Plant Physiology and Biochemistry 136 (2019) 118–126

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Role of *Azospirillum brasilense* in triggering different Fe chelate reductase enzymes in cucumber plants subjected to both nutrient deficiency and toxicity

Laura Marastoni, Youry Pii*, Mauro Maver, Fabio Valentinuzzi, Stefano Cesco, Tanja Mimmo

Faculty of Science and Technology, Free University of Bozen-Bolzano, I-39100, Bolzano, Italy

ARTICLE INFO

Keywords:
Iron chelate reductase oxidase
FRO gene family
Azospirillum brasilense
Cu toxicity
Cu and Fe deficiency
Cucumis sativus L

ABSTRACT

Azospirillum brasilense was reported to up-regulate iron (Fe) uptake mechanisms, such as Fe reduction and rhizosphere acidification, in both Fe sufficient and deficient cucumber plants (*Cucumis sativus* L.). Strategy I plants take up both Fe and copper (Cu) after their reduction mediated by the ferric-chelate reductase oxidase (FRO) enzyme. Interestingly, in cucumber genome only one *FRO* gene is reported. Thus, in the present study we applied a bioinformatics approach to identify the member of cucumber *FRO* gene family and allowed the identification of at least three *CsFRO* genes, one of which was the already identified, *i.e. CsFRO1*. The expression patterns of the newly identified transcripts were investigated in hydroponically grown cucumber plants treated with different Fe and Cu nutritional regimes. Gene expression was then correlated with morphological (*i.e.* root architecture) and physiological (Fe(III) reducing activity) parameters to shed light on: i) the *CsFRO* homologue responsible of the increased reduction activity in Fe-sufficient plants inoculated with *A. brasilense* cucumber plants, and ii) the possible effect of *A. brasilense* in ameliorating the symptoms of Cu toxicity in cucumber plants.

The data obtained showed that all the *CsFRO* genes were expressed in the root tissues of cucumber plants and responded to Cu starvation, combined Cu/Fe deficiency and Cu toxicity. Only *CsFRO3* was modulated by the *A. brasilense* in Fe-sufficient plants suggesting for the first time a different specificity of action of the three isoenzymes depending not only on the nutritional regime (either deficiency or toxicity) but also on the presence of the PGPR. Furthermore, results suggest that the PGPR could even ameliorate the stress symptoms caused by both the double (*i.e.* Cu and Fe) and Cu deficiency as well as Cu toxicity modulating, on one hand, the growth of the root system and, on the other hand, the root nutrient uptake.

1. Introduction

The increasing World population requires an increase in agricultural productivity, as well as, an enhancement in the food quality (Cassman, 1999; Matson et al., 1997; Tilman et al., 2002). So far, these challenges have been met by increasing the application of chemical fertilizers (Zhang et al., 2010). However, in the last decades, the agricultural productivity has reached a plateau irrespective from the external inputs, suggesting that the limiting factor might be represented by the nutrient use efficiency of crop plants (Taiz, 2013). In addition, metal toxicity is also becoming a huge agronomic problem, mainly due to the intense anthropogenic activities (Wuana and Okieimen, 2011). Interestingly, in the last years several studies have shown that the use of plant growth-promoting rhizobacteria (PGPR) might represent a useful tool to reduce the effects of heavy metal toxicity in plants (Tak et al., 2013), yet also improving the mineral nutrition of plants (Pii et al., 2015b).

Indeed, PGPR are soil bacteria that efficiently colonize the rhizosphere, *i.e.* the thin layer of soil in close contact with roots and influenced by their biological activities (Hinsinger et al., 2005), and can establish profitable relationships with plants (Alegria Terrazas et al., 2016; Crecchio et al., 2018; Glick, 2012; Lugtenberg and Kamilova, 2009; Parray et al., 2016; Pii et al., 2015b). Emerging evidence shows that PGPR can also interfere with the biochemical and/or molecular mechanisms devoted to the uptake of mineral nutrients in plants (Bashan, 1990; Bashan et al., 1989; Bertrand et al., 2000; Canellas et al., 2013, 2002; de Santiago et al., 2009; Pii et al., 2016, 2015b; Zhang et al., 2009; Zhao et al., 2014). In particular, recent works highlighted that the PGPR *Azospirillum brasilense* can restore iron (Fe)-deficiency symptoms of cucumber (*Cucumis sativus* L.) plants grown on

* Corresponding author.
E-mail address: youry.pii@unibz.it (Y. Pii).

https://doi.org/10.1016/j.plaphy.2019.01.013
Received 12 October 2018; Received in revised form 10 January 2019; Accepted 10 January 2019
Available online 11 January 2019

	Cu (μM)	0	0	0.2	25	50
	Fe (μM)	0	80	80	80	80
- *A. brasilense*	WinRhizo Scan					
	Weight	1.76 ± 0.15^{a,*}	4.66 ± 0.43^{b,NS}	2.12 ± 0.32^{a,NS}	4.86 ± 0.28^{b,NS}	1.88 ± 0.39^{a,**}
	Length	131.95 ± 13.09^{d,NS}	91.26 ± 12.11^{bc,*}	110.13 ± 11.00^{c,**}	81.76 ± 8.27^{b,***}	39.17 ± 12.53^{a,**}
	Tips	128.20 ± 14.51^{ns,*}	54.60 ± 7.28^{ns,NS}	116.00 ± 14.56^{ns,**}	69.60 ± 5.63^{ns,NS}	103.20 ± 42.82^{ns,NS}
+ *A. brasilense*	WinRhizo Scan					
	Weight	3.00 ± 0.50^{a}	5.26 ± 0.55^{b}	2.60 ± 0.48^{a}	4.48 ± 0.80^{b}	2.92 ± 0.39^{a}
	Length	141.13 ± 27.96^{b}	72.54 ± 7.40^{a}	140.00 ± 15.20^{b}	47.95 ± 7.06^{a}	68.02 ± 5.42^{a}
	Tips	189.40 ± 40.47^{b}	66.60 ± 9.69^{a}	168.20 ±24.25^{b}	62.60 ± 5.63^{a}	140.20 ± 17.40^{ab}

Fig. 1. Cucumber root biomass and morphology of plants grown for 4 days with or without inoculation. The data reported are referred to root dry weight (Weight, mg), total root length (Length, cm) and number of tips (Tips, n). The different Cu and Fe concentration are referred to the different nutritional regimes: combined deficiency (0 μM Cu, 0 μM Fe) - Cu deficiency (0 μM Cu, 80 μM Fe) – control (0.2 μM Cu, 80 μM Fe) – moderate Cu supply (25 μM Cu, 80 μM Fe) – high Cu supply (50 μM Cu, 80 μM Fe). Small letters indicate the ANOVA significance between the different Cu and Fe concentrations, whereas the * and capital letters report the *t*-test significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the *A. brasilense* treatments. Data are expressed as mean ± SE, n = 5.

calcareous soils (Pii et al., 2015c) by enhancing the response to the nutrient shortage at molecular level (Pii et al., 2016). Cucumber plants take up Fe from the growth media *via* an acidification/reduction mechanism (Marschner and Römheld, 1994); the inoculation with *A. brasilense* up-regulates both these activities, independently from the Fe nutritional status of plants (Pii et al., 2016). Whilst in Fe deficient cucumber plants treated with *A. brasilense* the enhanced acidification and reduction activities were due to an over-expression of *CsFRO1* and *CsHA1* (the plasma membrane H^+-ATPase), this phenomenon was not observed in Fe sufficient plants (Pii et al., 2016). In fact, in this latter condition, the enhanced reduction activity was not sustained by an increased expression of *CsFRO1*, suggesting that the reduction of Fe^{3+} might be carried out by other molecular entities (Pii et al., 2016). Consistently, the genome of *Arabidopsis thaliana* was shown to encode for an Fe reductase gene family composed of eight members, several of which are expressed in the root tissue and are responsive to Fe deficiency (Mukherjee et al., 2006). However, so far, information concerning *FRO* isoforms in *C. sativus* is not available.

