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Sources and availability of copper and iron affect growth and development of crops

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SUMMARY

Plants, as sessile organisms, are subject to a multitude of abiotic and biotic environmental challenges. Their acclimation is required for survival and reproductive success in non-optimal and heterogeneous environments; in particular, they have to adapt to the pedoclimatic conditions (Schulten and Krämer, 2017). Many soils are either globally or locally deficient in mineral nutrients or exhibit mineral imbalances that can lead to a harmful excess of a specific mineral. Indeed, the current stage of development of human society is characterized by processes of intensive agriculture, urbanization and industrialization. This has led to anincreased emission of organic and inorganic substances into the environment, which may give rise to the phenomenon of pollution and toxicity (Riffaldi *et al.,* 2006).

Plants must respond to such challenging conditions by modulating nutrient acquisition through mobilization and uptake, by managing nutrient distribution and utilization and by implementing storage or detoxification of ions in excess (Hawkesford and De Kok 2006; Jeong and Guerinot 2009; Masclaux-Daubresse *et al.,* 2010; Plaxton and Tran, 2011; Marschner, 2012).Besides these processes, plants exposure to toxic levels of minerals can have profound effects on plant metabolism, growth and development (Barberon *et al.,* 2016).

Iron (Fe) is a transition metal that is required by plants mainly for its fundamental roles in cell redox chemistry. Iron is crucial in the active sites of numerous enzymes involved in processes such as mitochondrial respiration, photosynthesis, oxidative stress protection, and various metabolic pathways(Puig*et al.,* 2007; Burkhead*et al.,* 2009).Irondeficiency is one of the most widespread nutrient imbalances in agriculture. Under Fe-deficiency conditions, chloroplast development and chlorophyll biosynthesis are impaired, resulting in the typical symptom of interveinal leaf chlorosis, so-called iron chlorosis. Moreover, a general limitation of plant biomass accumulation, a reprogramming of metabolism and an induction of Fe-acquisition mechanisms are also observed (Marschner, 2012).

Copper (Cu) was first identifyas a plant nutrient in the 1930s (Arnon and Stout, 1939; Hossain *et al.,* 2017) and, like Fe, it isa transition metalthatparticipatestooxido-reductivereactionsin plantsof considerablebiological importancesuch as photosynthesisandmitochondrial respiration (Yurela, 2005). Theoxidases of electron transport chainsin chloroplastsand mitochondriarequire Cu as cofactor to be able to transfer electrons. Copper metalloenzymes play important roles in photosynthesis, mitochondrial respiration, cell wall biosynthesis and superoxide scavenging(Braz*et al.,* 2005).Copper plays also a role in the metabolism of carbon and nitrogen and allows the transmission of hormonal signals(Peñarrubia*et al*., 2015). At the cellular level, Cu has an essential role in signaling the transcription, in oxidative phosphorylation and in the Fe mobilization (Braz*et al,* 2005; Adress*et al.,* 2015).

Typical symptoms of Cu shortage include stunted growth, leaf deformation, necrosis of apical meristems and chlorosis of young leaves (Rahimi and Bussler, 1973).Copperdeficiency occurs on calcareous soils, in which Cu availability is low due to its insolubility at high pH orin soils with a high content of organic

matter, due to the high affinity of Cu to organic compounds.On the other hand, toxic effects of Cu in plants can be observed by reduced yield, poor seed germination, stunted leaf and root growth, and ultrastructural and anatomical alterations leading also to the formation of reactive oxygen species (ROS).Plant phenotypes associated with Cu toxicity share similarities with those related to Fe-deficiency, such as the presence of leaf chlorosis, decreased leaf chlorophyll content and enhanced oxidative stress (Pätsikkä*et al.,* 2002).

For some metabolic functions, organisms may alternatively use Fe-containing proteins or Cu-containing proteins to catalyze biochemical reactions, depending on the bioavailability of each metal (Puig*et al.,* 2007).The reason could be related to changes across the geological erasin the concentration of soluble transition metalsin the biosphere along the increase of O_2 content within the atmosphere.In order to overcome Fe limitation, plants have evolved different mechanisms to acquire Fe from sparingly available Fe sources (Giehl*et al.,*2009). This oxidative atmosphere led to a decreased Fe solubility due to the formation of Fe oxides and to the progressive release of soluble Cu(II) from insoluble Cu sulfide salts (Burkhead*et al.,* 2009). Consequently, Fe has been progressively substituted in biological molecules by Cu which is able to perform similar functions.The best-adapted organisms developed new strategies to solubilize and acquire Fe^{3+}, but they also incorporate Cu in multiple processes requiring higher redox potentials.

The Cu-uptake mechanisms in plants have not been completely elucidated, however, common features between Fe-uptake and Cu-uptake mechanisms has been suggested (Ryan *et al.,* 2013).To acquire Fe,*non-graminaceous* plants have developed a reduction-based mechanism, so called *Strategy I,* which involves the solubilization of ferric Fe via protons released into the rhizosphere, followed by the subsequent reduction of Fe (III) by FERRIC REDUCTION OXIDASE 2 (FRO2) (Robinson *et al.,* 1999; Connolly *et al.,* 2003). Ferrous iron is then transported across the root plasma membrane of outer root cell by IRON REGULATED TRANSPORTER 1 (IRT1) (Vert*et al.,* 2002). It is known that chelate reductase membrane protein may be able of donating electrons to other transition metals thanFe (III), asCu (II, III, IV) (Uren, 1982; Marschner*et al.,* 1986). Ferric-reductase of the yeast, *Saccharomyces cerevisiae,* could reduce Cu^{2+} to Cu^+ (Lesuisse and Labbe, 1992) and it wasshowed that, in pea roots, Fe or Cu deficiency increased the activity of the inducible reductase system for the reduction of chelated Fe^{3+} and Cu^{2+} (Welch *et al.,* 1993). Furthermore, through Cu speciation and isotopicfractionation, it was observed that tomato roots took up preferentially the light Cu isotope (Ryan *et al.,* 2013). Such observation has been proposed to indicate that a reductive step is involved in the Cu acquisition into the root (Bernal *et al.,* 2012; Jouvin*et al.,* 2012; Ryan *et al.,* 2013).

In response to Fedeficiency, grass plants activate the so-called *Strategy II,* which is based ontheincrease of biosynthesis and release of phytosiderophores (PS). These latter compounds are able toform stable chelates with Fe^{3+} and, in the form of Fe(III)-PS complex, which it is taken up by roots (Hördt*et al.,* 2000). Phytosiderophorerelease occurs also under Cu deficiency and has been speculated to be a general adaptive plant response to enhance the acquisition of this metalby grasses (Gries*et al.,* 1998; Awad and

Römheld, 2000; Schenkeveld*et al.*, 2014).Moreover, Chaignon*et al.* (2002) observed that under Fe starvation, the release of PS and the accumulation of Cu by wheat grown on a Cu-contaminated soil were enhanced. Moreover, up to date the possibility that roots can absorb also other Cu sources such as soluble Cu-complexes with low-molecular-weight organic moleculescannot be excluded (Brunetto *et al.,* 2016).

The common and specific aspects of Fe and Cu acquisition mechanismsin plants is a topic of great interest for plant nutritionists, as well documented by recent reviews(Aguirre and Pilon, 2015; Waters *et al.,* 2014). However,littleis known about the crosstalk between these two microelements and their reciprocal interaction or antagonism, especially at molecular level.

Considering these aspects, this PhD thesis investigates the interactions between Cu and Fe acquisition in crops with the purpose to understand if their status and supply in different forms and amounts could affecttheir mutual acquisition. For this reason, maize (*Zea mays*)tomato (*Solanum lycopersicum*) and melon (*Cucumis melon)* were chosen because maize is one of the most widespread cultivated cereals and tomato and melon aremodel plants especially in Fe-deficiency studies.

Firstly, plants were grown under hydroponic conditions that caused Cu-and Fe-deficient symptoms due to the lack of Cu or Fe supplyorthe excess of Cu.Shoot-and root-morphological responses, leaf antioxidant enzymeactivity (SODs, CAT, APX and POX) as well as nutrients contentwere evaluated.It was found that maize is more tolerant to Cu toxicity than tomato which is more sensitive to Cu deficiency.

Secondary, with the purpose to evaluate the availability of different Fe and Cu compounds, different sources of Fe and Cu were supplied to maize plants while the Fe and Cu deficiencies were studied in tomato. In maize, the toxicity symptoms of Cu occur only when $CuSO_4$was supplied at a high concentration, independently of the source of Fe, and consisted in morphometricalterationsand changes in antioxidant enzymes'activities. In tomato, Cu- and Fe-deficiencies clearly induced morphological and physiologicalalterationswhile the molecular characterization did not bring any conclusive results and therefore it is not possible at now toidentify components involved in the crosstalk between Fe and Cu acquisition mechanisms and regulation.

Finally, the research on interactions between Cu and Fewas carried on two melon genotypes (Edisto, as wild type, and *fefe*, as a Fe-deficiency unresponsive mutant) using the split roots technique with the purpose to investigate the local and systemic signals involved in the regulation of Cu and Fe uptake by *Strategy I* plants. Physiological evidence showed local response to Fe deficiency while molecular analyses highlighted the presence of a systemic regulation.

On the whole, these results propose a putative interaction between Fe and Cu adsorption in crops and underline the need of metabolomic and isotopic studies that allow a more complete evaluation of the effects of the imbalance of these two elements in the plant metabolism.

CONTENTS

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1. Introduction

Bioavailability of nutrients

The survival and productivity of crops are strictly dependent on their ability to adapt to environmental conditions. This adaptation is the result of the interactions between roots and biotic and abiotic components of the soil, which lead to changes in both the morphology and physiology of the root system and the chemical, physical and biological characteristics of the soil. The processes underlying these interactions concern a limited area of the soil present in the surrounding area of the root, called *rhizosphere* (Hiltner, 1904); in this environment, there is an intense exchange activity concerning nutrients, energy and molecular signals that make its physic, chemistry, biochemistry and biology profoundly different from those occurring in the undisturbed soil, called *bulk soil* (Pinton *et al*., 2001). This causes, in the rhizospheric environment, a complex situation that involves the movements of gas, water and solutes, the dynamics of the microbial populations and the organic components as well as the alteration of the minerals present in the soil, with consequent formation of distinctive gradients (Figure 1). Indeed, availability of micronutrients to plants is regulated by various soil factors such as texture, soil reaction, organic matter, clay content, soil moisture, nutrient interactions in soil, microbial activity, redox potential and aeration (Plaxton and Tran, 2011; Marschner, 2012).
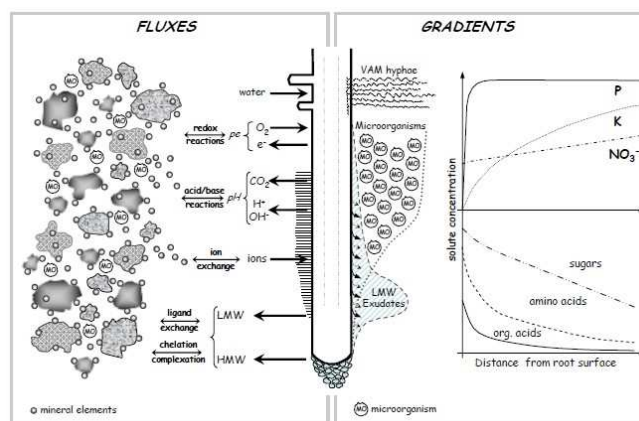
Figure1. Schematic representation of the main flows and gradients present at the rhizosphere (Pinton *et al.,* 2009).

