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Effects of foliar application of organic acids on strawberry plants

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ABSTRACT

The large economic costs and environmental impacts of iron-chelate treatments has led to the search for alternative methods and compounds to control iron (Fe) deficiency chlorosis. Strawberry plants (*Fragaria* x *ananassa*) were grown in Hoagland's nutrient solution in a greenhouse with two levels of Fe: 0 and 10 μM Fe(III)-EDDHA. After 20 days, plants growing without Fe showed typical symptoms of Fe deficiency chlorosis in young leaves. Then, the adaxial and abaxial sides of one mature or one young leaf in each plant were brushed with 10 mM malic (MA), citric (CA) or succinic (SA) acids. Eight applications were done over a two-week period. At the end of the experiment, the newly emerged (therefore untreated), young and mature leaves were sampled for nutritional and metabolomic analysis, to assess the effectiveness of treatments. Leaf regreening was monitored using a SPAD-502 apparatus, and the activity of the ferric chelate-reductase activity (FCR) was measured using root tips. Iron deficiency negatively affected biomass and leaf chlorophyll but did not increase FCR activity. Application of succinic acid alleviated the decrease in chlorophyll observed in other treatments, and the overall nutritional balance in the plant was also changed. The concentrations of two quinic acid derivatives increased under Fe deficiency and decreased in plants treated with succinic acid, and thus they are proposed as Fe stress markers. Data suggest that foliage treatments with carboxylates may be, in some cases, environmentally friendly alternatives to Fe(III)-chelates. The importance of Fe mobilization pathways in the formulation of new fertilizers is also discussed.

1. Introduction

Iron acquisition and transport in plants follow two general strategies (Marschner et al., 1986). Strategy I, also known as the reduction strategy, occurs in non-grass species, and involves a reduction of Fe(III) to Fe (II) before Fe root uptake by means of a membrane-bound ferric chelate reductase (FCR). The roots of plants that follow this strategy generally acidify the rhizosphere by secreting protons and release organic acids and other substances (Römheld, 1987). A different strategy, based on Fe chelation (Strategy II), is confined to grasses (*Poaceae* family) and relies on the secretion of phytosiderophores (PS) to the rhizosphere, followed by binding of Fe(III) and uptake of the Fe(III)-PS complex(es) via a high affinity system (Hindt and Guerinot, 2012).

Organic acids play important roles in the Fe bioavailability in the rhizosphere and Fe translocation within the plant (López-Millán et al., 2001; Abadía et al., 2002). The main organic acids involved in Fe plant metabolism are citric (CA), malic (MA) and succinic (SA) acids (Marschner et al., 1986). CA and MA are intermediates in the Krebs cycle that produces cellular energy by oxidative phosphorylation. The Fe (III)-citrate complex is one of the mobile forms of Fe inside the plant and plays an important role in the long-distance Fe transport between different plant compartments (Hell and Stephan, 2003; Durrett et al., 2007; Rellán-Álvarez et al., 2010). MA plays diverse roles in plants, providing osmotic balance in the vacuole, acting in pH regulation (Wedding, 1989) and as an energy source in mitochondria (Casati et al., 1999). SA is a precursor molecule used as a carbohydrate skeleton for

Abbreviations: FCR, Ferric chelate reductase.

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chlorophyll biosynthesis and is readily converted to MA in the Krebs cycle. Under Fe deficiency conditions, the concentrations of CA and/or MA are generally increased in many plant species and organs (López-Millán et al., 2000; Abadía et al., 2002; Zocchi, 2006; Zocchi et al., 2007; Rellán-Álvarez et al., 2010; Correia et al., 2014; Gayomba et al., 2015), as part of a general strategy that involves a complex network of metabolic pathways.

Iron foliar fertilization can be an inexpensive, more environmental-friendly alternative to soil treatments with synthetic Fe(III) chelates for the control of Fe deficiency chlorosis in fruit trees (Pestana et al., 2003; Álvarez-Fernández et al., 2004; Fernández and Brown, 2013). For instance, $FeSO_4$ has been tested as a foliar fertilizer in many studies. Increases in leaf Chl after foliar fertilization with $FeSO_4$ have been reported in kiwifruit (Rombolà et al., 2000), citrus (Pestana et al., 2001), peanut (Akhtar et al., 2019), peach (Larbi et al., 2003), pear (Álvarez-Fernández et al., 2004) and strawberry (Pestana et al., 2012). Although positive effects of foliar application of Fe sources used in combination with specific carboxylates has been already reported (Abadía et al., 2002; Álvarez-Fernández et al., 2004; Ghazijahani et al., 2014), there is still limited information on the effects of organic acids *per se*.

The role of carboxylates in the metabolism of Fe deficient plants is supported by previous studies showing that when they are applied to leaves of different Fe-deficient crops, including peach (Tagliavini et al., 2000), pear (Álvarez-Fernández et al., 2004), kiwifruit (Tagliavini et al., 1995; Rombolà et al., 2000) and orange trees (Pestana et al., 2001), a partial regreening of chlorotic leaves occasionally occurs. It has been suggested that the application of carboxylates to plant foliage could increase plant mineral acquisition uptake via root exudation of these compounds (Jafari and Hadavi, 2012), and an increase in carboxylate exudation in response to foliar treatments has been found (An et al., 2014). The role of these exudates in the uptake of minerals from the soil is well known (Bais et al., 2006). In *Lilium*, spraying MA led to an increase in leaf chlorophyll (Chl) and a change in the carbohydrate partitioning in favour of underground sinks (Darandeh and Hadavi, 2012). Of course, carboxylate treatments have synergisms with Fe ones, and the joint foliar application of $FeSO_4$ and CA extends the vase life in *Polianthes tuberosa* (Eidyan et al., 2014). Carboxylate treatments also have additional metal-related effects, and for instance in seedlings of larch (*Larix olgensis*), a forestry species used to remediate polluted soils, an exogenous application of SA alleviated the oxidative damage produced by Pb stress (Song et al., 2018).

The main aim of this study was to analyse the re-greening pattern in leaves of Fe-deficient strawberry plants after a localized leaf application of three organic acids, CA, MA and SA, in two different leaf types, mature green and young chlorotic ones. Since no Fe was present in the nutrient solution when the compounds were applied to leaves, any regreening observed should be ascribed to a remobilization of pre-existing endogenous Fe pools. Once remobilized, Fe (and probably other metals) would be expected to re-translocate towards strong sinks such as the new leaves. By testing the carboxylate applications in two different types of leaves, young and mature, we aimed to understand how Fe is transported under these conditions. Other objectives were to evaluate the relative effectiveness of the three carboxylates used on the physiological Fe-stress responses of strawberry plants, and to characterize the metabolite profiles in leaves of strawberry plants growing under different Fe levels.

