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REGULAR ARTICLE

Effects of individual and combined metal foliar fertilisers on iron- and manganese-deficient *Solanum lycopersicum* plants

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Abstract
Aims Using Fe- and Mn-deficient *Solanum lycopersicum* plants, we investigated the effects of Fe and Mn foliar fertilisers, either individually or combined, on plant biomass, leaf chlorophyll and metal micronutrient levels in leaves and roots.
Methods Plants were grown in hydroponics with different combinations of 0 or 45 μM Fe and 0 or 4.6 μM Mn. Foliar fertiliser formulations (9 mM $FeSO_4$ and/or 3 mM $MnSO_4$, supplemented with a surfactant) were applied in three consecutive doses. Fertilisation was applied to the first three leaf levels, while upper leaf levels were left untreated.
Results Iron and Mn deficiency led to characteristic symptoms. Foliar treatments increased concentrations of Fe and Mn, biomass and chlorophyll in treated leaves, although re-greening was incomplete. Approximately 11–12 % of the Fe increase was in roots (likely mediated via phloem transport), but no Fe increase occurred in untreated leaves. Regarding Mn, a 2 % increase occurred in untreated leaves, but no increase occurred in roots.
Conclusions Iron fertilisation was effective not only in leaves treated with the fertiliser but also in roots, whereas Mn fertilisation had major effects on treated leaves and minor effects on untreated ones. The combined application of Fe + Mn was not detrimental to Fe- or Mn-deficient plants.

Keywords Foliar fertilisation · Iron · Manganese · Multi-elemental fertilisers · Tomato

Abbreviations

Chl	Chlorophyll
EDTA	Ethylenediamine tetraacetic acid
ROS	Radical oxygen species
SPAD	Soil–plant analysis development

Introduction

Foliar fertilisation is an important agricultural practice for the sustainable and productive management of crops, where nutrients are applied directly to the plant foliage, including leaves and stems (Fernández et al. 2013). Foliar fertilisation has often been used to correct deficiencies of micronutrients, such as Fe, Mn, Cu and Zn (Fernández and Brown 2013). There is an increased

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interest in foliar fertilisation, since when applied adequately it can correct nutrient deficiencies in a more rapid and efficient way than soil fertilisation. This agronomic practice involves lower inputs than those used in soil fertilisation and therefore can be economically profitable and environmentally friendly (Álvarez-Fernández et al. 2003; Fernández and Eichert 2009).

The application of micronutrients to leaves can increase both yield and quality of produce in many crops, including vegetables (Roosta and Mohsenian 2012). cereals (Cakmak et al. 2010; He et al. 2013) and fruit trees (Papadakis et al. 2007; Hasani et al. 2012). especially when nutrient availability in the soil is low due to high pH, low organic matter content or high Ca carbonate content (Fernández and Ebert 2005; Rodríguez-Lucena et al. 2010). Interaction between elements included in the fertiliser formulations is a factor that may influence the plant availability of nutrients. The interaction between Fe and Mn at the root level has been extensively studied, and the antagonistic effect between Mn(II) and Fe(II) in root uptake (Heenan and Campbell 1983; Drakatos et al. 2002; Moosavi and Ronaghi 2011) is now supported by molecular evidence that both metals share membrane transporters (Morrissey and Guerinot 2009; Socha and Guerinot 2014). However, the possible interactions between micronutrients when using foliar fertilisers have been little studied.

Iron and Mn are essential nutrient elements that play key roles in plants. Iron is involved in chlorophyll (*Chl*) synthesis, photosynthetic electron transport processes and protection against radical oxygen species (ROS; Morales et al. 1998; Broadley et al. 2012). whereas Mn participates in photosynthesis and also in ROS detoxification as a component of Mn-superoxide dismutase (Shenker et al. 2004; Broadley et al. 2012). The first symptoms of leaf chlorosis due to Mn deficiency may be similar to those of Fe deficiency, and both often occur in high-pH soils. Farmers should try to resolve micronutrient deficiencies at an early stage, but since an accurate diagnosis can be difficult, they may choose to apply fertiliser formulations containing a combination of nutrients as a precautionary practice. In the numerous studies on foliar fertilisation published until now, there is only limited information about the efficiency of multi-elemental foliar products (Fernández et al. 2013; Singh et al. 2013; Rawashdeh and Florin 2015). The effects of individual and combined foliar fertilisation with several micronutrients (Fe, Mn and Zn) have been studied recently in *Triticum aestivum*. In one case, results indicated that the application of Fe, Mn and Zn as a combined treatment decreased yield components significantly, while the application of Fe and Mn in combination resulted in the best yields (Bameri et al. 2012). In a separate study, the application of Fe + Mn or Fe + Mn + Zn led to the best grain yields (Zain et al. 2015). Two further studies investigated the effects of foliar applications of Fe, Mn, Zn and B, either individually or combined, on *Solanum lycopersicum* and *Beta vulgaris* growth parameters. Both studies concluded that the multi-elemental foliar fertilisation (Fe + Mn + Zn + B) produced the highest yield (Naga Sivaiah et al. 2013; Gobarah et al. 2014). However, a study has questioned the effectiveness of multi-elemental foliar fertilisation (Castillon and Le Souder 2011). When Cu and Mn foliar treatments were applied individually in cereals, there was a significant increase in yields, whereas the combination of both elements was detrimental to grain production (Castillon and Le Souder 2011). Therefore, to achieve a better understanding of foliar fertilisation with micronutrients, it is necessary to assess whether the possible interactions between micronutrients may affect their uptake, transport and functionality in the plant.

The goal of this study was to test the hypothesis that using a combination of Fe and Mn in foliar fertilisation could decrease the treatment efficiency in Fe- and Mn-deficient *S. lycopersicum* plants. We investigated the effect of individual and combined Fe and Mn foliar treatments on Fe-deficient, Mn-deficient and Fe-, Mn-deficient plants in terms of biomass, leaf chlorosis recovery and metal concentrations and total contents in leaves and roots.

Materials and methods

Plant material and experimental design

Tomato (*Solanum lycopersicum* Mill cv. Tres Cantos) plants were grown in a growth chamber with a photosynthetic photon flux density at leaf height of 400 μmol m^{-2} s^{-1} photosynthetically active radiation, 70 % relative humidity and a 16-h, 25 °C/8-h, 25 °C, day/night regime. Seeds were germinated and grown in vermiculite for 10 days in germination solution (5 % Hoagland in distilled water) in two different conditions, one for Fe-deficient and control plants and another (without Mn) for Mn-deficient plants. Seedlings were pre-grown in 10-L boxes (30 plants per box) for an

additional 10-day period in half-strength Hoagland nutrient solution (in mM: 7.5 NO_3^-, 1 PO_4^{-2}, 1.05 SO_4^{-2}, 3.5 K, 2.5 Ca, 1 Mg; and in μM: 23.2 B, 1.2 Zn, 0.185 Cu, 0.06 Mo, 4.6 Cl, 46 Na). Pre-growth solutions contained 45 μM Fe and 4.6 μM Mn for Fe-deficient and control plants and 45 μM Fe and 0 μM Mn for Mn-deficient and Fe-,Mn-deficient plants. The source of Fe was Fe(III)-ethylenediamine tetraacetic acid (EDTA). Plants were then grown for 9 more days in 4-L plastic pots (four plants per pot) containing half-strength Hoagland solution, pH 5.5, with 0 or 4.6 μM Mn and 0 or 45 μM Fe. Therefore, treatments were 45 μM Fe/0 μM Mn (hereafter called -Mn), 0 μM Fe/4.6 μM Mn (-Fe), 0 μM Fe/0 μM Mn (-Fe,-Mn) and 45 μM Fe/4.6 μM Mn (Fe + Mn). The Mn and Fe concentrations in the nutrient solutions of the Mn- and Fe-deficient plants were between 30 and 100 nM, respectively (approximately 2–6 μg L^{-1}, measured using inductively-coupled plasma mass spectrometry (ICP-MS).

Foliar fertilisation was carried out with a total of 1.8 mL per plant in three consecutive doses at days 9, 10 and 13 after imposing the nutrient solution metal treatments (in each individual treatment event, 600 μL were applied per plant). The fertiliser solutions contained 9 mM $FeSO_4$ and/or 3 mM $MnSO_4$, supplemented with 0.2 % of a non-ionic, organo-silicon surfactant (Break-Thru S 233, Evonik Industries AG, Essen, Germany). An Fe/Mn ratio of 3/1 was used in the combined treatments because the Fe/Mn ratio in young leaves of *S. lycopersicum* in the conditions used is approximately 2.5 (see below). The concentration of Fe used in this study, 9 mM, was higher than the 2 mM concentration used in previous foliar fertilisation studies by our group and others (Rombolà et al. 2000; Fernández et al. 2006; El-Jendoubi et al. 2014). We used 9 mM $FeSO_4$ because in a number of experiments with different plant species (not shown) we confirmed that when applied in combination with appropriate surfactants this higher Fe concentration was not toxic to leaves. A total of 905 μg Fe and 297 μg Mn per plant was applied summing up the three fertilisation events, and this is in line with the total metal contents in control plants (924 μg Fe and 393 μg Mn; Table 2).

The fertiliser was applied to the first three leaf levels (L1–L3) of metal deficient-*S. lycopersicum* plants using a paintbrush on both the adaxial and abaxial leaf sides (Supplementary Fig. 1). The upper leaf levels (L4–L6) were left untreated in order to study the Fe and Mn transport from the treated to the untreated plant parts. During the foliar fertiliser application, the upper leaves and the surface of the pot containing the roots were covered with aluminium foil to avoid any possible spill from the fertiliser solution (Supplementary Fig. 1). Positive (Fe + Mn) and negative controls without Fe (-Fe), Mn (-Mn) or without both metals (-Fe,-Mn) were always used.

