Effects of iron chlorosis and iron resupply on leaf xylem architecture, water relations, gas exchange and stomatal performance of field-grown peach (*Prunus persica* (L.) Batsch.)

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Abstract

There is increasing evidence suggesting that iron (Fe) deficiency induces not only leaf chlorosis and a decline of photosynthesis, but also structural changes in leaf morphology, which might affect the functionality of leaves. In this study we investigated the effects of Fe deficiency on the water relations of peach (*Prunus persica* (L.) Batsch.) leaves and the responses of previously chlorotic leaves to Fe resupply via the root or the leaf. Iron deficiency induced a decline of maximum potential PSII efficiency (F_V/F_M), of rates of net photosynthesis and transpiration and of water use efficiency. Iron chlorosis was associated with a reduction of leaf xylem vessel size and of leaf hydraulic conductance. In the course of the day, water potentials in chlorotic leaves remained higher (less negative) than in green leaves. In chlorotic leaves, normal stomatal functioning was disturbed, as evidenced by the lack of opening upon withdrawal of external CO_2 and stomatal closure after sudden illumination of previously darkened leaves. We conclude that the Fe deficiency-induced limitations of xylem conductivity elicited a water saving strategy, which poses an additional challenge to plant growth on high pH, calcareous soils. Fertilisation with Fe improved photosynthetic performance but the proper xylem structure and water relations of leaves were not fully restored, indicating that Fe must be available at the first stages of leaf growth and development.

Abbreviations: A, net CO_2 uptake rate; ABA, abscisic acid; C_a, external CO_2 concentration; C_i, internal CO_2 concentration; Chl, chlorophyll; E, transpiration rate; F_0, minimum chlorophyll fluorescence; F_M, maximum chlorophyll fluorescence; K_L, leaf hydraulic conductance; g_S, stomatal conductance; PPFD, photosynthetic photon flux density; RH, relative humidity; WUE, instantaneous water use efficiency; Ψ_L, leaf water potential, $\Psi_{L,\ eq}$, leaf water potential in equilibrium with the supporting branch; Ψ_B, branch water potential;

Introduction

Iron (Fe) is a vital element for plant growth and development, since it is essential for the proper functioning of multiple metabolic and enzymatic processes such as those related to oxygen and electron transport, nitrogen fixation, DNA and chlorophyll biosynthesis and photosynthesis (Briat 2007; Jeong and Guerinot 2009).

Despite the ubiquitous presence of this element in the earth's crust, the low solubility of Fe compounds in many soils especially under high pH, aerobic conditions limits the bioavailability of Fe and induces the occurrence of Fe deficiency symptoms in plants, which are primarily observed in young leaves (Nikolic and Römheld 2003; Kim and Guerinot 2007; Jeong and Connolly 2009). Iron chlorosis is chiefly associated with plant growth on high pH, calcareous soils, and to the presence of high bicarbonate concentrations which can inhibit Fe uptake mechanisms (Lucena 2006; Lucena et al. 2007).

Due to the reduced availability of Fe for plant uptake in many soils, Fe chlorosis is an important limiting factor for crop production in many areas of the world, primarily under arid and semiarid climates (Fernández and Ebert 2005). The occurrence of Fe deficiency is therefore a problem of economic significance, since crop quality and yields can be severely affected, and the use of expensive corrective methods is required to preserve agricultural returns (Fernández and Ebert 2005; Fernández et al. 2008a). Iron supply to the root system chiefly in the form of expensive commercial Fe(III)-EDDHA-based products, is currently the most reliable and widely-used technique to control Fe deficiency under severe soil conditions (Lucena 2006; Rojas et al. 2008). Foliar iron sprays can also help alleviate Fe chlorosis symptoms and can be used as a strategy complementary to the application of root Fe fertilisers (Fernández et al. 2008a; Fernández and Eichert 2009).

As a consequence of the strong reduction of chlorophyll contents, Fe chlorosis markedly reduces the leaf photosynthetic rate (Larbi et al. 2006). Similarly, light absorption, photosystem II and Rubisco carboxylation efficiencies were described to be reduced under Fe deficiency (Larbi et al. 2006). The lower photosynthetic rates of severely Fe chlorotic leaves result in lower stomatal apertures and thus transpiration rates (Larbi et al. 2006; Fernández et al. 2008b). However the reduction in photosynthetic rate was frequently reported to exceed the concomitant reduction of transpiration rates, resulting in a reduced water use efficiency (WUE) as compared to green leaves (e.g., Larbi et al. 2006).

The reasons for the reduced WUE in chlorotic leaves are not yet fully understood, but there is some evidence suggesting that this may at least in part be due to an increased cuticular transpiration rate. In peach leaves, a reduction in abaxial cuticular weight and in the amount of soluble cuticular lipids per unit surface was observed (Fernández et al. 2008b), which might be the reason for the reduced resistance of the cuticle of chlorotic leaves against water loss reported in a number of studies (Hutchinson 1970; Anderson 1984; Fernández et al. 2008b).

On the other hand, there are hints suggesting that in chlorotic leaves the regulation of stomata may be disturbed, a phenomenon which may also contribute to reducing WUE. In a previous study we

observed that detached chlorotic pear and peach leaves, but not green leaves, showed an Iwanoff-effect (Fernández et al. 2008b), i.e. a transient opening of stomata immediately after detachment. This was ascribed to the observed increase in size of epidermal cells, which thus, probably exerted less counter-pressure against the guard cells and enabled the passive opening of stomata after detachment. It is to date not known, however, if and how the altered morphology of leaf epidermal cells affects stomatal control *in vivo*, but it is tempting to speculate that it might interfere with the fine-tuning of stomatal aperture finally resulting in disturbances of water relations.

Effects of nutrient deficiencies on plant water relations, stomatal behaviour or hydraulic architecture have been described for a few plant species, but mostly with regard to nitrogen (N) and phosphorous (P) nutrition (Radin and Parker 1979; Radin and Ackerson 1981; Radin 1990; Bucci et al. 2006; Samuelson et al. 2008; Ward et al. 2008). In a previous study (Fernández et al. 2008b) we observed that in Fe chlorotic leaves the vascular bundle appeared to be disorganised and heterogeneous in size and shape, making it likely that Fe deficiency might also affect leaf hydraulics.