2. Material and methods

2.1. Plant material and growth conditions

The experiment was conducted with strawberry (*Fragaria* x *ananassa* Duch., cv. 'Diamond') plants in a greenhouse. Bare-root plants (without leaves and with a root length of approximately 18 cm; Fig. 1), were disinfected by soaking in a solution containing 2 g L^{-1} of fosetyl-aluminium for 2 h and then washed in tap water. Plants were transferred to 20 L polyethylene vessels (six plants per vessel) filled with full-strength Hoagland solution containing (in mM): 5.0 $Ca(NO_3)_2$, 5.0 KNO_3, 1.0 KH_2PO_4, 2.0 $MgSO_4$, and (in μM): 46.0 H_3BO_3, 0.8 $ZnSO_4$, 0.4 $CuSO_4$, 9.0 $MnCl_2$ and 0.02 $(NH_4)_6Mo_7O_{27}$. Six plants were grown with 10 μM Fe(III)-EDDHA (Fe10), while 42 plants were grown in the absence of Fe (Fe0). The initial pH of nutrient solutions was adjusted to 6.0 ± 0.1 using NaOH 0.1 M (the electrical conductivity -EC- was 2.1 ± 0.1 dS m^{-1}). The pH and EC of the solutions were monitored every two days. The nutrient solution was frequently aerated, using alternated cycles of 15 min with and without aeration, programmed with a timer. The experiment was performed between March and May in a glasshouse under natural photoperiod conditions. The air temperature was ≤25 °C and the average relative humidity was 65%.

After 20 days, plants had 3.0 ± 0.0 leaves, irrespective of the Fe level. At this stage, young leaves of control plants grown with Fe (Fe10; positive control) in the nutrient solution were green, while plants grown without Fe (Fe0) developed leaf chlorosis (869 ± 96 and 394 ± 79 μmol

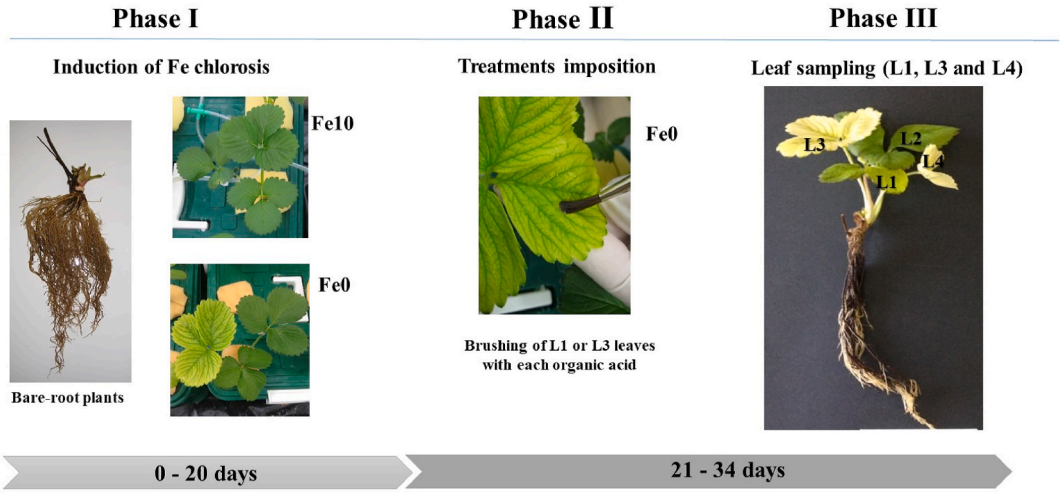

Fig. 1. Different phases of the experiment. Phase I: Induction of Fe deficiency chlorosis after 20 days of growth in Fe free nutrient solutions. At the end of this phase, typical symptoms of Fe deficiency chlorosis were observed in Fe0 plants but not in Fe10 ones. Phase II: L1 (mature leaves) or L3 (young leaves) were brushed with 10 mM succinic (SA), citric (CA) or malic (MA) acids, and treatments were repeated eight times in a two-week period. Phase III: After 34 days, the L1, L3 and newly emerged (L4) leaves were sampled and used for analysis.

Chl m^{-2}, respectively). Then, the Fe0 plants were kept in the zero Fe solution and submitted to different treatments: six were not treated (negative control; Fe0), and the remaining plants were used for the foliar brushing treatments, which consisted in the application of three carboxylates, MA, CA or SA. Leaves treated were the mature (L1) and young (L3) ones (Fig. 1). Middle leaves (L2, situated between L1 and L3) were not treated in this study. In a given plant, each carboxylate was applied with a paintbrush to one mature (L1) or one young (L3) leaf. A total of 36 plants were treated (three carboxylates x two leaf types x 6 replications). The same volume was applied in each foliar application, and each leaf received a volume of ca. 500 μl. Thus, the treatments were the following: Fe0, Fe10, L1 treated with MA, CA or SA, and L3 treated with MA, CA or SA. Each organic acid solution was prepared at 10 mM concentration using PA reagents (MA from Merck, Hohenbrunn, Germany, and CA and SA from Sigma Aldrich, Steinheim, Germany). A surfactant (0.1% Tween 20) was added to all solutions to obtain a homogeneous distribution of the product on the leaf surface. No effect was observed on the leaf Chl contents when a solution including the surfactant alone was applied (data not shown). Each treatment was repeated eight times in a two-week period, by brushing both the abaxial and adaxial surfaces of the same leaves treated initially (El-Jendoubi et al., 2014). Brushing was made in both leaf sides to spread the solutions more homogeneously and ensure the uptake of the tested solutions (strawberry has stomata in both leaf sides, but the stomatal density is higher in the abaxial side). At the end of the experiment, the L1, L3 and newly emerged (untreated; L4), leaves were sampled and analysed (Fig. 1).

2.2. Leaf chlorophyll estimation

Leaf chlorophyll (Chl) concentrations were estimated using a portable SPAD-502 apparatus (Minolta Corp., Osaka, Japan). The SPAD-502 readings were taken in the different leaf types three times per week, using the average of at least three measurements per leaf. Leaf SPAD readings (X) were converted to total Chl concentrations (in μmol m^{-2}) using an equation obtained in a previous study with the same strawberry cultivar: $Chl = 0.375X^2 + 6.630X + 71.554$ (Gama et al., 2016).