Sampling and growth parameters

Plant sampling was carried out 8 days after the first foliar fertilisation (at day 17). The whole plant was sampled for analysis, including the roots, leaves that had received foliar fertilisation (L1–3) and stems of these leaves as well as the upper plant parts that were not treated with the foliar fertiliser (leaves from L4–6 and their stems). Roots were washed with distilled water to remove traces of nutrient solution. Leaves and stems were analysed without any washing, with the rationale that i) any fertiliser left could still be available in changing ambient humidity conditions (Fernández and Eichert 2009) and that ii) leaf washing with HCl may be capable of removing metals from the inside of leaf cells close to the surface (e.g., from trichomes, epidermal cells, etc.). In separate experiments, it was found that approximately 9 % of the total leaf Fe in *S. lycopersicum* leaves foliar fertilised with Fe can be removed by 0.1 N HCl (this was done analysing the leaf material after washing as well as after the washing solution; results not shown). Prior to mineral analysis, all plant tissues were put in an oven at 60 °C for 72 h until constant weight. A total of two batches of plants and three replications per treatment and batch were used.

Assessment of leaf re-greening after foliar fertilisation using SPAD

Leaf *Chl* was estimated in each individual leaf level (L1–L6) using a SPAD 502 apparatus (Minolta Co., Osaka, Japan). Leaf re-greening was assessed before fertilisation (at day 8) and then three more times (at days 11, 12 and 16) after applying the foliar fertilisers. Leaf SPAD measurements were taken in the treated (L1–L3) and untreated (L4–L6) leaves. The SPAD data shown are the average of four measurements of each leaf level in a total of six plants (two batches of plants and three plants per treatment). It was not possible to assess the SPAD in some of the youngest leaf levels (L6 and most

of the L5 leaves) in some treatments, due to the very small leaf size.

Photosynthetic pigment analysis

At the end of the experiment (at day 16, 3 days after the last of the three fertilisation events), five disks per leaf level were sampled using a calibrated 0.5-cm-diameter cork borer in the same area used to measure SPAD. Fresh disks were wrapped in aluminium foil, frozen in liquid N_2 and stored at -20 °C until analysis. Leaf pigments were extracted with acetone in the presence of Na ascorbate and stored following the procedure described by Abadía and Abadía (1993). Pigment extracts were thawed on ice, filtered through a 0.22-μm polytetrafluoroethylene PTFE filter and analysed by high-performance liquid chromatography using a Waters 600 pump and 996 photodiode array detector (Waters Co., Milford, MA, USA) (Larbi et al. 2004). Total *Chl* (*Chl a* + *Chl b*), neoxanthin, violaxanthin (V), lutein epoxide (taraxanthin), antheraxanthin (A), lutein, zeaxanthin (Z), ß-carotene, total amount of violaxanthin cycle pigments (V + A + Z), *Chl a*/*Chl b* ratios and (A + Z)/(V + A + Z) ratios were determined. Two batches of plants and three replications per treatment and batch were analysed.

Chlorophyll fluorescence

At day 16, the dark-adapted, maximum potential photosystem II efficiency was calculated as the ratio of variable to maximum *Chl* fluorescence (F_V/F_M), as described by Larbi et al. (2004).

Analysis of micronutrient concentrations

Plant samples (0.2 g dry weight (DW) of tissue) were digested using a microwave system (Milestone Ethos Plus, Bergamo, Italy) with 6.4 mL HNO_3 (26 %, TraceSelect Ultra, Sigma-Aldrich, Madrid, Spain) and 1.6 mL H_2O_2 (30 %). The microwave digestion programme was 5 min at 100 °C, 10 min at 170 °C and 35 min at 180 °C. The digest was filtered through a 0.45-μm PTFE filter and diluted to 10 mL in water (type I reagent grade), and metals (Fe, Mn, Cu and Zn) were determined by flame atomic absorption spectrometry (AAS) using a Solaar 969 apparatus (Unicam Ltd., Cambridge, UK). Two batches of plants and three replications per treatment and batch were analysed.

Metal micronutrient contents were obtained by multiplying metal concentrations and DW values. A mass balance was carried out using the content of all plant parts (roots, treated leaves and their stems and untreated leaves and their stems) before and after fertiliser application.

Perls' Fe staining of transversal leaf sections

Leaves were first washed twice with type I reagent grade water and blotted dry with filter paper. Then, leaf pieces (2 cm^2) from the midst of leaf areas adjacent to main veins were embedded in 5 % agar and sectioned transversally (70 μm thickness) using a vibrating blade microtome (VT1000 S, Leica Microsystems GmbH, Wetzlar, Germany). Fresh sections were incubated with a 4 % $K_4[Fe(CN)_6]$, 4 % HCl solution for 30 min at room temperature. Negative stain controls were run by incubating fresh sections with 4 % HCl. Finally, sections were washed thrice with ultrapure water, and bright light images (2592 × 1994 pixels) were taken using an inverted microscope (DM IL LED, Leica) with a charge-coupled device camera (Leica DFC 240C).

Statistical analyses

Statistical analysis was carried out with SPSS for OSX (v. 21.0) and analysis of variance (ANOVA) or Welch's tests ($p \leq 0.05$), using a Levene test for checking homogeneity of variances. *Post hoc* multiple comparisons of means were carried out using Tukey or Games–Howell tests ($p \leq 0.05$).

Results

Effects of foliar fertilisation on root, stem and leaf DW

Leaves (L3) from the -Fe, -Mn and -Fe,-Mn plants are shown in Fig. 1. All metal-deficient plants showed decreases in the root, leaf and stem DW compared to the metal-sufficient controls (Fig. 2). In the case of the Fe-deficient plants, foliar fertilisation with Fe (without or with Mn) led after 8 days to increases in root DW up to values similar to those found in the control plants (Fig. 2a). Treated leaves (L1–L3) and their stems had larger DW than Fe-deficient leaves, but values did not reach the DW values observed in control plants. Conversely, the untreated upper leaf levels (L4–L6) and their

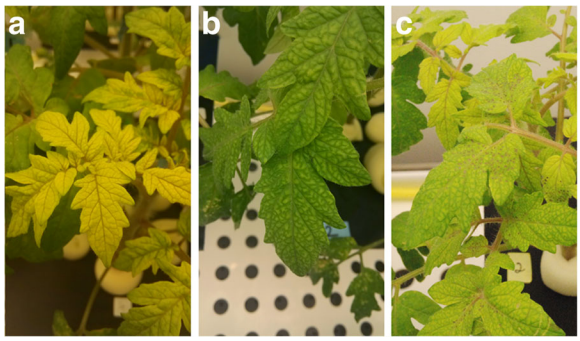

Fig. 1 Leaves (L3) of *Solanum lycopersicum* plants deficient in Fe (**a**), Mn (**b**) and Fe + Mn (**c**)

stems did not increase in DW with respect to those found in Fe-deficient leaves.

In the case of the Mn-deficient plants, foliar fertilisation with Mn (without or with Fe) increased root DW, but values did not reach those found in the control plants (Fig. 2b). Treated leaves and their stems increased in DW with respect to those in the Mn-deficient treatment, but only in the case of the combined fertilisation (Fe + Mn) did values reach close to those of the controls. In the case of the untreated leaves in the upper levels, DW values reached with both foliar treatments were similar to those found in the control plants. The stems of the untreated upper leaf levels also increased in DW significantly with respect to those found in the Mn-deficient plants, but values were lower than those found in the controls.

In the double deficient (-Fe,-Mn) plants, the combined Fe + Mn foliar fertilisation did not change significantly the DW of roots, treated leaves and their stems and stems of untreated leaves (Fig. 2c). The only significant change with the combined Fe + Mn foliar fertilisation was an increase in the DW of the untreated upper leaves.

Effects of foliar fertilisation on leaf re-greening

Leaves (L1–L6) from the foliar-treated -Fe, -Mn and -Fe,-Mn plants at day 17 (8 days after the first foliar treatment) are shown in Fig. 3. The re-greening was followed at every leaf level (L1–L6) using a SPAD device for 8 days after the first foliar fertilisation treatment (Fig. 4a–d). All metal-deficient plants showed decreases in SPAD values (Fig. 4a–c) compared to the sufficient controls (Fig. 4d). In general, significant re-greening was produced by foliar treatments in treated leaves (L1–L3; Fig. 4a–c), and in some cases the final SPAD values were close to those found in control plants (Fig. 4d).

In the case of Fe-deficient leaves, the re-greening was marked in L2 and L3, which had SPAD increases of approximately 13–19 units; final values reached were similar with Fe and Fe + Mn fertilisers (Fig. 4a). Level 1 leaves showed a less marked re-greening, since they were developed when Fe was still available during the pre--treatment, so that the initial chlorosis was not as strong. The upper leaf levels (L4–L5), which were not treated with the fertiliser, did not re-green after 8 days and had SPAD values similar to those found in Fe-deficient plants (values for L5 are only included at day 8, when leaves where large enough for SPAD to be measured).

In Mn-deficient leaves, a re-greening effect was observed in the treated leaves (L1–L3), reaching SPAD values 11–17 units higher at the end of the experiment; values reached were similar with Mn and Mn + Fe fertilisers (Fig. 4b). No re-greening was observed in the untreated L4 leaves. However, the newly developed, untreated top leaves (L5; only included at day 8) reached similar SPAD values to those found in treated leaves, both in the case of Mn- and (Fe + Mn)-fertilised plants.

In the double deficiency (Fe and Mn) case, the combined Fe + Mn foliar fertilisation led to a SPAD increase of 15 units at the end of the experiment in the treated leaves (Fig. 4c). No re-greening was observed in any of the untreated leaves.

Effects of foliar fertilisation on leaf photosynthetic pigment concentrations

The photosynthetic pigments neoxanthin, V, A, lutein, *Chl b*, *Chl a* and ß-carotene were detected in all treatments, whereas Z was not detected in control and Fe-fertilised leaves. On the other hand, lutein epoxide was detected only in the Mn-deficient plants (Table 1). All metal-deficient plants showed decreases in photosynthetic pigment concentrations when compared to the metal-sufficient controls (Fig. 5).

