Abstract: Bare root transplants of strawberry (Fragaria x ananassa Duch. cv. 'Selva') were transferred to nutrient solutions with or without iron (Fe). After six weeks of growth, plants in the solution without Fe were chlorotic and had morphological changes in roots typical of Fe deficiency. At this point, four treatments were imposed with three replicates (plants) each: plants always grown without Fe (Fe0); always grown with 10 μM Fe (Fe10); chlorotic plants with leaves treated with ferrous sulphate every two days (Fe-leaves); chlorotic plants transferred to a solution with ferrous sulphate (Fe-solution). These plants were grown for nine days through which period chlorophyll (Chl) in leaves was estimated with a SPAD-502 apparatus. At the beginning and end of the experiment, root ferric chelate reductase (FC-R; EC 1.16.1.17) activity and the mineral composition of leaves and roots were measured. Six days after the addition of Fe to the solution (Fe-solution) leaves had Chl content similar to (Fe10). When Fe was applied to leaves (Fe-leaves), a slight regreening of new leaves was observed only at the end of the experiment. At this date, the FC-R activity remained the same in (Fe10) and increased even further in (Fe0). Plants in the Fe-solution treatment maintained an FC-R activity similar to the initial value in chlorotic plants, while the activity was drastically reduced in plants of treatment (Fe-leaves). The Fe concentration in leaves of (Fe0) and (Fe10) was similar, while application of Fe to leaves or nutrient solution resulted in an enhanced concentration of Fe in leaves. In contrast to what happened in Fe-solution, application of Fe to leaves did not lead to an increase in the concentration of Fe in roots. Root FC-R was correlated with the concentrations of Mn, Zn and Cu in leaves and with Cu in roots. Under our experimental conditions, FC-R activity in strawberry may be rapidly de-activated by pulses of Fe applied by foliar sprays. On the other hand, this de-activation mechanism is slower if Fe is applied directly to roots, suggesting a greater opportunity for plants to uptake more Fe.
Please kindly consider the manuscript *Recovery of iron deficiency by iron resupply to roots or leaves of strawberry plants* for publication in Plant Physiology and Biochemistry. In this manuscript we present information involving the responses of Fe-deficient plants resupplied with Fe, which may provide crucial information for the optimization of the Fe-fertilization strategies. The aims of the present study were to characterize the changes induced by Fe depletion on the chlorophyll content, root FC-R and mineral composition of roots and leaves and to compare two different ways to resupply Fe: by foliar or root fertilization. We also discuss the agronomical consequences of Fe resupply by fertilization.
• The FC-R activity was higher in chlorotic plants than in green plants.

• The concentrations of Cu, Mn and Zn in roots of chlorotic plants were higher than those presented by green plants.

• FC-R activity in strawberry may be rapidly de-activated by Fe applied by foliar sprays.

• The de-activation mechanism is slower if Fe is applied directly to roots.

• Slower de-activation mechanisms suggests a greater opportunity for plants to uptake more Fe.
Recovery of iron deficiency by iron resupply to roots or leaves of strawberry plants

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ABSTRACT

Bare root transplants of strawberry (Fragaria x ananassa Duch. cv. ‘Selva’) were transferred to nutrient solutions with or without iron (Fe). After six weeks of growth, plants in the solution without Fe were chlorotic and had morphological changes in roots typical of Fe deficiency. At this point, four treatments were imposed with three replicates (plants) each: plants always grown without Fe (Fe0); always grown with 10 μM Fe (Fe10); chlorotic plants with leaves treated with ferrous sulphate every two days (Fe-leaves); chlorotic plants transferred to a solution with ferrous sulphate (Fe-solution). These plants were grown for nine days through which period chlorophyll (Chl) in leaves was estimated with a SPAD-502 apparatus. At the beginning and end of the experiment, root ferric chelate reductase (FC-R; EC 1.16.1.17) activity
and the mineral composition of leaves and roots were measured. Six days after the addition of Fe to the solution (Fe-solution) leaves had Chl content similar to (Fe10). When Fe was applied to leaves (Fe-leaves), a slight regreening of new leaves was observed only at the end of the experiment. At this date, the FC-R activity remained the same in (Fe10) and increased even further in (Fe0). Plants in the Fe-solution treatment maintained an FC-R activity similar to the initial value in chlorotic plants, while the activity was drastically reduced in plants of treatment (Fe-leaves). The Fe concentration in leaves of (Fe0) and (Fe10) was similar, while application of Fe to leaves or nutrient solution resulted in an enhanced concentration of Fe in leaves. In contrast to what happened in Fe-solution, application of Fe to leaves did not lead to an increase in the concentration of Fe in roots. Root FC-R was correlated with the concentrations of Mn, Zn and Cu in leaves and with Cu in roots. Under our experimental conditions, FC-R activity in strawberry may be rapidly de-activated by pulses of Fe applied by foliar sprays. On the other hand, this de-activation mechanism is slower if Fe is applied directly to roots, suggesting a greater opportunity for plants to uptake more Fe.