The enhancement of the abovementioned root activities induced by *A. brasilense* inoculation (Pii et al., 2016) might also increase the bioavailable fraction of other mineral cations, as for instance copper (Cu) (Brunetto et al., 2016), which is an essential micronutrient for regular plant growth and development (Broadley et al., 2011). Copper is taken up by Cu Transport Protein 1 (COPT1) (Yuan et al., 2011), albeit other transporters (e.g. Zn/Fe Permeases - ZIPs, Natural Resistance Associated Macrophage Proteins - NRAMP and Iron Related Transporters - IRT) seem to be involved in this process as well (Tsai and Schmidt, 2017; Wintz et al., 2003; Yruela, 2005). In fact, it is well established that the main Fe transporter (IRT1) can also mediate the uptake of Cu, as well as of cadmium, cobalt and zinc (Korshunova et al., 1999). In addition, it has been shown that Cu can be taken up both as Cu^{2+} and Cu^{+}, suggesting that Plasma Membrane-bound Reductase might have a relevant role in these processes (Brunetto et al., 2016); consistently, the Fe reductase genes *FRO4* and *FRO5* was shown to be induced by Cu deficiency in *A. thaliana* plants (Bernal et al., 2012).

As mentioned earlier, the intense anthropogenic activities, such as the application of Cu-based fungicides in agricultural soils, is causing an increase in Cu concentration in the soils (Komárek et al., 2010) that might lead to toxicity phenomena in plants (Brunetto et al., 2016; Feigl et al., 2013). The use of PGPR has been proposed as a possible tool to ameliorate heavy metals toxicity stress (Kumar and Verma, 2018; Tirry et al., 2018). Indeed, the consociation between plants and soil microbiota, both arbuscular mycorrhizal fungi (AMF) and bacteria, might result in an alleviation of Cu toxicity symptoms in the host plants (Brunetto et al., 2016). Bacteria have been shown to reduce plants metal uptake (Vivas et al., 2006), to change Cu speciation in less toxic forms (Carlot et al., 2002) and to decrease the ethylene-mediated stress induction (Rajkumar and Freitas, 2008). For instance, *Pseudomonas putida* CZ1, a free-living PGPR, was reported to increase the fitness of *Elsholtia splendents* plants in Cu polluted soils, most likely because the microorganism was able to release chelating agents of Cu and to produce indole-3-acetic acid (Xu et al., 2015). In addition, at high Cu concentration this microbial strain can aggregate and adhere onto the root surface, facilitating the flux of Cu into the plant xylem and thus enabling the accumulation of Cu in plant shoot (Xu et al., 2015).

However, the activities of microorganisms in the rhizosphere might also produce an increase of the bioavailable fraction of different cations (e.g. Fe, Cu) (Alegria Terrazas et al., 2016; Pii et al., 2016), thus

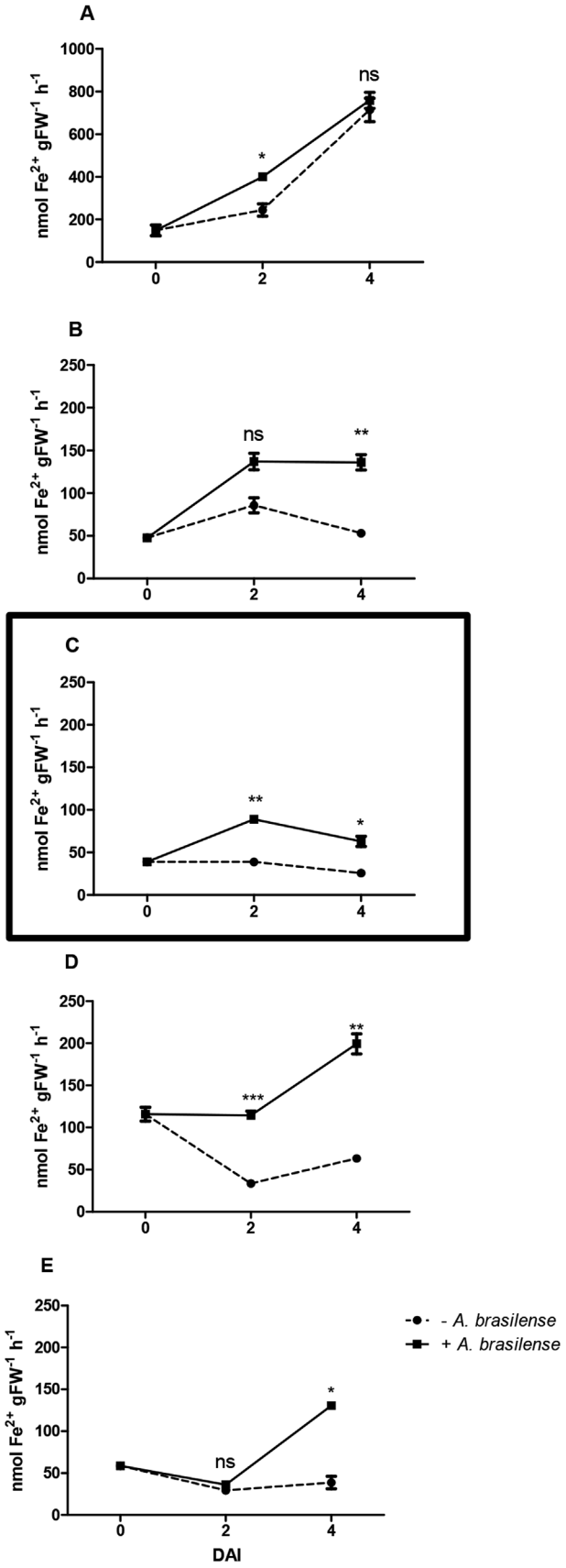

Fig. 2. Iron reductase activity at 0, 2 and 4 DAI. The capital letters are referred to the nutritional regimes: A. Combined deficiency (0 μM Cu, 0 μM Fe), B. Cu deficiency (0 μM Cu, 80 μM Fe), C. Control (0.2 μM Cu, 80 μM Fe), D. Moderate Cu supply (25 μM Cu, 80 μM Fe), E. High Cu supply (50 μM Cu, 80 μM Fe). The statistical significance is referred to a Student *t*-test between non-inoculated and inoculated plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Data are expressed as mean ± SE, n = 5.

possibly exacerbating toxicity phenomena in plants.

Considering the lack of knowledge about the ferric-chelate reductase (*FRO*) gene family in *C. sativus*, the aim of the present research were to characterize the *FRO* gene family and to identify which member is modulated by *A. brasilene* in cucumber plants subjected to different nutritional regimes (Cu, Fe and combined Cu/Fe deficiency and Cu toxicity conditions). In addition, biometric and physiological parameters, as for instance root morphology in terms of root weight, length and number of root tips, together with gene expression analysis and nutrient concentrations in roots will be employed to shed light on the responses induced by *A. brasilense* in cucumber plants subjected to different Cu and Fe nutritional regimes.

2. Materials and methods

2.1. Bacterial growth and plant inoculation

Azospirillum brasilense Cd (DSM-1843) growth and inoculation was performed as described by Pii et al. (2016). Briefly, the bacteria was grown for 4 days in LB medium (10 g L^{-1} triptone, 5g L^{-1} NaCl, 10 g L^{-1} yeast extract) with a continuous shaking at 30 °C. Bacteria were then centrifuged for 15 min at 4500 × *g* and washed three times with sterile saline solution (NaCl 0.85% w/v). Subsequently, plants were inoculated to reach final bacteria concentration of 10^6 cfu mL^{-1}. Control, non-inoculated, plants were treated with NaCl at the same concentration.