In the case of Fe-deficient plants, foliar fertilisation produced a significant increase in the concentration of photosynthetic pigments (total *Chl* and the carotenoids neoxanthin, lutein, ß-carotene and V + A + Z cycle pigments) in the treated leaves (L1–L3). However, values were still 30 % lower than the controls in the

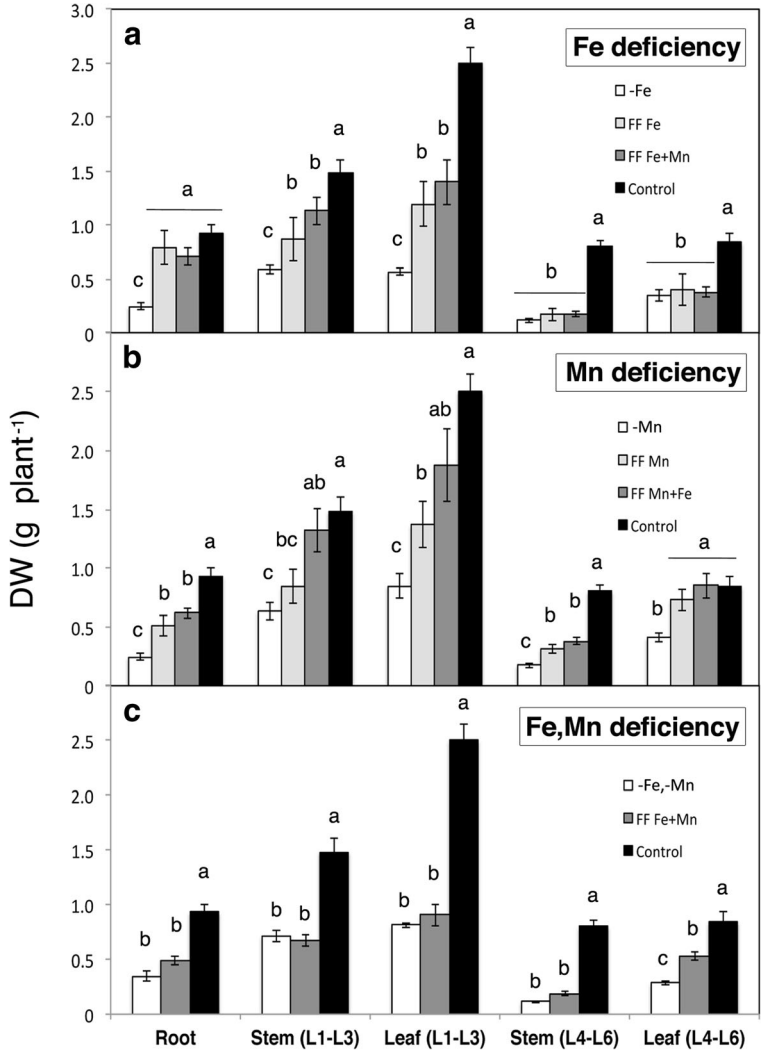

Fig. 2 Biomass (dry weight, in g plant^{-1}) of roots, stems (L1–L3), treated leaves (L1–L3), stems (L4–L6) and untreated leaves (L4–L6) of Fe-deficient (**a**), Mn-deficient (**b**) and Fe- and Mn-deficient *Solanum lycopersicum* plants (**c**) at day 17 (8 days after the first foliar treatment). Plants were treated (foliar fertilised (FF)) either with individual metals or combined. Data are means ± SE (n = 6 plants; two batches of plants and three samples per treatment). For a given plant tissue, columns marked with the same letter are not significantly different at the $p \leq 0.05$ level

case of total *Chl* and 50–60 % lower than the controls for the rest of photosynthetic pigments (Fig. 5a–e). There were no significant differences in photosynthetic pigment concentrations between the Fe and Fe + Mn treatments. In the upper untreated leaves (L4–L6), the photosynthetic pigment concentration values did not change with any of the fertiliser treatments. The *Chl a*/*b* ratios did not change significantly with the foliar treatments in the treated leaves, whereas the ratio increased in the untreated ones (Fig. 6a). The (A + Z)/(V + A + Z) ratios decreased significantly in all cases in the treated plants, with the decreases being more marked in the treated than in the untreated leaf levels (Fig. 6b).

In the case of Mn-deficient plants, foliar fertilisation led to a significant increase in the concentration of all photosynthetic pigments in both treated and untreated leaves, except for neoxanthin and V + A + Z cycle pigments in treated leaves when Mn was combined with Fe (Fig. 5a–e). There were no differences in photosynthetic pigment concentrations between the Mn and Mn + Fe treatments. The lutein epoxide peak present in the Mn-deficient leaves disappeared after Mn foliar fertilisation (Table 1). In the treated leaves, total *Chl* levels were similar to those in the metal-sufficient controls, but the recovery of the carotenoids was still incomplete. The *Chl* levels in the untreated leaves at the end of the experiment were lower than in the controls. The *Chl a*/*b* ratios in the L1–L3 leaves were lower in the Mn-deficient leaves than in the controls, and the ratios increased significantly upon foliar treatments toward the

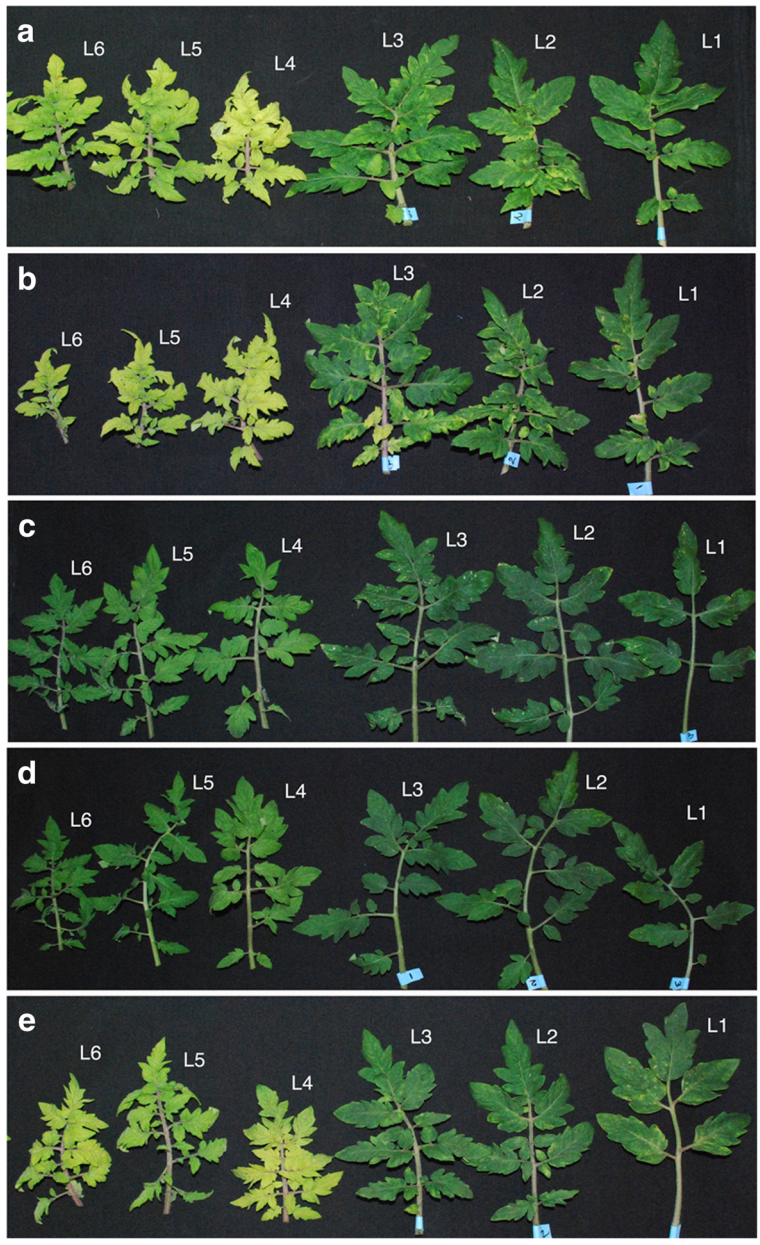

Fig. 3 Treated (L1–L3) and untreated (L4–L6) leaves from *Solanum lycopersicum* plants that were Fe deficient and treated with Fe (**a**) or Fe + Mn (**b**); Mn deficient and treated with Mn (**c**) or Mn + Fe (**d**); and Fe- and Mn-deficient and treated with Fe + Mn (**e**) at day 17 (8 days after the first foliar treatment)

control values (Fig. 6a). The (A + Z)/(V + A + Z) ratios decreased significantly with both treatments in the treated leaves and also in the untreated leaves after the Mn + Fe treatment (Fig. 6b).

In the case of double deficient (-Fe,-Mn) plants, foliar fertilisation in the treated leaves resulted in a significant increase of all photosynthetic pigment concentrations, and in the case of total *Chl*, ß-carotene and V + A + Z cycle pigment final values were similar to the control ones (Fig. 5a–e). In untreated leaves, the photosynthetic pigment concentrations did not change significantly with respect to those in the deficient plants, with the only exception of the V + A + Z cycle pigments, although final values were still lower than the controls. The *Chl a*/*b* ratio increased significantly with the Fe + Mn fertilisation in treated and untreated leaves (Fig. 6a). The (A + Z)/(V + A + Z) ratio decreased significantly after foliar fertilisation in treated leaves, reaching values similar to those of control plants, whereas in the untreated leaves this ratio remained unchanged (Fig. 6b).