Given the widespread occurrence of Fe deficiency in plants and the significance of Fe nutrition for plant production and physiology, a study was carried out to elucidate the effects of Fe chlorosis, Fe sufficiency and Fe resupply on stomatal functioning, leaf physiology and plant water relations working with peach trees grown on a calcareous, high pH soil. The specific research questions were: (1) Does Fe chlorosis affect the water relations, e.g via a lower WUE, effects on stomatal functionality or effects on hydraulics? (2) Does Fe chlorosis affect the functionality of stomata? (3) Does Fe resupply fully restore the functionality of leaves? Results are of physiological importance and provide insight into the effect of Fe supply via root and foliar application which should be considered to improve the efficiency of Fe fertiliser practices.

Materials and methods

Plant Material

This research was conducted in a commercial orchard located in the Jalón River Valley, in the Zaragoza province, Spain (41°40’ 28’’N, 1°13’34’’W, 278 m above sea level) planted with 14 year-old peach trees (*Prunus persica* (L.) Batsch, cv. Miraflores). Soil was calcareous, with approximately 30% total $CaCO_3$, 10% active $CaCO_3$, 7 mg kg^{-1} DTPA-extractable Fe, 2.6% organic matter and pH 7.8 in water. The flood-irrigated orchard had a planting density of 5 m x 4 m and was appropriately maintained in terms of pest and disease control.

Healthy trees were regularly treated with Fe(III)-EDDHA (100 g per tree Sequestrene 138 Fe G-100, Syngenta Agro S.A., Spain) and remained green for at least 2 seasons prior to the development of the trial. The product was applied by digging a circular 20 -30 cm drench approximately 1 m away from the trunk in May. Iron-chlorotic trees did not receive any exogenous Fe input in the last 3 years and developed Fe deficiency symptoms in springtime. Leaf emergence occurred in April and trees bound to be chlorotic developed strong Fe deficiency symptoms prior to Fe resupply.

On July 1st 2008, 4 previously chlorotic trees were resupplied with Fe, either by root treatment with Fe(III)-EDDHA (100 g per tree applied as described above) or by foliar application to individual branches (2 treatments with a 3-week interval) of 2 mM $FeSO_4$ (Panreac, Barcelona, Spain) in combination with 0.5 g l^{-1} of an organosilicone surfactant (Evonik Goldschmidt GmbH, Essen). Trees were regularly watered every 2 weeks until harvest (i.e., mid September) and leaf re-greening took place in the following weeks, with a more rapid response to the foliar (re-greening visible after 1 week) than to the root treatment (re-greening began to be evident only after 4 weeks). Chlorophyll (Chl) concentrations were estimated with a SPAD Chl meter (SPAD 502, Konica Minolta Sensing Europe B.V., MN Nieuwegein, NL), using 4 measurements per leaf.

Once Fe resupplied leaves were observed to be significantly green, physiological experiments were conducted (from mid August to mid September, 2008) with individuals of a similar maturity stage located at medium size shoots. Fully-expanded, comparable, non-damaged leaves of Fe sufficient (native green), Fe deficient (native chlorotic), root Fe-resupplied (Fe-root) and foliar Fe-resupplied (Fe-foliar) trees were selected. Leaves were of comparable insertion and sun exposition.

It must be stressed that despite re-greening occurred, Fe-resupplied leaves always presented some interveinal yellow patches and did never reach the same homogeneous and glossy appearance of native-green leaves. Severe leaf drop began by mid November, and during the experimental period leaves did not show signs of senescence.

Gas exchange measurements

Gas exchange measurements were performed in fully developed current-year attached leaves of recently flood-irrigated trees with a portable gas exchange system (CIRAS-2, PP Systems, Hitchin, UK). Net CO_2 uptake (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_S, mmol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and internal CO_2 concentration (C_i, μmol mol^{-1}) were registered. Unless otherwise stated, measurements were performed at controlled CO_2 external concentration (C_a = 380 μmol mol^{-1}) and at ambient temperature and relative humidity. Instantaneous WUE was calculated as the ratio A/E. Measurements were repeated at various dates and daytimes.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured on green and chlorotic areas of attached, fully-expanded leaves with a portable pulse amplitude modulation fluorometer FMS-II (Hansatech Instruments Ltd., Norfolk, UK). Plants were covered with a black bag and kept in darkness for 30 - 60 min to estimate the minimum (F_0) and maximum (F_M) Chl fluorescence. F_0 was measured by switching on the modulated light at 0.6 kHz in presence of far-red light (7 μmol m^{-2} s^{-1}) in order to fully oxidize the PSII acceptor side (Morales et al. 1998). Photosynthetic photon flux density (PPFD) was below 0.4 μmol m^{-2} s^{-1} at the leaf surface. F_M was measured at 20 kHz with a 0.8 s pulse of 6000 μmol m^{-2} s^{-1} of white light. The dark-adapted, maximum potential PSII efficiency was calculated as

F_V/F_M (Larbi et al. 2006) for Fe-chlorotic and -sufficient leaves (i.e., native green) and also for green and chlorotic areas of Fe-resupplied leaves. Prior to measurement, the average relative leaf Chl concentration was estimated with the SPAD-meter as described above.

Stomatal responses to external stimuli

Reactivity of stomata was analysed by exerting external stimuli known to induce an increase in stomatal aperture. Two experiments were conducted in which two different stimuli were used:

Experiment 1: Leaves were wrapped for 2 h in aluminium (Al) foil to induce stomatal closure. Thereafter, leaves were unwrapped, exposed to light (PPFD = 2000 µmol m^{-2} s^{-1}) and gas exchange parameters were monitored for 20 min.

Experiment 2: After measuring steady state gas exchange under in situ conditions, C_a was abruptly set to zero and the resulting changes in g_S were monitored for a period of up to 40 min.

Diurnal variations in leaf water potentials

Diurnal variations in leaf water potentials (Ψ_L, MPa) were measured with a Scholander pressure chamber (Scholander et al. 1965), following the methodological procedures described by Turner (1988). Measurements were performed at 9 a.m. 11 a.m. and 4 p.m. local time.

Estimation of leaf hydraulic conductance

The hydraulic conductance of the leaf (K_L) in previously heavily transpiring leaves was measured by a modified evaporative flux method (Wullschleger et al. 1998, Sack et al. 2002). K_L was calculated from the ratio between the whole leaf evaporative flux (E, mmol m^{-2} s^{-1}), and the pressure gradient established across the hydraulic pathway, i.e the difference between the water potentials of the leaf ($\Psi_{L,}$ MPa) and of the adjacent branch (Ψ_B, MPa), standardised by the leaf area (Sisó et al. 2001). Since the water vapour flux was estimated from g_S values (Pearcy et al. 1991), E values were obtained from g_S measurements taking into account the average leaf area and the water vapour mole fraction differences between the evaporating leaf surface and the bulk air (ΔW, Nardini et al. 1999). In this study, the average leaf and air temperature within the measuring chamber were adjusted to 25 ºC and the relative humidity to 50%.

