agronomy

MDPI

Article

Physiological Responses to Fe Deficiency in Split-Root Tomato Plants: Possible Roles of Auxin and Ethylene?

Silvia Celletti [1,*], Youry Pii [1], Fabio Valentinuzzi [1], Raphael Tiziani [1], Maria Chiara Fontanella [2], Gian Maria Beone [2], Tanja Mimmo [1], Stefano Cesco [1] and Stefania Astolfi [3]

1 Faculty of Science and Technology, Free University of Bozen-Bolzano, I-39100 Bolzano, Italy; youry.pii@unibz.it (Y.P.); fabio.valentinuzzi@unibz.it (F.V.); raphael.tiziani@natec.unibz.it (R.T.); tanja.mimmo@unibz.it (T.M.); stefano.cesco@unibz.it (S.C.)

2 Department for Sustainable Food Process, Faculty of Agriculture, Food and Environmental Sciences, Catholic University of the Sacred Heart, I-29122 Piacenza, Italy; mariachiara.fontanella@unicatt.it (M.C.F.); gian.beone@unicatt.it (G.M.B.)

3 Department of Agricultural and Forestry Sciences, University of Tuscia, I-01100 Viterbo, Italy; sastolfi@unitus.it

* Correspondence: silvia.celletti@unibz.it; Tel.: +39-0471-017-719

Received: 12 June 2020; Accepted: 9 July 2020; Published: 11 July 2020

Abstract: Iron (Fe) bioavailability in soils is often limited and can be further exacerbated by a non- homogeneous distribution in the soil profile, which has been demonstrated to vary both in space and time. Consequently, plants respond with morphological and physiological modifications at the root level involving a complex local and systemic signaling machinery. The present work unravels the role of two phytohormones (i.e., ethylene and auxin) and their integrated signaling in plant response to Fe deficiency. Inhibitors of auxin polar transport and of ethylene biosynthesis (*N*-1-naphthylphthalamic acid - NPA and aminoethoxyvinylglycine - AVG, respectively) were applied on tomato (*Solanum lycopersicum* L.) plants grown by the split-root technique, which allows to simulate condition of Fe heterogeneous distribution. Results showed that plants, exposed to an uneven Fe supply, triggered a complex auxin-ethylene signaling. A systemic action of auxin on *FERRIC REDUCTASE OXIDASE 1* (*SlFRO1*) expression was revealed, while ethylene signaling was effective both locally and systemically. In addition, the investigation of Fe concentration in tissues showed that when leaves overcame Fe deficiency a Fe "steady state" was maintained. Therefore, physiological adaptation to this heterogeneous Fe supply could be mediated by the integration of the complex signaling pathways prompted by both auxin and ethylene activities.

Keywords: auxin; ethylene; Fe deficiency; Fe signaling; split-root; tomato

1. Introduction

Mineral elements are crucial for plant growth and productivity, but they are often unevenly distributed within the upper layer of soil as well as along its profile, rather than homogeneously distributed. In particular, soil is characterized by areas defined as "nutrient hotspots", in which nutrients are more abundant [1–3]. Thus, in the natural growth conditions, plants are often exposed to a patchy distribution of nutrients [4,5], which may affect different aspects of plant growth, function and productivity with direct consequences on nutrient uptake and root growth and development. Therefore, understanding the responses of plants to soil heterogeneity is critical in order to develop strategies to optimize plant production.

Extensive research analyzing how plants cope with uneven nutrient availabilities in the soil has been previously performed [6–8]. It is well known that nutrient hotspots could likely increase the nutrient mass flux (nutrient movement *via* soil water driven by plant transpiration) to the root surface [9], and determine a tropic root growth towards the nutrient-rich areas [10]. Indeed, plants are able to efficiently exploit nutrient-rich patches by selectively increasing root growth proliferation [11,12]. However, nutrient uptake is highly affected not only by nutrient concentration, but also by its chemical form determining the available fraction for plant uptake. In particular, the nutrient bioavailability is influenced by physical, chemical, and biological proprieties of soil [13,14]. Iron (Fe) availability, for instance, depends primarily on the soil pH and redox potential [15]. Iron is the fourth most abundant element on the Earth's crust, but it is poorly available to plants, especially in alkaline soils [16]. The imbalance between Fe solubility in soils and the demand of Fe by the plant is the primary cause of leaf Fe chlorosis [17]. Iron plays a key role in crucial metabolic processes such as photosynthesis, respiration, and nitrogen assimilation, other than chlorophyll synthesis [18–20]. Being one of the main constraints for plant growth and productivity, Fe nutrition has been widely investigated with the aim of improving the nutrient use efficiency, and thus increasing agricultural productivity. Thus, a better exploitation of soil endogenous resources might lead to a reduction in the external Fe fertilizer applications, also resulting in the reduction of the environmental issues posed by such agronomic practices [21].

When plants are challenged with Fe limitation a reduction-based strategy, known as Strategy I (all the dicots and non-graminaceous monocots), or a chelation-based strategy, known as Strategy II (only monocots), allows to improve Fe mobilization and uptake by roots [22]. Tomato (*Solanum lycopersicum* L.), as Strategy I species, acidifies the rhizosphere through plasma membrane H^+-ATPase activity to solubilize Fe^{3+}, which is reduced to Fe^{2+} by the Fe^{3+}-chelate reductase (FCR) activity, and the resulting Fe^{2+} is transported into the root cells *via* the specific Fe transporter (*IRT1*) [23,24]. Physiological responses [25,26] are also associated with root morphological changes, including enhanced formation of subapical root hairs, transfer cells, and root branching [27–29]. Finally, various studies have highlighted that the regulation of Strategy I plant responses to Fe stress is mediated by phytohormones [30,31], by which Fe deficiency signals could be transmitted within the plant [30,32–34]. It has been suggested that a crucial role is thereby played by both auxin and ethylene, which might interact either synergistically or antagonistically in signaling processes [29,33–36].

Auxin and ethylene are supposed to be responsible for the generation of a long-distance signal, which is mediated by an unknown factor, moving from shoots to roots [37–39], and a local signal, which originates in the roots and directly activates the transcriptional network of FER [31,40]. The major role in the long-distance Fe signaling pathway has been attributed to auxin (indole-3-acetic acid, IAA), since its biosynthesis mainly occurs in aerial tissues (i.e., apical meristems) as well as at level of the root tips [41–43]; then, it is rapidly transported systemically from shoots to roots *via* the polar auxin transport (PAT) through the phloem, or, locally, by the cell-to-cell active transport [44,45]. Thus, auxin could represent the upstream signal in modulating Fe deficiency responses [46].

In the intricate signaling network of Fe availability, auxin does not operate independently, but it communicates Fe demand to the root apparatus and interacts with other hormonal molecules that act locally as enhancers of Fe deficiency responses, such as ethylene [47]. Ethylene is biosynthesized from *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC) [48,49], and it has been proposed as an essential component in the transduction of Fe deficiency signal, since Fe-deficient roots increase ethylene biosynthesis to induce the well-characterized adaptive changes in root architecture [50,51]. Consistently, plants treated with exogenous ACC, the precursor of ethylene, showed typical morphological changes of Fe deficiency responses [52,53].

Since roots can sense locally the changes in the external medium of Fe supply, the primary perception of Fe deficiency could originate in the roots. Iron deficiency could generate the transmissible information about the presence of nutrients from roots towards the leaves [54]. In addition, Fe from the phloem can act as signal to inhibit the expression of Fe-uptake genes [31,32,55,56].

In some experiments using the split-root system with various Strategy I plants (like tomato, Arabidopsis, and cucumber), it has been found that both expressions of Fe-uptake genes and activity of Fe^{3+}-chelate reductase enzyme was increased in the Fe-sufficient half of the root system [38,57,58]. It remains unclear how the signal of Fe deficiency is transmitted from the Fe-deficient to Fe-sufficient half of the root [38,59].

The present work addresses the role of ethylene and auxin and their integrated signaling in plant response to Fe deficiency. Inhibitors of auxin polar transport and of ethylene biosynthesis (*N*-1-naphthylphthalamic acid - NPA and aminoethoxyvinylglycine - AVG, respectively) were applied on tomato (*Solanum lycopersicum* L.) plants grown by the split-root technique. This latter technique is a useful tool to demonstrate root-to-shoot and shoot-to-root communication, allowing to discriminate between systemic and localized signals involved in the regulation of Fe deficiency responses. We further used stable labelled Fe isotope (^{57}Fe) to reveal the possible effect of Fe on regulating the complex machinery of Fe deficiency signaling and the intricate communication mechanisms between roots and shoots.