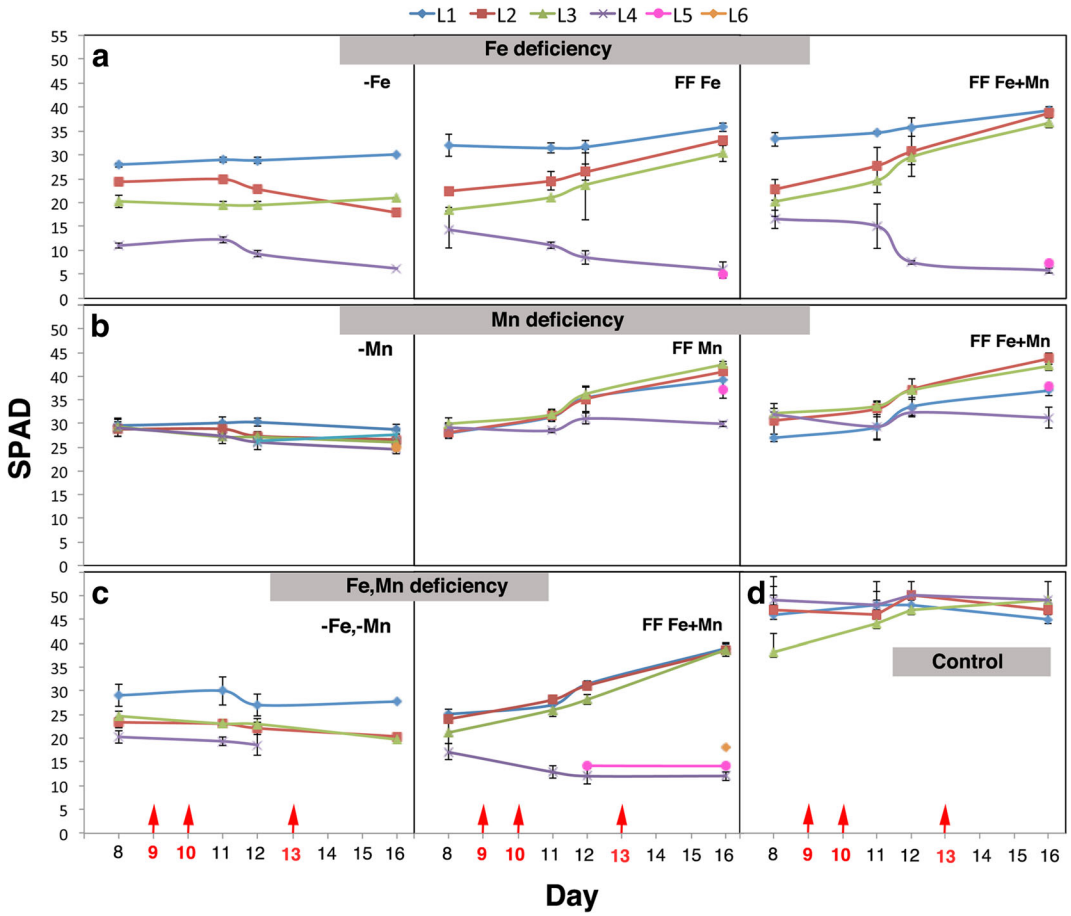

Fig. 4 Time course of soil–plant analysis development (SPAD) values in treated (L1–L3) and untreated (L4–L6) leaves during individual (foliar fertilised (FF) Fe, FF Mn) or combined (FF Fe + Mn, FF Mn + Fe) foliar treatments in Fe-deficient (**a**), Mn-deficient (**b**) and Fe- and Mn-deficient *Solanum lycopersicum* plants (**c**); leaf SPAD values of control plants (**d**) were also analysed. Values shown are for days 8 (the first foliar treatment) until 16. Fertilisation events are marked by arrows on the x-axes. Data are means ± SE ($n = 6$ plants; two batches of plants and three samples per treatment, with four readings per leaf)

Effects of foliar fertilisation on leaf chlorophyll fluorescence

Metal-deficient plants showed decreases in the F_V/F_M ratios when compared to the metal-sufficient controls, with the decreases being larger with Mn deficiency than with Fe deficiency (Table 1; values shown in the table are only for L1–L3 leaves). Foliar fertilisation led to F_V/F_M ratios similar to the control ones.

Effects of foliar fertilisation on Fe, Mn, Cu and Zn concentrations in plant organs

Iron deficiency caused significant decreases in the Fe concentrations in all *S. lycopersicum* plant parts, including roots and leaves in the lower (L1–L3) and upper levels (L4–L6) (decreases of 91, 71 and 47 %, respectively) (Table 2). In general, Mn, Cu and Zn concentrations increased in all plant parts with Fe deficiency when compared to Fe-sufficient controls; concentration increases (in roots/L1–L3 leaves/L4–L6 leaves) were 1.1-/5.0-/3.5-fold for Mn, 3.0-/1.8-/1.8-fold for Cu and 1.5-/3.3-/2.3-fold for Zn (all changes were statistically significant, with the only exception of Mn in roots).

Individual (Fe) or combined (Fe + Mn) foliar fertilisation increased the root Fe concentration of Fe-deficient plants, although the increase (82 %) was significant only with the Fe + Mn treatment, whereas the root concentrations of Mn, Cu and Zn were unchanged. In the treated leaves, foliar fertilisation with Fe (without or with Mn) increased markedly the Fe concentration (by 14- to 15-fold) and decreased the Zn concentration

Table 1 Maximum potential photosystem II (PSII) efficiencies, calculated as the ratio of variable to maximum chlorophyll fluorescence after dark adaptation (F_V/F_M), and lutein epoxide concentrations in leaves (at day 16). Values shown are for developed leaves L1–L3. Values significantly different at $p \leq 0.05$ are marked with different letters

	Lutein epoxide (μmol m^{-2})	F_V/F_M
Control	0 a	0.81 ± 0.01 a
-Fe	0 a	0.67 ± 0.01 b
FF Fe	0 a	0.83 ± 0.02 a
FF Fe + Mn	0 a	0.82 ± 0.01 a
-Mn	1.9 ± 0.1 b	0.54 ± 0.02 c
FF Mn	0 a	0.82 ± 0.01 a
FF Mn + Fe	0 a	0.82 ± 0.01 a
-Fe-Mn	0 a	0.68 ± 0.04 b
FF Fe + Mn	0 a	0.83 ± 0.01 a

(by 35–41 %) compared to Fe-deficient plants; Mn concentrations decreased significantly (33 %) only in the case of individual Fe fertilisation, whereas Cu concentrations did not change. In the untreated leaves, the only significant change upon fertilisation was a decrease in the Mn concentration (by 50–56 %), both in the Fe and Fe + Mn treatments.

Manganese deficiency caused significant decreases in Mn concentrations in all *S. lycopersicum* plant parts, including roots and leaves in the lower (L1–L3) and upper levels (L4–L6) (decreases of 93, 90 and 93 %, respectively) (Table 2). The concentrations of Fe, Cu and Zn increased significantly with Mn deficiency in roots (5.2-, 4.9- and 2.4-fold, respectively) and leaves in the upper levels (2.2-, 1.8- and 2.3-fold, respectively), whereas in the lower leaf levels the only significant increase was for Cu (1.9-fold).

Individual (Mn) or combined (Mn + Fe) foliar fertilisation did not change significantly the root Mn concentrations but decreased significantly the Fe concentrations (by 33–50 %); Cu and Zn concentrations decreased (by 52–54 %) only when Mn was applied alone (Table 2). In the treated leaves, both foliar fertilisation treatments increased significantly the Mn concentration (15- to 19-fold), whereas the Fe concentration increased (2.9-fold) with the Mn + Fe foliar treatment; the Cu concentrations decreased with both foliar treatments (by 38 %), whereas those of Zn did not change significantly. In the untreated leaves, foliar fertilisation increased significantly the Mn concentration (1.7- to 1.9-fold), but no statistically significant changes were found for Fe, Cu or Zn.

The combined Fe and Mn deficiency caused decreases in the Fe and Mn concentrations in all plant parts: decreases were (in roots/L1–L3 leaves/L4–L6 leaves) 55/61/78 % for Fe and 95/91/91 % for Mn (Table 2). In general, Cu and Zn concentrations increased in all plant parts with the combined Fe + Mn deficiency when compared to metal-sufficient controls: concentration increases were (roots/L1–L3 leaves/L4–L6 leaves) 2.2-/1.8-/1.9-fold for Cu and 2.0−/1.4−/1.9-fold for Zn (all changes were statistically significant, with the only exception of Zn concentrations in leaves L1–L3).

Foliar fertilisation with Fe + Mn decreased significantly the root Fe concentrations (by 54 %), without any significant change in Mn, Cu and Zn root concentrations (Table 2). In the treated leaves, foliar fertilisation increased significantly the Fe, Mn and Zn concentrations (by 13-, 26- and 2.7-fold, respectively), whereas Cu concentrations were unchanged. In the untreated leaves, foliar fertilisation increased significantly the Zn concentrations (1.6-fold), whereas no significant change was observed in Fe, Mn and Cu concentrations.

Effects of foliar fertilisation on Fe, Mn, Cu and Zn contents in plant organs

The effects of the deficiencies and those of the foliar fertiliser applications on the metal contents of the different plant organs were also assessed (Table 3; changes in stem metal contents are not discussed in detail, but they were measured and included in the table).

Iron deficiency caused significant decreases in the Fe contents in most *S. lycopersicum* plant parts, including roots, treated leaves and untreated leaves (decreases of 97, 91 and 79 %, respectively) (Table 3). Significant decreases were also found for the contents of Mn and Zn in roots (72 and 59 %, respectively) and in Cu in the L1–L3 leaves and stems (58 %). Individual (Fe) or combined (Fe + Mn) foliar fertilisation increased significantly the root Fe contents of Fe-deficient plants (4- to 5-fold, respectively) and also the root contents of Mn and Zn, whereas the content of Cu increased only in the case of the Fe + Mn treatment (Table 3). In the treated leaves and their stems, foliar fertilisation with Fe (without or with Mn) increased markedly the Fe content (23- to 34-fold and 3- to 8-fold, respectively), whereas Cu, Mn and Zn contents had only minor increases (2-fold or less).