2.2. Plant material and growth conditions

Cucumber (*Cucumis sativus* L. cv Chinese Long) plants were germinated on 0.5 mM $CaSO_4$ moist paper for 4 days and subsequently transferred in 1.5 L pots with an aerated 0.5 mM $CaSO_4$ solution for 1 day. Then, cucumber plants were grown in 1.5 L pots filled with an aerated solution; 5 different nutritional regimes have been used: control (0.2 μM Cu, 80 μM Fe), Cu deficiency (0 μM Cu, 80 μM Fe), moderate Cu supply (25 μM Cu, 80 μM Fe), high Cu supply (50 μM Cu, 80 μM Fe), combined deficiency (0 μM Cu, 0 μM Fe). Copper was supplemented as $CuSO_4 \cdot 5H_2O$ (Sigma-Aldrich), whereas Fe as FeNaEDTA (Sigma-Aldrich). The macro- and micronutrients composition of the nutrient solution was the following: $Ca(NO_3)_2$ 2 mM, KCl 0.1 mM, KH_2PO_4 0.1 mM, K_2SO_4 0.1 mM, $MgSO_4$ 0.5 mM, H_3BO_3 10 μM, $MnSO_4$ 0.5 μM, $CuSO_4$ 0.2 μM, $ZnSO_4$ and 0.5 μM $(NH_4)_6Mo_7O_{24}$ 0.01 μM. Plants were grown in a climatic chamber with light/dark period of 14/10 at 70% RH and 25 °C for 6 days. The nutrient solution was renewed just before the inoculation with *A. brasilense*, which was carried out after two days of growth in the nutrient solution; plants were harvested at 2 and 4 days after inoculation (DAI).

2.3. Root morphology

Total root length and number of root tips were measured using Winrhizo software (EPSON1680, WinRHIZO Pro2003b, Regent Instruments Inc., Quebec, Canada) at 0, 2 and 4 DAI.

2.4. Determination of Fe(III) reductase activity

The Fe (III) reducing activity of cucumber plants were measured

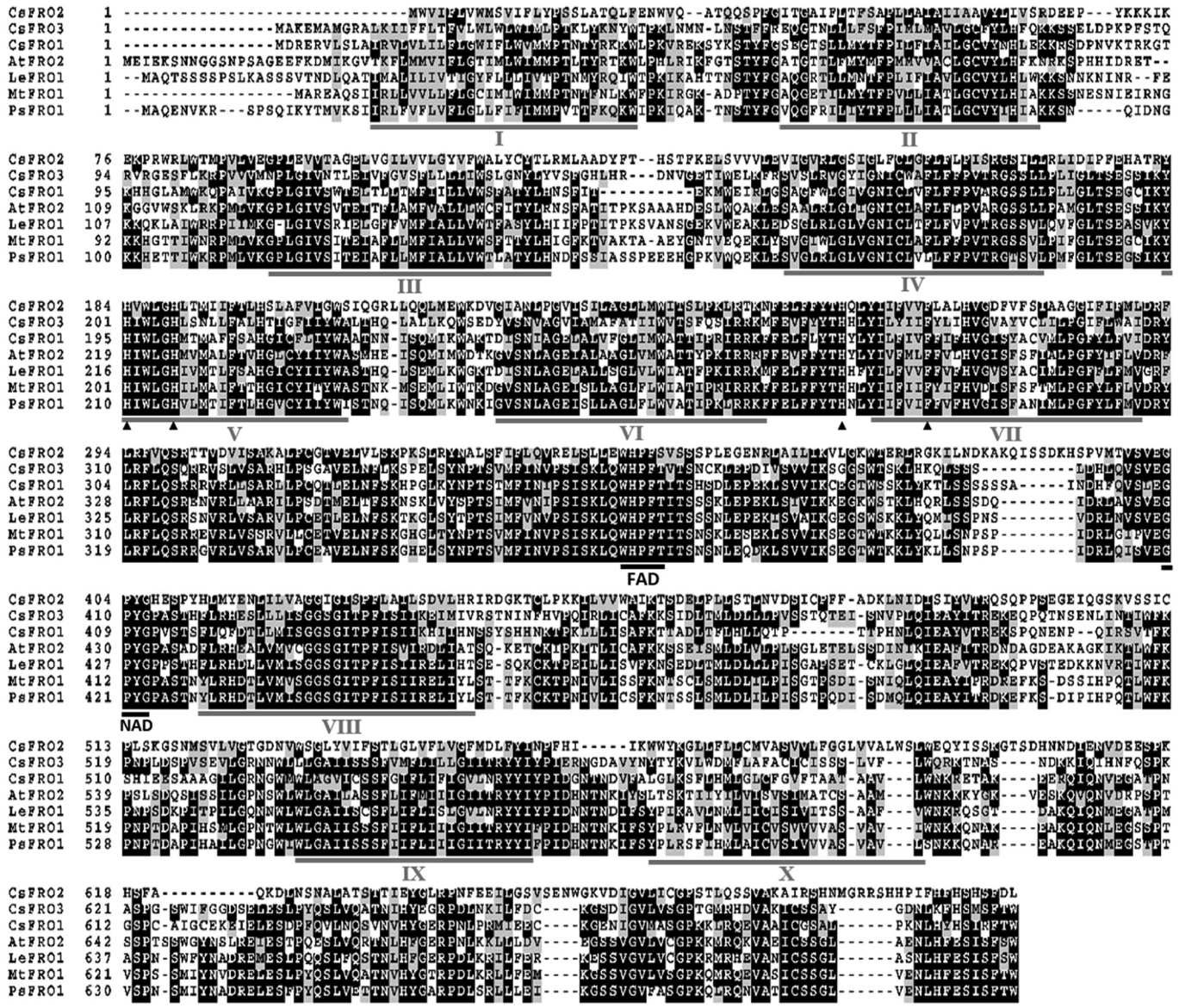

Fig. 3. Phylogenetic tree of FRO genes.

colorimetrically at 0, 2 and 4 DAI using the bathophenantroline disulfonate (BPDS) reagent (Vizzotto et al., 1999). As previously described by Pii et al. (2016), roots of intact cucumber plants were washed with $CaSO_4$ 0.5 mM and then submerged in the assay solution (10 mM MES NaOH pH 5.5, 0.25 mM Fe(III)-EDTA and 0.6 mM BPDS). The reaction was incubated for 60 min in the dark at 25 °C.

2.5. Mineral composition of plant tissues

Roots were oven dried at 65 °C for 5 days, subsequently they were digested with concentrated ultrapure HNO_3 (650 mL L^{-1}, Carlo Erba, Milano, Italy) using a single digestion chamber (SRC) microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA) and the mineral composition was obtained by Inductively-Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Arcos Ametek Spectro, Germany).

2.6. Bioinformatics analysis

The *FRO* sequences (Supplemental Table 1) of *Arabidopsis thaliana* (Wu et al., 2005), *Medicago truncatula* (Del C Orozco-Mosqueda et al., 2012), *Lycopersicum esculentum* (Li et al., 2004), *Pisum sativus* (Waters, 2002), *Cucumis sativus* (Waters et al., 2007), *Malus xiajinensis* (UNIPROT: B5KGU9), *Oryza sativa* (Ishimaru et al., 2006) and *Populus trichocarpa* (UNIPROT: B918N2) were used to obtain *CsFRO1* homologues by an amino acids sequence BLAST analysis, using the *Cucumis sativus* v 1.0 genome provided by Phytozome (http://phytozome.jgi.doe.gov/pz/portal.html). The sequences obtained were then screened for the number of transmembrane domains using BOXSHADE, the presence of the FAD (HPFT) and NAD (GPYG) binding domain as well as the four histidine residues which are characteristic of the FRO enzymes (Robinson et al., 1999; Waters, 2002). Subsequently the phylogenetic tree was fashioned using FigTree (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.7. RNA extraction and cDNA synthesis

The roots of cucumber plants for the RNA analysis were frozen in liquid nitrogen immediately after their excision. The tissues were then ground to fine powder and the RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma Aldirch). Then, 1 μg of RNA was treated with 1u of DNAse RQ1 (Promega, Madison, WI, USA). The cDNA synthesis was performed using ImProm-II Reverse Transcription System

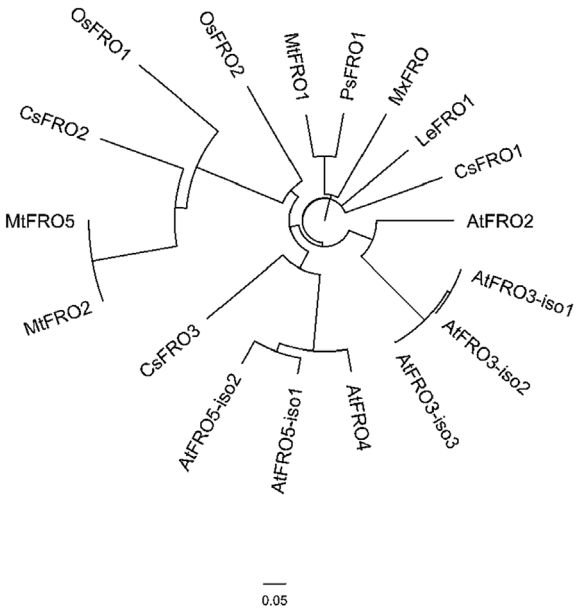

Fig. 4. Multiple sequence alignment. The conserved and similar amino acidic residues are highlighted in black and grey respectively. Triangles indicate the conserve H residues, black bars indicate the NAD and FAD binding domains and the grey bars underlined the transmembrane domains numbered with roman numbers.

