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ORIGINAL PAPER

Phytosiderophores released by graminaceous species promote ^{59}Fe-uptake in citrus

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Abstract Chlorosis-susceptible fruit trees growing on calcareous soils have been observed to recover in the presence of grass cover species. However, the physiological mechanisms behind this phenomenon are only scarcely understood. An investigation was carried out to verify whether citrus plants can use ^{59}Fe solubilized from a sparingly soluble source by the phytosiderophores (PS) released from graminaceous species. Experiments were performed in hydroponics, using two citrus rootstocks differing in their sensitivity to Fe-deficiency in the field (*Poncirus trifoliata* × *Citrus paradisi*, citrumelo ''Swingle'', highly susceptible, and *Citrus aurantium* L., moderately tolerant). Barley (*Hordeum vulgare* L., cv Europa) was used as a model species for PS-releasing graminaceous plants. Fe-deficient citrus plants increased ^{59}Fe-uptake from ^{59}Fe-hydroxide supplied inside a dialysis tube, when Fe-deficient barley plants or PS-containing barley root exudates were present in the uptake solution, this effect being particularly evident for the susceptible rootstock. ^{59}Fe-uptake from ^{59}Fe-hydroxide was also enhanced in Fe-deficient citrumelo ''Swingle'' in the presence of Fe-deficient *Poa pratensis* L. and *Festuca rubra* L., two perennial grasses normally grown in association with fruit trees; no effect was found when Fe-sufficient grasses were employed. The uptake of ^{59}Fe by the susceptible citrus rootstock increased in proportion to the amount of 2′-deoxymugineic acid (DMA), the major PS released by Fe-deficient *F. rubra*, present in the uptake solution. The beneficial effect of *F. rubra* or *P. pratensis* was evident from the leaf re-greening observed when Fe-deficient citrumelo ''Swingle'' plants were grown in association with the grasses in pots filled with a calcareous soil. Leaf re-greening was not observed when citrumelo ''Swingle'' plants and *yellow stripe 3* (*ys3*) maize (*Zea mays* L.) mutant plants, unable to release PS, were co-cultivated in pots filled with calcareous soil, unless exogenous PS were added to the soil. Results indicate that graminaceous cover species can improve the Fe-nutrition of fruit trees grown on calcareous soils by enhancing Fe-availability.

Keywords Citrus · Barley · Wild grasses · Cover crops · Iron deficiency · Phytosiderophores

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Introduction

Fruit tree species grown in alkaline and/or calcareous soils, such as peach, pear, citrus, and kiwifruit, often show symptoms of Fe chlorosis, with consequent, dramatic limitations in terms of plant vigour and productivity, fruit quality and productive period of the orchards (Pestana et al. 2003; Rombolà and Tagliavini 2006). At present, Fe chlorosis is mostly controlled by applications of synthetic Fe-chelates to the soil or the canopy (Tagliavini and Rombolà 2001). This treatment, however, is very expensive and its benefits are only temporary. The low stability of some of these compounds (citrate, gluconate, lignosulfonate) and synthetic chelates (HEDTA, EDTA, DTPA) makes them unsuitable for maintaining Fe in solution, particularly when applied to calcareous soils (Lucena 2003), where they are most needed. On the other hand, the use of more stable synthetic Fe-chelates such as Fe-EDDHA, Fe-EDDHMA, although quite effective, are not to be recommended because of their high cost and the environmental risks linked to their mobility along the soil profile (Cesco et al. 2000; Rombolà et al. 2002). It is therefore essential to study and develop new strategies to counteract Fe-deficiency that are economical, long-lasting and with a low environmental impact.

Various authors (Kamal et al. 2000; Tagliavini et al. 2000) have suggested the use of grasses as cover crops to prevent or cure Fe-chlorosis in orchards. The beneficial effects have been described for various crops, including peanut (Zuo et al. 2000), but the physiological mechanisms involved in the process are still rather unclear. Graminaceae (Poaceae) respond to low Fe-availability by releasing chelators (phytosiderophores, PS) with a high affinity for $Fe^{(III)}$ into the rhizosphere (Takagi 1976); these molecules solubilize Fe and are taken up by the plants as whole complexes (Römheld and Marschner 1986). Dicots and non-graminaceous monocots on the other hand respond to Fe-deficiency by increasing reduction of $Fe^{(III)}$-chelates at the rhizoplane, thus taking up Fe^{2+} (Schmidt 1999). Generally, grasses are more efficient than dicots in acquiring Fe from calcareous soils, since the mechanisms involved in their response to Fe-deficiency are less sensitive to high pH and bicarbonate levels (Römheld 1991).

Various authors (Awad et al. 1988; Treeby et al. 1989) have proved that PS released by graminaceous species can mobilize Fe from sparingly soluble soil sources; the $Fe^{(III)}$-PS complexes thus formed can be taken up by dicotyledonous plants after reduction (Römheld and Marschner 1986). These results might explain the beneficial effects of intercropping dicots with grasses: re-greening and increased Fe content, were observed in herbaceous dicots grown in solution culture (Hopkins et al. 1992), as well as under glasshouse or field conditions (Zuo et al. 2000).