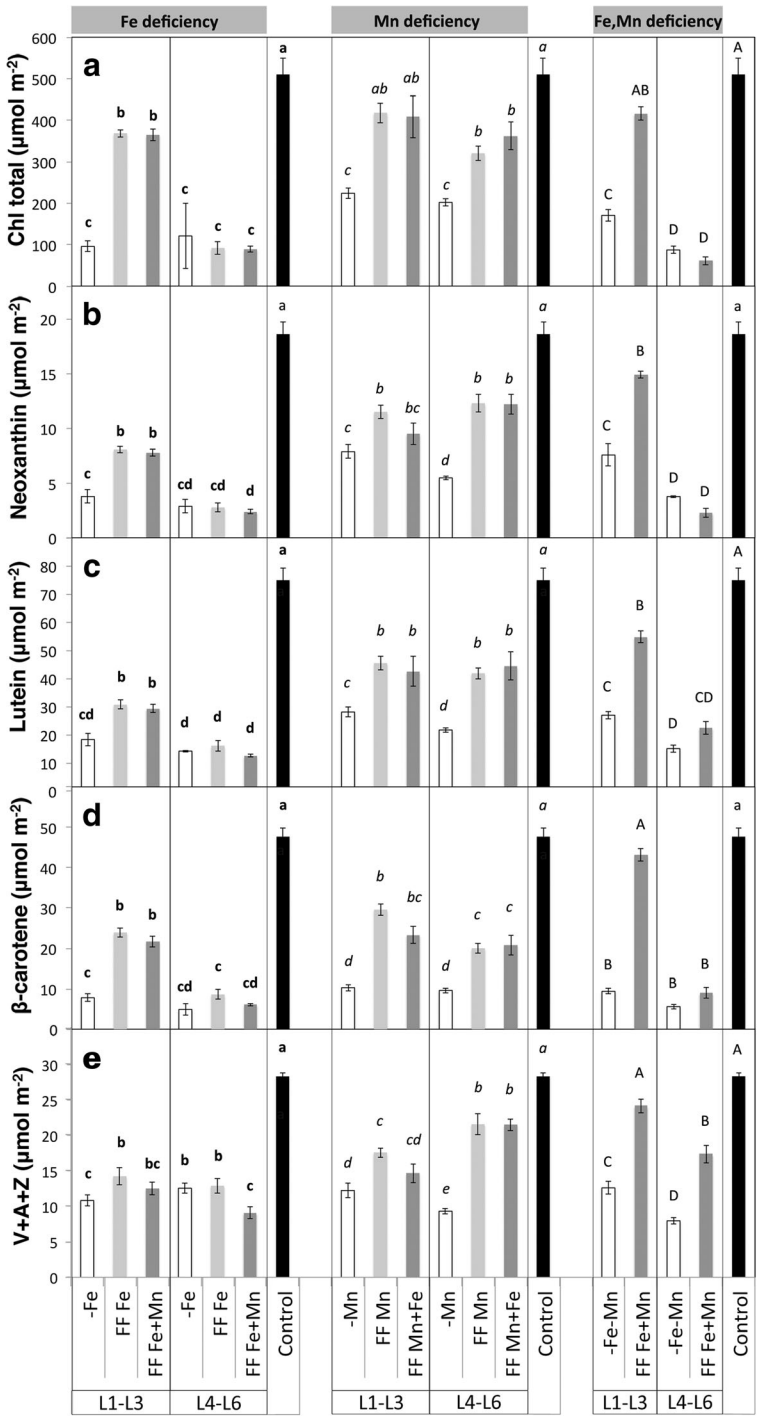

Fig. 5 Concentrations of photosynthetic pigments (in µmol m^{-2}; total chlorophyll (**a**), neoxanthin (**b**), lutein (**c**), ß-carotene (**d**) and violaxanthin + antheraxanthin + zeaxanthin (**e**) in treated (L1–L3) and untreated (L4–L6) leaves with individual (foliar fertilised (FF) with Fe, FF Mn) or combined (FF Fe + Mn, FF Mn + Fe) foliar treatments of Fe-deficient, Mn-deficient and Fe- and Mn-deficient *Solanum lycopersicum* plants. Samples were taken at day 16 (8 days after the first foliar treatment). Data are means ± SE (n = 6 plants; two batches of plants and three samples per treatment). For a given deficiency (Fe, Mn or Fe,Mn), columns marked with the same letter (in bold for Fe deficiency, italics for Mn deficiency and capitals for Fe,Mn deficiency) were not significantly different at $p \leq 0.05$

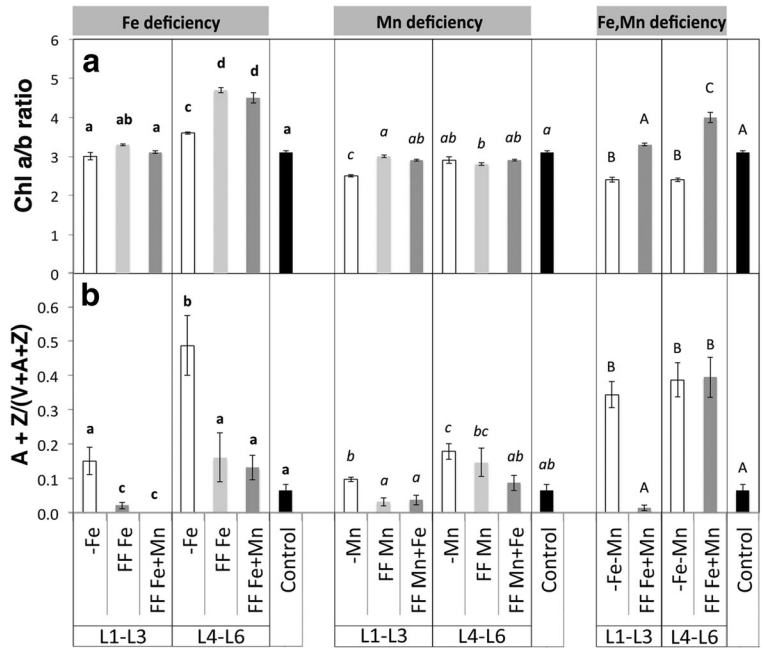

Fig. 6 Chlorophyll *a*/*b* (**a**) and (antheraxanthin + zeaxanthin)/(violaxanthin + antheraxanthin + zeaxanthin) molar ratios (**b**) in treated (L1–L3) and untreated (L4–L6) leaves with individual (foliar fertilised (FF) Fe, FF Mn) or combined (FF Fe + Mn, FF Mn + Fe) foliar treatments of Fe-deficient, Mn-deficient and Fe- and Mn-deficient *Solanum lycopersicum* plants. Samples were taken at day 16 (7 days after the first foliar treatment). Data are means ± SE ($n = 6$ plants; two batches of plants and three samples per treatment). For a given deficiency (Fe, Mn or Fe,Mn), columns marked with the same letter (in bold for Fe deficiency, italics for Mn deficiency and capitals for Fe,Mn deficiency) were not significantly different at $p \leq 0.05$

In the untreated leaves, no significant changes were observed for Fe, Mn, Cu and Zn contents.

Manganese deficiency caused significant decreases in Mn content in all *S. lycopersicum* plant parts, including roots, treated leaves and untreated leaves (98, 97 and 96 %, respectively) (Table 3). Significant decreases were also found for the contents of Zn in roots (38 %) and Cu in L1–L3 leaves (42 %). Individual (Mn) or combined (Mn + Fe) foliar fertilisation did not change significantly the root Mn content but increased significantly the Mn contents in all aerial parts, including treated (30- to 35-fold) and untreated leaves (4-fold) (Table 3).

The combined Fe and Mn deficiency caused decreases in the Fe and Mn contents in all plant parts: decreases were (in roots/treated leaves/untreated leaves) 83/83/91 % for Fe and 98/98/96 % for Mn (Table 3). Also, the combined Fe and Mn deficiency led to significant decreases for Cu in L1–L3 leaves (43 %) and for Zn in L1–L3 and L4–L6 leaves (36 and 42 %, respectively). Foliar fertilisation with Fe + Mn did not change significantly the Fe, Mn, Cu and Zn contents in roots (Table 3). Foliar fertilisation with Fe + Mn increased significantly the Fe, Mn and Zn contents in treated (by 15-, 31- and 3-fold, respectively) and untreated leaves (by 2-, 3- and 2-fold, respectively). Leaf Cu content did not change significantly with foliar fertilisation.

Perls' Fe staining of transversal leaf sections

Leaf sections taken 30 min after Fe foliar fertilisation showed a blue stain at the precise localisation of the phloem tissue (P) in the major leaf vein as well as in the vascular tissue coming from the leaf blade (marked with red arrows in Fig. 7a). Trichomes (T), xylem tissue (X), mesophyll tissue (M) and sclerenchyma support tissue (S) are also marked in the figure. This blue colour was absent in the controls (not shown). Blue Perls' staining (Prussian blue) is based on the formation of $Fe_4[Fe(CN)_6]_3 \times H_2O$ colloids resulting from the reaction of labile Fe forms occurring in the biological sample and exogenously $[Fe(CN)_6]_3$ in acidic media. Therefore, data support the presence in the phloem tissue of a labile Fe pool as a result of Fe foliar fertilisation. Sixty minutes after foliar fertilisation, the blue colour extends

Table 2 Concentrations of micronutrients (Fe, Mn, Cu and Zn; in μg g^{-1} dry weight) in roots, foliar fertilised leaves (L1–L3) and untreated leaves (L4–L6) of *Solanum lycopersicum* plants at the end of the treatments (at day 17, 8 days after the first foliar treatment). Data are means ± SE ($n = 6$ plants; two batches of plants and three samples per treatment). Values followed by the same letter within the same line (in bold for Fe treatments, italics for Mn treatments and capitals for Fe,Mn treatments) were not significantly different at $p \leq 0.05$