2. Materials and Methods

2.1. Plant Growth Conditions and the Split-Root System

Tomato (*Solanum lycopersicum* L., cv. Marmande) seeds were sown in plastic boxes, containing several layers of wet filter paper, and kept in the dark at 25 °C for 9 days to allow germination [60]. Afterwards, seedlings were selected based on uniformity of size to be transferred in plastic 1.5 L pots (10 seedlings/pot), filled with a continuously aerated nutrient solution (NS), composed as follows (mM): 2 $Ca(NO_3)_2$, 0.7 K_2SO_4, 0.1 KH_2PO_4, 0.1 KCl, 0.5 $MgSO_4$, and (µM): 10 H_3BO_3, 0.5 $MnSO_4$, 0.2 $CuSO_4$, 0.5 $ZnSO_4$, 0.01 $(NH_4)_6Mo_7O_{24}$, and 5 Fe^{3+}-EDTA [61].

After 2 weeks of low Fe availability (5 µM Fe^{3+}-EDTA), the root apparatus of each plant was divided into two approximately equal parts and separated into two compartments (split-root system); each compartment was filled with 1 L NS, containing different Fe concentrations, i.e., 40 µM Fe^{3+}-EDTA or 40 µM ^{57}Fe-EDTA for the Fe-sufficient (+Fe) and 0 µM for Fe-deficient (-Fe) condition (Figure 1A). The choice of the experimental cultivation scheme was based on our previous work [62].

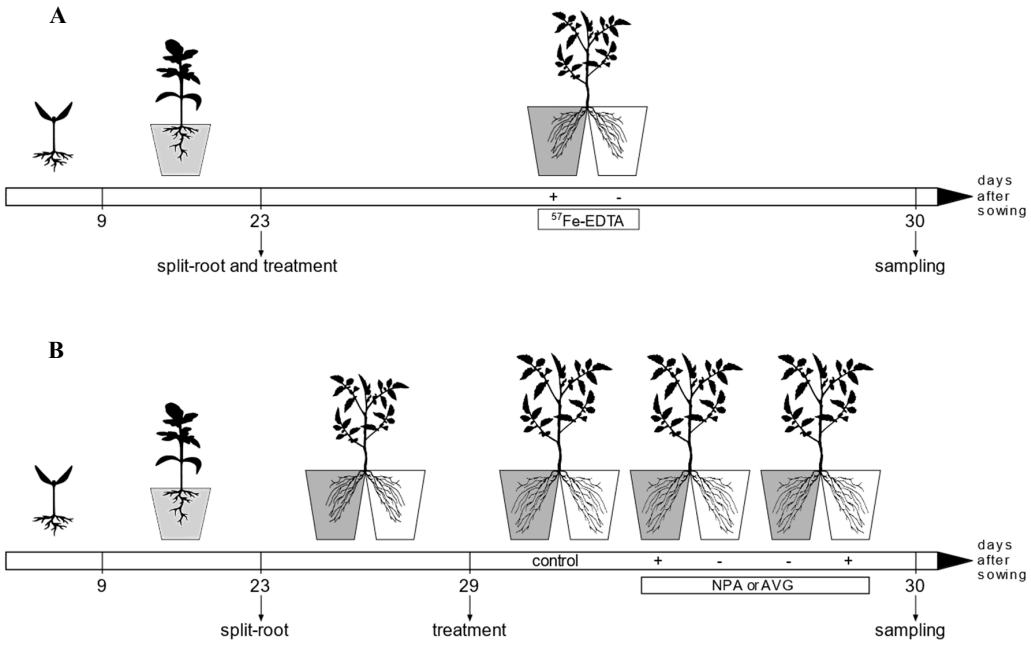

Figure 1. Schematic drawing of the experimental set up indicating: (**A**) the ^{57}Fe-EDTA treatment; (**B**) the supply of auxin and ethylene inhibitors.

Plants were grown hydroponically in a climatic growth chamber with day/night cycle of 14/10 h, temperature regime of 24/19 °C, light intensity of about 250 μmol m^{-2} s^{-1} at plant level, and relative humidity of approximately 70%. The NSs were renewed every 3 days.

2.2. Phytohormonal Inhibitor Treatments and Plant Sampling

The plants were cultured in the split-root system for 1 week in order to induce Fe deficiency response, as shown in Figure 1B, according to our previous work [62]. On the sixth day, plants were treated with *N*-1-naphthylphthalamic acid (NPA), the inhibitor of the polar transport of auxin, and aminoethoxyvinylglycine (AVG), inhibitor of ethylene synthesis (Figure 1B).

Both NPA and AVG were dissolved in dimethyl sulfoxide (DMSO) and added at the final concentration of 10 and 0.1 μM, respectively [63,64].

At the end of the experiment with 10 μM NPA or 0.1 μM AVG the different conditions were as follows: the 40/0 μM Fe^{3+}-EDTA treatment as control; the 40/0 μM Fe^{3+}-EDTA with NPA or AVG treatment added to Fe-sufficient part of the container and the 40/0 μM Fe^{3+}-EDTA with NPA or AVG treatment added to Fe-deficient part of the container (each side of "/" represents the two different compartments of the same container).

At harvest (30 days after sowing), the Fe^{3+}-chelate reductase activity was assayed *in vivo* on tomato roots. Shoot and root samples were separated, quickly frozen in liquid N_2, and stored at −80 °C for subsequent analysis.

2.3. Determination of Chlorophyll Content

After 7 days of growth in the split-root system, the chlorophyll content per unit area was measured on the youngest fully expanded leaves of each plant, by using a portable chlorophyll meter SPAD-502 (Minolta Co., Osaka, Japan), and recorded values were expressed as "Soil Plant Analysis Development" (SPAD) units.

2.4. Natural and ^{57}Fe Accumulation in Shoots and Roots

Uptake of ^{57}Fe by split-root tomato plants was determined by measuring the ^{57}Fe accumulation in shoots and in both Fe-deficient and Fe-sufficient portions of the root system (Figure 1A). Plants were grown for a week in a NS containing 40 μM ^{57}Fe-EDTA (94.87%) (ISC Science, Oviedo, Spain), titrated to pH 6 with 1 M KOH, as previously described [65]. At the end of the treatment with ^{57}Fe-EDTA (7 days after application), roots were washed for 15 min in 0.5 mM $CaSO_4$ to allow the removal of ^{57}Fe non-specifically bound to the cell wall [65]. Shoot and root tissues were then oven-dried at 60 °C for 48 h, weighed and then digested with concentrated HNO_3 [65% (*v*/*v*), Carlo Erba], using a single reaction chamber (SRC, UltraWAVE, Milestone Inc, Shelton, CT, USA). The mineralized solutions were spiked with 100 ppm ^{54}Fe (99.92%) standard solution (ISC Science, Oviedo, Spain) and Fe isotope ratios measurements were carried out by ICP-MS (Agilent 7900, Agilent Technologies, Santa Clara, CA, USA) [66–68]. Iron concentration was determined by isotope dilution analysis using a collision cell ICP-MS instrument with helium as collision gas. Instrument and octopole operating condition are summarized in Supplementary Materials Table S1. Isotope Pattern Deconvolution (IPD) is the most complete mathematical approach to perform multiple-spiking Isotopic Dilution (ID) [69,70], needed to quantify natural-abundance element and two enriched tracers [71]. In this work a mixture of natural-abundance Fe and ^{57}Fe-enriched Fe was determined by the addition of a second Fe tracer ^{54}Fe-enriched.

2.5. Determination of Fe^{3+}-Chelate Reductase Activity by Intact Roots

The Fe^{3+}-chelate reductase activity of tomato roots was assayed colorimetrically, using bathophenantrolinedisulfonate (BPDS) reagent [62,72]. Briefly, roots of intact plants were carefully rinsed in deionized water, incubated in darkness and at room temperature in a continuously aerated assay solution, with the following composition (mM): 0.5 $CaSO_4$, 10 MES-KOH (pH 5.5), 0.25 Fe^{3+}-EDTA, and 0.6 BPDS. After 60 min, the absorbance of the assay solution was determined at 535 nm with a spectrophotometer [73]. The reduced Fe was calculated on the basis of the concentration of Fe^{2+}-$BPDS_3$ complex, using the molar extinction coefficient of 22.1 $mM^{-1}cm^{-1}$, and was expressed in nmol Fe reduced $g_{root\ FW}{}^{-1}$ h^{-1}.