Understanding the processes that control the availability of nutrients can help to shed light on the phenomena responsible for the nutritional efficiency of the crops, such as the ability of the plants themselves to acquire essential mineral elements from the soil, whether they are naturally present in the soil or carried through fertilizations, and then accumulate biomass that will be a major determinant of the yield both in terms of quantity and quality.

Iron nutrition in plant

Iron (Fe) is an important micronutrient that playsa crucial role in plant due to its involvement as redox-active metal in photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis, pathogen defense, reproduction, production and scavenging of reactive oxygen species (Hansch and Mendel, 2009; Vigani*et al.,* 2013). As transition metal, Fe changes easily its oxidation state (Fe^{2+}/Fe^{3+}) under physiological conditions and is able to form octahedral complexes with different ligands. The redox potential of Fe (Fe^{2+}/Fe^{3+}) depends on the ligand and this variability clarifies the importance of Fe in biological redox systems and determines its versatile functions (Marschner, 2012).

Iron deficiency is a widespread limiting factor of the yield for a variety of field crops all around the world and generally results from the interactions between limited soil Fe bioavailability, pH of the soil and susceptible genotype cultivation (Hansen, 2007; Hsieh and Waters, 2016). In particular, the poor bioavailability of Fe for plant nutrition occurs quite frequently in calcareous soils(pH> 7.0)where the micronutrient is mainly found in poorly soluble Fe-oxide/hydroxide forms (Marschner, 2012).

Under Fe-deficiency conditions, chloroplasts development and chlorophyll biosynthesis are impaired, resulting in the typical symptom: interveinal leaf chlorosis. Plants respond to Fe deficiency also through a reduction in primary root elongation and formation of lateral roots (Landsberg, 1982). This behavior results also into an increase in root hair production, thickening of the root tips and formation of rhizodermal transfer cells (Römheld and Kramer, 1983). In some plants, the condition of limited availability of Fe determines the formation of *cluster roots* similar to those that can be develop under limiting availability of other nutrients in the soil solution, such as phosphorus (Hagström *et al.,* 2001).

Iron in soil

Iron is the fourth most abundant element of the earth's crust and has percentages ranging from 2 to 5% (weight/weight) of the soil. This element is present in the crystalline lattice of many minerals, as insoluble oxides and hydroxides (hematite, magnetite, siderite), ferromagnetic silicates (olivine, augite, biotite), amorphous oxides, and also in forms available for plant acquisition: in complexed forms bysoil organic components, adsorbed on colloids and, in limited quantities (10^{-20} to 10^{-10}M), in solution (Cornell and Schwertmann*et al.,*2003; Colombo *et al.,* 2014).

It has been observed that in well aerated soils and at pH values around neutrality, the concentration of free Fe in solution is less than 10^{-15} M (Marschner*et al.,* 1996), *i.e.* at least 6 orders of magnitude lower than the concentration required for optimal growth of plants. The following ionic forms belong to this soluble fraction: Fe^{3+}, Fe^{2+}, $Fe(OH)^{2+}$, $Fe(OH)^{+}$, $Fe(OH)_2^{+}$. The solubility of Fe can be considered dependent on the dissociation state of the hydroxide $Fe(OH)_3$, which formation follows this reaction:

$$Fe^{3+} + 3H_2O \longrightarrow Fe(OH)_{3(solid)} + 3H^{+}$$
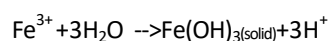
The equilibrium is greatly displaced towards the solid form Fe(OH)$_3$ and strictly depends on the pH, since an increase of pH leads to a decrease of Fe solubility. It has been observed that at each increment of a soil pH unit, the concentration of Fe in solution decreases 1000 times (Lindsay and Schwab, 1982).

In the pH range between 7.0 and 9.0, the species Fe(OH)$^{2+}$, Fe(OH)$_3$ and Fe(OH)$_4^-$ prevail, with a minimum of solubility between pH 7.4 and 8.5 (Gessa and Ciavatta, 2005). Therefore, acidic soils are characterized by a relatively high Fe concentration in soil solution, while opposite behavior occurs in alkaline (and above all calcareous) soils. The redox potential is also able to influence the solubility of Fe, since Fe^{2+} is much more soluble than Fe^{3+}. However, in well-aerated soils at pH between 6.0 and 8.0, the ferric form prevails over the ferrous form (Figure 2).
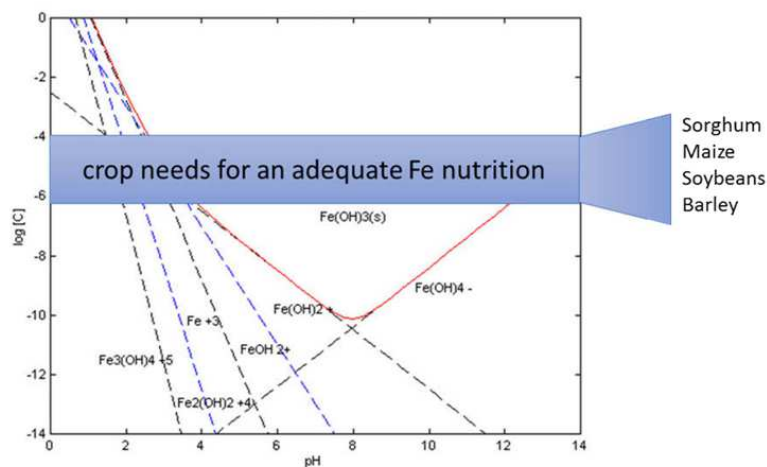
Figure2. Solubility of Fe is dependent on the dissociation of Fe(OH)$_3$ hydroxide. The equilibrium position lies to the right side and it is strictly dependent on pH (From Venuti, 2015).

In many soils, however, the total concentration of soluble Fe forms reaches values between 10^{-8} and 10^{-6} M; this depends on the presence of soluble and low molecular weight organic ligands which complex ionic Fe (Mimmo *et al.,* 2014). In the soil, and in particular at the rhizosphere level, there are different classes of organic compounds of microbial (siderophores) and plant (PS, carboxylic and phenolic acids) origin that are able to form complexes / chelates with Fe. Such complexes can facilitate the movement of Fe in the soil solution and become important source of Fe for root acquisition; low-molecular-weight humic substances, particularly the fulvic acids, are also able to complex Fe maintaining it in solution (Pinton*et al.,* 2001).

A limited availability of Fe in the soil may, however, lead to metabolic dysfunction of the crops and to the appearance of visible symptoms of nutritional deficiency. The Fe-deficiency is a yield-limiting factor and affects alkaline and an in particular the calcareous soils (Lucena, 2000), which covered more than 30% of the Earth's crust. Iron deficiency is the most important nutritional disorder that occurs in calcareous soils (Mortvedt, 1991). The pH, in these soils, is highly buffered due to the high amount of CaCO$_3$, thus limiting Fe solubility (Lindsay, 1979).

Fe acquisition and response to Fe deficiency

To ensure the supply of adequate amounts of Fe from the soil, plants have developed two different strategies that require the involvement of several biochemical mechanisms to promote the mobilization of the micronutrient in the rhizosphere and its transport into the root cells: *Strategy I* (reduction –based strategy) which occurs in dicots and non-*graminaceous* monocots and *Strategy II* that is found in grasses (Marschner*et al.,* 1987; Römheld, 1987; Schmidt, 1999; White and Broadley, 2009; Kobayashi and Nishizawa, 2012; Vigani*et al.,* 2013).

Strategy I

Strategy I (Römheld, 1987) is characterized by rhizosphere acidification by plasma membrane (PM) H^+-ATPase proteins, reduction of Fe (III) to Fe (II) by ferric chelate reductase proteins and uptake of Fe (II) by iron transporter proteins (Kobayashi and Nishizawa, 2012). Many molecular components of the *Strategy-I* Fe-uptake system have been well characterized. Iron solubility is improved by the acidification of the rhizosphere which follows the activation of the PM H^+-ATPasesbelonging to the HA family, such as CsHA1 and AtAHA2 (Santi *et al.,* 2005; Santi and Schmidt, 2009).The Fe(III)-reduction step is carried out by a plasma membrane-bound enzyme called FRO (ferric reductase oxidase, AtFRO2 in Arabidopsis and LeFRO1 in tomato; Guerinot, 2010, Brüggemann*et al.,* 1990; Holden *et al.,* 1991). Moreover, conditions of limited availability of Fe often induce an increase in the synthesis and release of chelating substances, such as organic acids (e.g.oxalic, malic and citric acids) and phenolic compounds (Lucena*et al.,* 2007; Gerke*et al.,* 1994; Jones*et al.,* 1996; Rodríguez-Celma*et al.*, 2013, Fourcroy*et al.*, 2014). In the last step, Fe^{2+} released from ferric complexes can be absorbed into the root cells by means of a trans-membrane iron transporter (IRT1,Eide*et al.,* 1996; Vert*et al.,* 2003). This latter protein shows high affinity for Fe^{2+}but not absolute specificity. It can mediate the transport of other bivalent cations than Fe, such as Zn^{2+}, Ni^{2+} , Pb^{2+}, $Cd^{2+}$and Mn^{2+} (Rogers *et al.*, 2000). *FRO* and *IRT* genes have been isolated and cloned from several species:*Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum, Cucumissativus*and *Cucumismelo* and their up-regulation occursin response to Fe-deficiency stress (Hindt and Guerinot, 2012; Waters *et al.,* 2007). *irt1*-knock-out *Arabidopsis*plantsshowed a strong chlorotic phenotype, indicatingthat this gene encodes a high-affinity Fe transporter, which is the main Fe-absorption system in the roots (Vert *et al.,* 2002).The functional characterization of *IRT1* was performed by restoring the growth of the yeast strain *Saccharomyces cerevisiae*fet3fet4which is defective for the main transmembrane transport of Fe in yeast (Eide*et al.,* 1996).Several studies have suggested that the rate-limiting step in the absorption of Fe is the reduction of Fe(III); for this reason, Conolly*et al.* (2003) have produced transgenic plants that overexpressed gene *FRO2*.Although these plants showed a greater tolerance to the low availability of Fe, the overexpression of *FRO2* only led to an increase in ferric-reductase activity in Fe-deficiency, suggesting a post-transcriptional regulation of *FRO2*controlled by the plant Fe nutritional status. In addition to *IRT1,* other genes (*NRAMP*) have been identified in *A. thaliana*:*NRAMP1*, *3, 4*, which are able to transport iron and could contribute to iron acquisition and

homeostasis (Curie *et al.,* 2001; Thomine*et al.,* 2000; Cailliatte*et al.,* 2010); recently *NRAMP1* has been proposed as a low affinity Fe transporter (Castaings *et al.,* 2016).