In the beginning of the experiments, g_S of five sun-exposed green or chlorotic leaves was measured. Immediately thereafter, Ψ_L of one leaf of each group was determined, while 20 to 30 leaves of each group were covered tightly with Al foil to inhibit transpiration and thereby induce the equilibration of Ψ_L with Ψ_B. During the next 4 h the resulting increase in Ψ_L was monitored by regular pressure chamber measurements of wrapped leaves.

The change of Ψ_L (y) over time (x) was plotted and curves of the type $y = y_0 + a \exp (b * x)$ were fit to the data, where y_0 represents the asymptotic water potential reached after equilibration ($\Psi_{L, eq}$), a is

the difference between the initial Ψ_L and $\Psi_{L,eq}$, and b is the time constant of exponential rise.

Ψ_B was estimated by two methods, using either $\Psi_{L,eq}$ obtained by curve fitting or the maximum predawn value of Ψ_L observed during the measurement campaign.

Microscopy and determination of cross-sectional area of xylem vessels

Transverse leaf sections were analysed after fixation in FAA (90% ethanol:water, 5% formaldehyde and 5% acetic acid), dehydration, embedding in paraffin and cutting with a microtome. Samples were observed under light microscope (Nikon E 800, Japan) and the structure of the xylem vessels (length, size and area of the 12 largest cells, homogeneity and level of organisation) was analysed using the programme NIS-Elements D (Nikon Corporation, Japan) with 15 repetitions.

Element concentrations in leaves

Mineral element concentrations were estimated 11 weeks after Fe resupply. Leaves were scrubbed in a 0.1% detergent solution and were thoroughly rinsed in 3 baths of double distilled water. Leaf tissues were subsequently dry-ashed prior to mineral element determination by Inductively Coupled Plasma (ICP, Perkin-Elmer, Optima 3000, Analysis Service of CEBAS-CSIC, Murcia, Spain). Only tissue Fe (mg kg^{-1} dry weight, DW) and K (% DW) concentrations of the 4 treatments evaluated are shown since they are especially relevant to indicate the Fe-status (Fernández et al. 2008a).

Data analysis

Statistical analyses were carried out using SPSS 14.0 (SPSS Inc., Chicago, IL). Effects of Fe treatments were analysed by t-tests of by ANOVA followed by a Tukey test where appropriate. Curve fitting was performed with SigmaPlot 10.0 (Systat Software, Inc., San Jose, USA). Standard error (SE) of K_L was calculated based on error propagation according to Sachs (1997). Unless otherwise denoted, data are expressed as means ± SE.

Results

Re-greening of leaves and element concentrations after Fe fertilisation

Supplying Fe via roots or leaves induced in both cases the re-greening of chlorotic peach leaves and of new growing tissues in the case of Fe-EDDHA treated trees. However, the concentration and homogeneous distribution of Chl as observed in healthy native green leaves was never achieved as derived from the yellow areas remaining in Fe resupplied leaves (Fig. 1). Chlorotic leaves had significantly lower tissue Fe concentrations than green leaves. Iron fertilisation via the root restored the Fe concentrations, while foliar Fe application caused an increase above the level of native green leaves (Table 1). On the contrary, K concentrations in native green leaves were significantly lower than in chlorotic leaves, while Fe treated leaves hat intermediate tissue K concentrations (Table 1).

Gas exchange measurements

Gas exchange rates of leaves were monitored at various dates and daytimes between July and September 2008. In Table 2 the diurnal variations of gas exchange parameters in green and chlorotic leaves measured on a typical sunny day in July are displayed. At any time, chlorotic leaves had a significantly lower E, A, g_S and WUE and higher C_i than green leaves. In both treatments a midday-depression of photosynthesis was observed, which was by far more pronounced in chlorotic leaves than in green leaves. In green leaves, the minimum g_S in the afternoon was 67% of the value measured in the morning, whereas in chlorotic leaves g_S dropped to 11% of the morning value.

Table 1 shows the effects of Fe fertilisation as measured 10 weeks after application. In chlorotic leaves all parameters were again significantly lower and C_i was higher than in green leaves. Leaves resupplied with Fe had significantly higher A, g_S and WUE values and lower C_i values than chlorotic leaves. In leaves treated with foliar Fe sprays, A and WUE were significantly lower than in native green leaves, whereas E, g_S and C_i were not significantly different. In leaves of trees to which Fe had been applied to the roots, all photosynthesis parameters were not significantly different from those of native green leaves, except for C_i which was significantly lower after root application.

It has to be considered, however, that due to the patchy re-greening of leaves after Fe treatment, which probably also induced a patchiness of stomatal apertures, the respective C_i values might be somewhat overestimated. Subsequently, for the more correct interpretation of gas exchange data, Chl fluorescence values for green and chlorotic areas of re-greened leaves were also obtained (Table 3).

Diurnal variation of leaf water potentials

A typical daily pattern of Ψ_L is shown in Fig. 2. In the morning (9 a.m.), Ψ_L was equal in all treatments and ranged from -0.79 to -1.00 MPa. Before midday (11 a.m.), Ψ_L in native green and Fe-treated leaves had dropped to -1.32 to -1.46 MPa, whereas in chlorotic leaves Ψ_L remained significantly higher (-1.06 MPa). In the afternoon (4 p.m.) native green leaves had the lowest Ψ_L of all treatments (-2.06 MPa), whereas in the other treatments Ψ_L remained at a significantly higher level (-1.25 to –1.53 MPa).

Chlorophyll fluorescence measurements

As expected from the low SPAD values measured, F_V/F_M values of chlorotic leaves were statistically lower than green native ones (Table 3). On the other hand, green areas of Fe-resupplied leaves also had increased F_V/F_M values, which were in the same range as native green leaves (Table 3). However, re-greened leaves failed to be homogeneous and the remaining chlorotic areas showed significantly lower F_V/F_M values, which were especially remarkable in Fe-sprayed leaves (Table 3).

Stomatal reactions on illumination of previously darkened leaves

After darkening leaves for 2 h, light was turned on and gas exchange was monitored. In Fig. 3 it can be seen that native green and Fe fertilised leaves (both types of application) responded with a transient decrease, followed by a more or less pronounced increase in g_S. On the contrary, in native chlorotic leaves g_S temporarily increased and then dropped to values of around 50% of the initial value.