2.6. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from approximately 0.1 g of root tissues of tomato plants exposed to different Fe supplies and subjected or not to NPA or AVG treatment, by using Spectrum Plant Total RNA Kit (Sigma-Aldrich Co. LLC), following the operating manual.

Afterward, 1 µg of each total RNA sample was treated with 10 U of DNAse RQ1 and 1 mg of the resulting DNase-treated RNA was utilized to synthesize cDNA by using ImProm-II Reverse Transcription System kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.7. Real-Time Reverse Transcription-PCR Analysis

Gene-specific primers were used for the target genes as well as for the housekeeping genes, as previously described by Coppa et al. (2018) [62] and Zuchi et al. (2015) [74]. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) experiments were performed in biological triplicates and the reaction was performed by using the SsoFast EvaGreen Supermix (Bio-Rad), as described in Valentinuzzi et al. (2015) [75].

The amplification efficiency was calculated from raw data using LinRegPCR software [76]. The relative expression ratio value was calculated for treated samples relative to the corresponding untreated sample at the same time-point according to the Pfaffl equation [77]. Standard error values were calculated according to Pfaffl et al. (2002) [78].

2.8. Statistical Analysis

The results are presented as means ± standard error (SE). Statistical analyses were carried out using R software. Statistical significance was tested by one-way ANOVA analysis with Tukey post hoc tests at $p < 0.001$ and by Student's *t*-test.

3. Results

3.1. Effect of Localized Fe Supply on Plant Growth and Chlorophyll Content

Root biomass accumulation, measured in terms of fresh weight, was significantly increased by approx. 40% in the compartment featuring Fe deficiency as compared to that Fe-resupplied (Figure 2A,B). Similarly, plants treated with either NPA or AVG, displayed a higher accumulation of biomass in the Fe-deficient root compartment, independently from the treated side of the root system (i.e., Fe-sufficient or Fe-deficient), as compared to the Fe-sufficient one (Figure 2A,B).

In addition, plants supplemented with NPA showed a slight increase in the allocation of biomass at shoot level (Figure 2C), whilst no significant differences were detected in plants treated with AVG (Figure 2D) as compared to control untreated plants.

Finally, the NPA treatment did not induce any alteration in the concentration of chlorophyll, estimated as SPAD units (Figure 2E); on the other hand, tomato plants supplemented with AVG displayed a slight increase in the chlorophyll concentration (Figure 2F).

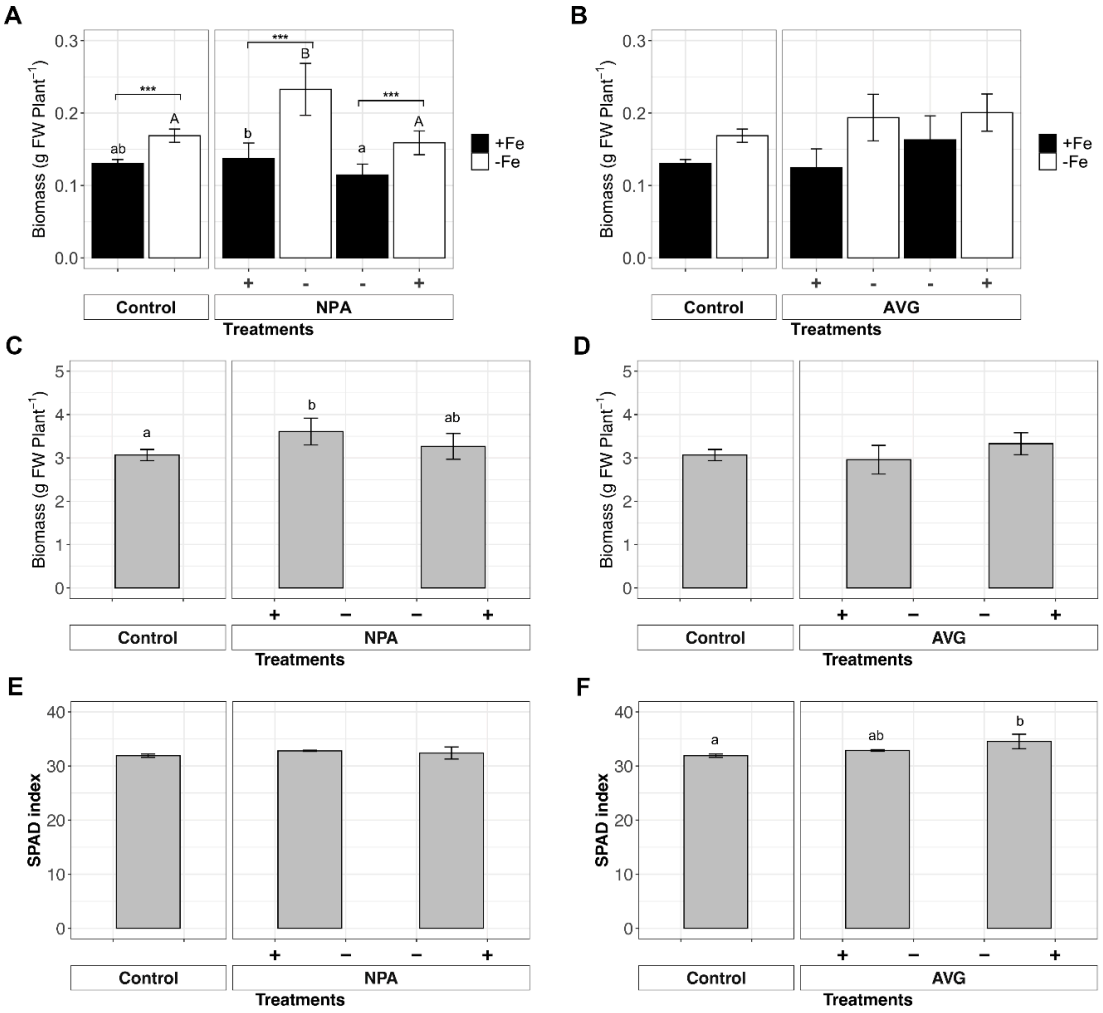

Figure 2. Growth parameters of split-root tomato plants: (**A**) root biomass of split-root tomato plants not-supplemented (control) or supplemented with polar auxin transport inhibitor naphthylphthalamic acid (NPA); (**B**) root biomass of split-root tomato plants not-supplemented (control) or supplemented with ethylene signaling inhibitor aminoethoxyvinylglycine (AVG); (**C**) shoot biomass of split-root tomato plants both not-supplemented and supplemented with polar auxin transport inhibitor NPA; (**D**) shoot biomass of split-root tomato plants both not-supplemented and supplemented with ethylene signaling inhibitor AVG; (**E**) "Soil Plant Analysis Development" (SPAD) index of tomato plants both not-supplemented and supplemented with polar auxin transport inhibitor NPA; (**F**) SPAD index of tomato plants both not-supplemented and supplemented with ethylene signaling inhibitor AVG. Data are reported as means ± SE of three independent biological replicates (each biological replicate was the mean of five plants) for each treatment. Statistical significance was tested by one-way ANOVA analysis with Tukey post hoc tests ($p < 0.001$). Different letters indicate statistically different values. The statistical significance between resupplied and not-resupplied root compartments was tested by Student's *t*-test (***, $p < 0.001$).

3.2. *Effect of Localized Fe Resupply on Fe-Starved Plants: ^{56}Fe and ^{57}Fe Accumulation in Shoots and Roots*

In the portion of the root system exposed to Fe shortage, the concentration of natural Fe (^{56}Fe) doubled, while that of ^{57}Fe was reduced by 93% with respect to the relative Fe-sufficient control portions of the same plant (Figure 3A,B, respectively) at 7 days after ^{57}Fe treatment (Figure 1A). On the other hand, ^{57}Fe accumulation in the Fe-sufficient (+Fe) root part was 15-fold higher (Figure 3B) compared to that of natural Fe (Figure 3A), and the natural Fe accumulation in the Fe-deficient (-Fe) root part was 2-fold higher (Figure 3A), as compared to that of ^{57}Fe (Figure 3B).