AtFRO2, *AtIRT1*, *AtRAMP1*, and numerous other genes are up-regulated under Fe deficiency by the basic helix-loop-helix (bHLH) transcriptional factor FIT (Colangelo and Guerinot, 2004; Jakoby*et al.,* 2004; Yuan *et al.,* 2005), a homolog of the tomato FER protein (Wang *et al.,* 2013). *FIT* gene expression is typically up-regulated by Fe deficiency (Colangelo and Guerinot, 2004; Lucena*et al.,* 2006). A group of four closely related Arabidopsis *bHLH* genes, *AtbHLH38/39/100/101,* are classified in clade Ib of the*bHLH* superfamily (Wang *et al.,* 2007; En-Jung Hsieh and Waters, 2016). The FIT protein regulates expression of its target genes as a heterodimer complex of FIT and a clade Ib*bHLH* protein (Yuan *et al.,* 2008, Wang *et al.,* 2013).A second regulatory system for Fe deficiency responses is mediated by the PYE, which is a bHLH protein specifically induced in root pericycle under Fe-deficient conditions (Ivanov *et al.,* 2012; Zhang *et al.,* 2015). Direct targets of PYE are the promotor of Fe acquisition-related genes, *Nicotianaminesynthase4*(*NAS4*), *FRO3*, *ZINC-induced facilitator1* (*ZIF1*), that are upregulated in Fe-deficient conditions (Long *et al.,* 2010).Usingco-expression analysis of *PYE*, Long*et al.* (2010) found that E3-ligase BRUTUS (BTS) is a putative negative regulator of Fe absorption genes.Moreover, Li *et al.,* (2016) have shown in *Arabidopsis* that other two basic helix-loop-helix-type transcription factors, bHLH34 and bHLH104, directly activate the transcription of the Ib subgroup *bHLH* genes, *bHLH38/39/100/101* while overexpression of *bHLH101* partially rescues the Fedeficiency phenotypes of *bhlh34bhlh104* double mutants.
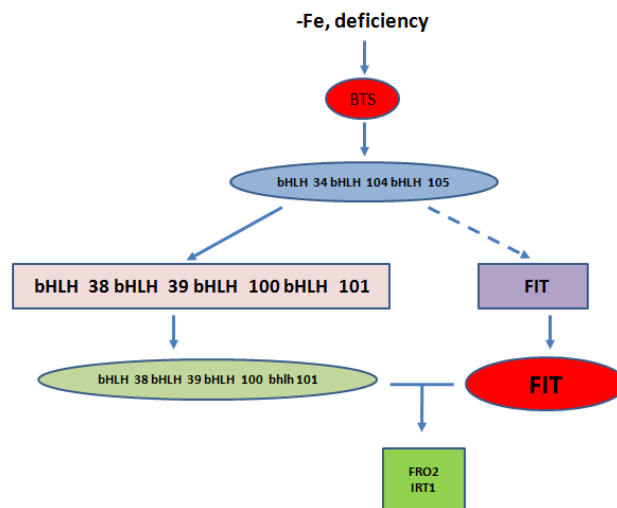
Figure 3.Scheme of Fe-deficiency-responsive signaling pathway. Iron deficiency stabilizes the interacts of BTS protein with bHLH104 and bHLH105. bHLH34, bHLH104, and bHLH105 can form homodimers or hetero-dimers and thus activate the transcription of bHLH38/39/100/101 and FIT. FIT, then,interacts with bHLH38/39/100/101 to activate the transcription of *FRO2* and *IRT1*. Arrows indicated direct induction while dotted arrows indicated indirect interaction. Squares are RNAs and circlesare protein(Adapted from Li *et al.,* 2016).

Iron-deficient *Strategy-I* plant species have long been known to increase efflux of roots exudates (see for review, Cesco *et al.,* 2010). Some species, such as *Arabidopsis thaliana*, mainly produce phenolic compounds (Fourcroy *et al.,* 2014; Schmidt *et al.,* 2014) while other species, including cucumber and melon, produce flavin compounds (Rodríguez-Celma *et al.,* 2011). Flavin and phenolic compounds mightbe able to reduce or complexextracellular Fe to facilitate its acquisition (Cesco *et al.,* 2010; Sisó-Terraza *et al.,* 2016).
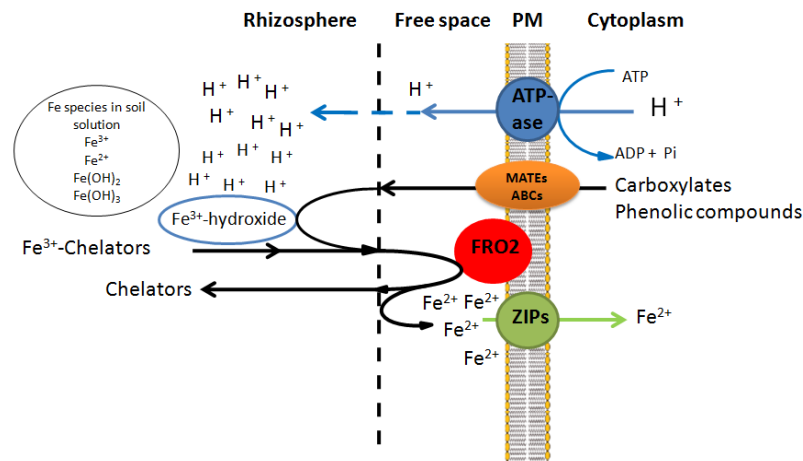
Figure 4.Model for root responses to Fe deficiency in dicots and non-*graminaceous* monocots (*Strategy I*). (Adapted from Römheld and Marschner, 1984).

Less is known about molecular Fe-deficiency responses in leaves. Only a few studies have profiled genome-wide gene expression in leaves (Waters *et al.,* 2012, Lauter *et al.,*2014). Several Fe-regulated genes respond to Fe deficiency in both leaf and root tissues, whereas some are specific to roots or leaves. While FIT is only expressed and regulated by Fe in roots, the transcripts of bHLH38/39/100/101 are up-regulated in both roots and leaves of Fe-deficient *Arabidopsis*plants (Rodríguez-Celma *et al.,* 2013, Hsieh and Waters *et al.,* 2016). In a leaf microarray study, Waters *et al.*(2012) showed that *iron responsive protein 1*(*AtIRP1, At1G47400*) and *3-ketoacyl-CoA synthase*(*AtKCS17*, *At4G34510*) were among the most strongly up-regulated genes in Fe-deficient Arabidopsis leaves.

Strategy II

Strategy II is theFe-acquisition mechanisms used by the *graminaceous* plants (*Poaceae*), which is based on the release into the rhizosphere of PS, which chelate and mobilize Fe in the soil. The PS are characterized by a very high affinity for Fe^{3+} with which they form extremely stable complexes ($Ksp≈10^{33}$) due to their six functional groups (three -COOH, two -NH, and one -OH)that can coordinate Fe (Ma and Nomoto, 1996). Thus, they could chelate Fe(III), and the Fe(III)-PS complexes are then taken up into roots (Römheld and Marschner, 1986). The biosynthetic pathway for mugineic acids (MAs), a class of PS compounds, in *graminaceous* plants has been elucidated (Mori and Nishizawa, 1987; Shojima*et al.,* 1990). S-adenosy-L-methionine (SAM), the precursor of MAs, is converted to 2'-deoxymugineic acid (DMA) via four sequential steps catalyzed by *S*-adenosylmethionine synthetase, nicotianamine synthase(NAS), nicotianamine aminotransferase(NAAT), and deoxymugineic acid synthase(DMAS), which produces, in the final phase, theDMA that is the precursor of all the MAs. Genes, encoding for these enzymes, are strongly induced in grass roots in response to the Fe deficiency. *TOM1* (mugineic acid transporter 1), belonging to the main facilitator superfamily (*MFS*), was identified and isolated from rice and barley and it is involved in the releaseof PS into the rhizosphere (Nozoye*et al.,*2011). The release of PS in barley follows a distinct diurnal rhythm, with a peak just after sunrise or initial illumination and it is influenced by the temperature (Takagi, 1984; Nozoye*et al.,* 2014). The Fe(III)-PS complex is taken into the root cells through a specific transporter, called YELLOW STRIPE 1 (YS1, Figure 5), which is localized at the root PM (Curie *et al.,*2001; Inoue *et al.,* 2009). The *YS1*gene was isolated from a maize mutant, *yellow stripe 1*, which shows leaf interveinal chlorosis due to its inability to take up the Fe(III)-PS complex (Curie *et al.,*2008).Phytosiderophores are also consideredto be important for the internal transport of various transition metals, especially Fe (Römheld and Marschner, 1986; Aciksoz *et al.,* 2011). *TOM2*,a rice homolog of*TOM1*, is involved in the internal transport of DMA (Nozoye *et al.,* 2015).Even if MAs are thought to be specific to *graminaceous* plants, nicotianamine (NA), a precursor of MA, has known to be produced in many crops, including non-*graminaceous* plants such as Arabidopsis, tomato, and tobacco (Stephan and Scholz,1993; Hashida*et al.,* 2007) and is believedto be involved in Fe transport (Kobayashi, and Nishizawa, 2012). Moreover, it has been shown that olive tree is able to biosynthesizeDMA (Suzuki *et al.,* 2016), which indicates that also dicot plants might produce and release PS. It is known that *Strategy-I* plants are able to use Fe bound to PS via a reduction of Fe and uptake of Fe^{2+}(Cesco*et al.,* 2006) or via homologs of *YS1* proteins that are present in dicot plant species and are able to transport Fe-PS (Xiong*et al.,* 2013).

Until recently, specific information on the regulation of genes involvedin the response of *Strategy-II* plantswas available.In rice, Hindt and Guerinot(2012) have shown that the response of the plant to Fe deficiency is regulated by the bHlH transcription factor *OsIRO2*. Indeed, under Fe deficiency,*OsIRO2* was up-regulated and seemed to correlate with the induction of those genes involved in PS synthesis and transport (Hindt and Guerinot, 2012). Furthermore, other genes like *OsIDEF1* and 2 (iron

deficiency-responsive element 1 and 2) encode for positive regulators of Fe starvation (Kobayashi and Nishizawa, 2012).

Figure 5. Model for root responses to iron deficiency in *graminaceous* species (*Strategy II*). (Adapted from Römheld and Marschner, 1984).

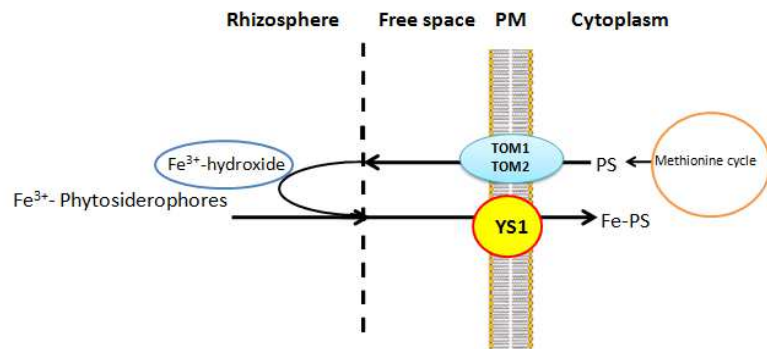# Copper nutrition in plants

Copper (Cu) is an essential micronutrient involved in many physiological processes of biological relevance such as photosynthesis and mitochondrial chain respiration but also in the hormone perception and in the oxidative stress responses (Himelblau *et al.,* 2000). Copper is a transition metal which can change its oxidative state (Cu^{2+}/Cu). Its multiple oxidation states explain the importance of Cu in biological redox systems (Arora *et al.,* 2002) and determines its versatile functions.