			Control				Fe deficiency		
			-Fe		FF Fe		FF Fe + Mn		-Mn
	Roots	Fe	692.0 ± 108.6**a***a*A	59.5 ± 3.8	**e**	93.1 ± 8.0	**cde**	108.1 ± 7.6	**cd**	3616.2 ± 188.3
		Mn	165.3 ± 26.8 **a***a*A	187.1 ± 17.4	**a**	141.6 ± 13.7	**a**	150.0 ± 18.3	**a**	11.0 ± 2.6
		Cu	21.7 ± 2.1 **a***a*A	65.0 ± 8.5	**b**	41.4 ± 6.7	**ab**	41.2 ± 4.3	**ab**	105.8 ± 15.6
		Zn	57.4 ± 2.2 **a***a*A	88.7 ± 5.5	**bc**	78.6 ± 12.8	**ab**	78.2 ± 9.9	**ab**	136.3 ± 12.3
L1-L3	Leaves	Fe	87.9 ± 12.0 **a***a*A	25.4 ± 3.2	**b**	372.9 ± 4.9	**c**	354.2 ± 20.2	**c**	129.3 ± 6.7
		Mn	59.9 ± 7.0 **a***a*A	300.0 ± 37.3	**e**	202.5 ± 7.2	**cd**	239.4 ± 9.9	**de**	6.1 ± 0.5
		Cu	11.9 ± 31.3 **a***a*A	22.0 ± 4.0	**b**	25.0 ± 1.0	**b**	20.6 ± 3.3	**b**	22.8 ± 2.5
		Zn	15.9 ± 2.7 **a***a*A	52.6 ± 7.7	**c**	31.1 ± 2.3	**ab**	34.4 ± 0.2	**ab**	25.9 ± 5.0
L4-L6	Leaves	Fe	98.9 ± 8.5 **a***a*A	52.8 ± 14.8	**bc**	32.1 ± 1.0	**b**	31.5 ± 0.9	**b**	218.7 ± 9.2
		Mn	40.2 ± 5.3 **a***a*A	141.6 ± 14.2	**c**	70.5 ± 3.9	**b**	62.7 ± 2.1	**b**	2.9 ± 0.2
		Cu	13.2 ± 1.8 **a***a*A	23.3 ± 0.5	**b**	24.0 ± 0.4	**b**	23.4 ± 0.2	**b**	24.2 ± 2.1
		Zn	22.9 ± 2.4 **a***a*A	53.2 ± 1.8	**bc**	58.4 ± 0.4	**c**	59.0 ± 2.7	**c**	51.9 ± 6.4

Fe deficiency	Mn deficiency				Fe,Mn deficiency			
	FF Mn		FF Mn + Fe		-Fe-Mn		FF Fe + Mn	
b	1802.7 ± 333.0	*d*	2435.9 ± 134.2	*d*	309.1 ± 30.0	B	143.7 ± 16.0	C
b	4.0 ± 0.3	*b*	4.8 ± 1.1	*b*	8.0 ± 1.3	B	4.3 ± 1.2	B
c	48.2 ± 9.5	*b*	66.5 ± 8.4	*bc*	48.5 ± 4.1	B	52.3 ± 5.3	B
bc	65.9 ± 7.0	*a*	84.9 ± 0.7	*b*	117.1 ± 1.0	C	99.5 ± 5.2	BC
ab	96.7 ± 11.0	*a*	374.3 ± 34.6	*d*	34.4 ± 0.6	B	444.3 ± 56.1	C
c	113.2 ± 8.9	*b*	93.5 ± 4.6	*ab*	5.1 ± 0.6	C	130.5 ± 13.7	B
c	14.1 ± 1.3	*ab*	14.1 ± 1.7	*ab*	22.0 ± 0.9	B	24.0 ± 1.6	B
ab	17.2 ± 1.0	*a*	16.4 ± 0.3	*a*	22.3 ± 2.9	A	60.3 ± 6.8	C
bc	178.0 ± 19.5	*b*	171.7 ± 14.6	*b*	22.1 ± 1.0	B	33.4 ± 2.7	B
b	5.4 ± 0.3	*c*	4.9 ± 0.3	*c*	3.7 ± 1.9	D	8.8 ± 1.8	D
b	20.3 ± 2.7	*ab*	19.6 ± 3.1	*ab*	24.5 ± 0.4	B	24.7 ± 1.5	B
bcd	37.6 ± 0.5	*c*	38.3 ± 1.8	*c*	42.7 ± 0.4	B	66.6 ± 3.4	C

Table 3 Contents of micronutrients (Fe, Mn, Cu and Zn; in μg) in roots, foliar fertilised leaves and stems (L1–L3) and untreated leaves and stems (L4–L6) of *Solanum lycopersicum* plants at the end of the treatments (at day 17, 8 days after the first foliar treatment). Data are means ± SE (n = 6 plants; two batches of plants and three samples per treatment). Values followed by the same letter within the same line (in bold for Fe treatments, italics for Mn treatments and capitals for Fe,Mn treatments) were not significantly different at $p \leq 0.05$

			Control	Fe deficiency						Mn deficiency
				-Fe		FF Fe		FF Fe + Mn		-Mn
	Roots	Fe	614.4 ± 76.8 **a***a*A	16.7 ± 3.1	**b**	63.8 ± 19.4	**c**	85.5 ± 4.7	**c**	880.3 ± 95.1
		Mn	148.8 ± 24.5 **a***a*A	42.1 ± 6.0	**b**	159.6 ± 35.1	**a**	120.4 ± 13.2	**a**	2.5 ± 0.5
		Cu	19.5 ± 1.8 **a***a*A	14.7 ± 2.5	**a**	31.7 ± 7.7	**ab**	33.1 ± 3.2	**b**	24.7 ± 2.8
		Zn	52.4 ± 3.8 **a***a*A	21.6 ± 1.1	**b**	58.8 ± 12.1	**a**	62.9 ± 7.5	**a**	32.4 ± 2.5
L1-L3	Leaves	Fe	161.4 ± 10.5 **a***a*A	15.2 ± 0.9	**b**	345.3 ± 33.2	**c**	510.0 ± 63.7	**c**	106.8 ± 8.2
		Mn	176.8 ± 26.3 **a***a*A	171.8 ± 32.9	**a**	151.8 ± 37.6	**a**	349.6 ± 52.0	**b**	5 ± 0.3
		Cu	31.7 ± 3.2 **a***a*A	13.2 ± 2.5	**b**	25.9 ± 4.3	**ab**	28.1 ± 1.4	**a**	18.5 ± 1.9
		Zn	28.4 ± 3.4 **a***a*A	30.5 ± 5.7	**a**	27.5 ± 2.7	**a**	50.9 ± 9.0	**b**	22.2 ± 4.8
	Stems	Fe	53.5 ± 4.8 **a***a*A	11.2 ± 3.5	**c**	34.7 ± 9.5	**ab**	85.1 ± 26.3	**a**	29.3 ± 4.8
		Mn	33.7 ± 3.7 **a***a*A	31.1 ± 8.1	**a**	36.9 ± 27.3	**a**	37.9 ± 3.1	**a**	1.5 ± 0.1
		Cu	11.5 ± 1.1 **a***a*A	5.4 ± 1.1	**b**	13.8 ± 9.5	**a**	15.3 ± 3.7	**a**	5.5 ± 0.5
		Zn	76.6 ± 8.4 **a***a*A	61.9 ± 10.1	**a**	49.3 ± 24	**ab**	86.6 ± 12.2	**a**	32.4 ± 5.4
L4-L6	Leaves	Fe	74.2 ± 13.4 **a***a*A	15.6 ± 3.1	**b**	9.8 ± 0.9	**b**	13.1 ± 1.9	**b**	90.8 ± 10.3
		Mn	28.0 ± 9.5 **a***a*A	48.3 ± 8.3	**ab**	21.5 ± 2.4	**a**	25.1 ± 0.3	**a**	1.1 ± 0.1
		Cu	13.2 ± 3.1 **a***a*A	7.9 ± 0.9	**a**	9.2 ± 1.9	**a**	9.5 ± 0.3	**a**	9.8 ± 1.0
		Zn	21.4 ± 2.8 **a***a*A	18.0 ± 2.2	**a**	21.1 ± 3.5	**a**	24.1 ± 1.8	**a**	21.9 ± 3.9
	Stems	Fe	21.0 ± 5.0 **a***a*A	9.7 ± 4.2	**a**	10.2 ± 4.5	**a**	7.6 ± 3.5	**ab**	9.0 ± 1.0
		Mn	6.3 ± 1.8 **a***a*A	5.8 ± 1.3	**a**	4.1 ± 1.8	**a**	1.9 ± 0.3	**b**	0.4 ± 0.1
		Cu	4.4 ± 1.1 **a***a*A	1.9 ± 0.3	**b**	3.6 ± 0.8	**a**	2.7 ± 0.2	**ab**	2.1 ± 0.5
		Zn	16.0 ± 5.1 **a***a*A	12.3 ± 2.0	**a**	18.1 ± 6.4	**a**	11.9 ± 1.2	**a**	9.3 ± 2.8

Mn deficiency				Fe,Mn deficiency			
	FF Mn		FF Mn + Fe	-Fe-Mn		FF Fe + Mn	
a	937.0 ± 228.8	*ab*	1510.8 ± 314.0	*b*	105.0 ± 9.0	B	72.8 ± 7.6	B
b	2.0 ± 0.3	*b*	2.5 ± 0.3	*b*	2.9 ± 0.8	B	2.2 ± 0.2	B
a	24.3 ± 5.2	*a*	29.1 ± 8.7	*ab*	16.9 ± 3.1	A	26.8 ± 2.7	A
b	32.7 ± 4.8	*b*	41.0 ± 7.4	*ab*	40.5 ± 4.9	AB	51.1 ± 3.7	A
ab	132.5 ± 22.3	*a*	669.7 ± 71.0	*c*	28.0 ± 0.8	B	432.8 ± 61.9	C
b	151.6 ± 19.4	*a*	173.9 ± 29.4	*a*	4.1 ± 0.6	C	127 ± 15.5	AB
b	19.1 ± 2.7	*b*	25.3 ± 3.8	*ab*	17.9 ± 1	B	23.3 ± 1.8	AB
a	23.1 ± 2.5	*a*	30.5 ± 5.1	*a*	18.3 ± 2.7	B	58.5 ± 6.9	C

Table 3 (continued)