**Keywords:** Chlorophyll, ferric chelate reductase, iron chlorosis, iron fertilization, micronutrients, principal component analysis.

**Abbreviations:** BPDS, Fe(II)-bathophenantrolinedisulfonate; Chl, Chlorophyll; DW, dry weight; EC, electrical conductivity; EDDHA, ethylenediamine-N-N’bis(o-hydroxyphenylacetic) acid; EDTA, ethylenediamine-tetraacetic acid; FC-R, ferric chelate reductase; FW, fresh weight; MES, 2-(N-morpholino)ethanesulfonic acid; PCA, principal component analyses; SPAD, soil and plant analyser development.
1. Introduction

Iron deficiency (iron chlorosis) is an important nutritional disorder in fruit trees that results not from a low level of Fe in soils but from impaired acquisition and use of the metal by plants. The most evident effect of Fe deficiency is the decrease in photosynthetic pigments, resulting in a relative enrichment of carotenoids over chlorophylls (Chl), and leading to the yellow colour characteristic of chlorotic leaves [1].

Plants have mechanisms that may promote the availability of Fe in the rhizosphere and plant. Dicot and monocot species, with the exception of members of the Poaceae family, have developed a strategy (Strategy I) that includes the induction of a root ferric chelate reductase (FC-R; EC 1.16.1.17) that converts Fe(III) to Fe(II) which can then be taken up by a Fe(II) transporter [2, 3]. Excretion of organic acids from roots to the rhizosphere can further improve Fe availability, and the accumulation of these compounds in Fe-deficient plants can also stimulate long-distance transport of the metal [4].

Calcium carbonate, present in great amounts in calcareous soils, results in high levels of bicarbonate ions, which are the main cause of Fe deficiency. Countries in southern Europe, such as Portugal, Spain, Italy and Greece, have large areas of calcareous soils with established orchards, where Fe chlorosis is a major factor that limits yield and profit for the farmer [5, 6]. Crops commonly affected by Fe deficiency when grown in calcareous soils include apple, blueberry, cherry, citrus, corn, grape, turf and pasture grasses, peach, pear, plum, quince, sorghum, soybean, and strawberry, resulting in impaired growth and yield [5, 7-11]. On calcareous soils, strawberry production may be seriously affected by induced Fe-deficiency. This species exhibit a wide genotypic variation in the tolerance to Fe deficiency, and in susceptible cultivars Fe correction is necessary. The Fe is frequently applied by fertigation or via foliar sprays. The use of Fe sulphate may be a cheap and environmental-friendly alternative to Fe-chelates [6, 7, 12]. Applying Fe directly to leaves can bypass the inhibitory effects of soil bicarbonate on Fe uptake and transport to the shoot [13, 14].
Iron resupply to chlorotic plants may induce metabolic changes within a few days or weeks, depending on the parameter and plant material. For example, Fe resupply leads to increases in Chl concentration and photosynthetic rate [15, 16] and decreases in the concentration of organic acids [4, 17]. Additionally, the reduction of the carboxylate concentration in xylem sap and in leaf apoplast was also reported in sugar beet [18] and fruit trees [19]. At root level, the deactivation of some acquisition mechanisms, including FRO (ferric reductase oxidase) and IRT (iron-regulated transporter) was reported [1, 4, 20, 21].

In a recent review paper, [1] stated that studies involving the responses of Fe-deficient plants resupplied with Fe are still scarce and may provide crucial [15] information for the optimization of the Fe-fertilization strategies. The aims of the present study were to characterize the changes induced by Fe depletion on the Chl content, root FC-R and mineral composition of roots and leaves and to compare two different ways to resupply Fe: by foliar or root fertilization. Finally, we discuss the agronomical consequences of Fe resupply by fertilization.

