, and Zn in leaves and K and Fe in stems, variations in U. laevis were larger than in U. minor. For both species, the relative changes followed a similar trend for all the elements excepting Zn, which increased in U. laevis chlorotic plants and decreased in U. minor (Tables 2 and 3). Leaf N concentrations decreased with lower root Fe supply levels, especially in U. laevis. On the contrary, stem N concentrations were higher in seedlings of the 0 μM Fe treatment. Phosphorus tissue concentrations increased in association with lower root Fe doses. Potassium leaf and stem concentrations were generally lower in 0 μM Fe treated elm seedlings, which also had the lowest leaf Ca concentrations (Table 2). Concerning the micronutrients, approximately five and eight times higher Mn concentrations were determined in leaves and stems of Fe-deprived versus 20 μM Fe treated U. laevis seedlings, indicating the accumulation of Mn under Fe-limiting conditions. However, in U. minor the lack of Fe in the growing medium approximately doubled the Mn concentration of leaves and stems as compared to the 20 μM Fe treatment. Root Fe shortage also led to the accumulation of B in leaves and of Cu in leaves and stems while no clear trend was observed for Zn concentrations in leaves and stems in relation to the different Fe treatments (Table 3).

Individual U. laevis and U. minor seed (extracted from the samara) weighed an average of 6.9 and 5.4 mg DW, respectively. Differences between U. laevis and U. minor were only recorded for the macronutrients (g kg⁻¹ DW): N (75.8 versus (vs.) 62.2) K (11.7 vs. 17.3), Ca (1.0 vs. 2.5), and Mg (1.7 vs. 3.0), and the micro-elements (mg kg⁻¹ DW): Fe (80.3 vs. 37.7) and Zn (76.9 vs. 56.5). Seeds of both elm species had a Mn concentration around 49.3 mg kg⁻¹ DW, which provides evidence for the accumulation of Mn in seedlings during hydroponic culture.

DISCUSSION

In this study we investigated the root response mechanisms under Fe-limiting conditions of a putative calcicoles versus a calcifuge elm species, based on their natural distribution in the soils of the Iberian Peninsula. Calcifuge plants and lichens do not occur in calcareous soils, where their growth and development is largely limited by Fe and P deficiency (Zohlen, 2002; Zohlen and Tyler,
Table 2 | Macronutrient leaf and stem concentrations (mean ± SE; the same letters within the same tissue and element correspond to Tukey’s HSD 95% homogeneous groups).