Mn deficiency					Fe,Mn deficiency			
	FF Mn		FF Mn + Fe		-Fe-Mn		FF Fe + Mn	
ab	41.2 ± 6.1	*a*	78.9 ± 15.1	*c*	1.3 ± 0.1	B	32.7 ± 5.7	C
b	3.9 ± 0.6	*c*	8.5 ± 1.5	*d*	0.3 ± 0.1	B	8.1 ± 1.6	C
b	7.9 ± 1.0	*ab*	11.9 ± 2.0	*a*	1.2 ± 0.1	B	7.3 ± 0.7	C
b	34.0 ± 10.4	*b*	61.3 ± 9.8	*a*	14.3 ± 0.5	B	76.7 ± 4.6	A
a	127.8 ± 15.5	*ab*	142.2 ± 12.2	*bc*	6.4 ± 0.7	B	12.2 ± 2.6	C
b	3.9 ± 0.5	*c*	4.2 ± 0.6	*c*	1.0 ± 0.5	B	3.1 ± 0.4	C
ab	14.5 ± 1.8	*a*	15.9 ± 1.8	*a*	7.0 ± 0.5	AB	9.1 ± 1.3	A
a	27.4 ± 3.5	*ab*	32.6 ± 4.5	*ab*	12.3 ± 0.9	B	25.5 ± 4.2	A
b	32.2 ± 11.5	*a*	24.5 ± 2.0	*a*	19.7 ± 0.2	A	3.7 ± 0.8	B
b	1.1 ± 0.2	*c*	0.9 ± 0.1	*c*	1.9 ± 0.3	B	0.5 ± 0.2	C
ab	3.0 ± 0.4	*a*	3.4 ± 0.3	*a*	14.4 ± 0.2	C	1.9 ± 0.1	B
ab	14.2 ± 1.0	*a*	17.0 ± 1.4	*a*	73.0 ± 2.1	C	9.8 ± 1.6	AB

to the xylem tissue and also to part of the sclerenchyma tissue (Fig. 7b).

Discussion

Establishment of metal deficiencies in Solanum lycopersicum

To assess the effects of individual (Fe or Mn) and combined (Fe + Mn) foliar treatments, *S. lycopersicum* plants with well-established Fe and Mn deficiencies were obtained. Iron-, Mn- and Fe-,Mn-deficient plants showed large decreases in the concentrations and contents of their corresponding depleted metal(s) as well as significant biomass decreases in all plant parts. Metal-deficient plants also showed decreases in leaf photosynthetic pigments (leaf chlorosis), accompanied by increases in the (A + Z)/(V + A + Z) ratio, a well-known index of photosynthetic stress, in young leaves; in the case of Fe-,Mn-deficient plants, the increase in this ratio also occurred in older leaves. In the case of Mn deficiency, the *Chl a*/*b* ratio in old leaves (and also in the young ones in the -Fe,-Mn combined deficiency) was lower than that of the controls, a finding that is in good agreement with the preferential decrease caused by Mn deficiency in the inner light harvesting *Chl*-protein complexes (Abadía et al. 1986). These complexes are located close to the Mn-containing photosystem II reaction centre and have little *Chl b* compared to the peripheral light harvesting complexes, which are enriched in *Chl b*. Furthermore, Mn-deficient leaves had two additional distinct characteristics: a decrease in variable *Chl* fluorescence (F_V/F_M ratio) more marked than that observed in Fe-deficient leaves and an appearance of an unusual pigment, lutein epoxide, that does not occur in the controls nor in the Fe-deficient leaves (Table 1). The significance of this finding will be explored in further studies. Decreases in the F_V/F_M ratio with Mn deficiency in *Hordeum vulgare* have been described in detail in several papers (Husted et al. 2009; Schmidt et al. 2013).

All deficiencies (-Fe, -Mn and -Fe,-Mn) led to general increases in the concentrations of other metals: Fe-deficient plants showed marked increases in Mn, Cu and Zn concentrations in most plant organs (except for Mn in roots); Mn-deficient plants showed increases in Fe, Cu and Zn concentrations in roots and young leaves (and also in older leaves except in the case of Zn); and Fe-,Mn-deficient plants showed increases in the

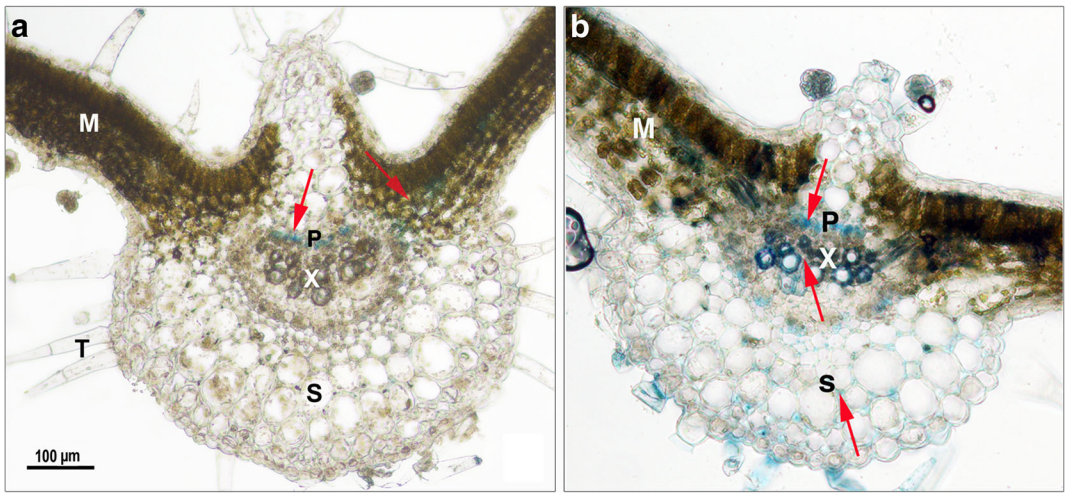

Fig. 7 Perls' iron staining in transversal leaf sections taken 30 min (**a**) and 60 min (**b**) after Fe foliar fertilisation. Phloem tissue (P), trichomes (T), xylem tissue (X), sclerenchyma support tissue (S) and mesophyll tissue (M) are marked in the figure. Red arrows mark the blue Perls' stain in the phloem tissue and vascular tissue at 30 min (**a**) and in the phloem, xylem and sclerenchyma tissues at 60 min (**b**)

concentrations of Cu and Zn in most plant organs (except for Zn in older leaves). This metal enrichment is likely due to the increased abundance (as a result of the deficiency of Fe or Mn) of the corresponding plasma membrane metal transporters, which have a poor metal specificity and are capable of taking up other divalent metals in addition to the one involved in the deficiency (e.g., IRT1 in the case of Fe, IRT1 and/or NRAMP1 in the case of Mn; Morrissey and Guerinot 2009; Socha and Guerinot 2014).

Effects of foliar fertilisation on Fe-deficient plants

In the case of Fe-deficient plants, both foliar treatments (Fe alone or Fe + Mn) led to similar effects: plants responded by leaf re-greening, with general increases in Fe concentrations, total Fe contents and biomass in the treated plant parts as well as in the roots. At the end of the experiment, leaf SPAD values in fertilised plants had increased approximately 2-fold compared to the initial values, although SPAD values were still lower than those found in the Fe-sufficient plants. This re-greening is comparable to those found in previous studies with *B. vulgaris* (6-fold; El-Jendoubi et al. 2014), *Prunus persica* (2-fold; Fernández et al. 2006; El-Jendoubi et al. 2014) and *Pyrus communis* (3-fold; Álvarez-Fernández et al. 2004).

Iron applied as $FeSO_4$ to leaves led to a significant increase in root Fe content. Although the largest part of the plant in which the Fe increase after the foliar treatments (Fe/Fe + Mn) compared to the Fe-deficient plants was in the leaves treated with the fertiliser (82/78 %), Fe content increases also occurred not only in the stems of the treated leaves (6–12 %) but also in the roots (11–12 %) (Fig. 8). This was associated with increases in root Fe concentrations and root biomass. Similar translocation from foliar fertilised leaves to the roots, but to a lower extent, was found in *Helianthus annuus*, *S. lycopersicum* and *Cucumis sativus* plants, where 5, 2–4 and 1–5 % of the total ^{59}Fe taken up using ^{59}Fe (III)-humic substances, ^{59}Fe-lignosulfonates and ^{59}Fe (III)-EDTA, respectively, was found in roots (Nikolic et al. 2003; Rodríguez-Lucena et al. 2009). Also, ^{57}Fe was detected in roots of *C. sativus* plants after ^{57}Fe foliar treatments (Rodríguez-Lucena et al. 2010). The root Fe increases (in μg) were much higher in our experiment probably because we used a fertiliser with 9 mM Fe, a concentration much higher than those used in the other studies, which ranged from 5 μM to 1 mM Fe. Transport of Fe to roots has been also reported recently after foliar fertilisation in *Panax ginseng* plants (Zhang et al. 2013). However, this may be species dependent, since no Fe mobilisation from treated leaves to roots was found in citrus plants (Papadakis et al. 2007). The involvement of phloem sap in Fe transport immediately after fertilisation is supported by Fig. 7a, although no specific Fe chemical form has been identified so far in the phloem sap of any dicotyledonous species (Álvarez-Fernández et al. 2014). Furthermore, not only phloem tissue

but also xylem and sclerenchyma tissue were loaded with labile Fe forms 1 h after fertilisation (Fig. 7b), and this is likely behind the increase in the Fe content of the stems after foliar fertilisation.

However, foliar fertilisation did not affect the Fe concentrations, Fe contents, biomass and SPAD levels of the leaves not directly treated with the fertiliser, indicating that the Fe applied as $FeSO_4$ was not transported in significant amounts to upper untreated leaves. A poor mobility of the Fe applied to leaves as $FeSO_4$ was also found when treating only the distal part of *B. vulgaris* and *P. persica* leaves, since very minor increases in Fe concentrations were found in the basal, non-fertilised leaf parts (El-Jendoubi et al. 2014). The mobility could be better using other Fe sources, since in *H. annuus*, *S. lycopersicum* and *C. sativus* plants up to 2.5–14 % (depending on the formulation) of the total ^{59}Fe taken up using ^{59}Fe(III)-humic substances, ^{59}Fe (III)-lignosulfonates and ^{59}Fe(III)-EDTA was found after 3 or 7 days in the untreated new leaves (Nikolic et al. 2003; Rodríguez-Lucena et al. 2009).