2. Results
2.1. Effects of iron depletion on strawberry plants

Iron deficiency was related to low leaf Chl concentration in strawberry plants (Fig. 2). After 36 days, plants grown in absence of iron (Fe0) showed the first symptoms of iron deficiency in youngest leaves that became chlorotic (139 ± 24 µmol Chl m⁻²), whereas control plants (Fe10) remained green during all the experimental period, presenting a chlorophyll content of approximately 395 ± 38 µmol m⁻². Leaf fresh and dry weights (Table 1) were lower in chlorotic plants (Fe0), compared to green plants (Fe10).

Roots of Fe deficient plants were smaller, with less fresh and dry weights (Table 1), but more ramified than control plants (Fe10). The FC-R activity (Fig. 3) was higher (approximately 2.5-fold) in chlorotic plants (20 ± 2 nmol Fe(II) min⁻¹ g⁻¹ FW) than in green plants (8 ± 1 nmol Fe(II) min⁻¹ g⁻¹ FW).
Iron deficiency also induced changes in mineral composition of strawberry leaves and roots (Table 1). Chlorotic leaves (Fe0) had higher concentration of Zn compared to Fe10 plants. The concentrations of Cu, Mn and Zn in roots of chlorotic plants (Fe0) were significantly higher than those presented by green plants. The concentration of Fe was lowest in leaves and roots of plants grown without Fe in nutrient solution during all the experimental period (Fe0).

2.2. Effects of iron resupply to chlorotic strawberry plants

Iron resupply to chlorotic plants caused an increase in Chl values of young and mature leaves (Fig. 2). Leaf re-greening was evident six days after the addition of Fe to nutrient solution (Fe-solution), but in sprayed plants the re-greening was only partial and the Chl content in mature leaves never reached the values observed in plants grown with Fe during all the experimental period (Fe10).

At the end of the Fe-resupply experiment, the FC-R activity remained similar in (Fe10) plants but increased even further in root tips of (Fe0) plants (Fig. 3). Roots of plants recovered with Fe applied to the nutrient solution (Fe-solution) maintained a FC-R activity similar to that observed at the beginning of the experiment (chlorotic plants; Fe0), while the activity was drastically reduced when Fe was applied to leaves (Fe-leaves). Iron concentration in leaves receiving Fe was higher, compared to other treatments (Fe0 and Fe10). The application of Fe to the solution induced higher concentrations of Fe in roots than application to leaves (Fe-leaves). In the latter treatment, roots had values of Fe similar to those of chlorotic plants (Table 1).

2.3. Relationships between nutrients, chlorophyll and FC-R activity

To investigate relationships between root FC-R activity, Chl content and mineral composition of plants, principal component analyses (PCA) were carried out. Three factors with eigenvalues greater than one were extracted by PCA, explaining 87% of the variability in the data in relation to the mineral composition of roots. In the projection of the first two factors,
which explained 77% of the variability in data, the FC-R activity was positively associated with the concentration of Cu in roots and Chl was negatively related with the concentrations of Cu, Mn and Zn in roots (Fig. 4).

Four factors with eigenvalues greater than one were extracted by PCA, explaining 86% of the variability in parameters in relation to the mineral composition of leaves. In the projection of the first two factors (explaining 57% of the data variability) the root FC-R activity was positively associated with the concentration Cu, Mn and Zn in leaves, and contrasted with the Fe concentration by factor 1 (Fig. 5). Chl was negatively related with the concentration of Zn in leaves, and separated from this by factors 3 and 4 (data not shown). No significant correlations either with FC-R or with Chl content were obtained with the remaining nutrients.

3. Discussion

Iron fertilisation improves yield and fruit quality in several crops and is a standard practice in fruit production regions [5]. The recovery of chlorotic strawberry plants was possible by adding Fe(II)-sulphate to roots or to leaves. As Fe concentrations in strawberry leaves were severely decreased by Fe deficiency, a rapid Fe resupply was observed, both when the nutrient was applied to leaves or to the nutrient solution. However, when Fe was supplied to leaves the regreening was only partial, and Chl values were lower in new developed leaves compared to plants always grown with Fe (Fe10). Partial re-greening following foliar sprays was previously observed in orange and peach trees [10, 11, 22]. To be effective, the exogenous Fe applied to leaves must cross the foliar barrier to enter cells and must then be integrated in plant metabolism [14]. The foliar treatment may have been more effective if a surfactant and an adjuvant had been used, but the interactions between Fe salts and these products still needs to be clarified [10] and therefore they were omitted in the present experiment.