2.3. Determination of the root Fe-chelate reducing capacity

The activity of the root ferric chelate reductase (FCR; EC1.16.1.17) was measured at the end of the experiment (after 34 days) in all plants, using bathophenanthroline disulfonate (BPDS), which forms a red Fe_3BPDS_2 complex with Fe(II), as in Bienfait et al. (1983). In each plant, a single root tip, approximately 2 cm in length (8.3 ± 1.0 mg), was excised with a razor blade and incubated in an Eppendorf tube, in the darkness and for 1 h, with 900 μL of micronutrient-free half-strength Hoagland's nutrient solution, containing 300 μM BPDS, 500 μM Fe (III)-EDTA and 5 mM MES, pH 6.0. Then, the FCR activity was measured at 535 nm, using a spectrophotometer (CADAS 100 UV-VIS Photometer; Dr. Lange, Düsseldorf, Germany), using a molar extinction coefficient of 22.14 mM^{-1} cm^{-1}. The root tip was then blotted on paper and fresh weight (FW) determined. Values shown for FCR activity were the mean of at least five root tips (plants) in each treatment, and values are given on a FW basis. Blank controls without root tips were also used to correct for any non-specific photoreduction.

2.4. Mineral composition analysis

At the end of the experiment (at day 34), five plants from each treatment were collected and separated into leaves, crowns (whole stems above the roots), and roots. The mineral composition of the L4 leaves was measured separately, and the L2 leaves were not analysed. The plant material was washed first with tap water, followed by deionized water with a non-ionic detergent, and then with 0.01 M HCl. Finally, three rinses were made with distilled water. The plant material was dried at 60 °C to constant weight, ground and ashed at 450 °C, followed by digestion with 1 M HCl. Nitrogen was analysed by the Kjeldahl method and P was determined colorimetrically by the molybdovanadate method. Potassium was determined by emission spectrophotometry and K, Ca, Mg, Mn, Zn, Cu and Fe were determined by atomic absorption spectrophotometry (SolaarM Series, Pye Unicam, Cambridge, UK) following standard methods (AOAC, 1990). The nutrient contents were calculated using the concentration and dry weight (DW) values.

2.5. Metabolomic analysis

2.5.1. Compound extraction

At the end of experiment, four leaf disks (1.0 cm diameter) were taken from each leaf type (L1, L3 and L4) in all plants using a calibrated cork borer, weighed, and immediately stored at −80 °C. Leaf tissues were ground with a mortar and a pestle and using liquid N_2 and homogenized in Milli-Q water (Millipore, Bedford, MA, USA) using a 1:10 sample:water ratio. The homogenates were centrifuged using a Hettich Mikro 200R (Andreas Hettich GmbH & Co., Germany) at 3070×*g* for 10 min, and the supernatant was filtered (0.45 mm PTFE filter) and stored at −80 °C before analysis. The number of biological replications was six.

2.5.2. LC-MS and LC-HRMS^2 analyses

All leaf extracts were analysed by liquid chromatography-mass spectrometry (LC-MS), using an Agilent 1200 LC coupled to an ion trap MS (HCT Ultra, Bruker Daltonics, Bremen, Germany). Chromatographic separation was achieved using a PRP-1 reversed phase LC column (150 × 2.1 mm, 5 μm particle size) at 25 °C. The mobile phase was built with water (A) and acetonitrile (B), both with 0.1% formic acid. The gradient (in v/v %) started with 90% A and 10% B, increased linearly to 30% B after 30 min, and then to 100% B in 4 min. The system was kept at 100% B for an additional 4 min period, switched to the initial conditions in 1 min and this composition kept for 4 min before the next run. The flow rate was 0.4 mL min^{-1}, and the injection volume was 5 μL. The metabolite profiles were obtained using the AutoMS mode. Typical spray and ion optics conditions were: capillary voltage, 3.5 kV; drying gas (N_2) temperature, 330 °C; drying gas flow, 7 L min^{-1}; nebulizer gas pressure, 35 psi; capillary exit voltage, 104 V, and skimmer voltage, 32 V.

Selected extracts were also analysed by high resolution mass spectrometry (LC-HRMS^2), using an Ultimate 3000 UHPLC (Thermo Fisher Scientific, Waltham, MA, U.S.A.) coupled to an Orbitrap Elite MS (Thermo Fisher Scientific) with a heated electrospray ionization source (HESI-II). The chromatographic separation was performed using a Thermo Scientific Accucore RP-18 (100 × 2.1 mm, 2.6 μm particle size) column. The mobile phase was prepared with water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient (in v/v %) started with 100% A for 2 min, increased linearly to 30% B in 13 min, and then to 100% B in 16 min. The mobile phase was kept at 100% B for 4 min, switched to the initial conditions in 1 min and this composition kept for 4 min before the next run. The flow rate was 0.3 mL min^{-1}, and the injection volume was 5 μL. MSn data were acquired using the following ionization parameters: spray voltages, 3.7 (positive polarity) and 4.0 kV (negative polarity); sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; heater temperature, 300 °C; capillary temperature, 350 °C, and S-Lenses RF level, 64.9%. The scan range was 100–1000 *m/z*. Fragmentation spectra were obtained by running the system in data dependent mode, using dynamic exclusion in negative and positive polarities. LC-MS profiles were analysed, and compounds annotated using Compound Discoverer 3.3 (Thermo Fisher Scientific), which has access to over than 270 online mass spectral databases, including mzCloud, (Thermo Fisher Scientific), Plantcyc and HMDB, and to the Arita's lab Flavonoid Structure Database (http://metabolomics.jp/wiki/Main_Page).

2.6. Statistical analysis

Analysis of variance (ANOVA; F test) was carried out for all treatments and conditions, and means were compared using the Duncan multiple range test at $p < 0.05$ (IBM SPSS® software, version 20). For each treatment, regression analysis between leaf Chl and days (from 20 until 34 days) was carried out, and the best fitted models were chosen.

3. Results

At the beginning of the experiment, all bare-root strawberry plants had a similar size, with a crown diameter of 1.35 ± 0.26 cm and a root FW of 8.16 ± 2.45 g. After 20 days of growing without Fe (Phase I – Induction of Fe chlorosis in Fig. 1), plants showed typical symptoms of Fe deficiency chlorosis, characterized by yellowing of the mesophyll in young leaves. Conversely, during Phase I control plants (Fe10) were green and healthy.