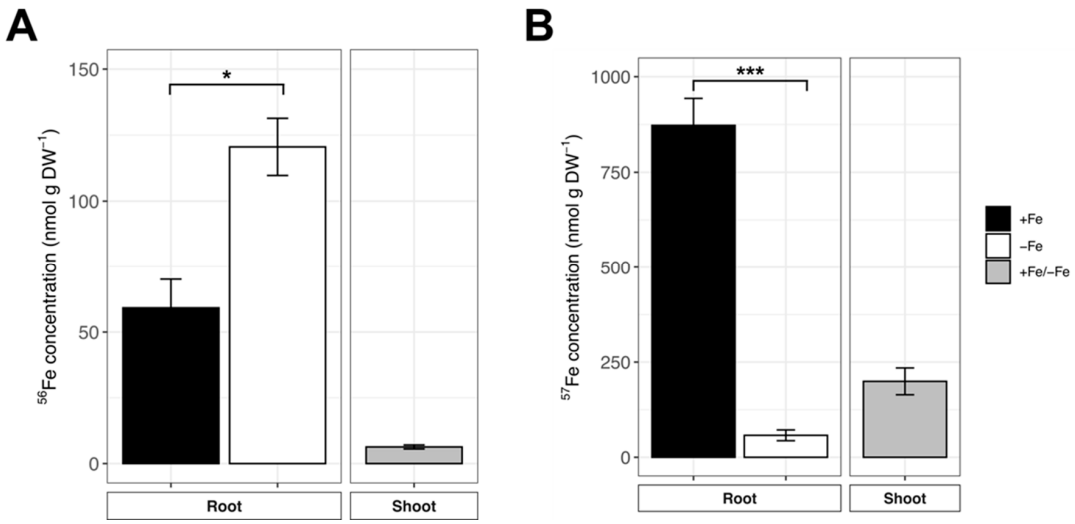

Figure 3. Natural (^{56}Fe) and labelled (^{57}Fe) Fe quantification in tomato split-root plants after Fe^{3+}-EDTA resupply: (**A**) ^{56}Fe quantification in root and shoot tissue of split-root tomato plants at 7 days after the resupply. The resupply was carried out only in one compartment corresponding to the black bars. Data are presented as means ± SE (n = 3). The statistical significance between resupplied and not-resupplied root compartments was tested by Student's t-test (*, $p < 0.05$); (**B**) ^{57}Fe quantification in root and shoot tissue of split-root tomato plants at 7 days after the resupply. The resupply was carried out only in one compartment corresponding to the black bars. Data are presented as means ± SE (n = 3). Grey bars (+Fe/-Fe) indicate shoot tissues of plants that were resupplied with ^{57}Fe only in one root compartment. The statistical significance between resupplied and not-resupplied root compartments was tested by Student's t-test (***, $p < 0.001$).

In the shoots the concentration of ^{57}Fe reached values approximately 32-fold higher than those measured for natural Fe in the same tissue of the same plant (Figure 3A,B, respectively).

3.3. Effect of Localized Fe Resupply on Fe-Starved Plants: Fe^{3+}-Chelate Reductase Activity

The *in vivo* assay of Fe^{3+}-chelate reductase enzyme (FCR) on split-root tomato plants showed a significant increase of this activity in roots resupplied with 40 µM Fe^{3+}-EDTA as compared to -Fe roots, independently from the treatment applied (Figure 4A,B). In particular, Fe reduction rate was decreased by about 40% in Fe-deficient roots as compared to those Fe-resupplied (+Fe) (Figure 4A,B). Interestingly, when plants were treated with NPA the reductase activity was reduced as compared to control plants (Figure 4A), albeit to a different extent as a function of the compartment supplied with the auxin transport inhibitor. In fact, if NPA was added to the Fe-resupplied compartment, the reductase activity showed a decreasing trend, yet not statistically significant as compared to controls, but, when NPA was applied to the -Fe root compartment, Fe reduction activity was significantly inhibited in both compartments, as compared to the respective controls (Figure 4A). Conversely, when plants were supplemented with AVG, the inhibitor of ethylene synthesis, the resupplied root compartment showed a Fe^{3+} reduction activity higher than the respective untreated control irrespective of the treated root part (i.e., resupplied or not resupplied) (Figure 4B). In particular, when AVG was added to the deficient compartment, the Fe-deficient roots displayed a significantly higher FCR activity as compared to the respective control (Figure 4B); on the other hand, the supply of AVG to the resupplied root portion did not impact the Fe^{3+} reduction activity of the Fe starved root apparatus compared to the control (Figure 4B).

3.4. Effect of Localized Fe Resupply on Fe-Starved Plants: SlFRO1 Gene Expression

Figure 5 shows the expression profile of *SlFRO1* (*FERRIC REDUCTASE OXIDASE 1*) gene, which encodes for Fe^{3+}-chelate reductase enzyme. In particular, the expression of the gene followed the same profile reported for enzyme activity in all conditions, except in the NPA-treated plants (Figure 5).

In fact, in control conditions, the expression level of *SlFRO1* was upregulated in the portion of root system exposed to Fe sufficiency with respect to the -Fe root portion. The same trend was observed also in the other conditions imposed, except for the Fe-sufficient root system supplemented with NPA. Surprisingly, NPA treatment resulted in higher *SlFRO1* expression in the Fe-deficient compartment, irrespective of the treated root part (i.e., resupplied or not resupplied) (Figure 5).

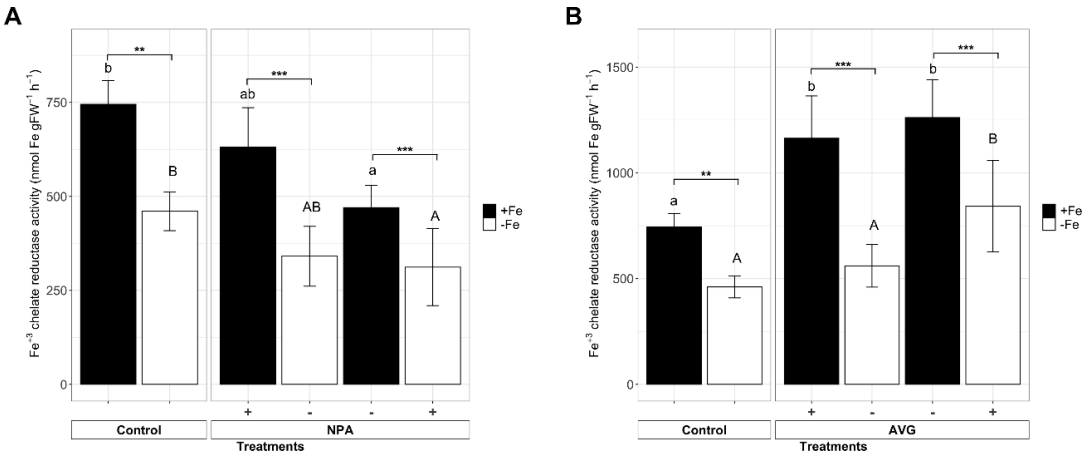

Figure 4. Iron (Fe^{3+})-chelate reductase activity (FCR): (**A**) FCR activity in split-root tomato plants both not-supplemented and supplemented with polar auxin transport inhibitor NPA; (**B**) FCR activity in split-root tomato plants both not-supplemented and supplemented with ethylene signaling inhibitor AVG. Data are reported as means ± SE of three independent biological replicates (each biological replicate was the mean of five plants) for each treatment. Statistical significance was tested by one-way ANOVA analysis with Tukey post hoc tests ($p < 0.001$). Different letters indicate statistically different values. The statistical significance between resupplied and not-resupplied root compartments was tested by Student's *t*-test (**, $p < 0.01$; ***, $p < 0.001$).