(Promega, Madison, WI, USA).

2.8. Real-Time RT PCR

The Real-Time RT PCR primers were designed to be 20 bp long (Supplemental Table 2) and to amplify fragments of 100 bp (Supplemental Table 2). The specificity of each primer was tested firstly performing a BLAST directly on the cucumber genome mRNA prediction and subsequently carrying out the melting curve. The elongation factor and the ubiquitin primers were obtained by Pii et al. (2016). The Real-Time RT PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad), the Qiagen Rotor Gene Q Real-Time PCR. The amplification efficiency was obtained through LinReg PCR (Ramakers et al., 2003) and the relative expression and the standard error was calculated according to Pfaffl et al., 2002.

2.9. Statistical analysis

The data are reported as mean of at least 3 replicates ± standard error (SE) or standard deviation (SD). The statistical significance was tested by Student t-test or ANOVA using GraphPad Prism 5.00.288 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1. Physiological characterization

The biomass and the architecture of the root system were determined in cucumber seedlings at harvest. In non-inoculated samples, the root biomass was the highest in plants grown in Cu deficiency (0 μM Cu and 80 μM Fe) and in moderate Cu supply (25 μM Cu and 80 μM Fe), whilst it was not significantly altered in combined deficiency (0 μM Cu

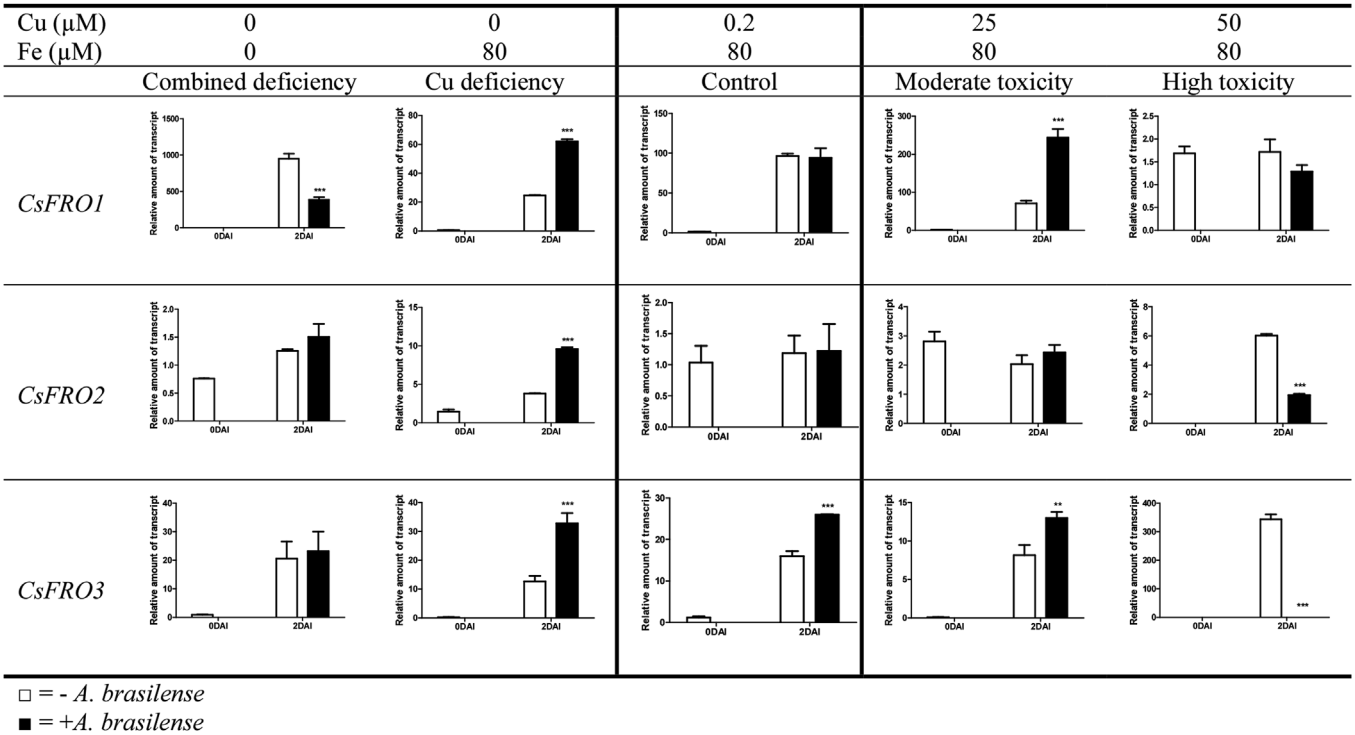

Fig. 5. *CsFRO* gene expression in root tissues of both non-inoculated (white) or inoculated (black) cucumbers at 0 and 2 DAI. The gene expression was reported as relative amount of transcript compared to the expression at 0DAI of cucumber plants grown with 0.2 and 80 μM Cu and Fe respectively. The different Cu and Fe concentration are referred to the different nutritional regimes: combined deficiency (0 μM Cu, 0 μM Fe) - Cu deficiency (0 μM Cu, 80 μM Fe) – control (0.2 μM Cu, 80 μM Fe) – moderate Cu supply (25 μM Cu, 80 μM Fe) – high Cu supply (50 μM Cu, 80 μM Fe). The statistical significance is referred to a Student *t*-test between non-inoculated and inoculated plants (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) at 2DAI. White columns are referred to non-inoculated plats while black to the inoculated cucumber. Data are expressed as mean ± SD, n = 9.

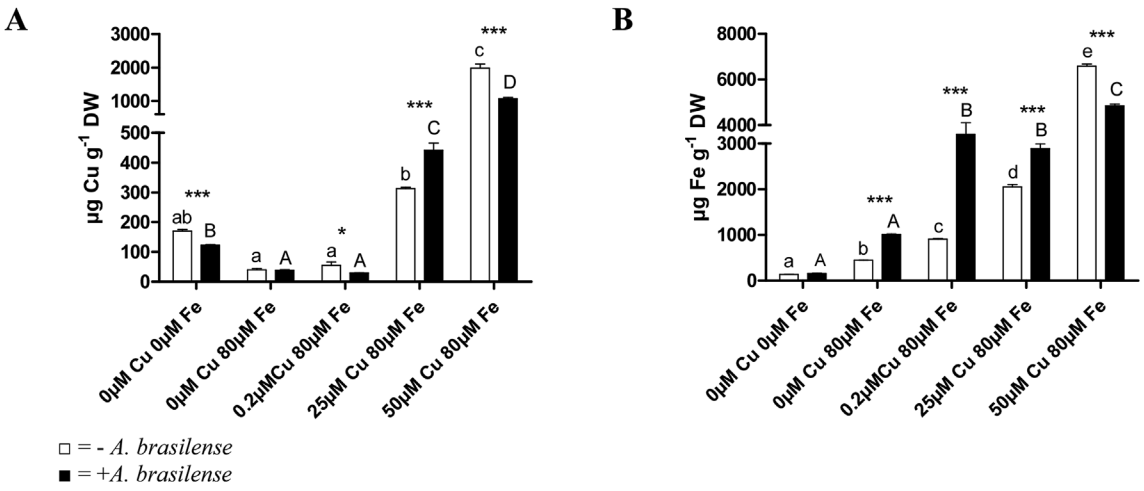

Fig. 6. Cu and Fe accumulation in cucumber root tissues at 4 DAI. Concentrations are expressed as µg per g of DW (Dry Weight). The different Cu and Fe concentration are referred to the different nutritional regimes: combined deficiency (0 µM Cu, 0 µM Fe) - Cu deficiency (0 µM Cu, 80 µM Fe) – control (0.2 µM Cu, 80 µM Fe) – moderate Cu supply (25 µM Cu, 80 µM Fe) – high Cu supply (50 µM Cu, 80 µM Fe). The statistical significance was tested with the ANOVA between the non-inoculated (small letters) and inoculated (capital letters) cucumber plants. The significance of the difference between inoculated and non-inoculated cucumber plants was calculated through a Student T-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The data are reported as mean ± SE ($n = 3$).