Plant Cu levels ranging from 2 to 50 µg g^{-1} DW (ppm) with 6 µg g^{-1} considerates as adequate in the shoots (Burkhead *et al.,* 2009). However, the amount of Cu contained in healthy plants varies considerably within this range and depends both on the species and the Cu-feeding status (Cohu and Pilon, 2009). Copper has been described as poorly mobile from old to young tissue, especially under deficiency (Loneragan, 1981) and it accumulates mostly at the root level as the acidic polysaccharides have a very strong ability to complex Cu. Thus, plants require Cu as an essential micronutrient for normal growth and development and when this ion is not available plants develop specific deficiency symptoms; most of which affect young leaves and reproductive organs as Cu-deficient wheat plants developed small anthers and pollen grains were fewer in number and with less viability (Hauser and Morrison 1964; Marschner *et al.,* 1995). Moreover, Cu-deficient plants show a change in the expression of a series of genes and activation of morphological changes such as in root and leaf architecture, with a significant reduction of crop yield (Burkhead *et al.,* 2009).

Copper in soil

With an atomic weight of 63.5 g and density of 8.96 g cm^{-3}, Cu is among one of the oldest known metals and is the 25th most abundant element in the Earth's crustwith an average concentration of around 70 ppm (Hodgson, 1963). Copper is much more abundant in basaltic than granitic rocks and has a tendency to be excluded from carbonate rocks(Krauskopf, 1972). While the predominant minerals of Cu in the earth's crust are sulfides (largely in the +1 oxidativestate), the metallic form of Cu (calcite) is also common in reducing environments. However, upon exposure to conditions at the earth's surface, Cu^{1+} and Cu^0 are oxidized to the cupric (Cu^{2+}) oxidation state (McBride, 1981). In aerobic soil environments, the primary sulfide minerals are dissolved by weathering process. The dissolution of chalcopyrite can be written as follows:

$$4\ CuFeS_2 + 17\ O_2 + 10H_2O \dashrightarrow 4\ Cu^{2+} + 4\ Fe\ (OH)_3 + 8\ SO_4^{2-} + 8\ H^+$$
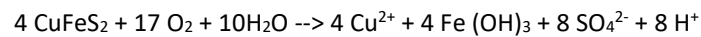
This element is also present in the crystal structure of many minerals including oxides, carbonates, silicates, sulphates and chlorides (tenorite, malachite, azurite, atacamite). However, it also occurs in available forms for plants absorption: strongly bound to humic substances, adsorbed on solid surfaces and in limited quantities, in solution (Fernandes and Henriques, 1991;Harter, 1991; McBride, 1981). Plant roots are usually exposed to a variable but adequate availability of Cu in the soil, since typically the concentration of Cu in the soil solution ranges from 10^{-9} to 10^{-6}M (Marschner, 2012). Copper solubility in soils is greatly dependent on soil pH and dissolved organic matter (DOM) content (Bravin$et\ al.,$ 2012) and becomes readily unavailable with increasing pH. With a lowering pH, amount of dissolved Cu increases and the free Cu ion activity is higher (Adriano, 2001; Brun$et\ al.,$ 2001). Additionally, with increasing pH, competitive adsorption arises between organic matter in the soil phase and DOM, generally leading to an increase in Cu concentration in the soil solution due to a higher dissolved organic carbon content (Carrillo-González and González-Chávez, 2006). Thus, upon increasing pH, Cu ion activity considerably decreases at the expense of organically bound complex species in the soil solution (Sauvé $et\ al.,$ 1997). When the pH approaches8, the overall solubility of cupric ions (Cu^{2+}) decreases to a minimum. At the same time, the formation of carbonate and anionic hydroxyl complexes becomes important (Figure 6). Thus, it has been suggested that a major inorganic form of complexed Cu(II) in neutral and alkaline soil solution is CuCO$_3^0$ (Sanders and Bloomfield, 1980; Sposito and Bingham, 1981).
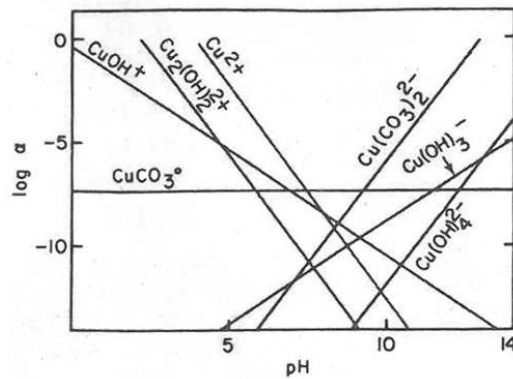
Figure 6. Activities of cupric ions (Cu^{2+}) species in equilibrium with Cu oxide/hydroxides and carbonates as function of pH (25°C, ionic strength=0, $logP_{CO2}$=-3.52; Adapted from Schindler, 1967).

The solubility of Cu^{2+}in soils, as expressed by total dissolved Cu rather than uncomplexed Cu^{2+}, approaches a minimum near pH 7 and increases above this pH. However, a number of studies have produced evidence that the natural cupric ions in soil solutions at higher pH does not exist as inorganic complexes but is largely complexed with soluble organics (Hodgson *et al.*, 1965; Marschner, 2012). It is evident that most of the Cu in soils is highly insoluble and can only be extracted by strong chemical treatments which dissolve various mineral structures or solubilize organic matter. Nevertheless, a significant pool of diffusible Cu exists, probably in organic complexes, which is in equilibrium with the very low level of free Cu in soil solution. Most of the soluble Cu in surface soils is organically complexed, a fact which causes the total Cu in soil solution to be greater than expected if soluble organics were not present (Carrillo-González and González-Chávez, 2006).

Literature reported contradictory results concerning the effect of pH on Cu uptake by plants. In very acidic soils, plant Cu concentration increased compared to calcareous soils in rape (*Brassica napus L.*) and tomato (*Solanum lycopersicum*)(Chaignon *et al.,*2002; Cornu *et al.,*2007). Conversely, Cu accumulation in maize was as high in calcareous soils as in acid soils (Brun*et al.,*2001) and, according to Zhao *et al.,* (2006) increasing soil pH may even increase Cu toxicity at a given free-Cu^{2+} activity. This suggestion lead to the apparent paradox of increasing Cu toxicity with increasing pH, presumably due to decreased competition between proton and Cu for adsorption onto root cell walls.

Root and microbialexudatesand synthetic chelators affect Cu availability
The activities of plants into the rhizosphereare mainly related with the release of root exudates, which include inorganic and organic compounds with low and high molecular weight, such as protons, carbohydrates, organic acids, amino acids, PS, phenolic compounds, mucilage and enzymes (Dakora and Phillips, 2002). Furthermore, root exudates, especially those of low molecular weight, can be used by microorganisms as a readily accessible source of carbon and energy in the rhizosphere, where the concentration of such compounds is higher than that of bulk soil (Hinsinger *et al.,* 2009). Regarding Cu biogeochemical cycles in the soil, it is interesting to note that the bioavailable fractions of Cu are considerably influenced by biological activities of the root, principally the release of exudates and in

particular of low-molecular-weightorganiccompounds (Brunetto *et al.,* 2016). This phenomenon has been mainly studied for its potentialities to modulate microbial growth, mobilization of poorly soluble nutrients and detoxify harmful heavy metals (Dakora and Phillips, 2002; Pii *et al.,* 2015).Regarding the latter case, the exudates, mainly organic and phenolic acids, are designed to chelate heavy metals, such as Cu, in the rhizosphere or in the apoplast, preventing plasma membrane transport and accumulation into the cytosol of root cells (Kochian *et al.,* 2004).

Therefore, the qualitative and quantitative composition of root exudates can play a fundamental role in alleviating the toxicity of Cu in plants. Low-molecular-weight organic compounds may be involved in both external and internal tolerance mechanisms. Root exudates released by plants might have a role in immobilizing and decreasing the bioavailability of toxic metals; this mechanism might be further influenced by the activity of rhizosphere microorganisms (Leyval *et al.,* 1997).The effect of ectomycorrhizae on metal uptake by trees has been reviewed by Wilkins (1991). He concluded that mycorrhizae can reduce concentrations of Zn, Ni and Cu in shoot tissues.Bacteria may also interact with plant roots and affect their growth in a variety of ways.Ma *et al.* (2009)show that *Achromobacter xylosoxidans* protects plant of *Brassica juncea* from Cu toxicity and also enhances the Cu accumulation in plant tissue with concurrent stimulation of plant growth.

Phytoremediation is a promising approach for cleaning up soils contaminated with heavy metals using hyperaccumulating plants (Jiang *et al.,* 2004). In plants, the mechanism of hyperaccumulation is defined by an extracellular and intracellular metal chelation with natural compounds, translocation of chelated heavy metals through the vascular system and compartmentalization of these metals into shoots and leaves (Raskin *et al.,* 1994). More than 200 plant species are known to hyperaccumulate heavy metals, but their biomass production is generally considered too low to make these plants feasible candidates for soil remediation.Synthetic chelators, e.g. ethylenediamine tetraacetate (EDTA), have been used to artificially enhance heavy metals solubility in soil solution from the soil solid phase and thus to increase heavy metal phytoavailability. The use of chelators is especially important for induced phytoextraction of Cu, since in general the Cu concentration of plants is more internally rather than externally regulated (Edebali *et al.,* 2016).
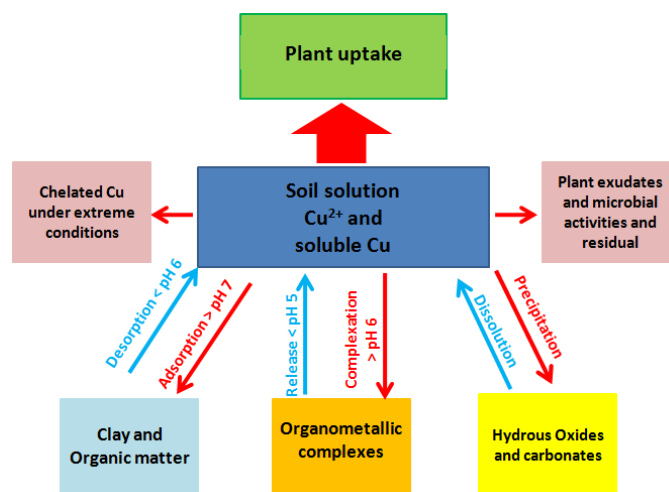
Figure 7. Schematic distribution of Cu between solid phase and soil solution depends on precipitation/dissolution, adsorption/desorption and redox reactions.