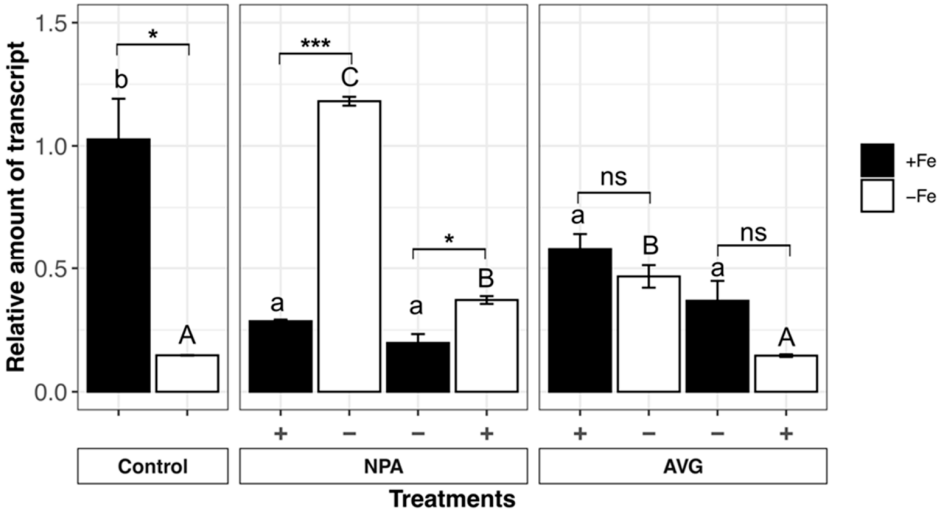

Figure 5. Quantitative real time RT-PCR analyses of *SlFRO1* expression in roots: the expression levels of *SlFRO1* was assessed by qRT-PCR. The data were normalized to the internal control Tomato *LeEF-1* mRNA for *elongation factor 1 alpha*. The relative expression ratios were calculated using resupplied control roots as a calibrator sample. Data are reported as means ± SE of three independent biological replicates (each biological replicate was the mean of five plants) for each treatment. Statistical significance was tested by one-way ANOVA analysis with Tukey post hoc tests ($p < 0.001$). Different letters indicate statistically different values. The statistical significance between resupplied and not-resupplied root compartments was tested by Student's *t*-test (*, $p < 0.05$; ***, $p < 0.001$).

4. Discussion

Iron (Fe) is an essential nutrient to plants [15] and, despite its relatively high concentrations in the soil, the bioavailability to plants can be often limited, depending on chemical and physical characteristics of the soils themselves [79,80]. In addition, the bioavailability of nutrients to plants can be also affected by the distribution of mineral elements in the soil, which has been demonstrated to be variable in space and time [1,4,5]. In the specific case of Fe, a few pieces of research have been aimed at investigating the effects of a heterogeneous nutrient supply in dicot plants. The Fe transporter (*Iron-Regulated Transporter 1*, *IRT1*) and the Fe-chelate reductase (FCR) enzyme have been demonstrated to be modulated by both local and systemic regulation pathways in Arabidopsis [19]. In addition, the use of the split-root technique in cucumber plants allowed to highlight that both the transcripts coding for *CsFRO1* and *CsIRT1* as well as the FCR activity were upregulated in the resupplied portion of the root system, whereas they were repressed in the non-resupplied one [58,81]. These observations suggested that Fe acts as local messenger for the upregulation of the uptake system [82]; nonetheless, the downregulation of Fe deficiency response observed in the non-resupplied portion of the root is thought to be mediated by a systemic signal, which could be represented by either the increase in the Fe translocation from the shoots [81] or by other mobile chemical effectors, as for instance phytohormones [29,31,83,84], or by the integration/interplay of both. As already demonstrated by Valentinuzzi et al. (2020) [81], upon resupply, the translocation mechanisms are activated by the availability of the micronutrient, thus causing the allocation of Fe to the starved organs (i.e., leaves and non-resupplied roots). In particular, it was established that, upon resupply, Fe was distributed in the short period (i.e., 12 h) to both starved roots and shoots to overcome the Fe deficiency threshold; however, in the longer period (i.e., 24–48 h), Fe was then preferentially delivered to leaves [81]. Indeed, the resupply data presented in this study (Figures 1A and 3) confirm previous observations, albeit disclosing interesting pieces of information concerning Fe dynamics in split-root systems. In fact, the Fe concentrations detected in the shoots and in the non-resupplied roots of tomato plants after 7 days of treatment with ^{57}Fe (Figure 3) were comparable to those previously obtained in cucumber plants after 48 h resupply [81]. This observation suggests that Fe redistribution after the resupply might reach a "steady state" in a short time period (e.g., 24 – 48 h), that is also maintained in the long term (e.g., 7 days). Consistently, this evidence also highlights the importance of shedding light on the signaling events taking place within the short time period after the resupply in order to deepen our understanding of Fe dynamics in plants.

The role of auxin in the response to Fe deficiency has been earlier addressed for its contribution in altering the root morphology in response to the nutritional stress [52]. However, contrarily to previous observations [85], the presence of auxin transport inhibitor NPA did not affect the growth of the root system in the split-root tomato plants, as well as other biometric parameters, independently from the treated root portion (resupplied or not resupplied) (Figure 2A,C,E). The lack of NPA effects on biometric parameters of tomato plants could be ascribable to the short time period of the treatments. More recently, auxin has also been shown to be involved in regulating the molecular response towards Fe starvation, through the transcriptional modulation of *Fer-like Iron Deficiency-Induced Transcription Factor* (*FIT*) and *FRO2* in Arabidopsis [63]. Indeed, the FCR enzyme activity was influenced by NPA (Figure 4A); in particular, when the inhibitor was added to the non-resupplied portion of the root system, a significant reduction in the FCR activity was observed in both the resupplied and non-resupplied tracts of the root system. Interestingly, this decrease was also mirrored by lower *SlFRO1* mRNA levels as compared to control plants, at least in the resupplied root portion (Figure 5); surprisingly, an increase in *SlFRO1* expression was displayed locally (non-resupplied roots). In spite of the slight and not significant reduction of FCR activity observed in the tomato plants treated with NPA in the resupplied root portion (Figure 4A), *SlFRO1* expression was strongly repressed locally (i.e., in the resupplied root tract) and upregulated in the non-resupplied compartment (Figure 5). The latter observation further confirms the implication of auxin in influencing the regulations of *FRO1* [86], yet disclosing a possible different role depending on the nutritional conditions of the root system

portion. Furthermore, the role of auxin could be affected by the presence of other hormones and/or signaling substances involved in the participation of the intricate Fe deficiency signaling [33].

Similarly to auxin, ethylene, which is produced in roots experiencing Fe deficiency, has been demonstrated to play a role in the activation of Fe deficiency response [50,87–89]. In fact, the supplementation of ethylene synthesis inhibitors (e.g., cobalt, AVG) can prevent most of the physiological responses to Fe deficiency in Strategy I plants, as for instance the FCR activity [30,50,56,87,90]. In our experimental system, the treatment with ethylene inhibitor AVG did not induce any alteration in the biomass accumulation in tomato plants (Figure 2B,D,F). Nonetheless, the presence of the inhibitor, independently from the root tract treated, caused an unexpected increase in the FCR activity in the resupplied roots as compared to control (Figure 4B) [30,50,56,87,90]. However, the higher Fe^{3+} reduction rates observed in the resupplied root tracts as compared to control were not sustained by an enhanced *SlFRO1* expression (Figure 5), as also previously observed in literature [30,50,56,87,90]. Interestingly, the effects of AVG on *SlFRO1* expression in the non-resupplied root tract was dependent on the treatment; in fact, when the inhibitor was provided in the Fe resupplied root portion, the gene was induced in the Fe starved root portion (Figure 5). On the other hand, if AVG was added to the non-resupplied root zone, *SlFRO1* was repressed both locally and in the other half of the root system (i.e., the resupplied one).

In conclusion, the data presented here disclose a very complex activity of both auxin and ethylene in plants provided with a heterogeneous Fe supply. Indeed, both a systemic and local action has been revealed for the two phytohormones. Fully active hormone signaling pathways seemed to be required for the expression of *SlFRO1* gene in the resupplied root tracts; nonetheless, the inhibition of *SlFRO1* expression in the Fe-sufficient root compartment induced its upregulation in the starved compartment, suggesting that both auxin and ethylene, and/or other secondary messengers, might be involved in this modulation. However, when the signaling pathways were inhibited in the non-resupplied root compartment, auxin showed just a systemic influence on *SlFRO1* expression, whereas the ethylene signaling was effective at both local and systemic level. In addition, the investigation of the Fe concentration within tissues highlighted that, even when provided with heterogeneous Fe distribution, plants can take up the micronutrient from the nutrient hot-spots and preferentially translocate it towards shoots, thus rapidly reaching and subsequently maintaining a "steady state". Indeed, the data presented here indicate that the physiological adaptation to such heterogeneous Fe supply can be mediated by the integration of the complex signaling pathways elicited by both auxin and ethylene activities.