3.1. Chlorophyll concentration

The relationship between leaf Chl concentration and time (days) in treated young leaves (L3), was assessed using regression analysis (Fig. 2). Control plants (Fe10) remained green over the experimental period, and the leaf Chl level remained quite constant (878.5 ± 45.5 μmol Chl m^{-2}; data not shown in the Figure). Regressions with negative slopes were obtained for all other treatments. However, in young leaves treated with SA, Chl levels reached a minimum after 31 days (161.6 ± 23.1 μmol Chl m^{-2}) and then increased 1.4-fold (to 219.9 ± 18.5 μmol Chl m^{-2}) by day 34.

The Chl concentrations in newly emerged, untreated leaves (L4) at the end of the experiment (on day 34) are shown for all treatments in Fig. 3. As expected, the total Chl concentration in L4 leaves of control plants (Fe10) was much higher than those in the other treatments (842.9 ± 26.5 μmol Chl m^{-2}, data not included in the Figure). In plants where the young leaf was brushed with SA (YL + SA), the Chl concentration was significantly higher when compared to the negative control (Fe0) and any of the other treatments. The lowest values were observed for the ML + SA and YL + CA treatments, which led to Chl concentrations lower than those in the Fe0 control. The ML + MA treatment gave Chl values significantly higher than those in the Fe0 control.

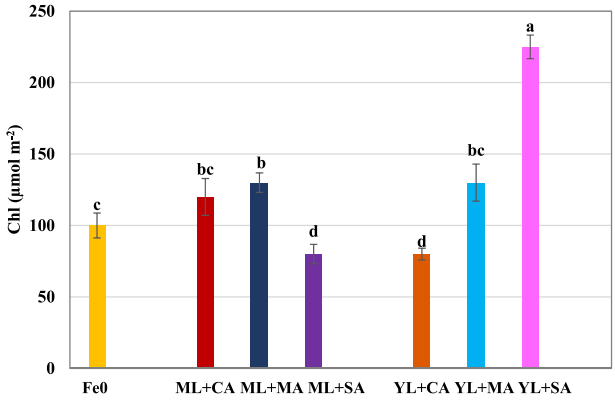

Fig. 3. Total leaf chlorophyll (Chl) concentration (in μmol m^{-2}) in newly emerged, untreated leaves (L4) for all treatments at the end of experiment (on day 34). The value for the Fe10 treatment is not included in the Figure and was 842.9 ± 26.5 μ mol Chl m^{-2}. Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) were brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA. Data shown are means ± SE (n = 6). Data in columns with the same letter were not significantly different (at $p < 0.05$), using the Duncan multiple range test.

3.2. Root ferric chelate reductase activity

At the end of the experiment, all plants growing in the absence of Fe (treated or not by brushing leaves with carboxylate solutions) showed subapical root swelling, and this feature was absent in the Fe10 plants. On this date, FCR activity measured in root tips was significantly higher in the Fe10 plants than in the Fe0 ones (5.75 ± 1.22 and 3.20 ± 0.72 nmol Fe(II) min^{-1} g^{-1} FW, respectively) (Fig. 4). In the other treatments, the FCR activity was in the range from 1.17 ± 0.15 to 3.85 ± 0.58 nmol Fe(II) min^{-1} g^{-1} FW (these two values were observed in plants where the mature leaves were treated with CA and MA, respectively). Plants where mature leaves were brushed with CA had FCR values not significantly different to those in Fe0 plants and other carboxylate treatments, with the only exception of plants where the mature leaves were treated with

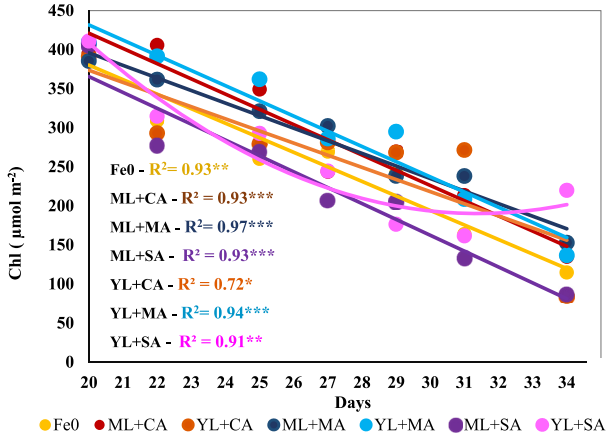

Fig. 2. Total leaf chlorophyll (Chl) concentrations in carboxylate-treated young leaves (L3) (in μmol m^{-2}). The values for the Fe10 treatment are not included in the Figure and were 878.5 ± 45.5 μmol Chl m^{-2} (average for the period 20–34). Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) were brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA. For each adjusted model: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

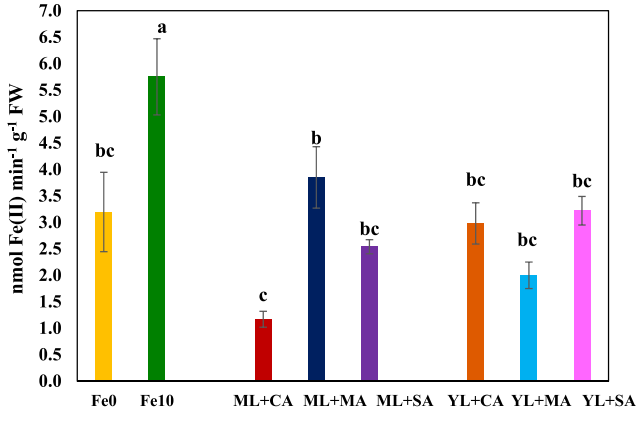

Fig. 4. Root ferric chelate reductase (FCR) (in nmol Fe(II) min^{-1} g^{-1} FW) for all treatments at the end of experiment (on day 34). Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) was brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA. Data shown are means ± SE (n = 6). Data in columns with the same letter were not significantly different (at $p < 0.05$), using the Duncan multiple range test.

MA.