and 0 µM Fe) and in high Cu supply (50 µM Cu and 80 µM Fe) as compared to control plants (0.2 µM Cu and 80 µM Fe) (Fig. 1). The inoculation with *A. brasilense* increased the root biomass of cucumber plants, yet resulting significant only in plants treated with combined deficiency and high Cu supply (Fig. 1).

The total root length of non-inoculated cucumber plants increased in combined deficiency, whereas decreased in both moderate and high Cu supply as compared to the control (Fig. 1). The inoculation with *A. brasilense* modified the total root length compared to the non-inoculated cucumbers increasing it in control condition and at high Cu supply and decreasing the length in Cu deficiency and in moderate Cu supply (Fig. 1).

The number of root tips did not change among non-inoculated samples, whereas it did in *A. brasilense*-inoculated samples, particularly in control and combined deficiency nutritional regimes (Fig. 1).

3.2. Fe reductase activity

The reductase activity of cucumber plants grown in deficiency conditions, especially in the combined one, increased with the duration of treatments (Fig. 2A and B) as compared to control plants (Fig. 2C). On the contrary, in Cu toxicity the reducing activity decreased at 2 DAI and increased at 4 DAI, even though only for the inoculated plants (Fig. 2D and E).

At 2 days after treatments, the inoculation with *A. brasilense* caused a significant increase in the reductase activity of cucumber plants grown in combined deficiency (Fig. 2A), in Cu deficiency (Fig. 2B), control conditions (Fig. 2C), and in moderate Cu toxicity (Fig. 2D), as compared to non-inoculated samples. Similarly, at 4 DAI *Azospirillum* induced an up-regulation of the reductase activity of cucumber plants, except for those grown in the combined Cu and Fe deficiencies (Fig. 2).

3.3. Isolation of CsFRO gene family

Ferric Reductase Oxidases (*FRO*) are known to be a gene family in plants, characterized by a different number of members according to the plant species. For instance, *A. thaliana* and *M. truncatula* feature 8 and 6 members, respectively (Del C Orozco-Mosqueda et al., 2012; Wu et al., 2005). In cucumber plants only one gene has been characterized to date, therefore a bioinformatics approach was undertaken to possibly reveal unknown components of *FRO* gene family. Genes encoding putative FRO enzyme were identified in the genome of *C. sativus* (www.phytozome.net) on the basis of protein sequence homology by running a BLASTP algorithm (Altschul et al., 1997) with members of the *FRO* family from *A. thaliana* (Wu et al., 2005), *M. truncatula* (Del C Orozco-Mosqueda et al., 2012), *L. esculentum* (Li et al., 2004), *P. sativus* (Waters, 2002), *C. sativus* (Waters et al., 2007), *M. xiajinensis* (UNIPROT: B5KGU9), *O. sativa* (Ishimaru et al., 2006) and *P. trichocarpa* (UNIPROT: B918N2). This approach allowed the retrieval of 20 protein sequences: a phylogenetic approach allowed the separation between the members of *FRO* and the correlated *rboh* (respiratory burst oxidase homologue) genes family (Supplemental Fig. 1), which was defined as the closest family to the *FRO* gene family in *A. thaliana* (Mukherjee et al., 2006). Afterwards, the hallmarks of the FRO enzymes, *i.e.* the number of amino acid residues and of transmembrane domains, the presence of the FAD and NAD binding domains as well as the four histidine (H) residues, were used to further screen the sequences obtained (Fig. 3). This allowed the identification of three putative members of cucumber *FRO* gene family: *Cucsa.166120.1*, which showed 100% homology with the already identified *CsFRO1* (Waters et al., 2007), *Cucsa.260.380.1* and *Cucsa.108040.1*, hereafter referred to as *CsFRO2* and *CsFRO3*. The newly identified genes *CsFRO2* and *CsFRO3* encode for 701- and 703-residues long proteins; they are both characterized by 10 transmembrane domains and feature the FAD and NAD binding domains, between the 7th and 8th transmembrane domains, as well as the four H residues distributed in the 5th and 7th helixes (Waters, 2002).

By running a phylogenetic analysis together with already characterized FRO enzymes, *CsFRO2* clustered with *MtFRO5*, already known to be induced by Fe deficiency (Del C Orozco-Mosqueda et al., 2012), and *MtFRO2* (Fig. 4) whereas, *CsFRO2* grouped with *AtFRO4* and the two *AtFRO5* isoforms (Fig. 4), which are known to be expressed in the root tissue of Fe deficient *Arabidopsis* plants (Mukherjee et al., 2006).

3.4. Quantitative gene expression analysis

The inoculation with *A. brasilense* has already been shown to induce an enhancement of *FRO* gene expression in cucumber plants at 2 DAI, whilst it was strongly down-regulated in Fe sufficient plants at 4 DAI (Pii et al., 2016). Accordingly, all the members of the *CsFRO* gene family resulted expressed in the roots of cucumber plants grown in control condition at both 0 and 2 DAI. Nonetheless, among the three *FRO* genes, *CsFRO2* was the more expressed in control conditions at 0 DAI (Supplemental Fig. 2). Except for the Cu toxicity condition, *CsFRO1*

gene resulted significantly induced in cucumber roots only at 2 DAI (Fig. 5). Interestingly, the inoculation with *A. brasilense* further up-regulated the expression, albeit only in Cu deficiency and in moderate toxicity (Fig. 5). The *CsFRO2* transcript was not strongly modulated by the nutritional regimes imposed, except for Cu toxicity, whereas it resulted significantly up-regulated by the PGPR inoculation in Cu deficient plants and, on the contrary, down-regulated in Cu toxicity (Fig. 5). Differently, the relative abundance of *CsFRO3* transcript showed a time-dependent dynamic, generally increasing at 2 DAI; in addition, the inoculation induced a significant over-expression of the gene in control, in Cu deficiency and in moderate toxicity conditions (Fig. 5).

3.5. Cu and Fe accumulation in root tissues

Root Cu concentration was maintained constant in plants cultivated in combined and single Cu deficiency as well as in control conditions, whereas it increased in moderate Cu supply reaching the highest value in high Cu supply grown plants (Fig. 6A). Differently, the root Fe concentration increased with increasing Cu and Fe concentrations (Fig. 6B). The inoculation with *A. brasilense* reduced root Cu concentration in double deficiency, control condition and high Cu supply, whereas elicited Cu accumulation in cucumber roots grown in moderate Cu supply (Fig. 6A). In the case of Fe, *A. brasilense* led to an increased micronutrient concentration in the roots treated with 80 μM Fe, except for the plants grown with high Cu supply (Fig. 6B).