The study of the effect of hormones on plants has aroused great interest among scientists for their role in stimulating plant growth, development, and fruit-set. For this reason, they are widely used by farmers to improve both production and quality of vegetables, including tomatoes [91]. Therefore, from a practical and applicative point of view, the insights in this work could contribute to a more detailed understanding of how hormone modulation can influence the physiology of mineral nutrition in field cultivated plants, having, as a consequence, significant impacts on both the yield and the quality of agricultural products.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/7/1000/s1, Table S1: Instrumental operating conditions of ICP-MS with octopole reaction cell.

Author Contributions: Conceptualization, Y.P., T.M., S.C. (Stefano Cesco) and S.A.; Data curation, S.C. (Silvia Celletti) and Y.P.; Formal analysis, S.C. (Silvia Celletti), F.V., R.T. and M.C.F.; Funding acquisition, T.M. and S.C. (Stefano Cesco); Investigation, S.C. (Silvia Celletti), F.V., R.T. and M.C.F.; Methodology, Y.P., T.M., S.C. (Stefano Cesco) and S.A.; Writing—original draft, S.C. (Silvia Celletti) and Y.P.; Writing—review and editing, G.M.B., T.M., S.C. (Stefano Cesco) and S.A. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by grants from the Free University of Bolzano (TN2053 and TN200E).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hendriks, M.; Visser, E.J.W.; Visschers, I.G.S.; Aarts, B.H.J.; de Caluwe, H.; Smit-Tiekstra, A.E.; van der Putten, W.H.; de Kroon, H.; Mommer, L. Root responses of grassland species to spatial heterogeneity of plant-soil feedback. *Funct. Ecol.* **2015**, *29*, 177–186. [CrossRef]

2. Reis, F.V.P.; Gutiérrez-Ginés, M.J.; Smith, C.M.S.; Lehto, N.J.; Robinson, B.H. Mānuka (Leptospermum scoparium) roots forage biosolids in low fertility soil. *Environ. Exp. Bot.* **2017**, *133*, 151–158. [CrossRef]
3. Weiser, M.; Koubek, T.; Herben, T. Root Foraging Performance and Life-History Traits. *Front. Plant Sci.* **2016**, 7. [CrossRef] [PubMed]
4. Cain, M.L.; Subler, S.; Evans, J.P.; Fortin, M.J. Sampling spatial and temporal variation in soil nitrogen availability. *Oecologia* **1999**, *118*, 397–404. [CrossRef]
5. Farley, R.A.; Fitter, A.H. The responses of seven co-occurring woodland herbaceous perennials to localized nutrient-rich patches. *J. Ecol.* **1999**, *87*, 849–859. [CrossRef]
6. Hodge, A. Plastic plants and patchy soils. *J. Exp. Bot.* **2006**, *57*, 401–411. [CrossRef]
7. Gross, K.L.; Peters, A.; Pregitzer, K.S. Fine root growth and demographic responses to nutrient patches in four old-field plant species. *Oecologia* **1993**, *95*, 61–64. [CrossRef]
8. Wijesinghe, D.K.; John, E.A.; Beurskens, S. Root System Size and Precision in Nutrient Foraging: Responses to Spatial Pattern of Nutrient Supply in Six Herbaceous Species. *J. Ecol.* **2001**, *89*, 972–983. [CrossRef]
9. York, L.M.; Carminati, A.; Mooney, S.J.; Ritz, K.; Bennett, M.J. The holistic rhizosphere: Integrating zones, processes, and semantics in the soil influenced by roots. *J. Exp. Bot.* **2016**, *67*, 3629–3643. [CrossRef]
10. Gersani, M.; Sachs, T. Development correlations between roots in heterogeneous environments. *Plant Cell Environ.* **1992**, *15*, 463–469. [CrossRef]
11. Drew, M.C. Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytol.* **1975**, *75*, 479–490. [CrossRef]
12. Cahill, J.F.; McNickle, G.G. The Behavioral Ecology of Nutrient Foraging by Plants. *Annu. Rev. Ecol. Evol. Syst.* **2011**, *42*, 289–311. [CrossRef]
13. Fageria, N.; Stone, L. Physical, chemical, and biological changes in the rhizosphere and nutrient availability. *J. Plant Nutr.* **2006**, *29*, 1327–1356. [CrossRef]
14. Delgado, A.; Gómez, J.A. The Soil. Physical, Chemical and Biological Properties. In *Principles of Agronomy for Sustainable Agriculture*; Springer International Publishing AG: Cham, Switzerland, 2016; pp. 15–26.
15. Marschner, P. *Mineral Nutrition of Higher Plants*, 3rd ed.; Academic Press: London, UK, 2012.
16. Römheld, V.; Marschner, H. Evidence for a Specific Uptake System for Iron Phytosiderophores in Roots of Grasses. *Plant Physiol.* **1986**, *80*, 175–180. [CrossRef]
17. Rout, G.R.; Sahoo, S. Role of iron in plant growth and metabolism. *Rev. Agric. Sci.* **2015**, *3*, 1–24. [CrossRef]
18. Imsande, J. Iron, sulfur, and chlorophyll deficiencies: A need for an integrative approach in plant physiology. *Physiol. Plant.* **1998**, *103*, 139–144. [CrossRef]
19. Kobayashi, T.; Nishizawa, N.K. Iron Uptake, Translocation, and Regulation in Higher Plants. *Annu. Rev. Plant Biol.* **2012**, *63*, 131–152. [CrossRef]
20. Connorton, J.M.; Balk, J.; Rodríguez-Celma, J. Iron homeostasis in plants-a brief overview. *Metallomics* **2017**, *9*, 813–823. [CrossRef]
21. Bindraban, P.S.; Dimkpa, C.; Nagarajan, L.; Roy, A.; Rabbinge, R. Revisiting fertilisers and fertilisation strategies for improved nutrient uptake by plants. *Biol. Fertil. Soils* **2015**, *51*, 897–911. [CrossRef]
22. Romheld, V.; Marschner, H. Iron deficiency stress induced morphological and physiological changes in root tips of sunflower. *Physiol. Plant.* **1981**, *53*, 354–360. [CrossRef]
23. Eide, D.; Broderius, M.; Fett, J.; Guerinot, M.L. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5624–5628. [CrossRef] [PubMed]
24. Vert, G.; Grotz, N.; Dédaldéchamp, F.; Gaymard, F.; Guerinot, M.L.; Briat, J.F.; Curie, C. IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell* **2002**, *14*, 1223–1233. [CrossRef] [PubMed]
25. Colangelo, E.P.; Guerinot, M. Lou The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell* **2004**, *16*, 3400–3412. [CrossRef] [PubMed]
26. Brumbarova, T.; Bauer, P. Iron-mediated control of the basic helix-loop-helix protein FER, a regulator of iron uptake in tomato. *Plant Physiol.* **2005**, *137*, 1018–1026. [CrossRef] [PubMed]
27. Jin, C.-W.; Chen, W.-W.; Meng, Z.-B.; Zheng, S.-J. Iron Deficiency-induced Increase of Root Branching Contributes to the Enhanced Root Ferric Chelate Reductase Activity. *J. Integr. Plant Biol.* **2008**, *50*, 1557–1562. [CrossRef] [PubMed]
28. Santi, S.; Schmidt, W. Laser microdissection-assisted analysis of the functional fate of iron deficiency-induced root hairs in cucumber. *J. Exp. Bot.* **2008**, *59*, 697–704. [CrossRef] [PubMed]