3.3. Biomass

The total root, crown and leaf biomass (in g DW plant^{-1}) at the end of the experiment (at day 34) are shown in Table 1. Regarding root biomass, plants where the new leaves were treated with CA (YL + CA) had smaller values when compared to those in the Fe10 treatment and in those where the mature leaves were treated with SA or CA (ML + SA; ML + CA) or the young leaves were treated with MA (YL + MA). In the case of crowns, the only significant difference in biomass was between the Fe0 and the Fe10 treatments. In the case of leaves, the highest biomass values were for the Fe10 plants and the lower for the Fe0 plants, which were similar to those plants where the young and mature leaves were treated with SA (YL + SA; ML + SA), mature leaves were treated with MA (ML + MA) and young leaves with CA (YL + CA). The root to shoot ratio (R/S) of plants where young leaves were treated with CA (YL + CA) was significantly lower than the other treatments.

When considering only the newly emerged leaves (L4), the biomass was highest in control plants (Fe10) with values of 0.47 g DW plant^{-1} (Fig. 5). Intermediate values were observed in plants where the young leaves were treated with MA (0.32 g DW plant^{-1}) and lower values were observed in the rest of the treatments (0.16–0.28 g DW plant^{-1}).

Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) was brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA. Data shown are means ± SE (n = 5). Data in columns with the same letter were not significantly different (at $p < 0.05$), using the Duncan multiple range test.

3.4. Mineral composition

The macronutrient and micronutrient concentrations in newly emerged leaves (L4) are shown in Table 2. The N concentrations in these leaves were not determined in Fe10 and Fe0 plants. Nitrogen concentrations were in the range 12.8–40.9 g kg^{-1} DW in the remaining treatments. The lowest N concentrations were observed with the SA treatments, both in young (YL + SA) and mature (ML + SA) leaves (12.8 and 21.0 g kg^{-1} DW, respectively). Phosphorus concentrations were in the range 6.8–10.9 g kg^{-1} DW, and the treatments that decreased most N concentrations led to the highest P concentrations (10.1 and 10.9 g kg^{-1} DW for YL + SA and ML + SA, respectively). Intermediate values were found in the YL + CA treatment, with the remaining treatments leading to lower values. Potassium concentrations were in the range 27.4–57.8 g kg^{-1} DW and were lower in the ML + CA and ML + MA treatments than in the untreated Fe0 control. Magnesium concentrations were in

Table 1
Biomass parameters (in g plant^{-1}) at the end of experiment (at day 34). The "Leaves" column includes all leaf types. In the root to shoot ratio, the sum of the dry weight (DW) of crowns and leaves was considered as corresponding to the shoot. Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) was brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA.

Treatments	Roots	Crowns	Leaves	Root/Shoot
g DW plant^{-1}				
Fe0	0.62 ± 0.08 bc	0.69 ± 0.12 c	0.88 ± 0.02 de	0.40 ± 0.05 ab
Fe10	1.07 ± 0.16 ab	1.78 ± 0.32 a	1.82 ± 0.04 a	0.29 ± 0.03 b
ML + CA	0.94 ± 0.13 ab	1.18 ± 0.16 abc	1.25 ± 0.06 bc	0.38 ± 0.03 ab
ML + MA	0.79 ± 0.11 bc	1.44 ± 0.18 ab	1.10 ± 0.06 bcde	0.31 ± 0.03 ab
ML + SA	0.88 ± 0.07 ab	1.05 ± 0.13 abc	0.98 ± 0.05 cde	0.45 ± 0.03 a
YL + CA	0.38 ± 0.06 c	1.42 ± 0.26 abc	1.15 ± 0.05 bcd	0.15 ± 0.02 c
YL + MA	1.28 ± 0.28 a	1.40 ± 0.27 abc	1.42 ± 0.05 b	0.44 ± 0.07 a
YL + SA	0.71 ± 0.09 bc	1.01 ± 0.29 bc	0.81 ± 0.06 e	0.41 ± 0.07 ab

Means with different letters are significantly different at $p < 0.05$ (Duncan test).

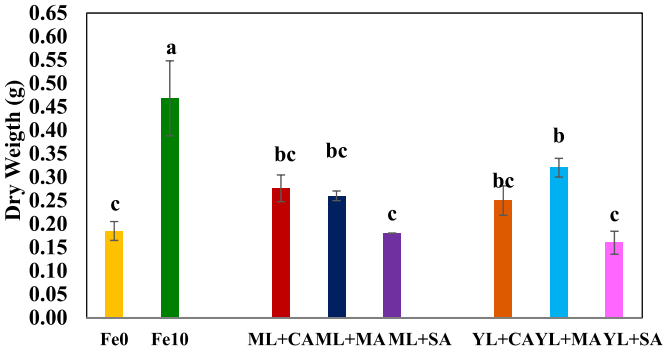

Fig. 5. Dry weight (g) of newly emerged leaves (L4; non-treated) for all treatments at the end of experiment.

Table 2
Macronutrient (in g kg^{-1}) and micronutrient (in mg kg^{-1}) concentrations in newly emerged leaves (L4) at the end of experiment. Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) was brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA.

Treatments	N	P	K	Mg	Ca
Fe0	n.d.	8.2 ± 0.4 bc	57.8 ± 12.7 a	6.4 ± 0.5 a	15.6 ± 1.1 a
Fe10	n.d.	6.9 ± 0.1 c	35.1 ± 2.3 ab	4.1 ± 0.2 cd	11.3 ± 0.8 bcd
YL + CA	40.9 ± 1.9 a	8.6 ± 0.4 b	37.9 ± 2.8 ab	3.8 ± 0.3 d	10.7 ± 1.1 cd
YL + MA	32.7 ± 0.3 ab	7.4 ± 0.5 bc	40.1 ± 2.2 ab	4.6 ± 0.2 bcd	14.7 ± 0.6 ab
YL + SA	12.8 ± 1.0 c	10.1 ± 0.6 a	41.5 ± 2.8 ab	4.0 ± 0.2 d	9.2 ± 0.9 d
ML + CA	36.9 ± 0.5 a	7.6 ± 0.9 bc	32.3 ± 7.7 b	4.9 ± 0.4 bc	13.5 ± 1.6 abc
ML + MA	36.9 ± 0.3 a	6.8 ± 0.1 c	27.4 ± 5.2 b	5.1 ± 0.2 b	13.7 ± 0.6 abc
ML + SA	21.0 ± 7.0 bc	10.9 ± 0.5 a	38.6 ± 1.2 ab	3.9 ± 0.4 d	9.2 ± 0.6 d
	Cu	**Zn**	**Mn**	**Fe**	
Fe0	0.22 ± 0.01 b	36.8 ± 3.2 ab	232.5 ± 36.5 b	123.3 ± 16.9 ab	–
Fe10	0.08 ± 0.01 b	28.1 ± 2.4 b	79.4 ± 4.0 c	140.0 ± 9.1 a	–
YL + CA	11.60 ± 0.90 a	36.9 ± 0.9 ab	570.9 ± 37.6 a	86.1 ± 15.5 bc	–
YL + MA	0.11 ± 0.01 b	31.2 ± 2.7 b	165.0 ± 14.2 bc	88.7 ± 6.1 bc	–
YL + SA	12.60 ± 1.00 a	40.5 ± 3.0 a	171.8 ± 15.5 b	127.7 ± 19.7 ab	–
ML + CA	0.14 ± 0.01 b	29.7 ± 1.8 b	235.4 ± 37.4 b	88.2 ± 7.1 bc	–
ML + MA	0.15 ± 0.01 b	29.0 ± 2.7 b	245.1 ± 14.2 b	71.2 ± 6.1 c	–
ML + SA	10.80 ± 2.70 a	35.3 ± 2.2 ab	218.3 ± 2.3 b	93.2 ± 8.4 bc	–