This investigation was aimed at assessing whether Fe-deficient citrus plants can effectively utilize ^{59}Fe solubilized from a sparingly soluble form (^{59}Fe-hydroxide) by the PS released from the roots of graminaceous species. Two citrus rootstocks differing in their sensitivity to Fe-deficiency were used in nutrient solution experiments, in combination with barley, a model donor for PS. Furthermore, two perennial grasses normally grown in association with fruit trees, *Poa pratensis* and *Festuca rubra*, were tested for their capacity to improve Fe-uptake by the susceptible citrus rootstock. The ability of the susceptible citrus rootstock to use Fe mobilized by the PS released from the roots of graminaceous species was also evaluated in pot experiments by growing tree plants with either perennial grasses or *yellow stripe 3* (*ys3*) maize mutant plants, which are unable to release PS.

Materials and methods

Plant material and growth conditions

Nutrient solution culture

Citrus rootstocks (*Poncirus trifoliata* × *Citrus paradisi*, genotype ''Swingle'', highly susceptible to iron chlorosis; *Citrus aurantium* L., moderately tolerant to iron chlorosis) seeds were germinated on filter paper moistened with 1 mM $CaSO_4$ for 6 and 20 days, respectively. The seedlings were then transferred in a continuously aerated

nutrient solution as reported by Pinton et al. (1999) for a further 31 days.

Barley seedlings (*Hordeum vulgare* L., cv Europa), germinated for 4 days on filter paper moistened with 1 mM $CaSO_4$, and *Festuca rubra* L. and *Poa pratensis* L. seedlings, germinated for 10 days on close-knitted nets suspended over short tubes filled with 1 mM $CaSO_4$, were transferred for a further 10 days to an aerated nutrient solution as described by Zhang et al. (1991).

Iron, when present (Fe-sufficient plants), was provided as FeEDTA at a final concentration of 40 μM. The seedlings were grown in nutrient solutions under the following conditions: 16/8 h day/night regime; 220 μmol m^{-2} s^{-1} light intensity; temperature (day/night) 25/20°C; RH 70–80%. In order to improve the Fe status of sufficient plants and avoid latent Fe-deficiency, (*epi*-HMA)-containing root exudates collected from Fe-deficient barley plants were mixed with 20 μM $FeCl_3$ and then added to the nutrient solution of Fe-sufficient barley, *F. rubra* and *P. pratensis* plants for 3 days prior to the experiment.

At the end of the growth period in the nutrient solution deprived of Fe, all plants revealed visible deficiency symptoms (leaf chlorosis). SPAD index was determined using a portable SPAD-502 meter (Minolta, Osaka, Japan).

Soil culture

Citrumelo ''Swingle'' plants grown for 31 days in a nutrient solution lacking Fe were transferred into plastic pots (ø33 mm × 200 mm height) filled with 250 g of a calcareous soil (pH 8.2, total $CaCO_3$ 72.9%, active $CaCO_3$ 12.0%, DTPA-Fe 7.8 μg g^{-1}, water content at the field capacity, i.e. 19.5%) mixed with quartz sand at a 9:1 ratio. The soil was air-dried and sieved through a 2-mm sieve before use. The pots were arranged in a complete randomized design with six replicates for each treatment. Two weeks later, *F. rubra* or *P. pratensis* seeds were sown in some pots, whereas in others, *ys3* maize (*Zea mays* L.) mutants, germinated for 4 days on filter paper moistened with 1 mM $CaSO_4$, were planted (two seedlings per pot). The control (citrus alone) and intercropped citrus plants were grown for a further 5 weeks at a 16/8 h day/night regime (25/20°C, respectively) and RH of 70–75%. Soil water content was maintained at the field capacity by daily watering with a Fe-free nutrient solution (Pinton et al. 1999) containing 1 g l^{-1} $CaCO_3$. A number of pots containing citrumelo ''Swingle'' and *ys3* plants were watered with a similar solution that also contained 175 μM *epi*-HMA (PS collected from Fe-deficient barley plants). At the first day (beginning of the experiment) the appropriate nutrient solution was added to the soil of each pot up to the field capacity and the weight of the pot measured. Fluctuations in soil water content were determined by weighing daily the pots and then adding a new Fe-free nutrient solution up to the initial weight, assuming that daily biomass accumulation were negligible.

In order to collect samples of the soil solutions, some pots were irrigated with 10 ml of the Fe-free nutrient solution exceeding the field capacity: leachates were collected, filtered at 0.2 μm and Fe concentration was determined by ICP-OS.

Leaf chlorophyll levels were estimated in the three youngest leaves at the end of the growth period, using a SPAD-502 meter (Minolta, Osaka, Japan). Fe concentration in leaves of citrumelo ''Swingle'' plants was determined by ICP-OS, after ashing the tissues at 550°C, and suspending in 1% (w/v) HNO_3.