Copperdeficiency

Symptoms of Cu deficiency are observed in plants grown in soils with low nutrient availability such as calcareous and ferruginous soils or soils with a high content of organic substances (Allowayand Tills, 1984). It could also occur in acid sandy and humus podzols and can be also provokedby the application of nitrogen fertilizer. Copper deficiency in plant tissues is assessed in <3-5 µg ofCug^{-1}dry weight and is highly dependent on the species, the type of organ, the stage of development and the availability of nitrogen (Thiel and Finck, 1973; Reuter and Robinson, 1997; Adress*et al.,* 2015). Wheat and rice, two major food crops, are both highly sensitive to Cu deficiency, however several other species can be affected if grown in condition of low available Cu (Table 1) (Graham, 1979; Follet, *et. al.,* 1981;Loneragan *et al.,* 1981).

Typical symptomsofCudeficiency includestunted growth, leafdeformation, necrosisofapical meristemsandchlorosistheyoung leaves(Rahimi and Bussler, 1973). In particular, symptoms of Cudeficiency in maize (*Zea mays*)are related with bluish-green leaves which become chlorotic near the tips, chlorosis develops downward along both sides of the midrib, followed by dark brown necrosis of the tips. Usually new leaves fail to unroll and maintain a needlelike appearance of the entire leaf, or occasionally of the half leaf, with the basal portion developing normally (Alloway and Tills, 1984).Instead, in tomato (*Solanum lycopersicum*) plants,Cu deficiency is related with stunted growth of shoots, poor root development, dark-bluish green foliage, curling of leaves, and no-flower formation (Sommer, 1931; Bailey and McHargue, 1943; Follet*et. al.,* 1981).Secondary symptoms of deficiency are generally caused by necrosis of the meristematic apexes and are the lack of tillering in cereals and the withering foliage that is interpreted as the result of decreased flow in xylem due to a poor lignification of the vasculature (Rahimi and Bussler, 1973;Pissarek, 1974)and the collapse of the phloem walls (Graham, 1979).Coppershortage has also a serious effect on embryonic development, the pollen viability and the production of seeds and fruits(Burkhead*et al.,*2009). Failure to produce seeds may be caused by

a lack of sufficient photosynthetic production or translocation, or to the absence of fertilized embryos (Graham, 1975; Dell, 1981).

HIGH SENSITIVITY CROPS		
Weat	Lettuce	Onion
Rice	Carrot	Citrus fruits
Oats	Table beet	Sudan grass
Lucerne	Spinach	
MEDIUM SENSITIVITY CROPS		
Barley	Cloves	Tomato
Maize	Cucumber	Pome and stone fruits
Broccoli	Parsnips	Vines
Cabbage	Radish	Sorghum
Cauliflower	Sugar beet	Pineapples
Celery	Turnip	Tung-oil
LOW SENSITIVITY CROPS		
Asparagus	Rye	Rape
Beans	Pasture grasses	Lupins
Peas	Soybeans	
Potatoes		

Table1. The crop sensitivity to copper deficiency (from: Follet, *et. al.,* 1981; Graham, 1979).

Figure8. Left panel: In the left part of the field, highly chlorotic barley plants due to severe Cu deficiency; right part, mature barley on peat soil after Cu fertilization. **Right panel:** Cu-deficiency symptoms in barley.
Both pictures are provided courtesy of the International Plant Nutrition Institute (IPNI) and its IPNI Crop Nutrient Deficiency Image Collection.

Copper toxicity

In recent years, Cu pollution in agricultural soils, due to heavy use of pesticides, fungicides, industrial effluent and wastewater irrigation, presents a major concern for a sustainable agri-food production especially in developing countries (Adress *et al.,* 2015).In viticulture, Cu-based fungicides are used at typical applications of 2–4 kg Cu ha^{-1} year^{-1} , leading to Cu soil concentrations that may reach values higher than 3g Cu kg^{-1} of soil, which is over the concentration range tolerable for most crops and thus toxic for plant growth (Alloway, 2013).

Generally, total Cu content is highest in soils derived from basic and intermediate rocks and increases with depth in the soil (McBride, 1981). Phytotoxicity of Cu depends upon the metal solubility and availability in the soil. For most agricultural species, the Cu toxicity is evaluated in 20-50 µg Cu g^{-1} dry weight (Hodenberg and Finck, 1975; Robson and Reuter, 1981), however the response of various crops to elevated Cu concentrations depends upon crop species and cultivars. Wheat and sorghum (*Sorghum bicolor L.*) showed more sensitivity to Cu toxicity as compared to maize plants and indicated decreasing trend towards increasing Cu excess. Işeri *et al.* (2011) have shown that Cu was more toxic to cucumber (*Cucumis sativus L.*) roots as compared to tomato at the same Cu treatment. Toxic effects of Cu in plants can be observed as reduced yield, poor seed germination, stunted leaf and root growth, and ultrastructural and anatomical alterations leading to the formation of reactive oxygen species (ROS).

In plants, the uptake of other nutrients seems to be altered by the Cu excess. Cu toxicity appear to induce Fe deficiency which depends on the Cu-form available for the plant (Rahimi and Bussler, 1973Woolhause, 1983). The evidence of this condition is the chlorosis which is the result of the increase of peroxidase activity as well as the destruction of the membranes of thylakoids (Maksymiec, 1998). Plants that receive a large Cu intake show an accumulation of the metal in the roots, proportional to the content in the circulating solution, even if the translocation to the leaf apparatus remains extremely low (Adress *et al.,* 2015).

The mechanisms that plants could implement in Cu excess condition are the immobilization of Cu in the roots, the reduction of its availability by complexing it with root exudates, the limitation of the influx through the PM, the stimulation of the outflow from the cytoplasm involving Heavy-Metal ATPases (HMA)proteins, the compartmentalization into the vacuole and the chelation in the interface between the cell wall and the PM (Burkhead*et al.,* 2009; Yurela, 2009).

Figure 9. Examples of symptoms of Cu excess in the field. **Left panel:**spot of stunted-growth wheat where the tank for fungicide treatment was filled with Cu-salts (Michaud *et al.,* 2007).**Right panel:** interveinal chlorosis symptoms have been observed in durum wheat along rows across the field (Adress *et al.,* 2015).

Copper acquisition and response to Cu supply

Although several transporters of Cu in roots have been characterized in the last decades (Sancenón *et al.,* 2003;Wintz *et al.,* 2003; De Freitas *et al.,* 2003), up to now the molecular mechanisms involved in the overall acquisition (mobilization, probably reduction and uptake) by roots are still unclear. Several advances in understanding the regulation of Cu homeostasis have been obtained from studies where plants were subjected to Cu deficiency. Two different strategies of uptake are suggested under Cu shortage: increasing Cu acquisition by activating high-affinity Cu transporters and the increase of the synthesis and the release of compounds that are able to chelate the element into the nutrient solution (Himelblau *et al.,* 1998; Abdel-Ghany and Pilon, 2008). Indeed, a strong overlap between Fe and Cu uptake mechanisms has been suggested (Ryan *et al.,* 2013) as the mechanisms of Cu and Fe transmembrane transport into root cells share the limitation of forms that can be taken up by roots (Adress *et al.,* 2015;Brunetto *et al.,* 2016).

In dicotyledons and non-*graminaceous* monocotyledons - referred to *Strategy I* plants, when Fe acquisition mechanisms are considered - Fe-chelate reductase proteins (FRO) might be capable of donating electrons to other transition metals besides Fe (III), including Cu (II, III, IV) (Marschner *et al.,* 1982; Cohen *et al.,* 1997). Lesuisse and Labbe (1992) reported that the ferric-reductase of the yeast, *Saccharomyces cerevisiae,* could reduce Cu^{2+} to Cu^+. Welch *et al.* (1993) have hypothesized that this electron-transport system does not only reduce Fe^{3+} to Fe^{2+} but also can reduce Cu^{2+} to Cu^+. In particular, it hasbeen shownthat, in pea roots, either Fe deficiency or Cu deficiency increases the activity of the inducible reductase system(s) which reduce(s) either chelated Fe^{3+} and Cu^{2+} (Cohen *et al.,* 1997). Analysis of isotopic fractionation of Cu uptake inside the roots indicates the preferential uptake of the light Cu isotope. Such observation has supported the idea that Cu (II) reduction to Cu (I) occurs at the root level (Bernal *et al.,* 2012; Jouvin *et al.,* 2012; Ryan *et al.,* 2013). This role could be accomplished either by specific PM proteins, PM bound Cu-chelate reductases or by the aspecific activity of Fe-chelate reductases. Studies of FRO2 in *Arabidopsis thaliana* have suggested that it may have a role in the reduction of Cu^{2+} to Cu^+ in addition to its role in Fe reduction (Yi and Guerinot, 1996; Robinson *et al.,* 1999) and *FRO3* is upregulated during Cu deficiency (Burkhead *et al.*, 2009; Palmer and Guerinot, 2009). Bernal *et al.*(2012) have shown that FRO4 and FRO5 act redundantly to reduce Cu at the root surface. Other proteins have been shown to be involved in the transport of Cu across membranes; Fe transporter (IRT1) can also mediate the uptake of Cu, Cd, Co and Zn (Korshunova *et al.,*1999) and some HMA transporters can transport either Cu^{2+} or Cu^+(Pilon and Tapken*,* 2009).

It is also possible that Cu uptake may proceed without prior reduction of $Cu^{2+}$to Cu^+, perhaps via a ZIP-type transporter. Interestingly, expression of ZIP2 and ZIP4 is upregulated under Cu limitation (Wintz *et al.,* 2003). The role of the ZIP transporters in Cu(II) transport remains, however, controversial since none of these transporters rescue the Cu uptake deficiency of Cu uptake deficient *ctr1/ctr3* yeast mutants (Milner *etal.,* 2013).

Copper, in the free-ion Cu^+ form, can be taken up into the plant through high-affinity Cu^+ transporters like COPT (Copper Transporter protein) family which has been identified in plants by sequence homology with the eukaryotic Cu transporters named *Ctr* or by functional complementation in yeast (Labbé and Thiele, 1999; Harris 2000; Puig and Thiele 2002; Puig *et al.*, 2007). In *A. thaliana*, the COPT family has six members while in grapevine up to eight members have been identified (Puig *et al.,* 2002). COPT1 is PM-localized, and the gene encoding it is highly expressed in root tips. COPT1 is thought to make the predominant contribution to root Cu uptake as its antisense-silencing results in a decrease in Cu levels by 40-60% (Sancenón *et al.,* 2004).