Fig. 4 shows that in chlorotic leaves the decrease in C_i, which was caused by the light-induced activation of A (data not shown), was accompanied by a decrease in g_S, whereas in all other treatment the decrease of C_i coincided with an increase in g_S.

Stomatal reactions on withdrawal of external CO_2

After measuring steady state gas exchange under in situ conditions, external CO_2 was abruptly set to zero and the resulting changes in g_S were monitored for 20 minutes (Fig. 5). In a range of individual measurements it was observed that native green leaves responded with a pronounced increase in g_S, whereas native chlorotic leaves did hardly react. The effects of Fe fertilisation were variable, but usually reactions of Fe-treated leaves were in-between native chlorotic and native green leaves. An example for this behaviour is shown in Fig. 5. Here, CO_2 withdrawal caused an increase in g_S by ca. 50% in the native green leaf within 20 min, whereas the native chlorotic leaf did hardly respond. The reaction of leaves treated with foliar Fe was comparable to that of chlorotic leaves, whereas leaves of root-Fe-treated trees reacted similarly to native green leaves.

Leaf hydraulic conductivity

Fig. 6 shows the time course of Ψ_L within 4 h after the elimination of leaf transpiration. In green leaves the Ψ_L recovered much quicker than in native chlorotic leaves. The calculation of K_L yielded different values depending on how Ψ_B was estimated. Using the maximum Ψ_L observed at the day of measurement as an estimator yielded a K_L of 11.8 ± 0.4 mmol m^{-2} s^{-1} MPa^{-1} in green leaves and 6.8 ± 0.1 mmol m^{-2} s^{-1} MPa^{-1} in chlorotic leaves, values that are within the range described by Sack et al. (2002) and Brodribb and Holbrook (2003) for different plant species. Using $\Psi_{L,eq}$ obtained by curve fitting resulted in 12.3 ± 0.4 mmol m^{-2} s^{-1} MPa^{-1} in green and 7.6 ± 0.2 mmol m^{-2} s^{-1} MPa^{-1} in chlorotic leaves.

Xylem architecture

Fig. 7 shows cross-sections of the central vein of all treatments, in which the xylem vessels can be identified as radial bundles forming a semi-cycle. In the central vein of chlorotic leaves (Fig. 7B) the size of the entire xylem system was much smaller than in the mid rib of green leaves (Fig. 7A) and the walls of the individual xylem vessels appeared much thicker. The size of the entire xylem system in of the central rib of Fe-treated leaves (Fig. 7C,D) was in-between the respective size in veins of native

green and chlorotic leaves. In addition, it can be said the Fe-resupply increased to some extent the size of xylem vessels, which, however remained heterogeneous in shape and disposition (Fig. 7C,D) versus the structure of the "native green" mid-rib vascular bundle (Fig. 7A).

These microscopic observations are corroborated by measurements of the size and shape of xylem cross-sections (Table 4). Average cross-sectional areas of xylem vessels were significantly different between the treatments. In chlorotic leaves the average cross-sectional area of the largest xylem vessels was only 53% of that in green leaves, whereas in the central vein of Fe-resupplied leaves (via foliar or root Fe application) the areas were 61% and 78% of those recorded for the xylem vessels of green leaves, respectively. The same trend was observed in the lengths of xylem bundles, which were reduced by 40 %, 25% and 4 % in chlorotic, leaf and root treated leaves, respectively. Xylem cells of native green leaves were almost circular, whereas in other treatments, particularly in native chlorotic and after Fe fertilisation via the roots, the shape was more elliptical.

Discussion

Iron deficiency chlorosis is a common disorder affecting the physiology of plants. Despite many metabolic and physiological studies regarding the response of plants to Fe shortage have been carried out in the last decades, especially with annual plants, little is known about the effect of this physiological disorder on plant anatomy and morphology and the resulting implications on plant water relations.

While looking at plant hydraulics, gas exchange and leaf structure, we observed a broad range of physiological disorders in chlorotic peach leaves. Some of them have been reported previously (e.g. Larbi et al. 2006; Fernández et al. 2008b), such as the lower F_V/F_M values, lower rates of A, g_S and WUE, and higher C_i values as compared to green leaves. However, we found that Fe chlorosis led to disorders which cannot be explained by direct effects of reduced chlorophyll contents. These comprise stomatal reactions to external stimuli, which were obviously independent of CO_2 assimilation requirements and point to physiological alterations of stomatal control, as well as alterations in xylem vessel morphology, leaf hydraulic conductance and leaf water potentials. Collectively, these new findings suggest that Fe deficiency induced fundamental disturbances in the water relations of peach leaves.

Transpiration rates (E) were significantly lower in chlorotic leaves than in green leaves (Tables 1, 2), and in addition, E and g_S exhibited a pronounced midday (and afternoon) depression in chlorotic leaves (Table 2). Water potentials in chlorotic leaves, however, remained on a significantly higher level than in green leaves (Fig. 2), indicating that the midday depression of E and g_S in chlorotic leaves was not a direct consequence of a water shortage. Most likely, the reduction of transpiration rates was a consequence of a hindered resupply of water via the xylem. Microscopic examination of central vein cross-sections revealed that in chlorotic leaves the xylem system, which comprises a major resistance of water transport inside the leaf (Sack et al. 2004; Sack and Holbrook 2006), was underdeveloped

(Fig. 7, Table 4). We found that these morphological changes substantially hindered the water supply of the leaves, as evidenced by the rehydration kinetics (curve shape and slope) determined for green and chlorotic leaves (Fig. 6), which resembled the pattern reported by Brodribb and Holbrook (2003) for high and low conductivity *Simarouba glauca* leaves, respectively.

There is a body of literature supporting the conclusion that stomatal conductance can be directly controlled by plant hydraulics (e.g., Sperry 2000; Meinzer 2002; Brodribb and Jordan 2008). Nardini et al. (2001), for example, reported that in *Prunus laurocerasus* the onset of stomatal closure correlated with the onset of cavitation events in the xylem. According to the concept of "water balance" as used by Nardini et al. (1999), the ratio of K_L of green *versus* chlorotic leaves and the ratio of E between them, which reached a quite similar value (ca. 2 for both parameters), would indicate the need to reduce the evaporative flux in chlorotic peach leaves in order to prevent cavitation events associated with an excessive water potential drop thorough the xylem vessels of the leaf veins (Nardini et al. 2001).