Preparation of amorphous ^{59}Fe-hydroxide

Amorphous ^{59}Fe-hydroxide was obtained by precipitating $^{59}Fe(NO_3)_3$ at alkaline pH levels, with the addition of 1 N KOH (Guzman et al. 1994). The specific activity of the labelled iron was 144 KBq μmol^{-1} Fe.

Solubilization and uptake of ^{59}Fe

Experiments were carried out as described by Awad et al. (1988) with slight modifications (Fig. 1). One milliliter of suspension containing ^{59}Fe-hydroxide (2 μmol Fe) was transferred into a dialysis tube (ZelluTrans/Roth 6.0, ø16 mm, exclusion limit of 8÷10 kDa, ROTH, Karlsruhe, Germany) and mixed with 6 ml of a nutrient solution (Solution A) having the following composition (mM): K_2SO_4 0.7, KCl 0.1, $Ca(NO_3)_2$ 2.0, $MgSO_4$ 0.5, KH_2PO_4 0.1, Hepes–KOH 10

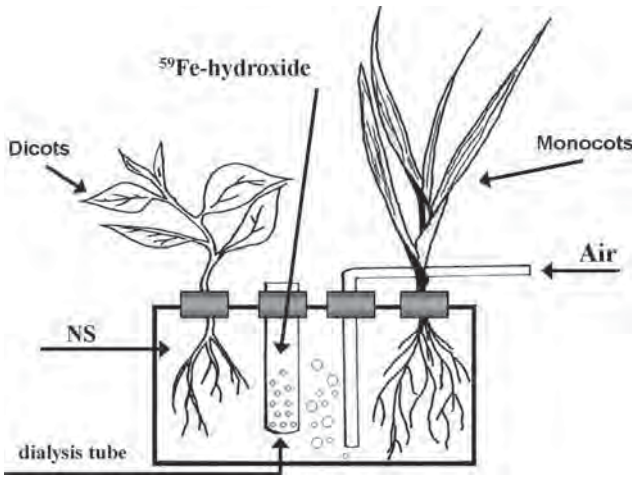

Fig. 1 Diagram of laboratory apparatus used to measure ^{59}Fe-uptake by roots of dicots and monocots

(pH 7.5). The dialysis tube was then transferred into 250 ml flasks containing 200 ml of solution A. The solution outside the dialysis tube was aerated by bubbling. Six barley plants were transferred into each flask, after rinsing the roots with Fe-free nutrient solution for 30 min. Uptake was measured both in the morning (2 h after the onset of light) and the evening (11 h after light onset), when barley plants release high and low levels of PS, respectively. The experiment was started by adding ^{59}Fe-hydroxide into the dialysis tubes, and lasted for 4 h. During this period, samples were collected every 30 min from the solution outside the dialysis tube and ^{59}Fe content was measured by liquid scintillation counting.

In order to examine the effect of barley root exudates on ^{59}Fe-uptake by citrus rootstocks, the above experiments were carried out using one citrus and six barley plants per flask, or citrus plants alone, having added exudates released by Fe-deficient barley plants during the morning (high PS release) to the solution used to measure ^{59}Fe-uptake.

At the end of the uptake period (4 h), the plants were transferred into a freshly prepared ^{59}Fe-free nutrient solution for 10 min and then harvested. Root apoplast ^{59}Fe was removed with 1.2 g l^{-1} sodium dithionite and 1.5 mM 2,2′-bipyridyl in 1 mM $Ca(NO_3)_2$ under N_2 bubbling, as described by Bienfait et al. (1985). Roots and shoots were oven-dried at 80°C, weighed, ashed at 550°C, and suspended in 1% (w/v) HCl for ^{59}Fe determination by liquid scintillation counting. The ^{59}Fe-uptake rate, measured as nmol ^{59}Fe, refers to the whole plant (root + shoot) and is expressed per g (dry weight) of roots per hour.

The effect of grass-borne root exudates on ^{59}Fe-uptake by citrumelo ''Swingle'' was examined using 1 citrumelo and 25 ÷ 30 *F. rubra* or *P. pratensis* plants immersed in 200 ml of solution A (pH 7.5) containing ^{59}Fe-hydroxide (2 μmol Fe); ^{59}Fe-uptake was measured after 24 h of root contact with the solution, as described previously. The effect of different levels of the phytosiderophore DMA (2′-deoxymugineic acid) was also determined using the same experimental procedure, adding the compound directly into the flask. In this case, the grass plants were absent. DMA was purchased from Toronto Research Chemicals Inc., North York, Ontario, Canada.

To characterize the response of the two citrus rootstocks to Fe-deficiency, ^{59}Fe-uptake was measured in separate experiments at pH 6.0 for 24 h using ^{59}Fe-hydroxide (2 μmol Fe) as a source of Fe.