Total leaf regreening was only observed when Fe was added to the nutrient solution. However, the response of strawberry plants to Fe deficiency or to Fe-resupply seems to be also
coordinated by shoots and not only by the Fe availability in the nutrient solution. This shoot-to-root coordination was firstly described by [2] in pea mutants. In our experiment, the range of root FC-R activities was similar to those reported for other species [9, 23-26]. Plants grown without Fe (Fe0) had chlorotic leaves and enhanced root FC-R activity (Fig. 3), while control plants (Fe10) had lower FC-R activity but remained green until the end of the experiment, and had no morphological or physiological changes, showing that this level of Fe in the nutrient solution was sufficient for strawberry plants. The root FC-R activity seems to be de-activated following Fe sprays and an increase in leaf Fe content was observed although leaves only became partially green (Table 1). This may explain the negative correlation between FC-R and leaf Fe concentration (Fig. 5). When Fe was added to the nutrient solution (Fe-solution) an high increase in root Fe concentration was observed (Table 1) and the FC-R activity was lower but not completely de-activated.

The agronomical implications of the de-activation of response mechanisms by foliar sprays are important, since most of the applications of micronutrients in crops are done via canopy (leaves and shoots). Additionally, an understanding of these mechanisms may help to sustain the re-greaning effects for a longer period of time. In fact, [1] recently proposed that following canopy sprays a high energetic consumption can be expected during this ‘on-off’ FC-R activation mechanism. Reciprocal grafting experiments with a pea mutant with elevated FC-R activity and wild type also showed that the control of FC-R activity was probably located in shoots [2, 27]. A decrease in mRNA expression of NtFRO1 and NtIRT1 was also observed upon foliar Fe resupply to tobacco plants, further stressing the importance of shoot signalling in the down-regulation of this enzyme [20].

The nature of the signal in Fe-deficient plants has not been clarified, although plant hormones, nitrogen monoxide, Fe-binding compounds and even Fe itself have all been suggested as possible messages used to down-regulate Fe-deficiency responses in roots [1]. When Fe was applied to the solution a total recovery from iron deficiency symptoms and an increase in Chl
synthesis was observed, showing a dependence on a fresh supply of Fe via xylem, but a slow de-activation of root FC-R. The possible different time course of the deactivation of FC-R activity may be related to the difference between a continuously available pool of Fe for root uptake and the pulses of Fe applied by foliar sprays.

Other cationic micronutrients (Mn, Cu and Zn) compete with Fe for ligands in plants. For example, Cu exists in multiple redox states, and acts as co-factor for components of the electron transport chain in mitochondria and chloroplasts [28]. Like Fe, Cu also has to be reduced before uptake by its respective transporter [29]. According to [28], FC-R is not up-regulated by Cu deficiency, but when induced by Fe deficiency, it is also able to reduce Cu. In fact, in the present experiment, Cu concentration in roots and leaves was linearly correlated with FC-R activity (Fig. 4 and 5), suggesting that the reductase was acting upon Cu and leading to enhanced uptake and translocation of this element.

The association of the root FC-R with the concentrations of Zn and Mn in leaves was significant, but the correlation with their concentrations in roots was not, although PCA analysis showed they were close to Cu in the upper right quadrant (Fig. 4). These results are in agreement with those of [30] who established a multivariate shoot ionomic signature for Arabidopsis, consisting of Mn, Co, Zn, Mo and Cd that is indicative of the nutritional status of plants in relation to Fe. When the Fe supply (via roots) was low, the shoots have enhanced concentrations of Mn, Zn, Co and Cd and less Mo.

In conclusion, under our experimental conditions, FC-R activity in strawberry may be rapidly de-activated by pulses of Fe applied by foliar sprays. On the other hand, this de-activation mechanism is slower if Fe is applied directly to roots, suggesting a greater opportunity for plants to uptake more Fe.