Furthermore, we found evidence that leaf chlorosis was also associated with alterations in stomatal reactivity to CO_2 and light. Chlorotic leaves did not respond to the withdrawal of external CO_2 (Fig. 5), and exposure to high light intensities caused stomatal movements in which the phases were reversed as compared to green leaves: Immediately after illumination stomata opened transiently and closed thereafter (Fig. 3). This is an odd stomatal behaviour, because one would rather expect a similar, but less pronounced reaction, i.e. a lower degree of opening in chlorotic than in green leaves due to their lower photosynthetic capacity, or at best, the absence of any stomatal reaction. The closure of stomata after illumination is even more unexpected because (i) this was accompanied by a substantial increase in A and a drop in C_i (Fig. 4), i.e. stomata closed in spite of an increased CO_2 demand of the leaves and although (ii) Ψ_L in chlorotic leaves were much higher than green leaves (Fig. 2) indicating that there was no absolute water shortage which could directly prevent the normal opening reaction of stomata (Hetherington 2001; Roelfsema and Hedrich 2005). Moreover, the closure of stomata as a reaction to illumination of leaves cannot be explained simply by the absence of the normal reaction to external stimuli, suggesting the involvement of an overruling closing signal. Plant stress is known to increase the sensitivity of guard cells to ABA (Hartung and Slovik 1991; Pou et al. 2008) and it was shown previously that Fe deficiency is associated with increased levels of ABA in maize plants (Battal et al. 2003). Therefore it is likely that the closure of stomata in Fe chlorotic peach leaves after illumination was caused by increased concentrations of ABA and/or a higher ABA sensitivity of guard cells. Further studies are required to substantiate this hypothesis.

Taken together, the findings that in chlorotic leaves (i) the xylem was underdeveloped, (ii) the water potentials were sustained on a significantly higher level than green leaves, (iii) stomata nevertheless closed in the course of the day and (iv) did not respond to stimuli known to induce opening strongly support the hypothesis that Fe deficiency activated a water-conservative strategy, probably triggered by the reduction of hydraulic conductivity. It is clear that such a strategy, in which the danger of

xylem cavitation due to limited water supply overrules the need for carbohydrate production, which is already strongly reduced due to the lack of Chl, presents an additional challenge for plant life under Fe deficiency.

The question remains, why xylem development was disturbed in Fe chlorotic plants. One simple explanation could be that because of the much lower photosynthetic productivity and the resulting reduction of carbohydrate availability less material was available for the build up of normal leaf structures. This explanation is in line with the increase in xylem bundle size observed after Fe supply of previously chlorotic leaves (Table 4, Fig. 7). It remains to be elucidated, if this was associated with overall leaf growth, which was not systematically evaluated in the present study. A more specific effect of Fe deficiency on xylem development could be a reduced synthesis of lignin, since Fe is an essential activator or co-factor in polyphenoloxidases and peroxidases (Quiroga et al. 2000). Further research on the presence, amount and localization of lignin in our system as well as on Fe-dependent activities of enzymes involved in lignification and thus proper xylem development should clarify this point.

Resupplying chlorotic leaves with Fe either as foliar sprays or root treatments, significantly improved tissue Chl concentrations and subsequently CO_2 assimilation and Chl fluorescence parameters. Re-greened leaves had a behaviour somewhere in between the one observed for native green and chlorotic leaves with regard to CO_2 assimilation, stomatal conductance, Chl fluorescence and xylem morphology, root treatments proving more effective in restoring leaf physiological parameter as compared to foliar Fe sprays. However, leaves treated with Fe in July, i.e. on the presence of Fe deficiency symptoms, never reached the glossy and homogeneous appearance of native green trees, neither the proper structure of the vascular bundle, indicating that Fe must be available for plant use at the first stages of leaf growth and development as suggested by Fernández et al. (2008b). Foliar Fe supply in combination with an organosilicone surfactant (Fernández et al. 2008a) led to significant re-greening and high tissue Fe concentrations, but the physiology of leaves remained closer to the performance of chlorotic ones. Results suggest that Fe was more bioavailable when supplied to the root as a stable Fe chelate as compared to the more local effect of foliar Fe sprays (Fernández et al. 2008a). In conclusion, our results show that Fe deficiency symptoms go far beyond the well-documented direct effects on Chl concentrations and photosynthetic performance. We found evidence for significant alterations of stomatal functionality and leaf hydraulic relations, which limit the photosynthetic performance in addition to the direct Chl dependent constraints. Feeding Fe after the appearance of deficiency symptoms may correct the direct effects of Chl depletion, but will probably be less effective in the restoration of proper leaf hydraulics. Our results thus suggest that Fe fertilisation must be timed early in the year, best before leaf initiation.

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Table 1. Effects of Fe fertilisation on gas exchange parameters and leaf Fe and K concentrations. Net photosynthesis (A, in μmol m^{-2} s^{-1}), transpiration rate (E, in mmol m^{-2} s^{-1}), stomatal conductance (g_S, in mmol m^{-2} s^{-1}) internal CO_2 concentrations ($C_{i,}$, in μmol mol^{-1}) water use efficiency (WUE, in mol CO_2 $mmol^{-1}$ H_2O), and leaf Fe (in mg kg^{-1} dry mass) and K concentrations (in % of dry mass) were measured in native green, native chlorotic, foliar Fe-treated and root Fe-treated leaves 10 weeks after the beginning of the experimental period. Measurements were taken at 2-3 p.m., when the prevailing environmental conditions were: T = 28-30°C, PPFD = 1.7-2.0 mmol m^{-1} s^{-1}, RH = 25-27%. Means ± SE are shown. Within a column values followed by the same letter are not significantly different (Tukey test, p = 0.05, n = 5).