Data shown are means ± SE (n = 5). Data in columns with the same letter were not significantly different (at $p < 0.05$), using the Duncan multiple range test. n. d. – not determined.

the range 3.8–6.4 g kg^{-1} DW and were highest in the untreated (Fe0) plants, with the rest of the treatments giving quite similar values. Calcium concentrations were in the range 9.2–15.6 g kg^{-1} DW, and showed only small variations between treatments, with the highest value in the untreated (Fe0) plants and the lowest in plants treated with SA (YL + SA and ML + SA).

Regarding micronutrients, the Cu concentrations were between 0.11 and 12.6 mg^{-1} kg DW and were significantly higher when young leaves were brushed with CA or SA (11.6–12.6 mg kg^{-1} DW), or when mature leaves were treated with SA (10.8 mg kg^{-1} DW). The plants of the remaining treatments showed lower foliar Cu values. Leaf Zn concentration was in the range 28.1–40.5 mg kg^{-1} DW and was higher in plants where SA was applied to young leaves, but similar in the other treatments, including the untreated controls (Fe0). Leaf Mn concentration was in the range 79.4–570.9 mg kg^{-1} DW, and the maximum values were found when CA was applied to young leaves, and the minimum in the Fe10 plants (79.4 mg kg^{-1} DW), with the remaining treatments showing intermediate values. Leaf Fe concentration was in the range 71.2–140.0 mg kg^{-1} DW, and the highest concentrations were observed in the Fe10, Fe0 and YL + SA treatments. In the remaining treatments, the Fe values were lower and similar to each other.

The nutrient contents in newly emerged (L4) leaves were calculated based on the nutrient concentration and the leaf biomass (Table 3 and Table 4). Nitrogen contents were in the range 3.0–10.2 mg $plant^{-1}$, with the lowest values in the YL + SA and ML + SA treatments (the Fe0 and Fe10 plants were not analysed). In the remaining treatments, the foliar contents of N were higher and similar to each other. Phosphorus contents were in the range 1.5–3.2 mg $plant^{-1}$ and were lower in the Fe0, YL + SA and ML + MA and ML + SA than in the Fe10 plants. Potassium contents were in the range 6.8–12.5 mg $plant^{-1}$, with higher values in the Fe10 plants compared with the rest of the treatments, except for the YL + MA one. Magnesium contents were in the range 0.6–1.9 mg $plant^{-1}$, and were higher in the Fe10, YL + MA and ML + CA treatments. Calcium contents were in the range 1.5–5.2 mg $plant^{-1}$, with Fe10 plants showing the highest value, but similar to the treatments where MA was applied to both leaf types (YL + MA: 4.8 g kg^{-1} DW and ML + MA: 3.5 g kg^{-1} DW) and to leaves where CA was applied to mature leaves (ML + CA: 3.8 g kg^{-1} DW).

Copper contents in L4 leaves were in the range <0.1–3.2 mg kg^{-1} DW. The highest Cu value was observed in the YL + CA plants, and the plants of treatments ML + SA and YL + SA showed intermediate values (1.8 and 2.0 mg kg^{-1} DW, respectively). The values of Cu in the remaining treatments were <0.1 mg kg^{-1} DW and similar to each other. Zinc contents in L4 leaves were within the range 6.2–12.8 mg kg^{-1} DW. Control plants (Fe10) had the highest Zn content (12.8 mg kg^{-1} DW), although it was not significantly different from those in the YL + CA and YL + MA plants (9.4–9.5 mg kg^{-1} DW). Manganese contents were between 26.7 and 145.0 mg kg^{-1} DW, and the highest value was in YL + CA plants, with values in the other treatments being similar to each other. Iron contents were within the range 16.0–64.8 mg kg^{-1} DW. The highest Fe content was observed in Fe10 plants and values in the other treatments were similar to each other (16.0–25.4 mg kg^{-1} DW).

Table 3
Macronutrient (in mg) and micronutrient (in μg) contents in the newly emerged leaves (L4) at the end of experiment. Fe0: zero Fe in the nutrient solution. In each plant, both sides of one mature leaf (ML) or one young leaf (YL) was brushed with a given carboxylate. The tested carboxylates were malic (MA), citric (CA) and succinic (SA) acids. The treatments are: Fe0, Fe10, YL + MA, YL + CA, YL + SA, ML + MA, ML + CA or ML + SA.