4. Discussion

The reduction of Fe(III) to Fe(II) as well as that of Cu(II) to Cu(I) at the root plasma membrane is crucial to allow the uptake of both these micronutrients in Strategy I plants (Kobayashi and Nishizawa, 2012; Peñarrubia et al., 2015). Yet, it has been demonstrated that the Fe chelate reductase enzyme (FRO) can indiscriminately catalyse the reduction of both cations (Welch et al., 1993). In a previous work, we have shown that in Fe deficient cucumber plants such enzymatic activities were sustained at transcriptional level by an up-regulation of *CsFRO1*, thus resulting in an increased Fe uptake from the growth medium (Pii et al., 2016). The inoculation with the PGPR *A. brasilense* has been demonstrated to induce the mechanisms belonging to the response towards Fe starvation of Strategy I plants, regardless the Fe nutritional status (Pii et al., 2016). However, in spite of the increase in the Fe reduction activity, *CsFRO1* expression was not modulated by the inoculation in Fe sufficient plants (Pii et al., 2016); in *A. thaliana* and *M. truncatula* genome, 8 and 6 *FRO* homologue genes have been characterized, respectively, and their expression was shown to be modulated depending on plant tissues and nutrient condition (Del C Orozco-Mosqueda et al., 2012; Wu et al., 2005). A bioinformatic approach led to the identification of a FRO gene family composed of three members in cucumber genome, the already known *CsFRO1* (Waters et al., 2007), and the two newly identified *CsFRO2* and *CsFRO3* (Fig. 4).

As already reported (Pii et al., 2016), Fe sufficient cucumber plants inoculated with *A. brasilense* showed an increase in the lateral root growth and an enhancement in the Fe reduction activity as compared to the non-inoculated plants (Figs. 1 and 2). The relative expression levels of both *CsFRO1* and *CsFRO2* were not affected by the inoculation in Fe sufficient plants (Fig. 5), whilst *CsFRO3* resulted up-regulated (Fig. 5). These data further confirmed that, in Fe sufficiency, the up-regulation of Fe reduction activity induced by *A. brasilense* was not ascribable to the induction of the known molecular entities belonging to the canonical response to the micronutrient starvation (*i.e.* induction of *CsFRO1*).

The *Azospirillum*-induced root activities (*i.e.* reduction and acidification) can indeed influence the rhizosphere bioavailability of other mineral nutrients, as for instance Cu (Brümmer, 1986). Despite being a plant essential micronutrient (Broadley et al., 2011), high Cu concentrations can be toxic for plants (Adrees et al., 2015). Considering that the development of sustainable agronomic practices based on the use of PGPR is recognized as a task to meet the challenges of the near future in terms of agricultural productivity (Crecchio et al., 2018; Pii et al., 2015b), the understanding of the influence of *A. brasilense* on Cu nutrition (both deficiency *and* toxicity) might be of crucial importance. Indeed, the PGPR increased the lateral roots development of Cu deficient cucumber plants and this effect was further enhanced in combined Fe and Cu deficiency (Fig. 1). Indeed, a more developed root system can enable plants being more efficient in nutrients interception, thus having an influence on the plants nutritional status as well as on the ionomic profile (Adesemoye and Kloepper, 2009; Pii et al., 2015a). Interestingly, *A. brasilense* enhanced cucumber root length also in plants exposed to Cu toxicity compared to the non-inoculated plants, thus suggesting that the PGPR might alleviate the Cu toxicity symptoms (*i.e.* reduction of root growth) normally shown by non-inoculated plants (Fig. 1). It has been widely demonstrated that some plant species accumulate Cu mainly in the apoplasm (Kopittke and Menzies, 2006; Lu et al., 2017; Zhao et al., 2010); therefore, the increase of the root surface induced by *Azospirillum* inoculation might serve as possible strategy to cope with Cu excess, also considering the ability of *Azospirillum* to thrive in the presence of high Cu concentrations in the growth medium (Supplemental Fig. 3).

The Cu uptake at root level involves several molecular entities; nonetheless, COPT represents a specific family of Cu transporters, which are responsible for Cu(I) transport across the plasma membrane (Peñarrubia et al., 2015). Therefore, Cu(II) needs to be reduced to Cu(I) by the root plasma membrane reductase FRO before being taken up (Printz et al., 2016). Consistently, all the members of the cucumber *FRO* gene family were expressed in control condition, Cu deficiency and toxicity (Fig. 5). Moreover, in Cu deficiency all the three *CsFRO* isoforms were over-expressed in inoculated plants compared to the non-inoculated ones (Fig. 5). Indeed, also in *A. thaliana* plants *FRO4* and *FRO5* were shown to be responsive and up-regulated in the root tissue of Cu deficient plants (Bernal et al., 2012). On the contrary, the combined deficiency had an inhibitory action on the *CsFRO1* gene expression induced by *Azospirillum*, whereas no effects of the PGPR were reported for *CsFRO2* and *CsFRO3* (Fig. 5). These findings might suggest that the presence of at least one of the two cations (*i.e.* Cu and/or Fe) is required for this PGPR activity. In Cu toxicity, *A. brasilense* inoculation caused a significant inhibition of *CsFRO2* and *CsFRO3* compared to the non-inoculated cucumber plants (Fig. 5), that was in line with the lack of variation of the Fe reduction activity at root plasma membrane between inoculated and non-inoculated plants (Fig. 2E).

In Cu toxicity, non-inoculated plants did not modulate FRO activity (Fig. 2). However, both root Cu and Fe concentrations increased with increasing Cu concentration in nutrient solution (Fig. 6), suggesting a synergic relation between the two nutrients. Furthermore, by modulating the root biological processes, the PGPR *A. brasilense* could also affect the nutrient uptake mechanisms, influencing both Fe and Cu root concentrations. Indeed, *A. brasilense* increased the Fe accumulation in roots of Cu deficient cucumber plants as compared to the non-inoculated ones, whilst reduced the root Cu concentration in those plants exposed to the double deficiency (Fig. 6). In control condition, the PGPR enhanced the accumulation of Fe and reduced the concentration of Cu in the root tissues, whereas, in extreme Cu toxicity, the inoculation reduces both Fe and Cu concentration in roots. These results are in agreement with the PGPR modulation of plant nutrient homeostasis reducing the accumulation of toxic elements or altering the uptake of essential nutrients (Paul and Lade, 2014).

5. Conclusions

In conclusion, the data hereby presented highlight that in cucumber plants the Fe chelate reductase enzymes are encoded by a gene family composed of three members, all of them expressed in the root tissue and responding to both Cu starvation, combined Cu/Fe deficiency and Cu

toxicity. Interestingly, only *CsFRO3* showed a modulation upon *A. brasilense* inoculation in Fe sufficient control plants. Overall, these observations might suggest a different specificity of action of the three isoenzymes. In addition, the experiments carried out in both Cu starvation and Cu toxicity showed that *A. brasilense* might play a role in alleviating this abiotic stress in cucumber plants, by modulating, on one hand, the growth of the root system and, on the other hand, the root uptake of Cu. Nevertheless, further studies using soil-grown plants will be necessary to assess the efficacy of *A. brasilense* inoculation in alleviating Cu stresses in cucumber plants.

Authors' contribution

Designing of the research: LM, YP, TM, SC.
Performance of the research: LM, MM, FV.
Data analyses, collection, or interpretation: LM, MM, FV, YP, TM, SC.
Writing the manuscript: LM, YP, TM, SC.

Acknowledgments

The research was supported by grants from Free University of Bolzano (TN2017 e TN2053).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.013.

List of abbreviations

Fe	Iron
Cu	Copper
FRO	Ferric-chelate Reductase Oxidase
Cs	*Cucumis sativus*
COPT1	Cu Transport Protein 1
ZIP	Zn/Fe Permeases
IRT1	Iron Related Transporter 1
PGPR	Plant Growth-Promoting Rhizobacteria
DAI	Days After Inoculation

References

Adesemoye, A., Kloepper, J., 2009. Plant–microbes interactions in enhanced fertilizer-use efficiency. Appl. Microbiol. Biotechnol. 85, 1–12. https://doi.org/10.1007/s00253-009-2196-0.

Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., Zia-ur-rehman, M., Irshad, M.K., Bharwana, S.A., 2015. The effect of excess copper on growth and physiology of important food crops : a review. Environ. Sci. Pollut. Res. 22, 8148–8162. https://doi.org/10.1007/s11356-015-4496-5.