COPT2 is found in both green tissues and in some parts of the root tissue. Perea-Garcia *et al.,* (2013) suggested the implication of COPT2 in the Cu uptake, and it might be also involvedin phosphate sensing and Fe homeostasis. COPT6is primarily expressed in shoots and may have a role in Cu distribution from green tissues to reproductive organs (Garcia-Molina *et al.,* 2013).
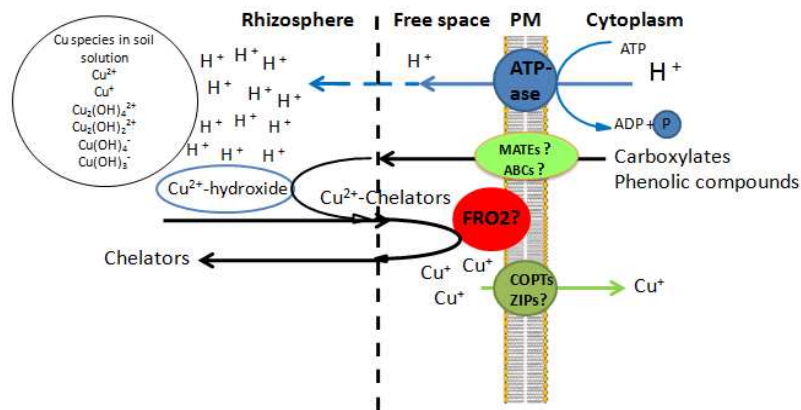
Figure 10. A conceptual model of the uptake mechanisms of Cu in (Fe)*Strategy I* plants

In grass species - Fe *Strategy-II* plants - Cu might be taken up as Cu-PS, via yellow stripe-like (YSL) members (Wintz *et al.,* 2003; Marschner, 2012). Schenkeveld *et al.* (2014) investigated metal mobilization by PS and found that Cu was quantitatively the most important elements competing with Fe for complexation by DMA.Chaignon *et al.* (2002) observed that under Fe starvation the increase level of both PS release and the accumulation of Cu occurred inwheat grown on a Cu-contaminated soil.*In Arabidopsis,* at least 8YSLproteins seem to be involved mainly in the allocation to the shoot and not in the uptake of this micronutrient (Curie *et al.,* 2001*;* Wintz *et al.,* 2003;). However, some evidence shown that plants couldreleasein the rhizosphere alarge number of exudates (citrate, malate, oxalate) which bind Cu thanks to their high affinity with heavy metals (Jones and Darrah, 1994;Kochian *et al.,* 2004).
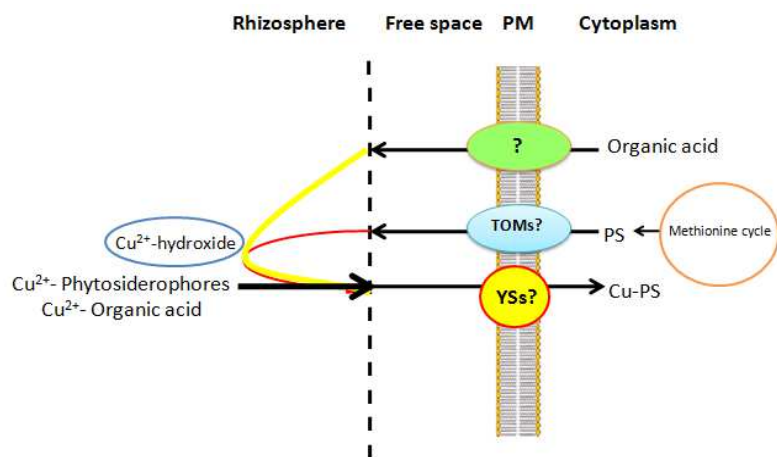
Figure11. Figure. A conceptual model of the uptake of Cu in (Fe)*Strategy II* plants.

Concerning the Cu translocation within plant tissues, Cu seems to be transported exclusively in complexed form (Graham, 1979), most likely with organic nitrogen ligands, as for instance amino acids (Kochian, 1991) and nicotianamine. In fact, Cu has a high affinity for peptide and sulfhydryl groups, as well as for carboxylic and phenolic groups. The internal distribution of Cu is carried out by metallo chaperones, low-molecular-weight proteins which form weak bonds with Cu ions(Huffman and O'Halloran, 2001).

Concerning regulation of Cu acquisition, recently, specific information on transcriptional factors involved in Cu response regulation became available. SQUAMOSA PROMOTER BINDING PROTEINS (*SBP*) constitute a transcription factor family exclusively found in green plants(Yamasaki *et al.,* 2009). Despite evolutionary divergence between the different family members, the tertiary structure of all *SBP* proteins encompasses the founding SBP-domain. It consists of a 76-amino-acid signature including a functional bipartite nuclear localization signal and a series of 8conserved cysteine and histidine residues organized in two unconventional zinc fingers (*ZF1* and *ZF2*)(Wintz *et al.,* 2003; Birkenbihl *et al.,* 2005). The *SPL7* (SBP-like7) transcription factor functions as a master regulator ofthe Cu-deficiency response in *Arabidopsis* (Yamasaki *et al.,* 2009). Recently, RNA-Seq revealed that *FRO4* and *FRO5* are strongly upregulated in roots under Cu limitation. In addition, induction of *FRO4* and *FRO5* in roots under Cu

limitation depends on the activity of SPL7 (Bernal *et al.,* 2012). *FRO4* and *FRO5* lie in tandem on chromosome 5 and share high sequence similarity at the amino acid level (Wilson,2014). *SPL7* was shown to be constitutively expressed in plants – although mainly in roots – independently of soil Cu availability. Consequently, the regulation of its activity should occur at the post-transcriptional level (Yamasaki *et al.,* 2009; Garcia-Molina *et al.,*2014). Immunolocalization of *SPL7* and an endoplasmic reticulum (ER)-marker revealed the possible dual subcellular localization of *SPL7* in both the nucleus and the ER (Garcia-Molina *et al.,* 2014). *SPL7* has furthermore been demonstrated to physically interact with ELONGATED HYPOCOTYL5 (*HY5*) which encodes a bZIP-type transcription factor that functions downstream of multiple photoreceptors to promote photomorphogenesis (Binkert *et al.,* 2014).

Copper and Iron interaction: evolutionary linking

There is a strong overlapping between Fe and Cu roles in cell metabolism. The colonization of earth by photosynthetic organisms has driven a progressive accumulation of oxygen in the environment. This oxidative atmosphere led to a decreased solubility of Fe by the formation of Fe oxides and to the progressive liberation of soluble Cu (II) from insoluble Cu sulfide salts (Burkhead *et al.,* 2009). Since then, Fe in biological molecules has been progressively substituted by Cu which is able to perform similar functions. The best-adapted organisms developed new strategies to solubilize and acquire Fe^{3+}, but they also replaced Fe by Cu in multiple processes requiring higher redox potentials. Copper proteins are, therefore, a more recent biochemical achievement that coincides with the appearance of multicellular organisms (Puig *et al.,* 2007). This may explain why many Cu-proteins have a functional counterpart that uses Fe as cofactor and why growth on a substrate with a toxic Cu level is commonly linked to a decreased Fe-content in roots and leaves (Burkhead *et al.,* 2009; Festa and Thiele, 2011). Consequently, plant phenotypes associated with Cu toxicity share similarities with those related to Fe-deficiency, such as the presence of leaf chlorosis, decreased leaf chlorophyll content and enhanced oxidative stress (Pätsikkä *et al.,* 2002).

Biochemical functions of copper

Copperis required in biological systems as a structural component and catalytical enzyme as a cofactor of proteins which have a considerable biological importance. The majority of this metal is bound to plastocyanin (usually more than 50%) which is involved in the electron transport process associated with photosynthesis, however, Cu is also associated with other proteins with different functions such as in the reduction of oxygen to water (*cytochrome oxidase*), the oxidation of phenols (*monophenoloxidase*), the degradation of amines and polyamides (*polyamineoxidase*), and also detoxification of ROS(*superoxide dismutase*) (Yurela, 2009).

Reactive oxygen species are produced in both unstressed and stressed cells. A direct effect of excess Cu in plants at the cellular level is oxidative stress with the increase of ROSproduction, whichmay damage nucleic acids, compromise the permeability of membranes due tothe peroxidation of lipids, and affectimportant cellular processes such as the photosynthesis, the mitochondrial respiration and other cellular mechanisms(Figure 12) (Azooz *et al.*, 2012; Adress *et al.,* 2015). Plant response to oxidative stress also depends upon species and cultivars (Yurela, 2009; Pantola and Shekhawat, 2012; Adress *et al.,* 2015); and,in general,development and growth which are strongly inhibited and accompanied bymorphological, anatomical and physiological changes (Bertrand and Poirier 2005; Yurela 2009). To counteract the oxidative stress, enzymes such as superoxide dismutase (SOD), peroxidase (POD)and catalase (CAT) are essential for their ability to convert the ROS species into less reactive molecules, preventing their effects at the cellular level (Drazkiewicz *et al.,* 2003; Meng *et al.,* 2007; Liu *et al.,* 2014).Indeed, the SODs are the first mechanism of defense against ROS. Based on the metal co-factor, SODs enzymes are classified into three groups that show different compartment locations in the cell (Alscher *et al.,* 2002).
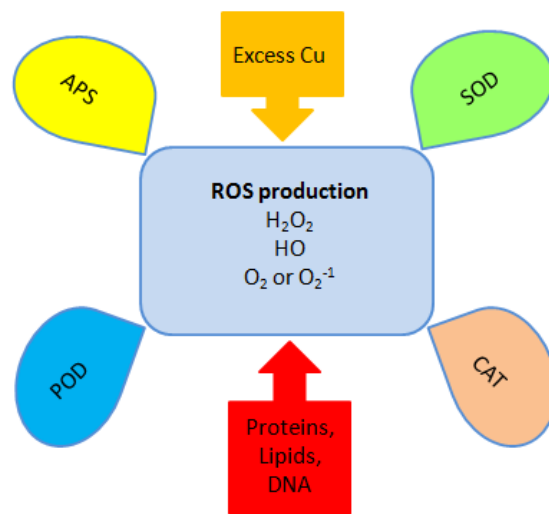
Figure 12. Exposure to excess Cu causesthe increase of ROS production which may be scavengedby antioxidant enzymes, such as catalases (CAT), superoxide dismutases (SOD), ascorbate peroxidases (APX) and peroxidases (POD), adapted from Adress *et al.,* 2015).

Superoxide dismutase proteins

Superoxide dismutases are a group of isozymes functioning as superoxide radical scavenger in the living organisms. They catalyze the disproportionation of superoxide radicals to hydrogen peroxide and dioxygen:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$
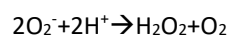
Superoxide dismutases were originally discovered by McCord and Fridovich in 1969. An impressive property of this enzyme is its ability to react with superoxide radicals at rates limited only by diffusion and enhanced by electrostatic guidance (Getzoff *et al.,* 1992), providing what must be a highly effective mean of removing superoxide radicals. Furthermore, SOD arelikely to play an important role in cellular defense against oxidative stress because theiractivity directly modulates the amounts of O_2^- and H_2O_2, the two Haber-Weiss reaction substrates (Van Camp *et al.,* 1996).

In plants, there are some evidence that SOD overexpression can protect plants against adverse environmental conditions (Bowler *at al.,* 1992; Gupta *et al.,* 1993), and their presence in all aerobic organisms and everysubcellular compartment where oxidative stress is likely to arise can be indicated as a proof of their importance (Bannister *at al.,* 1987; Fridovich, 1989).