Collection of root exudates and quantitative analysis of *epi*-hydroxymugineic acid (*epi*-HMA)

Root exudates released by Fe-deficient barley plants were collected after transferring plants to 100 ml aerated distilled water for 4 h during the morning (high PS release) or the evening (low PS release). Root exudates were filtered and then passed through a cation-exchange resin column

filled with Amberlite IR-120B resin (H^+-form; Sigma–Aldrich) (Ma et al. 1999). After washing with distilled water, the PS retained by the cation-exchange resin were eluted with 2 M NH_4OH and the eluate was concentrated to dryness in a rotary evaporator (40°C) (Ma et al. 1999). The residue was re-dissolved in 1 ml water and an aliquot of 100 µl was air-dried, derivatized with phenylisothiocyanate (PITC) and analysed (Howe et al. 1999) by HPLC (LC-1000, Jasco, Tokyo, Japan). The HPLC system was equipped with a C_{18} column (XTerra RP 18; 150 mm long, 4.6 mm i.d., 3 µm particle size; Waters), a Borwin-PDATM 1.50 version (JMBS, Grenoble, France) controller and a PU-1580 pump. The UV absorption spectra of eluate components were obtained using a Jasco model UV–VIS MD-1510 photodiode array detector. Purified *epi*-HMA was used as a standard.

$Fe^{(III)}$EDTA reduction by intact roots

Reduction of $Fe^{(III)}$EDTA by the roots of intact citrus plants was measured as described by Pinton et al. (1999) using the bathophenanthrolinedisulfonate (BPDS) reagent (Chaney et al. 1972). Roots were incubated for 30 min in an aerated solution containing 0.5 mM $CaSO_4$, 250 µM FeEDTA, 300 µM BPDS, 10 mM Mes–NaOH (pH 5.5) in the dark at 25°C.

Results

Effect of barley root exudates on ^{59}Fe-uptake by citrus rootstocks

The two citrus rootstocks, *Citrus aurantium* and citrumelo ''Swingle'', after 31 days of growth in a Fe-free nutrient solution revealed visible symptoms of deficiency (leaf chlorosis). At this stage however, only *Citrus aurantium* (moderately tolerant) was able to activate physiological responses to this form of stress, i.e. acidification of the nutrient solution and enhanced $Fe^{(III)}$-chelate reductase activity, resulting in higher ^{59}Fe-uptake levels from ^{59}Fe-hydroxide at pH 6.0 (Table 1). Similar results were obtained when the uptake by the two Fe-deficient rootstocks was measured at pH 7.5 using ^{59}Fe-hydroxide supplied inside a dialysis tube (Fig. 4, control).

Barley plants were used to investigate the contribution of grass species to Fe-uptake by citrus plants. The massive amounts of PS released by these plants into the soil follow a typical diurnal pattern.

Figure 2 shows that Fe-deficient barley plants could take up ^{59}Fe from ^{59}Fe-hydroxide supplied in a dialysis tube at higher rates than Fe-sufficient plants. This effect was conceivably due to the mobilization of ^{59}Fe by phytosiderophores (PS) released during the morning (abundant PS release); in fact, exudates collected before noon from Fe-deficient plants contained large amounts of *epi*-HMA (13.5 ± 0.8 µmol g^{-1} root DW), the major PS released by barley roots, and could promote uptake of ^{59}Fe in Fe-deficient plants when supplied in the evening (when PS release was scarce). On the other hand, *epi*-HMA was not detected in exudates collected in the evening from Fe-deficient plants and hardly detectable (≤0.05 µmol g^{-1} root DW) in those collected in the morning from Fe-sufficient plants. Moreover, during the period of high PS release, larger amounts of ^{59}Fe were present in the uptake solution of Fe-deficient plants than that of Fe-sufficient ones (Fig. 3).

Table 1 SPAD index, root $Fe^{(III)}$EDTA reducing activity (nmol Fe^{II} g^{-1} FW h^{-1}), pH value of the nutrient solution (NS) and ^{59}Fe-uptake (nmol ^{59}Fe g^{-1} root DW h^{-1}) in *Citrus aurantium* and citrumelo ''Swingle'' plants grown for 31 days in nutrient solution containing (+Fe) or lacking (–Fe) 40 µM FeEDTA

	Citrus aurantium		citrumelo ''Swingle''	
	+Fe	–Fe	+Fe	–Fe
SPAD index	69 ± 3	38 ± 9	42 ± 2	31 ± 4
$Fe^{(III)}$-reduction	28 ± 3	65 ± 10	37 ± 5	29 ± 8
pH of NS	6.7 ± 0.2	4.8 ± 0.3	6.7 ± 0.2	6.8 ± 0.3
^{59}Fe-uptake	20 ± 3	39 ± 4	19 ± 3	17 ± 2

Data are the means ± SE of three independent experiments (n = 4)