| Tissue | Species | Treatment | N       | P        | K        | Ca       | Mg       |
|--------|---------|-----------|---------|----------|----------|----------|----------|
| Leaf   | U. laevis | 0 μM Fe  | 25.8 ± 0.5 a | 9.3 ± 0.2 f | 16.8 ± 0.3 ab | 53.0 ± 1.1 d | 10.4 ± 0.2 e |
|        |         | 1 μM Fe  | 30.7 ± 0.3 bc | 6.0 ± 0.2 e | 17.5 ± 1.1 ab | 38.4 ± 1.7 cd | 6.8 ± 0.2 d |
|        |         | 5 μM Fe  | 32.1 ± 3.4 bc | 2.6 ± 0.1 ab | 14.0 ± 0.7 a  | 32.2 ± 2.0 abc | 5.0 ± 0.3 c  |
|        |         | 20 μM Fe | 40.1 ± 0.4 c  | 2.4 ± 0.1 ab | 15.9 ± 0.7 ab | 25.2 ± 1.2 a  | 3.5 ± 0.2 a  |
| Leaf   | U. minor | 0 μM Fe  | 35.3 ± 0.7 bc | 5.2 ± 0.1 de | 15.0 ± 0.3 a  | 40.0 ± 0.8 cd | 5.6 ± 0.1 cd |
|        |         | 1 μM Fe  | 34.3 ± 1.6 bc | 4.1 ± 0.5 cd | 17.2 ± 1.5 ab | 36.8 ± 1.0 bc | 6.5 ± 0.1 d  |
|        |         | 5 μM Fe  | 37.4 ± 1.0 bc | 2.4 ± 0.1 a  | 15.9 ± 1.2 ab | 32.6 ± 1.9 bc | 4.6 ± 0.2 bc  |
|        |         | 20 μM Fe | 39.7 ± 0.9 c  | 3.2 ± 0.2 bc | 19.3 ± 1.1 b  | 27.9 ± 1.5 ab | 3.6 ± 0.3 ab  |
| Stem   | U. laevis | 0 μM Fe  | 32.5 ± 0.6 b  | 4.7 ± 0.1 d  | 9.6 ± 0.2 a   | 20.1 ± 0.4 c  | 2.0 ± 0.0 bc  |
|        |         | 1 μM Fe  | 18.0 ± 0.4 a  | 3.6 ± 0.1 cd | 12.4 ± 0.2 ab | 14.1 ± 0.3 bc | 1.3 ± 0.0 abc |
|        |         | 5 μM Fe  | 19.5 ± 2.8 a  | 1.6 ± 0.1 ab | 12.5 ± 0.9 ab | 8.9 ± 0.7 ab  | 1.1 ± 0.1 ab  |
|        |         | 20 μM Fe | 16.6 ± 1.0 a  | 1.4 ± 0.1 a  | 11.9 ± 0.8 ab | 7.0 ± 0.6 a   | 0.9 ± 0.1 a   |
| Stem   | U. minor | 0 μM Fe  | 29.1 ± 0.6 b  | 3.3 ± 0.1 c  | 12.5 ± 0.3 a  | 13.0 ± 0.3 bc | 2.4 ± 0.0 c   |
|        |         | 1 μM Fe  | 27.4 ± 0.5 b  | 4.2 ± 0.1 cd | 24.0 ± 0.5 c  | 15.0 ± 0.3 c  | 4.0 ± 0.1 d   |
|        |         | 5 μM Fe  | 172 ± 1.2 a   | 1.5 ± 0.1 ab | 14.7 ± 0.9 ab | 7.3 ± 0.6 a   | 1.7 ± 0.1 c   |
|        |         | 20 μM Fe | 19.4 ± 1.5 a  | 2.0 ± 0.1 a  | 17.7 ± 1.5 cd | 7.4 ± 0.3 a   | 1.5 ± 0.1 bc  |

Table 3 | Micronutrient leaf and stem concentrations (mean ± SE; the same letters within the same tissue and element correspond to Tukey’s HSD 95% homogeneous groups).

| Tissue | Species | Treatment | Fe       | B        | Mn       | Cu       | Zn       |
|--------|---------|-----------|----------|----------|----------|----------|----------|
| Leaf   | U. laevis | 0 μM Fe  | 21.0 ± 0.4 a | 96.7 ± 1.9 e | 10274 ± 20.5 e | 17.1 ± 0.3 e | 91.3 ± 1.8 d |
|        |         | 1 μM Fe  | 23.8 ± 5.8 ab | 59.1 ± 2.7 d | 5071 ± 13.9 d | 10.7 ± 0.9 d | 72.2 ± 3.8 bc |
|        |         | 5 μM Fe  | 370 ± 3.6 bc | 29.8 ± 1.7 a | 256.0 ± 5.5 c | 5.4 ± 0.5 bc | 48.9 ± 2.1 ab |
|        |         | 20 μM Fe | 60.0 ± 2.7 d | 30.0 ± 1.8 a | 205.5 ± 7.3 b | 5.3 ± 0.4 bc | 776 ± 7.4 cd |
| Leaf   | U. minor | 0 μM Fe  | 10.8 ± 0.2 a  | 56.8 ± 1.1 cd | 303.8 ± 6.1 c | 8.7 ± 0.2 cd | 36.5 ± 0.7 ab |
|        |         | 1 μM Fe  | 11.9 ± 0.1 a  | 42.4 ± 4.4 bc | 278.1 ± 1.2 c | 7.0 ± 0.3 c  | 274 ± 2.6 a  |
|        |         | 5 μM Fe  | 38.8 ± 2.3 bc | 310.0 ± 2.5 ab | 160.8 ± 10.0 a | 4.6 ± 0.4 ab | 49.7 ± 3.6 ab |
|        |         | 20 μM Fe | 44.9 ± 1.5 c  | 258.0 ± 1.7 a | 1678 ± 11.1 ab | 3.5 ± 0.2 a  | 53.0 ± 3.7 abc |
| Stem   | U. laevis | 0 μM Fe  | 6.2 ± 0.1 b   | 13.1 ± 0.3 ab | 181.3 ± 3.6 e | 9.1 ± 0.2 c  | 43.9 ± 0.9 a  |
|        |         | 1 μM Fe  | 8.1 ± 0.2 bc  | 13.0 ± 0.3 ab | 61.0 ± 1.2 c  | 6.1 ± 0.1 bc  | 26.8 ± 0.5 ab |
|        |         | 5 μM Fe  | 10.8 ± 1.3 c  | 10.2 ± 0.8 a  | 21.9 ± 2.0 a  | 3.6 ± 0.4 ab  | 20.2 ± 1.7 a  |
|        |         | 20 μM Fe | 13.7 ± 1.0 cd | 9.8 ± 0.7 a   | 23.1 ± 2.5 ab | 3.2 ± 0.3 a  | 26.2 ± 2.7 ab |
| Stem   | U. minor | 0 μM Fe  | 4.3 ± 0.1 a   | 14.0 ± 0.3 ab | 79.8 ± 1.6 cd | 7.0 ± 0.1 c  | 29.7 ± 0.6 ab |
|        |         | 1 μM Fe  | 9.0 ± 0.2 c   | 20.6 ± 0.4 b  | 88.6 ± 1.8 d  | 7.7 ± 0.2 c  | 30.1 ± 0.6 ab |
|        |         | 5 μM Fe  | 9.9 ± 0.8 c   | 9.6 ± 0.5 a   | 31.7 ± 2.2 b  | 3.7 ± 0.2 ab  | 277 ± 1.7 ab  |
|        |         | 20 μM Fe | 15.0 ± 0.9 d  | 10.1 ± 0.6 a  | 32.2 ± 1.5 b  | 3.4 ± 0.2 a  | 32.2 ± 2.5 b  |