Treatments	N	P	K	Mg	Ca
Fe0	n.d.	1.5 ± 0.1 b	10.0 ± 3.0 b	1.2 ± 0.1 bc	2.9 ± 0.2 bcd
Fe10	n.d.	3.2 ± 0.5 a	16.3 ± 2.1 a	1.9 ± 0.3 a	5.2 ± 0.7 a
YL + CA	8.5 ± 1.7 a	2.2 ± 0.3 ab	9.2 ± 0.7 b	0.9 ± 0.1 bc	2.6 ± 0.2 bcd
YL + MA	10.2 ± 1.0 a	2.5 ± 0.6 ab	12.5 ± 2.1 ab	1.5 ± 0.3 ab	4.8 ± 1.1 a
YL + SA	3.0 ± 0.5 b	1.6 ± 0.2 b	6.8 ± 1.2 b	0.6 ± 0.1 c	1.5 ± 0.3 d
ML + CA	10.2 ± 1.1 a	2.1 ± 0.3 ab	9.0 ± 0.3 b	1.4 ± 0.2 ab	3.8 ± 0.6 ab
ML + MA	9.5 ± 0.3 a	1.8 ± 0.1 b	7.6 ± 0.5 b	1.3 ± 0.0 b	3.5 ± 0.3 abc
ML + SA	3.6 ± 0.4 b	1.9 ± 0.3 b	7.0 ± 1.2 b	0.7 ± 0.2 c	1.8 ± 0.4 cd
	Cu	**Zn**	**Mn**	**Fe**	
Fe0	0.04 ± 0.0 c	6.9 ± 1.0 b	42.7 ± 6.4 b	22.4 ± 1.7 b	–
Fe10	0.03 ± 0.0 c	12.8 ± 1.5 a	36.5 ± 1.1 b	64.8 ± 8.4 a	–
YL + CA	3.2 ± 0.6 a	9.5 ± 1.0 ab	145.0 ± 25.1 a	20.9 ± 4.0 b	–
YL + MA	0.03 ± 0.0 c	9.4 ± 1.0 ab	50.7 ± 13.0 b	25.4 ± 1.4 b	–
YL + SA	2.0 ± 0.3 b	6.4 ± 0.7 b	26.7 ± 2.0 b	19.1 ± 1.2 b	–
ML + CA	0.03 ± 0.0 c	8.1 ± 1.0 b	63.2 ± 8.8 b	23.2 ± 1.1 b	–
ML + MA	0.04 ± 0.0 c	7.3 ± 0.8 b	61.5 ± 5.7 b	17.8 ± 1.2 b	–
ML + SA	1.8 ± 0.2 b	6.2 ± 0.9 b	40.9 ± 8.1 b	16.0 ± 1.6 b	–

Data shown are means ± SE (n = 5). Data in columns with the same letter were not significantly different (at $p < 0.05$), using the Duncan multiple range test. n. d. – not determined.

Table 4
Relative abundance changes of metabolites in extracts of newly emerged leaves (L4) with average area ratios >4 and p-values ≤0.02. Samples from plants grown without Fe (Fe0), under Fe0 and brushed with SA (Fe0-SA) and in the presence of iron (Fe10) were used.

Nº	RT (min)	$[M-H]^-$	Putative identification	Fe0/Fe10 (p-value)	Fe0-SA/Fe10
1	2.0	343.07	Galloylquinic acid[a)]	4.5 (0.010)	0.9
2	3.2	639.12	Quercetin-glucoside-glucuronide[b)]	16.0 (0.002)	18.1
3	3.3	495.08	Digalloylquinic acid[c)]	6.2 (0.020)	2.2

[a] $MS^2(343) \rightarrow 191$ (Clifford et al., 2007).
[b] Arita Lab 6549 Flavonoid Structure Database.
[c] mzCloud match 93.4 (entry 47 in Supplementary Table S1).

3.5. Metabolites

Using LC-$HRMS^2$ and Compound Discoverer 3.3, a total of 48 compounds with mzCloud match values ≥ 85% were consistently observed in the extracts of all L4 strawberry leaves. These compounds were annotated as different phenolic and fatty acid compounds and their derivatives, flavonoids, nucleosides and their derivatives, and amino acids and low molecular mass acids. The list of compounds tentatively identified are shown in the Supplementary Table (S1).

The LC-MS metabolite profiles of the extracts of L4 leaves from the Fe0 and Fe10 treatments were compared (see example in Fig. S1). Only compounds showing different peak areas in the Fe0 and Fe10 samples (showing ratios Fe0/Fe10 > 4 or <0.25) and with p-values ≤0.02 were considered. This analysis reveals that three compounds, galloylquinic acid, quercetin-glucoside-glucuronide and digalloylquinic acid increased significantly under Fe deficiency (Table 4 and Fig. S2).

The relative abundance of these three compounds was also determined in L4 leaves of plants submitted to all carboxylate treatments. The comparison of these samples with those of the Fe10 treatment indicated that galloylquinic and digalloylquinic acids decreased in plants where leaves were treated with SA (YL + SA) when compared to the changes found in the Fe0 controls, whereas little change was observed for quercetin-glucoside-glucuronide (Table 4 and Fig. S3). Application of

other acids did not cause significant changes in the contents of these three compounds.

4. Discussion

The regreening of Fe-deficient, chlorotic leaves in a specific crop relies on the optimization of applied formulations, dosages, and application techniques. There are many studies reporting the regreening effects of foliar sprays with Fe compounds, including Fe-chelates, inorganic Fe salts and natural Fe-complexes (Rombolà et al., 2001; Fernández et al., 2006; Lucena et al., 2010; El-Jendoubi et al., 2014; Salahi et al., 2017; Santos et al., 2021). New molecules have also been synthesized and assayed, which enable Fe chelation, Fe uptake and leaf regreening (Suzuki et al., 2021).

In contrast, other compounds that do not contain Fe in easily assimilable forms can have a biostimulant role which can potentiate specific biochemical stress responses (Pestana et al., 2011; Billard et al., 2014; Brown and Saa, 2015; Van Oosten et al., 2017; Paradikovic et al., 2019; Saavedra et al., 2020). Furthermore, other studies have shown that the application of organic acids (including oxalic, CA, MA and salicylic acid) to leaves may alleviate Fe stress conditions and relieve metal toxicity, probably due to their role on plant growth regulation and metal homeostasis (Song et al., 2018, 2020). Previous research on tree crops has shown the effectiveness of dilute solution of carboxylates on Fe deficiency chlorosis recovery, likely via changes in the leaf apoplastic pH, which facilitates the release of previously immobilized Fe pools (e.g., Pestana et al., 2003). Besides Fe mobilization, possible changes on carbon metabolism by inducing increases in anaplerotic fixation of carbon via phosphoenolpyruvate carboxylase (PEPC) enzymes (Martínez-Cuenca et al., 2013) cannot be excluded. Enhancement of the PEPC root activity associated to higher concentration of CA in roots and lower xylem sap pH was already reported by Rombolà et al. (2002) in kiwifruit grown under Fe deficiency.

In this study, the absence of Fe in the nutrient solution led to a decrease in the Chl concentration and DW of young leaves, in line with previous results in strawberry plants (Pestana et al., 2012; Gama et al., 2016). Generally speaking, plant biomass and particularly leaf DW, were negatively affected in the Fe0 control and in some foliar carboxylate treatments. This decrease was apparently more intense when SA was applied to both leaf types, whereas the application of CA in young leaves (YL + CA) triggered an unbalance between below and aboveground biomass, leading to the lowest Root/Shoot ratios.