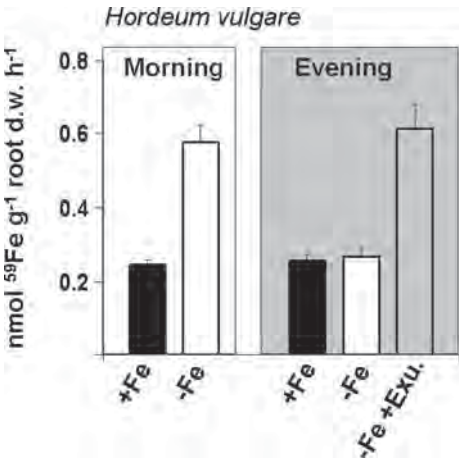

Fig. 2 Uptake of ^{59}Fe by Fe-sufficient (**+**Fe) and Fe-deficient (–Fe) barley plants supplied with ^{59}Fe-hydroxide inside a dialysis tube (2 μmol Fe/tube, pH 7.5). The experiments were run for 4 h in the morning (high PS release) and in the evening (low PS release). In the latter case, Fe-deficient barley exudates collected in the morning were also added to the uptake solution of Fe-deficient barley plants (–Fe + Exu.). Data are means ± SE of three independent experiments (n = 4)

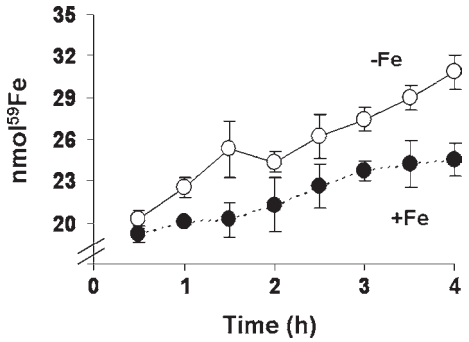

Fig. 3 Time-course of ^{59}Fe content measured in the uptake solution containing roots of intact Fe-sufficient (+Fe) or Fe-deficient (–Fe) barley plants during the experiments run in the morning (see Fig. 1). Iron was supplied as ^{59}Fe-hydroxide inside a dialysis tube (2 μmol Fe/tube, pH 7.5); at time intervals, samples from the solution outside the dialysis tube were collected and the amount of ^{59}Fe measured. Data are means ± SE of three independent experiments (n = 4)

The amount of ^{59}Fe taken up by Fe-deficient citrus plants increased when Fe-deficient barley plants were present in the uptake solution, particularly in the case of the susceptible rootstock (citrumelo ''Swingle'') (Fig. 4). This effect was not observed when the uptake experiments were run in the presence of Fe-sufficient barley plants (Fig. 4) or in the evening (data not shown). Enhanced ^{59}Fe-uptake by citrus rootstocks was also observed in a separate experiment, where exudates collected from Fe-deficient barley plants during the period of high PS release (morning) were added to the solution.

Effect of grass-borne root exudates on ^{59}Fe-uptake by citrumelo ''Swingle''

In order to investigate a system similar to that encountered in intercropped orchards, experiments were carried out using *P. pratensis* and *F. rubra* to improve Fe-uptake by citrumelo ''Swingle''.

Both grass species enhanced their capacity to take up ^{59}Fe when grown in a Fe-free nutrient solution as compared to their respective controls (118.7 ± 6.6 vs. 54.6 ± 2.5 nmol g^{-1} root DW h^{-1} for *P. pratensis* and 97.9 ± 5.0 vs. 48.7 ± 2.1 nmol g^{-1} root DW h^{-1} for *F. rubra*).

Plants of Fe-deficient citrumelo ''Swingle'' were able to take up a significantly larger amount of ^{59}Fe from ^{59}Fe-hydroxide when Fe-deficient *P. pratensis* or *F. rubra* plants were also present in the uptake solution (+87% and +73%) (Fig. 5). These experiments were run adding ^{59}Fe-hydroxide directly to the uptake medium, thus determining a much higher uptake in Fe-deficient citrus plants. Nevertheless, the beneficial effect could be observed only when Fe-deficient but not Fe-sufficient grasses were used (Fig. 5).

In the absence of the two grass species, a progressive rise in ^{59}Fe-uptake by the citrus rootstock could be obtained by adding increasing amounts of 2′-deoxymugineic acid (DMA), the major PS released by Fe-deficient *F. rubra*, to the uptake solution (Fig. 6).

Effect of graminaceous species on leaf re-greening of soil-grown citrumelo ''Swingle''

The beneficial effect of *P. pratensis* and *F. rubra* on the Fe-nutrition of citrumelo ''Swingle'' was also evident when the latter plants, following 31 days of growth in a Fe-free solution, were transferred for 5 weeks to pots filled with calcareous soil. When *P. pratensis* or *F. rubra* seeds were sown and grown