2004; Paul et al., 2009). Calcicole species however, thrive in soils containing a high CaCO₃ concentration, which implies that they were able to develop efficient mineral element uptake and homeostasis mechanisms as an adaptation to such rhizospheric environment. Since lime-induced chlorosis can sometimes be observed in wild plants grown in calcareous soils and problems of tissue Fe immobilization have been previously reported (Zohlen and Tyler, 1997; Zohlen, 2002), we analyzed for the first time the
Fe-reducing and acidification capacity of two Ulmus species of major ecological significance in azonal forests of Spain, which are naturally distributed in either siliceous (U. laevis) or calcareous (U. minor) soils.

The existing studies on calcifuge and calcicole species were always performed by growing plants in calcareous or acid soils (e.g., Zohlen, 2002; Zohlen and Tyler, 2004) and focused on the distribution and partition of Fe within the different plant tissues (Zohlen and Tyler, 2000). However, plant growth in a solid substrate poses some experimental constraints to analyse in detail the response of roots to Fe-limiting conditions, which led us to carry out the trials in hydroponic culture. While both species have high water requirements as compared to other Mediterranean plants (Galmés et al., 2012; Venturas et al., 2013b), U. laevis may be subjected to prolonged soil flooding periods (Collin et al., 2000) in contrast to the occasional flooding that U. minor may tolerate (García-Nieto et al., 2000).

The results show that lack of iron (T0) severely affected the growth and development of both elm species. By the end of the experimental period, seedlings grown without Fe were stunted and almost completely defoliated. This shows that Fe is essential and very limiting for Ulmus seedling growth and establishment as shown previously for other wild species (Hutchinson, 1967, 1970). Micronutrient contents in leaves and stems of elms grown with no Fe limitation (T20; Table 3) were within the range described for other temperate European trees (Hagen-Thorn and Stjernquist, 2005).

Seed nutrient contents may be key for the successful establishment of seedlings (Tyler and Zohlen, 1998), and we observed that U. laevis contained twice as much Fe as U. minor seeds, in accordance with the higher leaf Chl and Fe concentrations measured in fully expanded leaves. This indicates the higher nutrient requirements of U. laevis that may compete better than U. minor under flooding, in siliceous soils and with optimal growing conditions. However, for seedling establishment in calcareous soils, it will be important that the root uptake mechanisms of a species are effective for the acquisition of Fe. Our results indicate that under Fe-limiting conditions U. minor seems to be more Fe-efficient than U. laevis, and hence more competitive. Furthermore, it is likely that fitness and recruitment of calcifuge plants growing in calcareous soils is much reduced due to the occurrence of lime-induced chlorosis. This will yield calcifuge species more susceptible to be displaced by calcicole plants, following a process of natural selection. Thereby, observation of Fe-deficiency symptoms in natural populations is limited to the plants which may be able to withstand a certain degree of Fe-chlorosis when growing in a calcareous, high pH soil at the seedling stage, even though such seedlings may not be competitive.