Despite the decrease in leaf Chl in all carboxylate treatments, as shown by the negative slopes of the leaf Chl concentration vs. time relationships, slight increases in this parameter were observed in young (L3) leaves brushed with YL + SA after 29 days. Similarly, the Chl values in L4 leaves were also higher in the YL + SA plants, although they were still lower than those in the Fe-sufficient (Fe10) plants. Since young leaves (L4) emerging from the apical zone of the crown were never treated with carboxylates, the increase in Chl would imply that the SA treatment in L3 promotes Chl biosynthesis in the upper L4 leaf. Other carboxylate treatments did not cause a similar effect. The literature regarding the effects of carboxylates on Fe-deficient plants is still scarce, although positive effects of foliar applications of CA alone or in combination with Fe sources have been reported previously (Tagliavini et al., 1995, 2000; Pestana et al., 2001; Álvarez-Fernández et al., 2004).

The possible physiological link between SA and Fe deficiency chlorosis alleviation in untreated tissues deserves consideration. Organic acids can increase the pigment contents of plants growing in both normal and stressed environments (Song et al., 2020). For instance, Cd stress in *Salix variegata* caused a decrease in the pigment content and disruption of chloroplast structure, leading to a reduction in photosynthesis and biomass (Chen et al., 2020). However, after adding MA to the nutrient solution, shoot and total biomass increased due to an increase in leaf Chl, which was probably related to an increase in the expression of genes encoding enzymes responsible for pigment synthesis (Chen et al., 2020). Cadmium uptake was also enhanced upon application of CA to *Halimione portulacoides*, a species normally grown in saline and polluted environments (Duarte et al., 2007). Moreover, the authors found that Cd translocation to the aerial parts of the plant increased significantly. This ability to chelate specific metals may explain why the application of CA to young leaves (YL + CA) triggered significantly higher Mn and Cu concentrations and contents in L4 leaves. Citrate has a major role in long-distance Fe transport, but its chemical speciation is largely dependent of Fe availability (López-Millán et al., 2001). Apparently, the application of CA did not enhance Fe contents, likely due to the fact that Fe was absent in the nutrient solution.

Changes in the mineral composition of different plant organs with Fe deficiency have been studied in several crops and under contrasting experimental conditions (see Pestana et al., 2004; Gama et al., 2016) but in general, severe Fe stress led to reduced growth and nutritional imbalances (Pestana et al., 2003). Regarding the possible effects of foliar brushing treatments on the nutritional status of emerging leaves, the lack of significant differences between treatments in the concentrations of the major macronutrients (N, P and K) did not reveal a consistent response. The results obtained here are in accordance with previous findings with the same strawberry (Gama et al., 2016). However, Zn and Fe concentrations increased in L4 leaves upon application of SA to young leaves (YL + SA treatment) and led to lower Mg and Ca concentrations in the new leaves.

It is known that short term Fe stress does not induce permanent damages in the photosynthetic performance in the strawberry cultivar used ("Diamond"), allowing for a fast recovery upon supply of Fe external (Osório et al., 2014). This cultivar also shows a high growth rate, a trait which is associated to low FCR activity under Fe-depleted conditions (Pestana et al., 2012). This characteristic may explain the low values of the enzyme in Fe0 plants (and in the carboxylate leaf brushing treatments) compared to Fe10 plants. Thus, the carboxylate treatments apparently did not induce an elicitation of the FCR activity, probably excluding some biochemical signalling in leaf-to-root pathways.

Fe deficiency has been generally shown to increase the concentrations of carboxylates in roots, stem exudates and leaves of different plant species (Abadía et al., 2002; Correia et al., 2014). In this study, analysis of LC-HRMS^2 profiles showed the presence of phenolics and fatty acids and their derivatives, flavonoids, nucleosides and their derivatives, and amino acids, compounds previously observed to occur in strawberry leaves (Kårlund et al., 2016). Compounds showing significant changes in L4 leaves of Fe0 plants were quercetin-glucoside-glucuronide, galloylquinic acid and digalloylquinic acid. Quercetins are a special subclass of flavonoids commonly found in edible plants (Singh et al., 2021), and quercetin-glucuronides are typical compounds in the leaves of berries and fruits. Quercetin and kaempferol derivatives were the most abundant class of low molecular weight phenolic compounds in strawberry leaves (Kårlund et al., 2016).

Polyphenols such as quercetin and quinic acids derivatives, namely galloylquinic acids, can potentially bind Fe (Baratto et al., 2003; Perron and Brumaghim, 2009) and are known to facilitate its translocation within plants. In Fe0 plants, the quercetin-glucoside-glucuronide concentration increased 16-fold when compared to that in Fe10 plants, while the contents of galloyl and digalloyl quinic acids increased 4.5 and 6.2 times, respectively, suggesting that these compounds are related in some way to the Fe deficiency condition. As these compounds may interact with and bind Fe, their increase in Fe0 plants may potentially facilitate the translocation of Fe within the plant. Among the applied carboxylates, only SA changed significantly the relative abundance of two of these compounds in L4 leaves, namely galloylquinic acid and digalloylquinic acid (Table 4). This result is in agreement with the observed SPAD increases with SA application. However, it is not known how the application of SA to strawberry leaves triggers the decrease of both compounds in the new leaves (L4).

5. Conclusions

Data shown in this study indicate that Fe-deficient, chlorotic plants exhibited a higher concentration of chlorophyll in newly emerged leaves (L4) after brushing the young leaf (L3) with succinic acid, and to a lower extent the mature leaves (L1) with malic acid. The application of these carboxylates to Fe-deficient, chlorotic plants, without an additional source of Fe, was slightly effective in increasing chlorophyll levels in the newly emerged leaves. These results indicate the importance of endogenous metal complexing agents on the mobilization and translocation of internal Fe pools. In fact, Cu also increased in new leaves when SA acid was applied to mature leaves. Also, among a total of 48 metabolites found in strawberry leaves, two derivatives of quinic acid, galloylquinic and digalloylquinic acids, appear to be physiologically linked to Fe deficiency, suggesting they may be considered Fe stress markers for this crop.

Contributions

A. de Varennes, P.J. Correia, and M. Pestana designed the experiment. T. Saavedra wrote the manuscript and executed the experiments. F. Gama and M.A. Rodrigues executed the experiments. J.P. da Silva supervised the LC-MS and LC-HRMSn experiments and processed/analysed the data. All authors contributed to the discussion of the results.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.08.004.

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