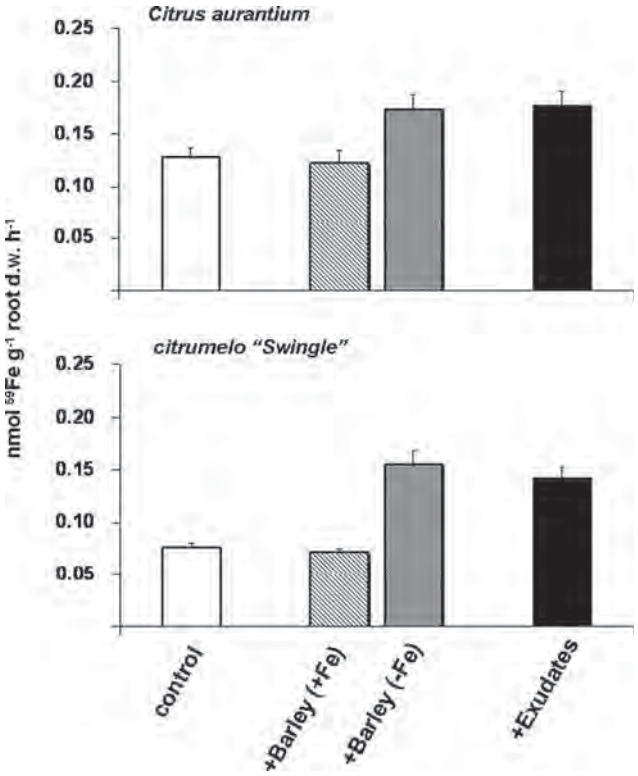

Fig. 4 Uptake of ^{59}Fe by Fe-deficient citrus rootstocks, the source of iron being ^{59}Fe-hydroxide inside a dialysis tube (2 μmol Fe per tube, pH 7.5). Measurements were also made in the presence of Fe-sufficient [**+**Barley(+Fe)] or Fe-deficient [**+**Barley(–Fe)] barley plants, and of root exudates collected from Fe-deficient barley plants in the morning (**+**Exudates). Data are means ± SE of three independent experiments (n = 4)

in the pots containing Fe-deficient citrus, the latter plants showed signs of recovery from leaf chlorosis (Fig. 7A). The SPAD index and Fe concentration were greater than those measured on the leaves of Fe-deficient plants grown alone (Table 2). The presence of grasses also induced greater water consumption (Table 2).

In order to verify how the presence of the grasses affected the amount of Fe in the soil solution, some pots were irrigated for one day with a Fe-free nutrient solution over the field capacity; the leachates were then collected and analysed. Table 2 shows that the leachates from pots containing citrus plants and grasses

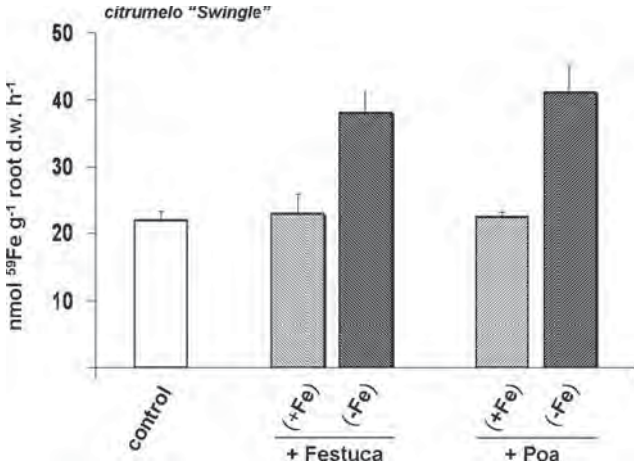

Fig. 5 Uptake of ^{59}Fe by Fe-deficient citrumelo ''Swingle'' plants either alone or in the presence of Fe-sufficient **(+**Fe**)** or Fe-deficient (–Fe) *F. rubra* and *P. pratensis* plants. Iron was supplied as ^{59}Fe-hydroxide (2 μmol Fe, pH 7.5). Data are means ± SE of three independent experiments (n = 4)

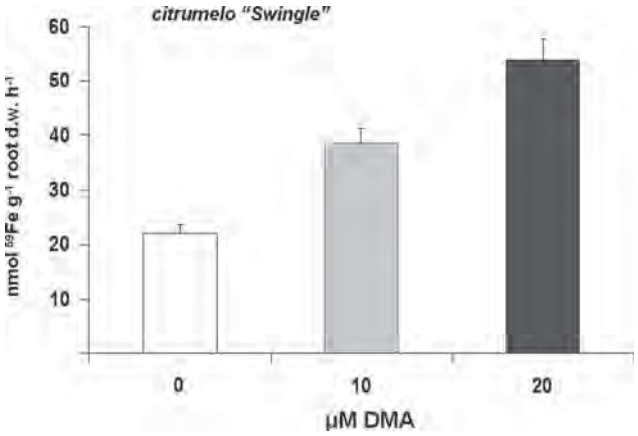

Fig. 6 Effect of increasing concentrations of DMA on ^{59}Fe-uptake by Fe-deficient citrumelo ''Swingle'' plants. The micronutrient was supplied as ^{59}Fe-hydroxide (2 µmol Fe, pH 7.5). Data are means ± SE of three independent experiments (n = 4)

contained larger amounts of Fe than those collected from the pots containing only the rootstock.