Alegria Terrazas, R., Giles, C., Paterson, E., Robertson-Albertyn, S., Cesco, S., Mimmo, T., Pii, Y., Bulgarelli, D., 2016. Plant-microbiota interactions as a driver of the mineral turnover in the rhizosphere. Adv. Appl. Microbiol. https://doi.org/10.1016/bs.aambs.2016.03.001.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Bashan, Y., 1990. Short exposure to *Azospirillum brasilense* Cd inoculation enhanced proton efflux of intact wheat roots. Can. J. Microbiol. 36, 419–425. https://doi.org/10.1139/m90-073.

Bashan, Y., Levanony, H., Mitiku, G., 1989. Changes in proton efflux of intact wheat roots induced by *Azospirillum brasilense* Cd. Can. J. Microbiol. 35, 691–697. https://doi.org/10.1139/m89-113.

Bernal, M., Casero, D., Singh, V., Wilson, G.T., Grande, A., Yang, H., Dodani, S.C., Pellegrini, M., Huijser, P., Connolly, E.L., Merchant, S.S., Kramer, U., 2012. Transcriptome sequencing identifies SPL7-regulated copper acquisition genes *FRO4/FRO5* and the copper dependence of iron homeostasis in *Arabidopsis*. Plant Cell Online 24, 738–761. https://doi.org/10.1105/tpc.111.090431.

Bertrand, H., Plassard, C., Pinochet, X., Touraine, B., Normand, P., Cleyet-Marel, J.C., 2000. Stimulation of the ionic transport system in *Brassica napus* by a plant growth-promoting rhizobacterium (*Achromobacter* sp.). Can. J. Microbiol. 46, 229–236. https://doi.org/10.1139/w99-137.

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., Zhao, F., 2011. Function of nutrients: micronutrients. In: Marschner, P. (Ed.), Marschner's Mineral Nutrition of Higher Plants, third ed. Academic Press, pp. 191–248. https://doi.org/10.1016/B978-0-12-384905-2.00007-8.

Brümmer, G.W., 1986. Heavy metals species, mobility and availability in soils. In: Bernhard, M., Brinckman, F.E., Sadler, P.J. (Eds.), The Importance of Chemical "Speciation" in Environmental Processes. Springer-Verlag Berlin, Heidelberg, pp. 169–192.

Brunetto, G., Bastos de Melo, G.W., Terzano, R., Del Buono, D., Astolfi, S., Tomasi, N., Pii, Y., Mimmo, T., Cesco, S., 2016. Copper accumulation in vineyard soils: Rhizosphere processes and agronomic practices to limit its toxicity. Chemosphere 162, 293–307. https://doi.org/10.1016/j.chemosphere.2016.07.104.

Canellas, L., Balmori, D., Médici, L., Aguiar, N., Campostrini, E., Rosa, R.C., Façanha, A., Olivares, F., 2013. A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). Plant Soil 366, 119–132. https://doi.org/10.1007/s11104-012-1382-5.

Canellas, L.P., Olivares, F.L., Okorokova-fac, A.L., 2002. Humic Acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots. Plant Physiol. 130, 1951–1957. https://doi.org/10.1104/pp.007088.loosens.

Carlot, M., Giacomini, A., Casella, S., 2002. Aspects of plant-microbe interactions in heavy metal polluted soil. Acta Biotechnol. 22, 13–20. https://doi.org/10.1002/1521-3846(200205)22:1/2<13::AID-ABIO13>3.0.CO;2–9.

Cassman, K.G., 1999. Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. Proc. Natl. Acad. Sci. U. S. A. 96, 5952–5959.

Crecchio, C., Mimmo, T., Bulgarelli, D., Pertot, I., Pii, Y., Perazzolli, M., Scagliola, M., Cesco, S., 2018. In: Lichtfouse, E. (Ed.), Beneficial Soil Microbiome for Sustainable Agriculture Production BT - Sustainable Agriculture Reviews 31: Biocontrol. Springer International Publishing, Cham, pp. 443–481. https://doi.org/10.1007/978-3-319-94232-2_9.

de Santiago, A., Quintero, J.M., Avilés, M., Delgado, A., 2009. Effect of *Trichoderma asperellum* strain T34 on iron nutrition in white lupin. Soil Biol. Biochem. 41, 2453–2459. https://doi.org/10.1016/j.soilbio.2009.07.033.

Del C Orozco-Mosqueda, M., Santoyo, G., Farías-Rodríguez, R., Macías-Rodríguez, L., Valencia-Cantero, E., 2012. Identification and expression analysis of multiple *FRO* gene copies in *Medicago truncatula*. Genet. Mol. Res. 11, 4402–4410. https://doi.org/10.4238/2012.October.9.7.

Feigl, G., Kumar, D., Lehotai, N., Tugyi, N., Molnár, Á., Ördög, A., Szepesi, Á., Gémes, K., Laskay, G., Erdei, L., Kolbert, Z., 2013. Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress. Ecotoxicol. Environ. Saf. 94, 179–189. https://doi.org/10.1016/j.ecoenv.2013.04.029.

Glick, B.R., 2012. Plant Growth-Promoting Bacteria: mechanisms and applications. Scientifica (Cairo) 2012, 1–15. https://doi.org/10.6064/2012/963401.

Hinsinger, P., Gobran, G.R., Gregory, P.J., Wenzel, W.W., 2005. Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. New Phytol. 168, 293–303. https://doi.org/10.1111/j.1469-8137.2005.01512.x.

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., Wada, Y., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S., Nishizawa, N.K., 2006. Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. Plant J. 45, 335–346. https://doi.org/10.1111/j.1365-313X.2005.02624.x.

Kobayashi, T., Nishizawa, N.K., 2012. Iron uptake, translocation, and regulation in higher plants. Annu. Rev. Plant Biol. 63, 131–152. https://doi.org/10.1146/annurev-arplant-042811-105522.

Komárek, M., Čadková, E., Chrastný, V., Bordas, F., Bollinger, J.C., 2010. Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. Environ. Int. 36, 138–151. https://doi.org/10.1016/j.envint.2009.10.005.

Kopittke, P.M., Menzies, N.W., 2006. Effect of Cu toxicity on growth of cowpea (*Vigna unguiculata*). Plant Soil 279, 287–296. https://doi.org/10.1007/s11104-005-1578-z.

Korshunova, Y., Eide, D., Gregg Clark, W., Lou Guerinot, M., Pakrasi, H., 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol. Biol. 40, 37–44. https://doi.org/10.1023/A:1026438615520.

Kumar, A., Verma, J.P., 2018. Does plant—Microbe interaction confer stress tolerance in plants: A review? Microbiol. Res. 207, 41–52. https://doi.org/10.1016/J.MICRES.2017.11.004.

Li, L.L., Cheng, X., Ling, H., 2004. Isolation and characterization of Fe(III)-chelate reductase gene *LeFRO1* in tomato. Plant Mol. Biol. 54, 125–136.

Lu, L., Xie, R., Liu, T., Wang, H., Hou, D., Du, Y., He, Z., Yang, X., Sun, H., Tian, S., 2017. Spatial imaging and speciation of Cu in rice (*Oryza sativa* L.) roots using synchrotron-based X-ray microfluorescence and X-ray absorption spectroscopy. Chemosphere 175, 356–364. https://doi.org/10.1016/j.chemosphere.2017.02.082.

Lugtenberg, B., Kamilova, F., 2009. Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918.

Marschner, H., Römheld, V., 1994. Strategies of plants for acquisition of iron. Plant Soil 165, 261–274. https://doi.org/10.1007/BF00008069.

Matson, P.A., Parton, W.J., Power, A.G., Swift, M.J., 1997. Agricultural intensification and ecosystem properties. Science (80-.) 277, 504–509. https://doi.org/10.1126/science.277.5325.504.