4. Methods

4.1. Plant material
Strawberry (Fragaria x ananassa Duch. cv. ‘Selva’) bare root plants (with root length of approximately 18 cm) without leaves were sterilised by immersion in a solution with 2.5g fosetyl-aluminium for 2 h followed by thorough washing in running water. Twenty four plants were then transferred to two 20 L polyethylene vessels filled with Hoagland nutrient solution containing (in mM): 5 Ca (NO$_3$)$_2$, 5 KNO$_3$, 1.0 KH$_2$PO$_4$, 2.0 MgSO$_4$; (in μM): 46.0 H$_3$BO$_3$, 0.8 ZnSO$_4$, 0.4 CuSO$_4$, 9.0 MnCl$_2$, and 0.02 MoO$_3$. Half of the plants were grown with 10 μM Fe (as Fe(III)-EDDHA - Fe10 plants) and half without Fe (Fe0). Initial pH was 6.0 ± 0.2 and electrical conductivity (EC) was 2.2 ± 0.1 dS m$^{-1}$. The aerated nutrient solution was replaced when the EC dropped to 1.7 dS m$^{-1}$. The experiments were done in a glasshouse under natural photoperiod conditions and temperature ≤ 25 ºC.

After 62 days, plants grown with Fe (Fe10) remained green (492 ± 38 µmol of Chl m$^{-2}$) but plants grown in absence of iron (Fe0) were chlorotic (148 ± 24 µmol Chl m$^{-2}$). At this date, plants were individually transferred to 1 L glass jars containing nutrient solution. Four plants were kept growing with 10 μM Fe in solution and another four were kept growing without Fe; the positive and negative controls, respectively. Six chlorotic plants were used to resupply Fe with two additional treatments: i) foliar sprays (Fe-leaves) - plants grown in nutrient solution without Fe were sprayed three times every two days with a solution of 1.8 mM of Fe as ferrous sulphate; ii) nutrient solution (Fe-solution) - plants were transferred to nutrient solution containing 0.75 mM of Fe as ferrous sulphate. In the (Fe-leaves) treatment, all leaves (about five) were sprayed in both faces (abaxial and adaxial) with a total volume of 83 mL (without wetting agents or surfactants) applied to each plant over the three sprays. In conclusion, four treatments were obtained, each with at least three plants (replicates): plants always grown without iron (Fe0); plants always grown with Fe (Fe10); chlorotic plants sprayed with Fe (Fe-leaves); chlorotic plants transferred to a solution with Fe (Fe-solution). Plants were kept in the four treatments during nine days in the same glasshouse.
4.2. Leaf chlorophyll determination

New leaves appeared about 15 days after the beginning of the experiment and from this date, total chlorophyll (Chl) concentration was estimated non-destructively in mature and youngest fully expanded leaves, using a portable SPAD-502 meter (Minolta, Osaka, Japan). Five readings per leaf were taken in at least three leaves per plant. SPAD readings were converted to total Chl using the equation:

\[ Y = 0.4453x^2 - 1.1114x + 32.562 \quad (r^2 = 0.98; \ n=31; \ p < 0.001) \]

where \( Y \) is the Chl content (\( \mu \text{mol m}^{-2} \)) and \( x \) the SPAD reading [9]. This calibration curve was established by reading with the SPAD-502 leaf disks with different degrees of Fe deficiency, extracting the pigments from the same leaf area with 100% acetone in the presence of Na ascorbate [31] and measuring Chl spectrophotometrically according to [32].

4.3. Ferric chelate-reductase activity of strawberry root tips

The activity of the root ferric chelate reductase (FC-R; EC 1.16.1.17) was evaluated in plants from both containers immediately before the four treatments were imposed and at the end of the experiment. The FC-R was measured by the formation of the \( \text{Fe(II)-bathophenantroline disulfonate (BPDS)} \) complex from \( \text{Fe(III)-EDTA} \) [33]. Previously, a preliminary test was done in order to visualize the localization of FC-R activity. Since the activity of the enzyme was clearly seen by the rose coloration of the root tips (Fig. 1), it was possible to use the following methodology. At least seven root tips were excised with a razor blade from plants of each treatment, in a total of at least 15 FC-R values per treatment. Each excised root tip (approximately 2 cm, \( 1.40 \pm 0.35 \) mg fresh weights) was incubated in an Eppendorf tube in the dark with 900 µl of micronutrient-free half Hoagland’s nutrient solution, containing 300 µM BPDS, 500 µM \( \text{Fe(III)-EDTA} \) and 5 mM MES buffer, pH 6.0. Readings were done after centrifugation at 535 nm, one hour after starting the incubation in a darkened
laboratory, by using a spectrophotometer (CADAS 100). Fe(II)-BPDS was quantified using a molar extinction coefficient of 22.14 mM cm$^{-1}$. Following each assay, roots were gently dried with a blotting paper and fresh weights (FW) were determined. All reductase values were calculated on a FW basis. Blank controls without root tips were also used to correct for any unspecific photoreduction.