The increases in total Fe contents (including all plant parts) were 401 and 637 μg in the Fe only and the Fe + Mn treatments, respectively. This indicates that approximately 44–70 % of the applied Fe was in the plants at the end of the experiment, with the highest value found in the combined Fe + Mn application. The remaining Fe was possibly lost during the treatment due to dripping from the treated leaf surfaces (the surface of the pots was fully covered to prevent incorporation of these metals into the nutrient solution, as mentioned above). It should also be taken into account that a small fraction of the leaf Fe in the treated leaves can be removed by 0.1 N HCl, supporting that this fraction could be located on the surface of the leaf.

The (A + Z)/(V + A + Z) ratio decreased not only in treated leaves but also in untreated ones (although to a lower extent), supporting that the stress was in the way of being relieved in these leaves. This is in line with previous studies showing that the (A + Z)/(V + A + Z) ratio is a very sensitive stress marker for Fe deficiency, since Fe treatments in part of the plant affect this ratio in other plant parts not directly treated. For instance, the (A + Z)/(V + A + Z) ratio decreased in untreated leaf areas of *B. vulgaris* and *P. persica* after Fe foliar fertilisation (El-Jendoubi et al. 2014), and the same also occurred soon after Fe root resupply to Fe-deficient plants (Larbi et al. 2006). The *Chl a*/*b* ratio increased in Fe-deficient untreated leaf levels after foliar fertilisation, and a similar increase in this ratio was previously attributed to the formation of new photosynthetic membrane during short-term Fe resupply to the roots (Nishio et al. 1985).

On the other hand, Fe or Fe + Mn foliar fertilisation also affected the concentrations of Mn and Zn in leaves. The Zn concentration of treated leaves decreased significantly after individual (Fe) or combined (Fe + Mn) foliar fertilisation, whereas Mn concentrations in all leaves also decreased with individual Fe fertilisation. Similar results were reported for leaves of *Glycine max* and *Lupinus albus* treated with Fe foliar applications (Moraghan et al. 1986; Moraghan 1992). In *G. max*, decreases in shoot Mn concentration were found when Fe was applied to the soil (Ghasemi-Fasaei et al. 2002), but in another study Fe foliar fertilisation did not change shoot Mn concentrations (Moosavi and Ronaghi 2011).

Effects of foliar fertilisation on Mn-deficient plants

In the case of Mn-deficient plants, both foliar treatments (Mn alone or Mn + Fe) led to similar effects: plants responded by leaf re-greening, with general increases in Mn concentrations and total contents, biomass and photosynthetic pigment concentrations in treated leaves. The only difference found between the two treatments used was that with the combined (Mn + Fe) fertiliser formulation, the biomass of the treated leaves and stems reached values similar to those in the controls, whereas with Mn alone the values were still lower than those in the controls (Fig. 2). This suggests that the additional supply of Fe during Mn resupply may allow for a more rapid recovery. In previous studies, biomass increases occurred when using formulations including Mn, Fe, Zn and B in *S. lycopersicum* and *B. vulgaris* (Naga Sivaiah et al. 2013; Gobarah et al. 2014). whereas the combination of Fe and Mn resulted in a better yield when compared to Mn alone in *H. vulgare* (Bameri et al. 2012). At the end of the experiment, leaf SPAD values in fertilised plants had increased approximately 30–50 % compared to the initial values, although SPAD values were still lower than those found in the Mn-sufficient plants. The fact that re-greening did not occur in leaf L4 (Fig. 4) suggests that Mn transport may be preferentially focussed to the youngest leaves. Upon fertilisation with both treatments, the *Chl a*/*b* ratio increased significantly and the (A + Z)/(V + A + Z) ratios decrease significantly, suggesting a stress recovery.

Manganese applied as $MnSO_4$ led to a small but significant increase in the Mn content of untreated

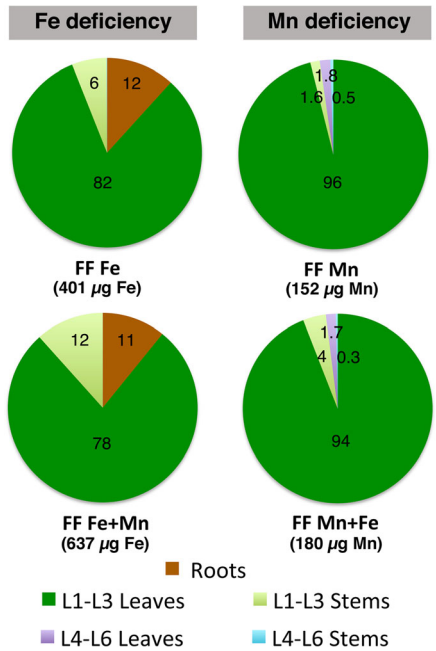

Fig. 8 Allocation of the increases in plant Fe and Mn upon foliar fertilisation. The total increases in Fe and Mn (shown in parentheses in the figure) were calculated from the initial plant contents before fertilisation, and the final contents after foliar fertilisation shown in Table 3. Allocations are given in percentages

leaves. Although the largest part of the plant in which the Mn increase after foliar treatments (Mn/Mn + Fe) compared to the Mn-deficient plants was in the leaves treated with the fertiliser (96/94 %), the contents of Mn increased not only in the stems of the treated leaves (2–4 %) but also in the leaves that were not directly treated with the fertiliser (2 %) (Fig. 8). This was associated with increases in Mn concentrations, biomass, SPAD values and photosynthetic pigment concentrations in the untreated leaves. This increase is likely to occur via xylem sap, as suggested by previous studies (White 2012). although no specific Mn form responsible for xylem sap transport has been identified so far (Álvarez-Fernández et al. 2014). In other studies, Mn was found to be transported from the basal treated leaves to untreated leaves but not to the roots in citrus plants (Papadakis et al. 2007). whereas in *P. ginseng* plants Mn was translocated from leaves to roots after foliar fertilisation (Zhang et al. 2013). These reports show that redistribution of Mn within plants is complex, and redistribution may depend on species, biological stage and physiological status of the plant.

The increases in total Mn content (including all plant parts) were 152 and 180 μg in the Mn only and the Fe + Mn treatments, respectively. This indicates that approximately 51–61 % of the applied Mn was in the plants at the end of the experiment, with the highest values found in the combined Fe + Mn application. It should also be taken into account that a fraction of the leaf Mn in the treated leaves can be removed by 0.1 N HCl at the end of the experiment, supporting that it could be located on the surface of the leaf.

Foliar fertilisation with Mn (Mn and Mn + Fe) also affected the concentrations of other metals. Root concentration decreases were found for Fe, Cu and Zn, suggesting that Mn may modify the status of other micronutrients in roots. Also, when data are expressed on a content per plant basis, Mn + Fe foliar fertilisation led to a 2-fold increase in Fe content in roots, in agreement with what happens after Fe foliar treatments in Fe-deficient plants, providing further support for the view that Fe applied to the foliage as $FeSO_4$ can be transported to roots.

Effects of foliar fertilisation on Fe-,Mn-deficient plants

In the case of Fe- and Mn-deficient plants, the combined Fe + Mn foliar fertilisation resulted in a significant re-greening, with increases in photosynthetic pigment concentrations and Fe and Mn concentrations and contents in the treated leaves. At the end of the experiment, leaf SPAD values in fertilised plants had increased by approximately 72 % compared to the initial values. However, biomass did not increase 8 days after foliar treatment, likely because the double deficiency affected the plant biomass to a large degree than individual deficiencies. The *Chl a/b* ratio increased after foliar treatment in all leaves, from the low values characteristic of Mn deficiency up to levels higher than the control ones; this is, as indicated above, an over-shooting effect characteristic of the recovery from Fe deficiency. The concentration of V + A + Z cycle pigments increased after foliar treatment in all leaves, but the (A + Z)/(V + A + Z) ratio decreased only in treated leaves, indicating that untreated leaves were still stressed.

The largest part (92–93 %) of the Mn and Fe increases after $MnSO_4$ and $FeSO_4$ applications corresponded to the leaves treated with the fertiliser. Minor increases in contents were observed in untreated leaves (approximately 1 % of the Fe and 2 % of the Mn), whereas no Mn or Fe increase was observed in roots.

On the other hand, Mn + Fe foliar fertilisation to Mn-,Fe-deficient plants did not change significantly the concentrations or contents of Cu and Zn in roots, whereas the Fe concentration in roots decreased

significantly (by 53 %), as it occurred after foliar fertilisation in Mn-deficient plants. However, Mn + Fe foliar fertilisation to Mn-,Fe-deficient plants led to increases in Zn concentrations and contents in treated and untreated leaves.

Concluding remarks

In summary, the main agronomic implication of the results is that using a combination of $FeSO_4$ and $MnSO_4$ in foliar fertiliser formulations does not seem to cause decreases in treatment efficiency in Fe- and Mn-deficient *S. lycopersicum* plants. The combined Fe + Mn treatment also works well in Fe-,Mn-deficient plants. Also, results provide further support for the view that Fe foliar fertilisation with $FeSO_4$ must cover as much foliage as possible, since effects are limited to the leaf area directly wetted with the fertiliser formulation. Furthermore, results show that a significant part of the Fe content increases after foliar fertilisation resides in the roots, thus emphasising the need for investigating what are the physiological consequences of foliar fertilisation on the modulation of the root responses to Fe deficiency.

On the other hand, the allocation of the metal increases after foliar fertilisation was different for Fe and Mn. Increases in Fe contents occurred in the roots (11–12 %), likely via phloem sap, but not in untreated leaves, whereas some increases in Mn contents (2 %) occurred in the untreated leaves but not in the roots. These percentages may underestimate slightly the real remobilisation capacity from the treated leaf areas to the roots or to the non-treated leaves, since they were calculated based on the total leaf metal contents, and a portion of the metals (usually below 10 %, depending on the specific conditions) can be immobilised at the leaf surface. Since all results were obtained using relatively young (1-month old) plants, we cannot exclude that the remobilisation of metals applied to older plant material could have been different.

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