Treatment	A	E	g_S	C_i	WUE	Fe	K
Green	15.9 ± 1.4 c	6.2 ± 0.5 b	529 ± 124 b	335 ± 3 b	2.6 ± 0.1 c	118 ± 9 b	1.51 ± 0.12 a
Chlorotic	4.0 ± 0.7 a	4.4 ± 0.3 a	243 ± 26 a	365 ± 5 c	0.9 ± 0.1 a	95 ± 8 a	3.19 ± 0.10 d
Fe-foliar	12.0 ± 0.3 b	5.6 ± 0.2 ab	450 ± 28 b	329 ± 1 b	2.2 ± 0.1 b	212 ± 3 c	2.43 ± 0.01 c
Fe-root	13.5 ± 0.8 bc	5.4 ± 0.2 ab	419 ± 28 b	306 ± 4 a	2.5 ± 0.1 bc	111 ± 9 ab	1.95 ± 0.06 b

Table 2. Diurnal variation of irradiance and gas exchange parameters measured in green and chlorotic leaves 2 weeks after the onset of the experiment. Photosynthetic photon flux density (PPFD, in mmol m^{-2} s^{-1}), transpiration rate (E, in mmol H_2O m^{-2} s^{-1}), net photosynthesis (A, in µmol CO_2 m^{-2} s^{-1}), internal CO_2 concentrations ($C_{i,}$, in µmol mol^{-1}), water use efficiency (WUE, in µmol CO_2 $mmol^{-1}$ H_2O), stomatal conductance (g_S, in mmol m^{-2} s^{-1}) and stomatal conductance relative to the value measured at 10 a.m. ($g_{S,\ rel}$ in %) are shown. Leaf temperatures ranged from 20.4 °C to 25.0 °C and were not significantly different between the treatments. Means ± SE are shown. Asterisks indicate significant differences between the leaf types at a given time (t-test, n = 3-4, n.s.: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Time of day	Leaf type (SPAD value)	PPFD	E	A	C_i	WUE	g_S	$g_{S,\ rel.}$
10	Chlorotic (6.8 ± 1.3)	1.08 ± 0.02	0.94 ± 0.02	4.6 ± 0.4	215 ± 18	5.0 ± 0.6	59 ± 2	(100)
	Green (36.8 ± 0.4)	1.08 ± 0.03	1.78 ± 0.40	17.6 ± 0.6	148 ± 22	11.2 ± 3.0	125 ± 15	(100)
	***	n.s.	*	***	*.	*	*	
13	Chlorotic (10.0 ± 1.0)	1.85 ± 0.01	0.83 ± 0.31	2.2 ± 0.6	382 ± 4	2.2 ± 0.1	16 ± 7	27 ± 13
	Green (36.9 ± 0.9)	1.86 ± 0.03	2.02 ± 0.21	19.0 ± 1.1	71 ± 9	9.6 ± 0.6	117 ± 14	94 ± 11
	***	n.s.	*	***	***	***	**	*
16	Chlorotic (8.7 ± 1.3)	2.00 ± 0.02	0.53 ± 0.17	0.9 ± 0.4	327 ± 33	2.5 ± 1.3	7 ± 7	11± 11
	Green (37.4 ± 1.0)	2.00 ± 0.02	1.68 ± 0.12	17.4 ± 1.3	91 ± 17	10.3 ± 0.5	84 ± 10	67 ± 8
	***	n.s.	**	***	**	**	**	**
18	Chlorotic (9.9 ± 0.6)	1.38 ± 0.02	0.57 ± 0.15	2.0 ± 0.4	304 ± 43	4.5 ± 1.4	25 ± 6	42 ± 10
	Green (39.1 ± 0.8)	1.38 ± 0.03	1.68 ± 0.06	16.9 ± 0.3	87 ± 8	10.1 ± 0.6	86 ± 3	69 ± 6
	***	n.s.	***	***	**	**	***	*

Table 3. SPAD units and F_V/F_M values of native green, native chlorotic, foliar Fe-treated and root Fe-treated leaves 12 weeks after the beginning of the experimental period. Data are means ± SE (n = 10). Within a column values followed by the same letter are not significantly different (Tukey test, p = 0.05,).

Treatment	Leaf areas	SPAD units	Fv/Fm
Native green	Green	41.6 ± 1.0 b	0.850 ± 0.018 d
Native chlorotic	Chlorotic	6.0 ± 1.2 a	0.702 ± 0.021 a
Fe-foliar	Green	31.9 ± 1.2 b	0.817 ± 0.021 cd
Fe-foliar	Chlorotic	17.0 ± 1.2 b	0.764 ± 0.021 b
Fe-root	Green	40.8 ± 1.2 b	0.835 ± 0.021 cd
Fe-root	Chlorotic	18.1 ± 1.1 b	0.777 ± 0.019 bc

Table 4. Length of the xylem bundles and size, shape and area of the largest xylem vessel observed in transverse leaf mid-vein leaf sections. Xylem cells were approximated as ellipses and the axes (a, b) were used to calculate ellipticity and area. Green and chlorotic leaves and leaves resupplied with Fe by foliar or root application were examined 12 weeks after the beginning of the trial. Data are means ± SE (n = 10). Within a column means marked by the same letter are not significantly different (Tukey test, p = 0.05).

Treatment	Length of xylem bundle (μm)	Major axis a (μm)	Minor axis b (μm)	Ellipticity a/b	Area (μm^2)
Green	124.8 ± 3.3 c	20.5 ± 0.1	18.5 ± 0.5	1.12 ± 0.02 a	297.9 ± 10.4 d
Chlorotic	75.2 ± 5.9 a	15.8 ± 0.1	12.8 ± 0.8	1.23 ± 0.03 b	158.8 ± 2.0 a
Fe-foliar	93.9 ± 2.7 b	15.9 ± 0.1	13.6 ± 0.1	1.17 ± 0.03 ab	169.8 ± 2.1 b
Fe-root	120.1 ± 9.1 c	19.7 ± 0.2	14.2 ± 0.1	1.39 ± 0.04 c	219.7 ± 3.5 c

Figure legends

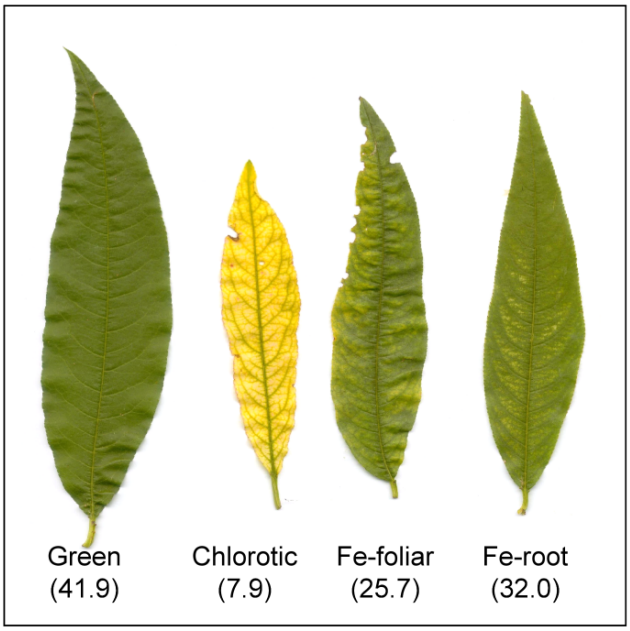

Fig. 1. Representative images of leaves used for physiological measurements 11 weeks after the beginning of the trial. Trees were either regularly supplied with Fe-EDDHA ("green") or untreated ("chlorotic") for three years before the measurement, or previously chlorotic leaves were resupplied with foliar-applied $FeSO_4$ ("Fe-foliar") or root-applied Fe-EDDHA ("Fe-root"). Values in brackets are SPAD values.