From the three major root Fe uptake mechanisms characterized in Strategy I plants, we analyzed the root acidification capacity and ferric reductase activity of both elm species in relation to different Fe treatments. Our results suggest that U. minor may be better adapted to Fe-limiting environments (i.e., high rhizosphere pH and a lower Fe supply) than U. laevis. This can be concluded since the root acidification capacity and reductase activity are higher for U. minor, which will subsequently be more efficient in solubilizing and taking up Fe from the rhizosphere. We did not compare the effectiveness of the root ferrous Fe transporter (IRT) between both species, but the increased tissue Mn concentrations determined particularly in U. laevis when subjected to Fe-limiting conditions, may provide indirect evidence for the high activity of this transporter and for the interactions between Fe and Mn nutrition (Conte and Walker, 2011; Marschner, 2012).

Iron deficiency changed the nutrient balance of U. laevis and U. minor seedlings as previously reported for other plant species (e.g., Fernández et al., 2008). We observed that despite growing under Fe-limiting conditions, U. minor was able to preserve a better nutrient balance as compared to U. laevis seedlings, which experienced more remarkable tissue nutrient variations in relation to Fe deficiency with especial regard to Mn, P, B, and Mg. U. minor plants supplied 5 μM Fe reached the largest size, tissue DW and Fe absorption rates, while higher root medium Fe doses improved growth and increased tissue Fe and leaf Chl concentrations in U. laevis. This suggests that the calcicole U. minor may be adapted to lower soil Fe availability levels as commonly found in calcareous, high pH soils in which the solubility of Fe can be extremely low (Lindsay and Schwab, 1982). In contrast, U. laevis appeared to physiologically benefit from receiving a 20 μM Fe concentration via the root system, which may be linked to the generally higher availability of Fe in siliceous soils.

Furthermore, after Fe-resupply, U. minor leaves fully re-greened following a homogeneous pattern in contrast to U. laevis in which re-greening chiefly occurred on the young leaves. The highest leaf and stem Fe concentration increments after Fe-resupply were also recorded for U. minor. Both Chl and tissue Fe results after Fe-resupply may be due to the lower mobility and symplastic uptake of Fe in U. laevis as compared to U. minor.

The root Fe uptake efficiency of U. minor and U. laevis may account for their natural distribution in calcareous and acid soils of the Iberian Peninsula, respectively. However, calcifuge species may be affected by the reduced soil availability of other nutrients such as P or Mn (Larcher, 2003). Additional factors such as water availability and drought resistance (Venturas et al., 2013b) may affect the distribution of elms, but our results suggest that the pollen record found in calcareous areas of Eastern Spain probably belongs to the calcifuge U. minor, since U. laevis is largely restricted to western Spain likely due to its calcifuge nature. In contrast, it cannot be a priori concluded whether the pollen record in Western Spain (non-carbonated soils) exclusively belongs to U. laevis, since calcicole plants may also thrive in siliceous soils.

Our results provide evidence for the important role of Fe as an essential element for growth and survival of a potentially calcifuge species (i.e., U. laevis). Phosphorus deficiency of calcifuge plants growing in calcareous soils has however, been suggested to be a major physiological problem (Tyler, 1996; Zohlen and Tyler, 2004). Soil pH and nutrient availability may determine the ecological distribution of plant species (Viani et al., 2014) but further studies correlating soil chemistry, species distribution and physiology shall be performed for clarifying the calcifuge or calcicole nature of U. laevis and U. minor.

**CONCLUSION**

The different response of U. laevis and U. minor seedlings to Fe-limiting conditions enabled us to interpret their natural
distribution in the soils of the Iberian Peninsula. Results concerning the root ferric reductase activity and proton extrusion capacity, together with tissue mineral element concentrations and plant responses to Fe-supply, provided evidence for the better Fe uptake and homeostasis of *U. minor* as compared to *U. laevis*. The development of hydroponic plant nutrition studies performed with forest species, complemented with field investigations may subsequently prove useful for improving their fitness and establishment during reforestations, and for characterizing nutrient absorption and homeostasis mechanisms of wild plants in relation to their surrounding environment.

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