29. Lucena, C.; Romera, F.J.; García, M.J.; Alcántara, E.; Pérez-Vicente, R. Ethylene participates in the regulation of Fe deficiency responses in strategy I plants and in rice. *Front. Plant Sci.* **2015**, *6*, 1056. [CrossRef]
30. Romera, F.J.; Lucena, C.; Alcàntara, E. Plant Hormones Influencing Iron Uptake in Plants. In *Iron Nutrition in Plants and Rhizospheric Microorganisms*; Springer: Dordrecht, The Netherlands, 2006; pp. 251–278.
31. Hindt, M.N.; Guerinot, M. Lou Getting a sense for signals: Regulation of the plant iron deficiency response. *Biochim. Biophys. Acta Mol. Cell Res.* **2012**, *1823*, 1521–1530. [CrossRef]
32. Romera, F.J.; García, M.J.; Alcántara, E.; Pérez-Vicente, R. Latest findings about the interplay of auxin, ethylene and nitric oxide in the regulation of Fe deficiency responses by strategy I plants. *Plant Signal. Behav.* **2011**, *6*. [CrossRef]
33. Romera, F.J.; Lucena, C.; García, M.J.; Alcántara, E.; Pérez-Vicente, R. The role of ethylene and other signals in the regulation of fe deficiency responses by Dicot plants. In *Stress Signaling in Plants: Genomics and Proteomics Perspective*; Springer International Publishing: Cham, Switzerland, 2017; Volume 2, pp. 277–300. ISBN 978-3-319-42183-4. [CrossRef]
34. Li, W.; Lan, P. The understanding of the plant iron deficiency responses in strategy I plants and the role of ethylene in this process by omic approaches. *Front. Plant Sci.* **2017**, *8*, 40. [CrossRef]
35. Bacaicoa, E.; Mora, V.; Zamarreño, Á.M.; Fuentes, M.; Casanova, E.; García-Mina, J.M. Auxin: A major player in the shoot-to-root regulation of root Fe-stress physiological responses to Fe deficiency in cucumber plants. *Plant Physiol. Biochem.* **2011**, *49*, 545–556. [CrossRef] [PubMed]
36. Muday, G.K.; Rahman, A.; Binder, B.M. Auxin and ethylene: Collaborators or competitors? *Trends Plant Sci.* **2012**, *17*, 181–195. [CrossRef] [PubMed]
37. Grusak, M.A.; Pezeshgi, S. Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the dgl mutant of pea. *Plant Physiol.* **1996**, *110*, 329–334. [CrossRef] [PubMed]
38. Vert, G.A.; Briat, J.F.; Curie, C. Dual regulation of the Arabidopsis high-affinity root iron uptake system by local and long-distance signals. *Plant Physiol.* **2003**, *132*, 796–804. [CrossRef] [PubMed]
39. Enomoto, Y.; Goto, F. Long-distance signaling of iron deficiency in plants. *Plant Signal. Behav.* **2008**, *3*, 396–397. [CrossRef] [PubMed]
40. Walker, E.L.; Connolly, E.L. Time to pump iron: Iron-deficiency-signaling mechanisms of higher plants. *Curr. Opin. Plant Biol.* **2008**, *11*, 530–535. [CrossRef]
41. Tivendale, N.D.; Ross, J.J.; Cohen, J.D. The shifting paradigms of auxin biosynthesis. *Trends Plant Sci.* **2014**, *19*, 44–51. [CrossRef] [PubMed]
42. Ljung, K.; Hull, A.K.; Celenza, J.; Yamada, M.; Estelle, M.; Normanly, J.; Sandberg, G. Sites and regulation of auxin biosynthesis in arabidopsis roots. *Plant Cell* **2005**, *17*, 1090–1104. [CrossRef] [PubMed]
43. Zhao, Y. Auxin Biosynthesis. *Arabidopsis Book* **2014**, *12*, e0173. [CrossRef]
44. Vanneste, S.; Friml, J. Auxin: A Trigger for Change in Plant Development. *Cell* **2009**, *136*, 1005–1016. [CrossRef]
45. Spalding, E.P. Diverting the downhill flow of auxin to steer growth during tropisms. *Am. J. Bot.* **2013**, *100*, 203–214. [CrossRef] [PubMed]
46. Kazan, K. Auxin and the integration of environmental signals into plant root development. *Ann. Bot.* **2013**, *112*, 1655–1665. [CrossRef] [PubMed]
47. Bienfait, H.F. Regulated redox processes at the plasmalemma of plant root cells and their function in iron uptake. *J. Bioenerg. Biomembr.* **1985**, *17*, 73–83. [CrossRef] [PubMed]
48. Yang, S.F.; Hoffman, N.E. Ethylene Biosynthesis and its Regulation in Higher Plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155–189. [CrossRef]
49. Kende, H. Ethylene Biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1993**, *44*, 283–307. [CrossRef]
50. Romera, F.J.; Alcántara, E. Iron-deficiency stress responses in cucumber (*Cucumis sativus* L) roots: A possible role for ethylene? *Plant Physiol.* **1994**, *105*, 1133–1138. [CrossRef] [PubMed]
51. Romera, F.J.; Alcantara, E.; De La Guardia, M.D. Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by Strategy I plants. *Ann. Bot.* **1999**, *83*, 51–55. [CrossRef]
52. Schmidt, W.; Tittel, J.; Schikora, A. Role of hormones in the induction of iron deficiency responses in Arabidopsis roots. *Plant Physiol.* **2000**, *122*, 1109–1118. [CrossRef]
53. Schmidt, W.; Schikora, A. Different pathways are involved in phosphate and iron stress-induced alterations of root epidermal cell development. *Plant Physiol.* **2001**, *125*, 2078–2084. [CrossRef]