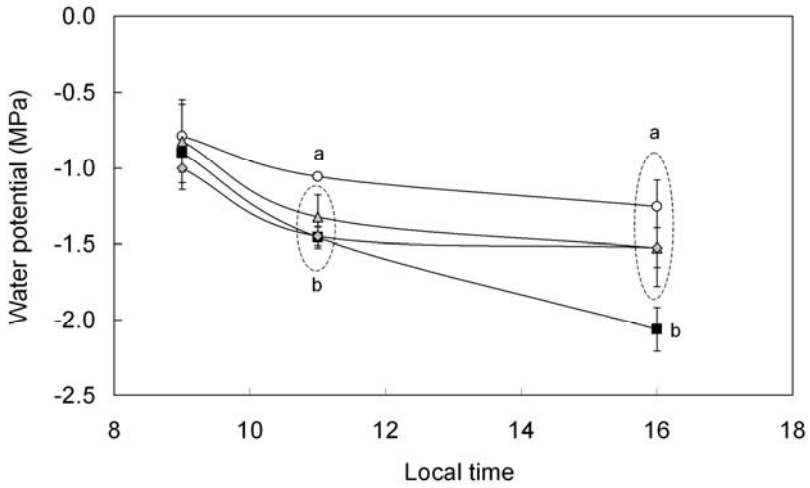

Fig. 2. Time course of leaf water potentials in native green (squares) or chlorotic leaves (circles) and in leaves of trees which were treated with Fe-EDDHA 11 weeks before the measurements via the leaves (triangles) or roots (rhombs). Values marked by different letters are significantly different (Tukey test, p = 0.05, n = 5).

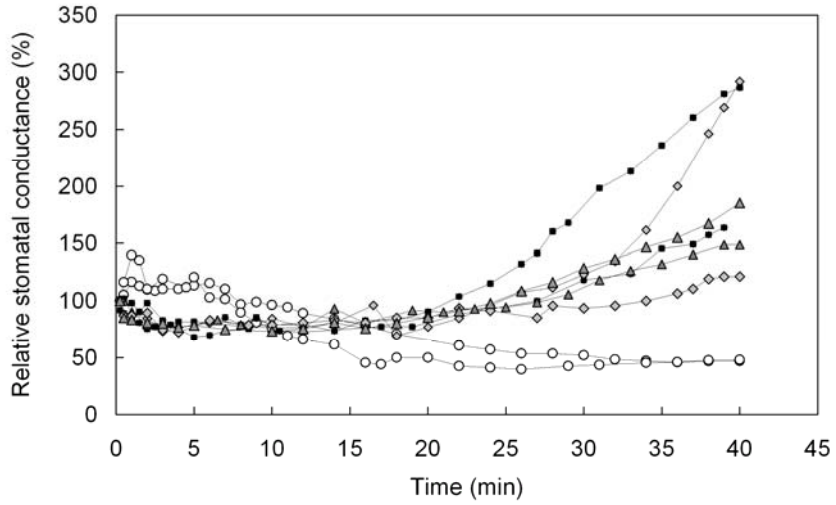

Fig 3. Time course of relative stomatal conductances of leaves exposed to light (PPFD = 2000 µmol m^{-2} s^{-1}) after 2 h in darkness measured in native green (squares) or chlorotic leaves (circles) and in leaves of trees which received Fe via the leaves (triangles) or roots (rhombs) 12 weeks before. Values are expressed in percent of the initial value measured at the onset of illumination (t = 0).

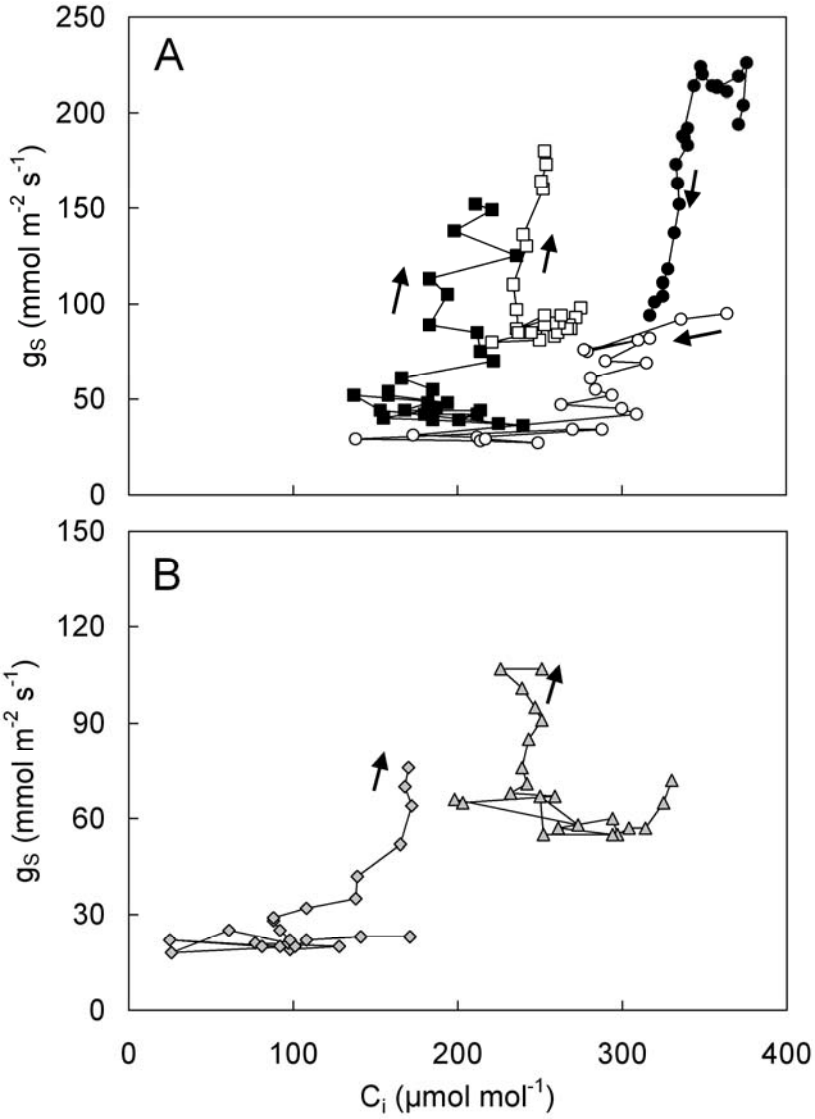

Fig. 4. Response curves of stomatal conductance (g_S) to internal CO_2 concentrations (C_i) in leaves previously darkened and subsequently exposed to light (PPFD = 2000 µmol m^{-2} s^{-1}). (A) native green (squares) and native chlorotic leaves (circles). (B): Leaves of trees fertilised with Fe by root (rhombs) or leaf application (triangles). Arrows indicate the time course of response. Measurements were carried out 11 weeks after Fe-resupply.