Mukherjee, I., Campbell, N., Ash, J., Connolly, E., 2006. Expression profiling of the *Arabidopsis* ferric chelate reductase (FRO) gene family reveals differential regulation by iron and copper. Planta 223, 1178–1190. https://doi.org/10.1007/s00425-005-0165-0.

Parray, J.A., Jan, S., Kamili, A.N., Qadri, R.A., Egamberdieva, D., Ahmad, P., 2016.

Current perspectives on Plant Growth-Promoting Rhizobacteria. J. Plant Growth Regul. 35, 877–902. https://doi.org/10.1007/s00344-016-9583-4.
Paul, D., Lade, H., 2014. Plant-growth-promoting rhizobacteria to improve crop growth in saline soils: a review. Agron. Sustain. Dev. 34, 737–752. https://doi.org/10.1007/s13593-014-0233-6.
Peñarrubia, L., Romero, P., Carrió-Segui, A., Andrès-Borderia, A., Moreno, J., Sanz, A., 2015. Temporal aspects of copper homeostasis and its crosstalk with hormones. Front. Plant Sci. 6, 1–18. https://doi.org/10.3389/fpls.2015.00255.
Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30https://doi.org/10.1093/nar/30.9.e36. e36–e36.
Pii, Y., Cesco, S., Mimmo, T., 2015a. Shoot ionome to predict the synergism and antagonism between nutrients as affected by substrate and physiological status. Plant Physiol. Biochem. 94, 48–56. https://doi.org/10.1016/j.plaphy.2015.05.002.
Pii, Y., Marastoni, L., Springeth, C., Fontanella, M.C., Beone, G.M., Cesco, S., Mimmo, T., 2016. Modulation of Fe acquisition process by *Azospirillum brasilense* in cucumber plants. Environ. Exp. Bot. 130, 216–225. https://doi.org/10.1016/j.envexpbot.2016.06.011.
Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., Crecchio, C., 2015b. Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. Biol. Fertil. Soils 51, 403–415. https://doi.org/10.1007/s00374-015-0996-1.
Pii, Y., Penn, A., Terzano, R., Crecchio, C., Mimmo, T., Cesco, S., 2015c. Plant-microorganism-soil interactions influence the Fe availability in the rhizosphere of cucumber plants. Plant Physiol. Biochem. 87, 45–52. https://doi.org/10.1016/j.plaphy.2014.12.014.
Printz, B., Lutts, S., Hausman, J.-F., Sergeant, K., 2016. Copper trafficking in plants and its implication on cell wall dynamics. Front. Plant Sci. 7, 1–16. https://doi.org/10.3389/fpls.2016.00601.
Rajkumar, M., Freitas, H., 2008. Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. Chemosphere 71, 834–842. https://doi.org/10.1016/j.chemosphere.2007.11.038.
Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. https://doi.org/10.1016/S0304-3940(02)01423-4.
Robinson, N.J., Procter, C.M., Connolly, E.L., Guerinot, M.L., 1999. A ferric chelate reductase for iron uptake from soils. Nature 397, 694–697.
Taiz, L., 2013. Agriculture, plant physiology, and human population growth : past , present , and future. Theor. Exp. Plant Physiol. 25, 167–181.
Tak, H.I., Ahmad, F., Babalola, O.O., 2013. Advances in the application of Plant Growth-Promoting Rhizobacteria in phytoremediation of heavy metals. Rev. Environ. Contam. Toxicol. 147, 33–51.
Tilman, D., Cassman, K.G., Matson, P. a, Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677. https://doi.org/10.1038/nature01014.
Tirry, N., Tahri Joutey, N., Sayel, H., Kouchou, A., Bahafid, W., Asri, M., El Ghachtouli, N., 2018. Screening of plant growth promoting traits in heavy metals resistant bacteria: Prospects in phytoremediation. J. Genet. Eng. Biotechnol. https://doi.org/10.1016/J.JGEB.2018.06.004.
Tsai, H.-H., Schmidt, W., 2017. One way. Or another? Iron uptake in plants. New Phytol. 214, 500–505. https://doi.org/10.1111/nph.14477.
Vivas, A., Biró, B., Németh, T., Barea, J.M., Azcón, R., 2006. Nickel-tolerant *Brevibacillus brevis* and arbuscular mycorrhizal fungus can reduce metal acquisition and nickel toxicity effects in plant growing in nickel supplemented soil. Soil Biol. Biochem. 38, 2694–2704. https://doi.org/10.1016/j.soilbio.2006.04.020.
Vizzotto, G., Pinton, R., Bomben, C., Cesco, S., Varanini, Z., Costa, G., 1999. Iron reduction in iron-stressed plants of *Actinidia deliciosa* genotypes: Involvement of PM Fe (III)-chelate reductase and H^+-ATPase activity. J. Plant Nutr. 22, 479–488.
Waters, B.M., 2002. Characterization of *FRO1*, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol. 129, 85–94. https://doi.org/10.1104/pp.010829.
Waters, B.M., Lucena, C., Romera, F.J., Jester, G.G., Wynn, A.N., Rojas, C.L., Alcántara, E., Pérez-Vicente, R., 2007. 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. 45, 293–301. https://doi.org/10.1016/j.plaphy.2007.03.011.
Welch, R.M., Norvell, W.A., Schaefer, S.C., Shaff, J.E., Kochian, L.V., 1993. Induction of iron(III) and copper(II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: Does the root-cell plasmalemma Fe(III)-chelate reductase perform a general role in regulating cation uptake? Planta 190, 555–561. https://doi.org/10.1007/BF00224795.
Wintz, H., Fox, T., Wu, Y.-Y., Feng, V., Chen, W., Chang, H.-S., Zhu, T., Vulpe, C., 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. J. Biol. Chem. 278, 47644–47653. https://doi.org/10.1074/jbc.M309338200.
Wu, H., Li, L., Du, J., Yuan, Y., Cheng, X., Ling, H.Q., 2005. Molecular and biochemical characterization of the Fe(III) chelate reductase gene family in *Arabidopsis thaliana*. Plant Cell Physiol. 46, 1505–1514. https://doi.org/10.1093/pcp/pci163.
Wuana, R.A., Okieimen, F.E., 2011. Heavy metals in contaminated soils: arReview of sources, chemistry, risks and best available strategies for remediation. ISRN Ecol. 2011, 1–20. https://doi.org/10.5402/2011/402647.
Xu, C., Chen, X., Duan, D., Peng, C., Le, T., Shi, J., 2015. Effect of heavy-metal-resistant bacteria on enhanced metal uptake and translocation of the Cu-tolerant plant, *Elsholtzia splendens*. Environ. Sci. Pollut. Res. 22, 5070–5081. https://doi.org/10.1007/s11356-014-3931-3.
Yruela, I., 2005. Copper in plants. Braz. J. Plant Physiol. 17, 145–156.
Yuan, M., Li, X., Xiao, J., Wang, S., 2011. Molecular and functional analyses of *COPT/Ctr-type copper transporter-like* gene family in rice. BMC Plant Biol. 11, 69–80. https://doi.org/10.1186/1471-2229-11-69.
Zhang, F., Shen, J., Zhang, J., Zuo, Y., Li, L., Chen, X., 2010. Rhizosphere processes and management for improving nutrient use efficiency and crop productivity : implications for China. In: Advances in Agronomy. Elsevier Inc, pp. 1–32. https://doi.org/10.1016/S0065-2113(10)07001-X.
Zhang, H., Sun, Y., Xie, X., Kim, M.-S., Dowd, S.E., Paré, P.W., 2009. A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms. Plant J. 58, 568–577. https://doi.org/10.1111/j.1365-313X.2009.03803.x.
Zhao, L., Wang, F., Zhang, Y., Zhang, J., 2014. Involvement of *Trichoderma asperellum* strain T6 in regulating iron acquisition in plants. J. Basic Microbiol. 54, S115–S124. https://doi.org/10.1002/jobm.201400148.
Zhao, S., Liu, Q., Qi, Y., Duo, L., 2010. Responses of root growth and protective enzymes to copper stress in turfgrass. Acta Biol. Cracov. Ser. Bot. 52, 7–11. https://doi.org/10.2478/v10182-010-0017-5.