The contribution of PS released by graminaceous plants to Fe nutrition of citrus was further evaluated by co-cultivating, citrumelo ''Swingle'' and *ys3*, a maize mutant that cannot release PS (Lanfranchi et al. 2002), in pots filled with calcareous soil. Under these condition, the plants were unable to recover, as proved by SPAD index values (Table 3), and the maize plants revealed visible symptoms of Fe-deficiency, too (Fig. 7B). Recovery was only achieved by daily applications of exogenous PS, collected from Fe-deficient barley plants, to the soil of control and *ys3*-intercropped citrus plants. In the latter case, leaf re-greening was also observed in the maize plants (Fig. 7B). Soil water consumption was increased when citrus and maize plants were grown in mixed culture, but unaffected by the addition of PS (Table 3).

Discussion

Re-greening of fruit trees observed in calcareous soils when grass is grown along the rows of trees (Kamal et al. 2000; Tagliavini et al. 2000) has been attributed to the capability of graminaceous plant species to increase the availability of Fe by releasing phytosiderophores (PS) into the soil (Ma et al. 2003), thus favouring the uptake of this micronutrient by the fruit trees.

Grass-borne PS can in fact solubilize Fe from sparingly soluble sources (Awad et al. 1988; Takagi et al. 1988); this action would in turn lead to the formation of $Fe^{(III)}$-PS complexes that can be used by dicots as a substrate for the reduction-based uptake of the micronutrient (Römheld and Marschner 1986).

This work was undertaken to assess the ability of graminaceous species to improve Fe acquisition in two rootstocks of citrus, a fruit tree that often shows symptoms of Fe chlorosis when grown in calcareous soils. Citrus genotypes display different degrees of susceptibility, which in turn has been inversely related to their ability to respond to Fe-deficiency by enhancing acidification of external medium and $Fe^{(III)}$-chelate reduction (Treeby and Uren 1993; Manthey et al. 1994). When Fe-starved, the moderately tolerant *Citrus aurantium* L. increased $Fe^{(III)}$-reduction and acidified the external solution to a greater extent than the Fe-sufficient controls, with a consequent stimulation of ^{59}Fe-uptake rates; on the other hand, the highly susceptible citrumelo ''Swingle'' failed to develop any physiological response to Fe-deficiency and could not increase its capacity to take up ^{59}Fe (Table 1).

Both citrus rootstocks enhanced ^{59}Fe-uptake from ^{59}Fe-hydroxide supplied inside a dialysis tube when the experiments were run in the presence of Fe-deficient barley plants or the latter's PS (*epi*-HMA)-containing exudates were added to the uptake solution (Fig. 4). No benefits were evident when Fe-sufficient barley plants were used. These results agree with the observations by Walter et al. (1995) on the diurnal release pattern of PS by Fe-deficient barley plants and suggest that citrus plants can use the Fe-PS complexes formed by the solubilizing action of PS as a source of Fe.

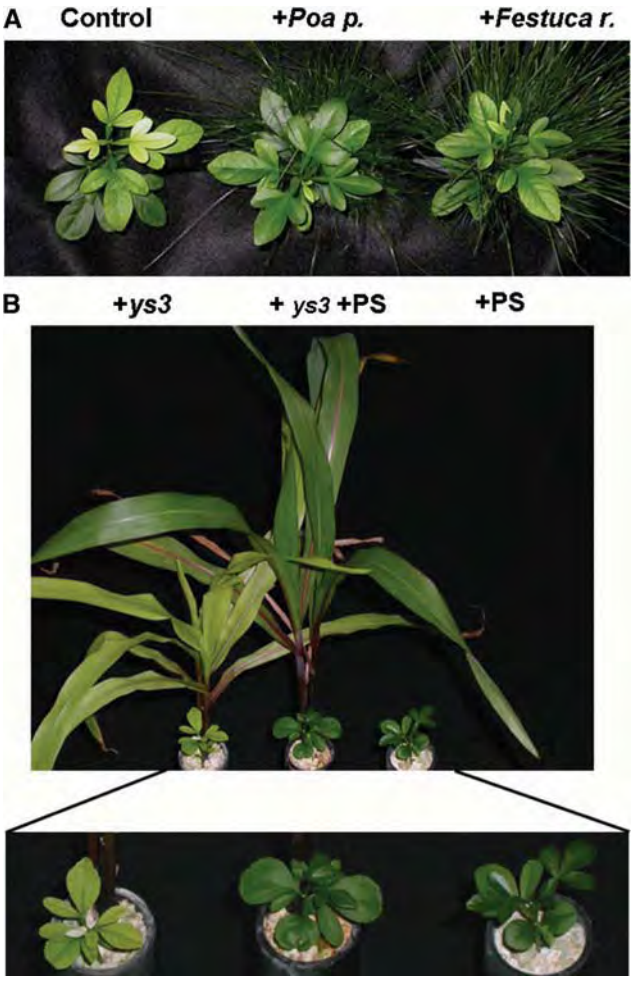

Fig. 7 Effect of co-cultivation with *P. pratensis*, *F. rubra* (panel A) or *ys3* maize (panel B) plants on leaf re-greening of Citrumelo ''Swingle'' grown for 5 weeks in pots filled with calcareous soil