54. Wu, T.; Zhang, H.-T.; Wang, Y.; Jia, W.-S.; Xu, X.-F.; Zhang, X.-Z.; Han, Z.H. Induction of root Fe(lll) reductase activity and proton extrusion by iron deficiency is mediated by auxin-based systemic signalling in Malus xiaojinensis. *J. Exp. Bot.* **2012**, *63*, 859–870. [CrossRef]
55. Maas, F.M.; van de Wetering, D.A.M.; van Beusichem, M.L.; Bienfait, H.F. Characterization of Phloem Iron and Its Possible Role in the Regulation of Fe-Efficiency Reactions. *Plant Physiol.* **1988**, *87*, 167–171. [CrossRef] [PubMed]
56. Lucena, C.; Waters, B.M.; Romera, F.J.; García, M.J.; Morales, M.; Alcántara, E.; Pérez-Vicente, R. Ethylene could influence ferric reductase, iron transporter, and H +-ATPase gene expression by affecting FER (or FER-like) gene activity. *J. Exp. Bot.* **2006**, *57*, 4145–4154. [CrossRef] [PubMed]
57. Schmidt, W.; Michalke, W.; Schikora, A. Proton pumping by tomato roots. Effect of Fe deficiency and hormones on the activity and distribution of plasma membrane H+-ATPase in rhizodermal cells. *Plant Cell Environ.* **2003**, *26*, 361–370. [CrossRef]
58. De Nisi, P.; Vigani, G.; Dell'Orto, M.; Zocchi, G. Application of the split root technique to study iron uptake in cucumber plants. *Plant Physiol. Biochem.* **2012**, *57*, 168–174. [CrossRef] [PubMed]
59. Schmidt, W.; Boomgaarden, B.; Ahrens, V. Reduction of root iron in Plantago lanceolata during recovery from Fe deficiency. *Physiol. Plant.* **1996**, *98*, 587–593. [CrossRef]
60. Pii, Y.; Borruso, L.; Brusetti, L.; Crecchio, C.; Cesco, S.; Mimmo, T. The interaction between iron nutrition, plant species and soil type shapes the rhizosphere microbiome. *Plant Physiol. Biochem.* **2016**, *99*, 39–48. [CrossRef]
61. Zhang, F.S.; Römheld, V.; Marschner, H. Role of the root apoplasm for iron acquisition by wheat plants. *Plant Physiol.* **1991**, *97*, 1302–1305. [CrossRef]
62. Coppa, E.; Celletti, S.; Pii, Y.; Mimmo, T.; Cesco, S.; Astolfi, S. Revisiting Fe/S interplay in tomato: A split-root approach to study the systemic and local responses. *Plant Sci.* **2018**, *276*, 134–142. [CrossRef]
63. Chen, W.W.; Yang, J.L.; Qin, C.; Jin, C.W.; Mo, J.H.; Ye, T.; Zheng, S.J. Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in arabidopsis. *Plant Physiol.* **2010**, *154*, 810–819. [CrossRef]
64. Pii, Y.; Astegno, A.; Peroni, E.; Zaccardelli, M.; Pandolfini, T.; Crimi, M. The Medicago truncatula N5 gene encoding a root-specific lipid transfer protein is required for the symbiotic interaction with Sinorhizobium meliloti. *Mol. Plant-Microbe Interact.* **2009**, *22*, 1577–1587. [CrossRef]
65. Martínez-Cuenca, M.R.; Iglesias, D.J.; Forner-Giner, M.A.; Primo-Millo, E.; Legaz, F. The effect of sodium bicarbonate on plant performance and iron acquisition system of FA-5 (Forner-Alcaide 5) citrus seedlings. *Acta Physiol. Plant.* **2013**, *35*, 2833–2845. [CrossRef]
66. Park, C.J.; Suh, J.K.; Lee, R.W.; Lee, S.H. Determination of Cd, Cu, Zn and Pb in rice flour reference materials by isotope dilution inductively coupled plasma mass spectrometry. *Anal. Sci.* **1997**, *13*, 429–432. [CrossRef]
67. Wu, J.; Boyle, E.A. Determination of iron in seawater by high-resolution isotope dilution inductively coupled plasma mass spectrometry after Mg(OH)2 coprecipitation. *Anal. Chim. Acta* **1998**, *367*, 183–191. [CrossRef]
68. Sariego Muñiz, C.; Marchante-Gayón, J.M.; García Alonso, J.I.; Sanz-Medel, A. Multi-elemental trace analysis of human serum by double-focusing ICP-MS. *J. Anal. At. Spectrom.* **1999**, *14*, 193–198. [CrossRef]
69. Meija, J.; Caruso, J.A. Deconvolution of isobaric interferences in mass spectra. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 654–658. [CrossRef] [PubMed]
70. Rodríguez-González, P.; Marchante-Gayón, J.M.; García Alonso, J.I.; Sanz-Medel, A. Isotope dilution analysis for elemental speciation: A tutorial review. *Spectrochim. Acta Part B At. Spectrosc. Spectrochim. Acta Part B At. Spectrosc.* **2005**, *60*, 151–207. [CrossRef]
71. Giner Martínez-Sierra, J.; Moreno Sanz, F.; Herrero Espílez, P.; Marchante Gayón, J.M.; García Alonso, J.I. Biosynthesis of sulfur-34 labelled yeast and its characterisation by multicollector-ICP-MS. *J. Anal. At. Spectrom.* **2007**, *22*, 1105–1112. [CrossRef]
72. Dell'Orto, M.; Santi, S.; De Nisi, P.; Cesco, S.; Varanini, Z.; Zocchi, G.; Pinton, R. Development of Fe-deficiency responses in cucumber (*Cucumis sativus* L.) roots: Involvement of plasma membrane H+-ATPase activity. *J. Exp. Bot.* **2000**, *51*, 695–701. [CrossRef]
73. Vizzotto, G.; Pinton, R.; Bomben, C.; Cesco, S.; Varanini, Z.; Costa, G. Iron reduction in iron-stressed plants of Actinidia deliciosa genotypes: Involvement of PM Fe(III)-chelate reductase and H+-ATPase activity. *J. Plant Nutr.* **1999**, *22*, 479–488. [CrossRef]

74. Zuchi, S.; Watanabe, M.; Hubberten, H.M.; Bromke, M.; Osorio, S.; Fernie, A.R.; Celletti, S.; Paolacci, A.R.; Catarcione, G.; Ciaffi, M.; et al. The interplay between sulfur and iron nutrition in tomato. *Plant Physiol.* **2015**, *169*, 2624–2639. [CrossRef] [PubMed]
75. Valentinuzzi, F.; Pii, Y.; Vigani, G.; Lehmann, M.; Cesco, S.; Mimmo, T. Phosphorus and iron deficiencies induce a metabolic reprogramming and affect the exudation traits of the woody plant *Fragaria* × *ananassa*. *J. Exp. Bot.* **2015**, *66*, 6483–6495. [CrossRef] [PubMed]
76. Ramakers, C.; Ruijter, J.M.; Lekanne Deprez, R.H.; Moorman, A.F.M. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **2003**, *339*, 62–66. [CrossRef]
77. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]
78. Pfaffl, M.W. Relative expression software tool (REST(C)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **2002**, *30*, e36. [CrossRef] [PubMed]
79. Colombo, C.; Palumbo, G.; He, J.Z.; Pinton, R.; Cesco, S. Review on iron availability in soil: Interaction of Fe minerals, plants, and microbes. *J. Soils Sediments* **2014**, *14*, 538–548. [CrossRef]
80. Mimmo, T.; Del Buono, D.; Terzano, R.; Tomasi, N.; Vigani, G.; Crecchio, C.; Pinton, R.; Zocchi, G.; Cesco, S. Rhizospheric organic compounds in the soil-microorganism-plant system: Their role in iron availability. *Eur. J. Soil Sci.* **2014**, *65*, 629–642. [CrossRef]
81. Valentinuzzi, F.; Pii, Y.; Carlo, P.; Roberto, T.; Fontanella, M.C.; Beone, G.M.; Astolfi, S.; Mimmo, T.; Cesco, S. Root-shoot-root Fe translocation in cucumber plants grown in a heterogeneous Fe provision. *Plant Sci.* **2020**, *293*, 110431. [CrossRef]
82. Gayomba, S.R.; Zhai, Z.; Jung, H., II; Vatamaniuk, O.K. Local and systemic signaling of iron status and its interactions with homeostasis of other essential elements. *Front. Plant Sci.* **2015**, *6*, 716. [CrossRef]
83. Liu, J.; Moore, S.; Chen, C.; Lindsey, K. Crosstalk Complexities between Auxin, Cytokinin, and Ethylene in Arabidopsis Root Development: From Experiments to Systems Modeling, and Back Again. *Mol. Plant* **2017**, *10*, 1480–1496. [CrossRef]
84. Li, S.-B.; Xie, Z.-Z.; Hu, C.-G.; Zhang, J.-Z. A Review of Auxin Response Factors (ARFs) in Plants. *Front. Plant Sci.* **2016**, 7. [CrossRef]
85. Molesini, B.; Pandolfini, T.; Pii, Y.; Korte, A.; Spena, A. Arabidopsis thaliana AUCSIA-1 Regulates Auxin Biology and Physically Interacts with a Kinesin-Related Protein. *PLoS ONE* **2012**, *7*, e41327. [CrossRef] [PubMed]
86. Connolly, E.L.; Campbell, N.H.; Grotz, N.; Prichard, C.L.; Guerinot, M. Lou Overexpression of the FRO2 Ferric Chelate Reductase Confers Tolerance to Growth on Low Iron and Uncovers Posttranscriptional Control. *Plant Physiol.* **2003**, *133*, 1102–1110. [CrossRef] [PubMed]
87. Romera, F.J.; Alcántara, E.; Agronomía, D. Ethylene involvement in the regulation of Fe-deficiency stress responses by Strategy I plants. *Funct. Plant Biol.* **2004**, *31*, 315–328. [CrossRef]
88. Molassiotis, A.; Therios, I.; Dimassi, K.; Diamantidis, G.; Chatzissavvidis, C. Induction of Fe(III)-chelate reductase activity by ethylene and salicylic acid in iron-deficient peach rootstock explants. *J. Plant Nutr.* **2005**, *28*, 669–682. [CrossRef]
89. Lingam, S.; Mohrbacher, J.; Brumbarova, T.; Potuschak, T.; Fink-Straube, C.; Blondet, E.; Genschik, P.; Bauer, P. Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in Arabidopsis. *Plant Cell* **2011**, *23*, 1815–1829. [CrossRef] [PubMed]
90. Waters, B.M.; Lucena, C.; Romera, F.J.; Jester, G.G.; Wynn, A.N.; Rojas, C.L.; Alcántara, E.; Pérez-Vicente, R. Ethylene involvement in the regulation of the H+-ATPase CsHA1 gene and of the new isolated ferric reductase CsFRO1 and iron transporter CsIRT1 genes in cucumber plants. *Plant Physiol. Biochem.* **2007**, *45*, 293–301. [CrossRef] [PubMed]
91. Pramanik, K.; Mohapatra, P.P. Role of Auxin on Growth, Yield and Quality of Tomato—A Review. *Int. J. Curr. Microbiol. Appl. Sci.* **2017**, *6*, 1624–1636. [CrossRef]