As previously said, theROS production increase under biotic and abiotic stresses (Gupta *et al.,* 2015; Choudhry *et al.,* 2017) andseveral studies have shown the change of SOD activity under nutrient shortage which seems to depend mostly to the plant species and the kind ofnutrient deficiency. In bean, leaves Mg deficiency inducesanincrease of Cu-SOD activity (Cakmak and Marschner, 1992), while in leaves of different wheat cultivars, Zndeficiency decreased SOD activity (Cakmak *et al.,* 1997). Moreover, if Fe deficiency induces contrasting response of SODs activity in rapeseedand wheat (Tewari *et al.,* 2003; Agarwal *et al.,* 2006), in lupinCu deficiency significantly decreased Mn-SOD and CuZn-SOD activities (Yu *et al.,* 1999).

Distribution of SODs in plants

At least, three isoforms of the enzyme exist, as classified by the metal ions present at the active site: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD). These different isoenzymes are distributed throughout different subcellular locations, presumably because O_2^- cannot cross membranes (Takahashi and Asada, 1983) and therefore must be detoxifying at its sites of production.

The Fe-SOD is a dimeric enzyme consisting of two identical subunits. In contrast to animals, where it is absent, it has now been found in several plant species and Fe speciation studies have indicated that, when it is present, it is located in the chloroplast (Alscher *et al.,* 2002).

Mn-SOD has been more thoroughly studied than Fe-SOD and appears to be found in the mitochondrial matrix of all plants so far subjected to detailed analysis, including maize (Baum and Scanalios, 1979), *Brassica campestris* (Salin and Bridges, 1981), tobacco (Bowler *et al.,* 1991), *Glycine max* (Puppo *et al.,* 1987), *Vigna radiata* (Reddy and Venkaiah 1982, 1984).

Plant Cu/Zn-SOD, like its counterparts in other eukaryotes, is a dimeric enzyme consisting of two identical subunits. Of the three different plant SODs, it is the one studied most intensively and has been found in many cellular locations. It seems probable that Cu/Zn-SOD is always present in the cytosol of plants because Mn-SOD and Fe-SOD appear to be organellar specific. A cytosolic location has been observed in maize (Scandalios, 1990), *N. plumbaginifolia*(Tsang *et al.,* 1991), tomato (Kwiatowski and Kaniuga, 1986), rice (Kanematsu and Asada, 1989) and spinach (Lumsden and Hall, 1974).
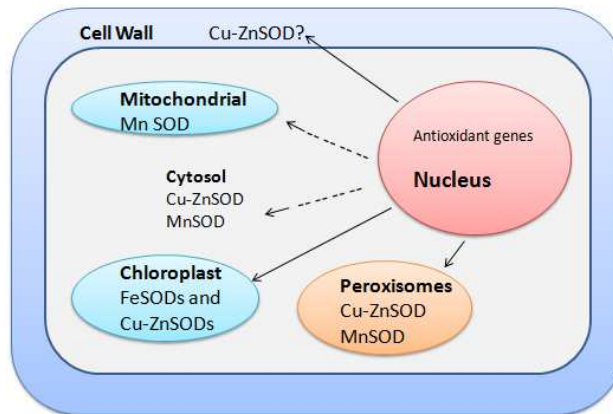
Figure 13. Locations of SODs throughout the plant cell (adapt form Alsher *et al.,* 2002)

Catalase proteins

Catalase is an antioxidant enzyme present in all aerobic organisms. It is present in all living organisms, ranging from unicellular prokaryotes to multicellular eukaryotes. The evolutionary design of protein catalysts started about 3.5 billion years ago when the ancestral planktonic bacteria began aerobic respiration (Sharma, 2003). As an enzymatic antioxidant, catalase has a key role to prevent cellular oxidative damage by efficiently degrading hydrogen peroxide (H_2O_2) into water and oxygen (Ahmad *et al.,* 2011). In plants, catalase scavenges H_2O_2 generated during mitochondrial electron transport, oxidation of fatty acids, and most important, photorespiratory oxidation either innormal or stressed conditions. Due to its efficient catalytic and regulatory properties among all antioxidant enzymes of the plant system, catalase has been purified and extensively characterized at the genomic, biochemical, and molecular level inplants. Recently, it has been established that catalase is present as multiple isoforms (CAT1, CAT2, CAT3) encoded by multiple genes (*Cat1*, *Cat2*, *Cat3*) expressed in organelles with temporal and stress specific manners(Sharma and Ahmad, 2014).

Although much effort has been made to study catalase among prokaryotes and animals, very little information is available on the activities of catalase in plants.

Oxidases and the role of ascorbate peroxidase

Oxidases are enzymes that reduce molecular oxygen to water according to the following reaction:

$$2AH_2 + O_2 \rightarrow 2A + 2H_2O$$
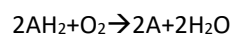
They are enzymes that contain several metals as cofactors and among them the most important are: cytochrome oxidase, ascorbate peroxidase (APX) and polyphenol oxidase. Ascorbate peroxidase has

been identified in many higher plants and comprises a family of isoenzymes with different characteristics. Ascorbate peroxidase has at least four atoms of Cu and catalyzes the reaction:

$$2 \text{ ascorbic acid} + O_2 \longleftrightarrow 2 \text{ monodehydroascorbate} + 2H_2O$$
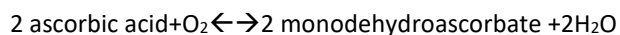
It is present in the cell wall and in the cytoplasm; its activity is also used as a diagnostic test for evaluating the degree of Cu deficiency. Ascorbate peroxidase utilizes ascorbate (AsA) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDAsA), a univalent oxidant of AsA. Monodehydroascorbate is also directly reduced to AsA by the action of NAD(P)H-dependent MDAsA reductase. Dehydroascorbate (DAsA) reductase utilizes glutathione (GSH) to reduce DAsA and thereby regenerate AsA. The oxidized GSH is then regenerated by GSH reductase, utilizing reducing equivalents from NAD(P)H. Thus, APXrole, in combination with the effective AsA–GSH cycle,is to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms (Ushimaru *et al.*, 1997; Shigeoka *et al.,* 2002). Ascorbate peroxidaseisoenzymes are distributed in at least four distinct cellular compartments: stromal APX and membrane-bound thylakoid APX, microbody (including glyoxysome and peroxisome) membrane-bound APX, and cytosolic APX (Ishikawa *et al*., 1998; Shigeoka *et al.,* 2002).
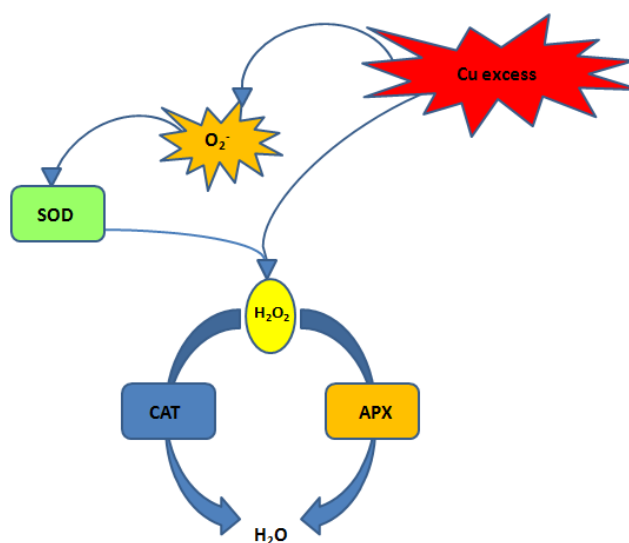
Figure 14. Mechanisms of antioxidant defense by different enzymes in plants.SODs catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 in all subcellular compartments. CAT and APX dismutases H_2O_2 in peroxisomes, glyoxysomes and related organelles (Garg and Manchanda 2009; Gill and Tuteja 2010).

2.OBJECTIVES

The aim of the present PhD thesis is to investigate the mechanisms involved in the acquisition of Fe and Cu into rootsand to unravel possible links between the effects of Fe and Cu availability and forms on the growth and development of crops.

In the first step, through a morphometric approach, the different effects of Cu imbalance were analyzed in maize and tomato evaluating parameters of plant growth and development. Next, in maize, which exhibited a higherCu-excess tolerance capability, several approaches have been carried on providing different sources and amounts of Cu and Fein order to clarifywhich are the mechanisms used by *graminaceous* plants to acquire Cu and itspossible interactions with the uptake of Fe. Moreover, Fe and Cu deficiencies wereinvestigated in tomato plants with the purpose of improving the knowledge on the physiological and molecular aspects of the response to these nutritional disorders.

During this PhD work, enzymes' activity related to the antioxidant system in the plants(SODs, CAT, APX) as well as nutrient content and acquisitionmechanisms were examined. Finally, in order to improve the knowledge about local and systemic signals involved in the regulation of the Fe and Cu status in *Strategy I* plants, the responses of two different genotypes of melon (EDISTO and *fefe*) were investigated *via*a split-root technique.

3.Overview of copper sensitivity by crops: a comparison between maize and tomato

Introduction

In plants, copper(Cu) is a micronutrient required for normal healthy growth and reproduction. Copper deficiency is assessed in <3-5 µg Cug^{-1}dry weight in plant tissues, while the toxicity is evaluated in >20-50 µg Cu g^{-1} dry weight; both states are highly dependent on the plant species, the type of organ considered, the stage of development and the availability of nitrogen (Thiel and Finck, 1973; Adress *et al.,* 2015)

Copper solubility in soil is highly dependent on soil pH (Bravin*et al.,* 2012).At acidic pH, dissolved Cu increases because of its weaker adsorptiononcolloid and organic matter surfaces, and the free-Cu ion activity is higher (Sauvé *et al.,* 1997; Adriano, 2001; Brun*et al.,* 2001).Copper deficiency is a very rare nutritional disorder and it occurs on calcareous soils, in which Cu is poorly available due to its poor solubility at high pH (Thiel and Finck, 1973;Adriano, 2001).Symptoms of Cudeficiency include stunted growth, leaf deformation, necrosis of apical meristems and chlorosis of the young leaves (RahimiandBussler,1973;Alloway and Tills, 1984; Adress*et al.,* 2015;).

Starting from the end of 19th century, Cu-based fungicide treatments (e.g.Bordeaux mixture: Ca(OH)$_2$+CuSO$_4$) have been widely applied to crops and in particular to vineyards to prevent and treat diseases, such asvine downy mildew (Chopin *et al.,* 2008). The extensive use in agriculture of Cu-containing agrochemicalshas contributed to the increase of Cu contamination in agricultural soils determining, in some cases, Cu toxicity in crops (Brun*et al.,* 2001;Schramel*et al.,* 2000; Chaignon*et al.,* 2003) with negative impacts on economics and environments.Elevated Cu concentrations, as free ions, can lead to great environmental problem by accumulating and contaminating soils, vegetation, animals or ground- and surface waters (Jung and Thornton, 1996; Chopin and Alloway, 2007). Indeed, typical symptoms of Cu excess on plants include stunted growth, chlorosis and senescence of leaves, and cracking of the root cell membranes (Kopittke and Menzies, 2006; Michaud *et al.,*2008). Several studies have shown that plant phenotypes associated with Cu toxicity share similarities with those related to Fe-deficiency, such as decreased leaf chlorophyll content and enhanced oxidative stresses(Pätsikkä *et al.,* 2002; Schaaf *et al.,* 2003; Grotz and Guerinot*,* 2012).