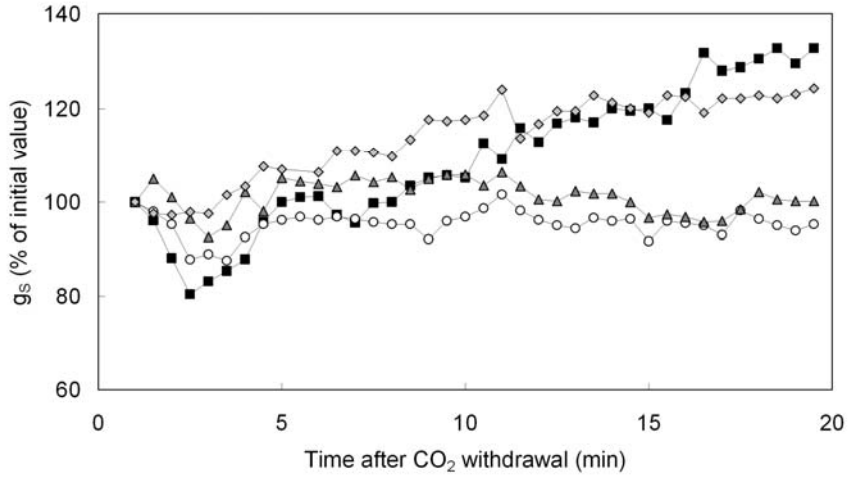

Fig. 5. Relative changes of stomatal conductance (g_S) after withdrawal of external CO_2 measured 10 weeks after beginning of the trial, in native green (squares) or native chlorotic (circles) leaves and in leaves after Fe supply to leaves (triangles) or roots (rhombs). The value of g_S measured after 60 s of CO_2 withdrawal was set to 100%.

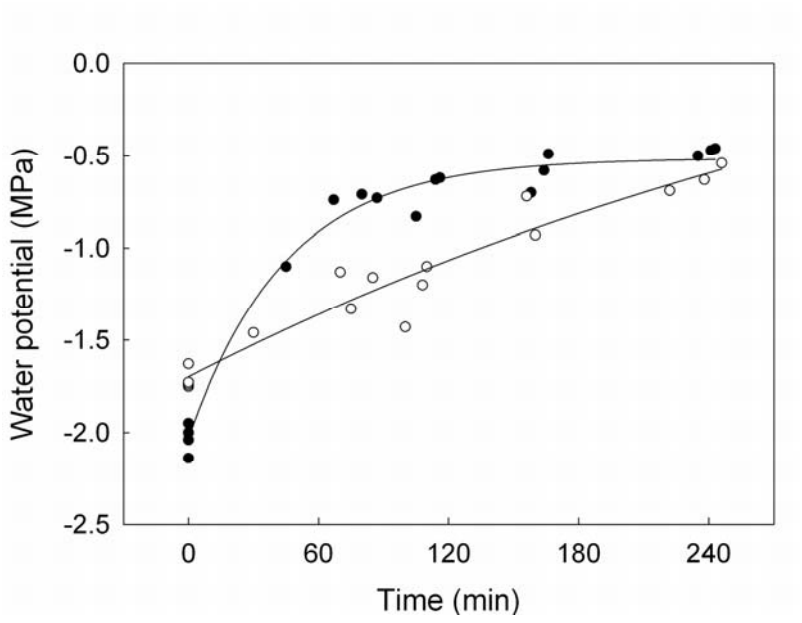

Fig. 6. Time course of recovery of leaf water potential in native green (filled circles) and chlorotic leaves (open circles) after cease of transpiration measured 10 weeks after beginning of the trial. At t = 0 leaves were tightly covered in aluminium foil and total leaf water potential was measured using a pressure chamber.

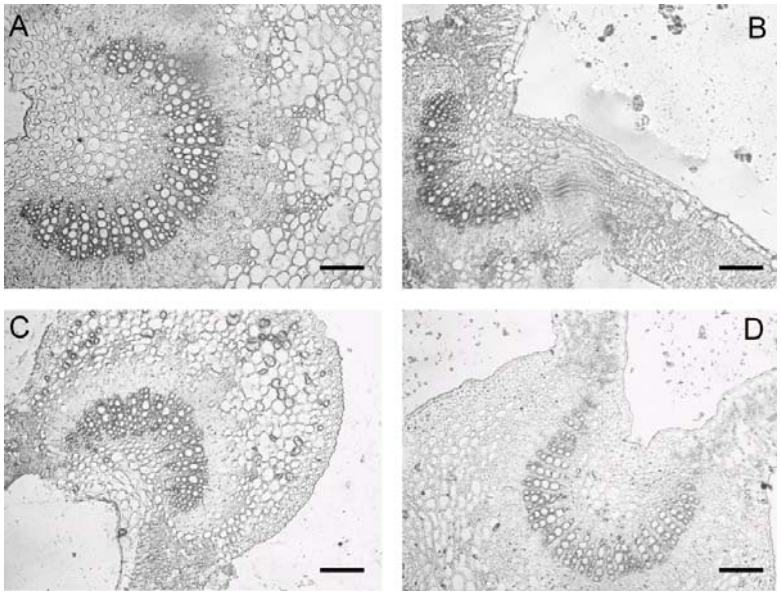

Fig 7. Cross sections of the mid vein taken at ½ of the leaf length. Xylem vessels form radial bundles which are arranged in semi-circles around the centre of the mid rib. Samples were taken 11 weeks after the beginning of the experimental period. (A) native green, (B) native chlorotic, (C) Fe applied to the leaves, (D) Fe applied to the roots. Bars: 100 µm.