4.4. Mineral composition analysis

At the end of the experiment, plants from each treatment were collected, separated into roots and shoots (leaves and petioles), washed with de-ionized water, dried at 60 ºC until constant weight and ground. The mineral composition was determined as described previously [34]. Nitrogen concentration was determined by the Kjeldahl method. Subsamples were dry-ashed at 450 ºC and digested in HNO$_3$ and HCl following the A.O.A.C. procedure [35]. The concentration of P was determined spectrophotometrically and those of K, Ca, Mg, Mn, Zn, Fe and Cu determined by atomic absorption spectrophotometry (Pye Unicam, Cambridge, UK).

4.5. Statistical analyses

The effects of treatments were evaluated by the general linear method (GLM) and the means compared using the Duncan Multiple Range Test at $P<0.05$. Principal Component analysis (PCA) was performed to detect key parameters contributing to data variability and to identify the relationships between FC-R, leaf Chl and mineral composition of plants. PCA is an exploratory multivariate statistical method that reduces several parameters to a small number of new derived variables that can reveal associations in the data that cannot be found by analysing each parameter separately. The procedure associated with PCA analysis is described with more detail in [34]. Linear correlations between studied parameters were determined and the Pearson correlation coefficients presented. Statistical analyses were carried out with the Statistica 10 software (StatSoft Inc.).
Acknowledgements

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References


[16] Larbi A., Abadía A., Morales F., Abadía J., Fe resupply to Fe-deficient sugar beet plants leads to rapid changes in the violaxanthin cycle and other photosynthetic characteristics.


Table 1. Mineral composition of Fe-sufficient (Fe10), Fe-deficient (Fe0) and Fe-resupplied to leaves (Fe-leaves) or to solution (Fe-solution) as Fe(II)-sulphate. Macronutrients (N, P, K, Mg, a) are in g kg$^{-1}$ dry weight (DW) and micronutrients (Cu, Zn, Mn and Fe) are in mg kg$^{-1}$ DW.

<table>
<thead>
<tr>
<th></th>
<th>Fe-deficient</th>
<th>Fe-sufficient</th>
<th>Fe-resupplied</th>
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<tr>
<td></td>
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<td>Fe10</td>
<td>Fe-solution</td>
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<tr>
<td>Leaves</td>
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<tr>
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<td>84 ± 13 c</td>
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Data are means ± standard error (SE) of at least 3 replicates. FW – fresh weight expressed in g; ns – not significant. For each row, values with the same letter were not significantly different (Duncan’s test, $P<0.05$).
Figure 1. The rose root tips indicate the localization of the FC-R activity.
Figure 2. Evolution of leaf chlorophyll (Chl) in the youngest and mature leaves during the experiment. For each date, values with the same letter were not significantly different (Duncan’s test, $P<0.05$).
Figure 3. Root ferric chelate reductase activities at the beginning and end of the experiment, in strawberry plants grown in the absence of Fe (Fe0), in the presence of Fe (Fe10), and in chlorotic plants with Fe added to the nutrient solution (Fe-solution) or applied to leaves (Fe-leaves). For each column, values with the same letter were not significantly different (Duncan’s test, \( P<0.05 \)).
Figure 4. Principal component analysis of nutrients in roots, root ferric chelate reductase activity (FC-R) and leaf chlorophyll (Chl). The Pearson correlation coefficients between parameters are shown in the insert.
Figure 5. Principal component analysis of nutrients in leaves, root ferric chelate reductase activity (FC-R) and leaf chlorophyll (Chl). The Pearson correlation coefficients between parameters are shown in the insert.