Interestingly, the effect was more evident in the susceptible rootstock (citrumelo ''Swingle'') that could therefore derive greater benefits from intercropping with graminaceous species than the moderately tolerant citrus. In this context, the susceptible rootstock was used for further investigations aimed at analysing the contribution of two perennial grasses, normally employed in association with fruit trees (Tagliavini et al. 2000), to its Fe-nutrition. Recent experiments performed on kiwifruit plants grown in chlorosis-inducing soils revealed that intercropping with various grass species (*Lolium perenne* L., *Poa pratensis* L., *Festuca rubra* L.) could relieve the

Table 2 SPAD index and Fe concentration in leaves of citrumelo ''Swingle'' plants grown either alone or in the presence of *P. pratensis* or *F. rubra* in pots filled with calcareous soil

Growth condition of citrumelo ''Swingle''	SPAD index	Leaf Fe (μg g^{-1} DW)	Daily fluctuations in soil water content (% of field capacity)	Fe in the leachate (μg l^{-1})
Alone	23.5 ± 8.1	56.8 ± 8.4	6.4 ± 3.6	4.7 ± 0.4
+*Poa pratensis*	50.2 ± 4.8	119.3 ± 2.7	29.6 ± 6.2	8.0 ± 2.2
+*Festuca rubra*	41.3 ± 3.8	87.3 ± 2.1	39.0 ± 9.2	7.8 ± 1.4

The table also reports the daily fluctuations in soil water content, calculated by weighing pots before and after the daily irrigation with a Fe-free nutrient solution containing 1 g l^{-1} $CaCO_3$, and Fe content in the leachates. Data are the means ± SE of two independent experiments (n = 8)

Table 3 SPAD index in citrumelo ''Swingle'' and *ys3* maize mutant plants co-cultivated in pots filled with calcareous soil and watered daily to field capacity with a Fe-free nutrient solution with or without 175 μM *epi*-HMA (PS)

Growth condition of citrumelo ''Swingle''	SPAD index citrumelo ''Swingle''	*Ys3*	Daily fluctuations in soil water content (% of field capacity)
Alone	24.0 ± 5.6	–	5.0 ± 2.4
+*ys3*	19.0 ± 6.0	14.1 ± 3.5	22.3 ± 6.4
+*ys3* + PS	40.6 ± 7.5	29.5 ± 5.5	26.3 ± 5.0
+PS	45.5 ± 11.8	–	7.2 ± 2.8

The daily fluctuations in soil water content are also reported. Data are the means ± SE of two independent experiments (n = 8)

symptoms of Fe-deficiency in *Actinidia* (Rombolà et al. 2003), thus suggesting possible interactions between the two species.

The nutrient solution approach employed in this research confirms that Fe-deficient *F. rubra* and *P. pratensis* can enhance ^{59}Fe-uptake by citrumelo ''Swingle'', whereas no benefits were observed when Fe-sufficient grasses were employed (Fig. 5). This effect can probably be ascribed to the ability of grass-borne PS to mobilize Fe. In fact, *F. rubra* is known to release PS (DMA) when grown in a Fe-free medium (Ma et al. 2003), a condition, we observed, that enhances the grass's ability to take up ^{59}Fe from ^{59}Fe-hydroxide. Moreover, when growing amounts of DMA were supplied to Fe-deficient citrus plants, the uptake rates for ^{59}Fe augmented progressively (Fig. 6). These results clearly indicate that fruit trees growing on calcareous soils can benefit, in terms of Fe-nutrition, from intercropping with perennial grasses that increase Fe-availability for root uptake. This conclusion is further supported by a pot experiments: leaf re-greening and increase in Fe concentration were observed when susceptible citrus rootstocks planted in a calcareous soil (Fig. 7 A, Table 2) were co-cultivated with *F. rubra* or *P. pratensis*; furthermore the levels of Fe in the soil solution were increased in the mixed cultivation (Table 2). The possibility that the beneficial effect of grasses may be due to reduced soil water content (Table 2) and therefore bicarbonate toxicity, could not, however, be ruled out. In order to better clarify the role of PS to the Fe-nutrition of soil-grown citrus, Fe-deficient plants were grown in pots filled with calcareous soil, together with maize mutants (*ys3*) that are unable to release PS (Lanfranchi et al. 2002). These experiments revealed that re-greening of citrus leaves could only be achieved by adding exogenous PS to the solution irrigating the soil (Table 3, Fig. 7B); moreover, soil water content was not significantly affected by applications of PS. These results support the idea that PS released by graminaceous plant species can contribute to the Fe-nutrition of fruit trees; nonetheless, it should be noted that other factors, such as changes in soil water content, differences in Fe-mobilizing capacity (Gries and Runge 1992) and the nutrient demand of grasses can affect the capacity of cover crops to provide Fe to fruit tree plants. Fe-availability in orchards could also be affected by grasses through a control of the nitrification process in the soil (Lata et al. 2004).

The intense intermingling of citrus and grass roots noticed in pots (not shown) suggests an interaction at the rhizosphere (Zuo et al. 2000), but this hypothesis needs to be confirmed in the field.

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