Maize (*Zea mays*L.) isone of the most widespread cultivated cereals, both for human and animal consumption. Symptoms of Cu deficiency in maize are bluish-green leaves which become chlorotic near the tips, chlorosis develops downward along both sides of the midrib (Rahimi and Bussler, 1973, Alloway and Tills, 1984). Also, new leaves fail to unroll and maintain a needlelike appearance of the entire leaf, or occasionally of half the leaf, with the basal portion developing normally (Alloway and Tills, 1984). Higher Cu concentrationsalter plant morphology, root and shoot elongation (Jiang *et al.,*2008). Furthermore, among the *graminaceous* species, wheat and sorghum showed more sensitivity to Cu stress as compared to maize plants (An *et al.,* 2006).

Tomato (*Solanum lycopersicum*L.) is a *Strategy-I* plantsfor the Fe uptake with a medium sensitivity to Cu deficiency which isassociated with stunted shoot growth, poor root development, dark-bluish green

foliage, curling of leaves, and no flower formation. Follet *et al.*(1981) and Chaigon *et al.*(2002) have investigated the effect of pH changes and nitrogen supply on Cu bioavailability and was found out that in tomato plants are more sensitive to Cu toxicity in acid soils.

Numerous studies were conducted in order to evaluate the Cu sensitivity of these two crops. In general, both plant species seem to show similar responses to Cu limitation, while the plant responses to Cu toxicity remains uncharacterized andisdependent to the pH conditions.For example, application of high Cuconcentration (over 3 mM) in nutrient solution at pH 5 reduces the length ofthe roots and leaves in maize plants (Benimali*et al.,* 2010), while, in tomato Cu toxicityaffects elemental composition and plant growth dependingon the pH of nutrient solution (Işeri*et al.,* 2011).

Aim of this chapter was to characterize the Cu deficiency and toxicity response in maize and tomato plants. In order to clarify how Cu concentration can affect the plant growth and development, maize and tomato plants were grown under different Cu availability and physiological, morphological and morphometric data were analyzed. Moreover, the plant response to Cu availability was analyzed in detail comparing plant responses to a well-known nutritional disorder, Fe deficiency.

Materials and methods

Plant material and growth conditions

Maize seeds (*Zea mays L.,* inbred line P0423, Pioneer Hybrid Italia S.p.A.) were germinated over aerated 0.5mM $CaSO_4$ solution in a dark growth chamber at 25°C. Six-day-old seedlings were then transferred for 15 days in a continuously aerated nutrient solution containing, μM: $Ca(NO_3)_2$1000; $CaSO_4$ 500; $MgSO_4$ 100; KH_2PO_4 175; KCl 5; H_3BO_3 2.5; $MnSO_4$ 0.2; $ZnSO_4$0.2; Na_2MoO_4 0.05, Fe and Cu were added to nutrient solution depending on nutritional treatment. Therefore, plants were growth in a nutrient solution with (see also Table 1): lowFe and Cufree (-Fe -Cu: Fe-EDTA 10 μMand $CuSO_4$ 0.00 μM), low-Fe (-Fe +Cu: Fe-EDTA 10 μM and $CuSO_4$ 0.05 μM), Cufree (+Fe -Cu: Fe-EDTA 100 μMand $CuSO_4$ 0.00 μM), Cu-excess (+Fe ++Cu: Fe-EDTA 100 μM and $CuSO_4$ 100 μM) or under complete nutrient solution containing both Fe and Cu as control (+Fe+Cu condition: Fe-EDTA 100 μM, $CuSO_4$ 0.05 μM). Nutrient solutions were renewed every three days and buffered at pH 6.0 with 0.6 mM MES-KOH. The controlled climatic conditions to the growth chamber were the following: day/night photoperiod, 16/8 h; light intensity, 220 μE $m^{-2}s^{-1}$; temperature (day/night) 25/20°C; RH 70 to 80%.

Treatment	Symbol	μM Fe-EDTA	μM CuSO₄
Control	+Fe +Cu	100	0.05
low-Fe deficiency	-Fe +Cu	10	0.05
Cu deficiency	+Fe -Cu	100	0
low-Fe and Cu deficiency	-Fe -Cu	10	0
Cu toxicity	+Fe ++Cu	100	100

Table 1. Composition of the nutrient solutions in maizein the last 15 days of growth.

Tomato seedling (*Solanum lycopersicum* L., cv. 'Marmande superprecoce' from DOTTO Spa, Italy), germinated for 7 days on filter paper moistened with 0.5 mM $CaSO_4$, were grown for 14 days in a continuously aerated nutrient solution:(mM): K_2SO_4 0.7, KCl 0.1, $Ca(NO_3)_2$, $MgSO_4$ 0.5, KH_2PO_4 0.1;(μ*M*):H_3BO_3 10, $MnSO_4$ 0.5, $ZnSO_4$ 0.5, $CuSO_4$ 0.2, $(NH_4)_6Mo_7O_{24}$ 0.01with 100 μM or 10 μM Fe-EDTA and 0.5 μM $CuSO_4$. Thereafter, some plants were transferred for two weeks to a low-Fe and Cu-free nutrient solution (-Fe -Cu: Fe-EDTA 10 μMand $CuSO_4$ 0.0 μM), to a low-Fe nutrient solution (-Fe +Cu:Fe-EDTA 10 μM, $CuSO_4$ 0.2 μM), to a Cu-free nutrient solution (+Fe -Cu:Fe-EDTA 100 μM and $CuSO_4$ 0.0 μM), to a Cu-excess nutrient solution (+Fe ++Cu: Fe-EDTA 100 μM, $CuSO_4$ 3 μM),and some tomato plants were transferred to a complete nutrient solution containing both Fe and Cu as control (+Fe+Cu: Fe-EDTA 100 μM, $CuSO_4$ 0.2 μM).Nutrient solutions were renewed every three daysand adjusted to pH 6.0 with 0.6mM MES-KOH. The controlled climatic conditions were the following: day/night photoperiod, 16/8 h; light intensity, 220 μE $m^{-2}s^{-1}$; temperature (day/night) 25/20°C; RH 70 to 80%.

Treatment	Symbol	μM Fe-EDTA	μM CuSO₄
Control	+Fe +Cu	100	0.2
low-Fe deficiency	-Fe +Cu	10	0.2
Cu deficiency	+Fe -Cu	100	0
low-Fe and Cu deficiency	-Fe -Cu	10	0
Cu toxicity	+Fe ++Cu	100	3

Table 2. Composition of the nutrient solutions in tomatoin the last two weeks of growth.

Characterization of plant growth and element analysis

Plants were harvested separating roots and shoots and fresh weight (FW) was assessed. Chlorophyll content was evaluated measuring light transmittance of fully expanded leaves using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan) and presented as SPAD index values.

Subsets of samples (three replicates per treatments) were oven dried at 105°C and nitric acid-digested in a microwave oven (MARS Xpress, CEM, Matthews, NC, USA).Some macro-andmicro-nutrients (Cu, Fe; Zn, Mn; Ca, Mg, P and S) were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista Pro axial, USA) analysisorinductively coupled plasma mass emission spectroscopy (ICP-MS, PerkinElmer Inc. - NexION™ 300).

Protein content and enzyme activities

One gram of fine leaf powder was homogenized in 5.0 mL of 100 mM potassium phosphatebuffer (pH 7.5) containing 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid(EDTA) and 4% (w/v) polyvinylpolypyrrolidone (PVPP) (Hippler*et al.,* 2016). The suspension wascentrifuged at 12,100 × g at 4 °C for 35 min, and the supernatant was stored at -80 °C for furtheranalysis. The total protein content was determined according to Bradford method (1976) using bovineserum albumin (BSA) as a standard.

The superoxide dismutase (SOD) activity was evaluated as described by Dourado *et al.* (2014).Electrophoresis was carried out under non-denaturing conditions in a 12-% polyacrylamide gels with equal amounts of protein (20μg) loaded onto each gel lane. After non-denaturing PAGE separation,the gel was rinsed in distilled deionized water and incubated in the dark in 50 mM potassiumphosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitrobluetetrazolium (NBT), and 0.3% N,N,N',N'-tetramethylethylene-diamine (TEMED). One unit ofbovine-liver SOD (Sigma, St. Louis, USA) was used as a positive control for activity. After 30minutes,gels were rinsed with distilled deionized water and then illuminated in water until thedevelopment of achromatic bands of SOD activity on a purple-stained gel.

Total SOD activity was assayed as described by Elavarthi and Martin (2010). The 2-mL assay reaction mixture contained50mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 μM NBT, and 0.025% Triton-X100. Forty microliters of diluted (2x) sample and 20 μL of 1-mM riboflavin were added and the reaction was initiated by illuminating the samples under a 15-W incandescent lamp.

During the 10-min exposure, the test tubes were placed in a box lined with aluminum foil. The box with the test tubes was placed on a slowly oscillating platform at a distance of approximately 12 cm from the light source. Duplicate tubes with the same reaction mixture were kept in the dark and used as blanks. Absorbance of the samples was measured at 560 nm immediately after the blocking of the reaction.

Catalase (CAT) activity was determined according to Kraus *et al.* (1995) with modifications (Azevedo*et al.,*1998).The reaction was initiated by addition of 20 μL of plant extract in a reaction mixturecontaining 1 mL of 100-mM potassium phosphate buffer (pH 7.5) and 2.5 μL H_2O_2 (30-%solution) at 25 °C. The

enzyme activity was determined by following the decrease in absorbance at240 nm, which is due tothe disproportionation of H_2O_2, for 1 min using a plant extract-free as blank. Catalase activity was calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

Peroxidase (POX) activity was determined following the method of Kar and Mishra (1976). The assaymixture contained a 25-mM phosphate buffer (pH 6.8),20 mM pyrogallol and 20 mMH_2O_2. The samples were incubated at room temperature for 1 min and then 0.5% H_2SO_4 (v/v) was addedto stop the reaction. The activity was estimated by measuring the absorbance at 420 nm for 1 min,and a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used.

Catalase and POXactivities were both expressed as μmol min^{-1} mg^{-1} protein.

Statistical analyses

Analyses were performed on three independent experiments (n = 3), a pool of three plants was used for each sample. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test (P<0.05, n=3). Statistical analyses were performed using SigmaPlot Version 12.0 software.

Results

Maize

In maize plants, the condition of Cu deficiency led to a significant reduction in the chlorophyll content in comparison with leaves of the Fe and Cu-sufficient plants (control plants), whileno significant differences of fresh and dry weights of roots and shoots were observed (Fig. 1). A stronger reduction in chlorophyll content was observed under Cu excess and under both deficiencies reaching, respectively,a reduction of two to three-foldin the SPAD index value. These changesin the chlorophyll content under Cu excess and under Fe and Cu deficiency were also associated with a significant limitation in the shoot growth (Fig. 1B,C and 2). The reduction of three-fold in chlorophyll content was also visible under Fe limitation (-Fe +Cu), although this nutritional condition did not show significant changes in dry weight in comparison to controlplants.
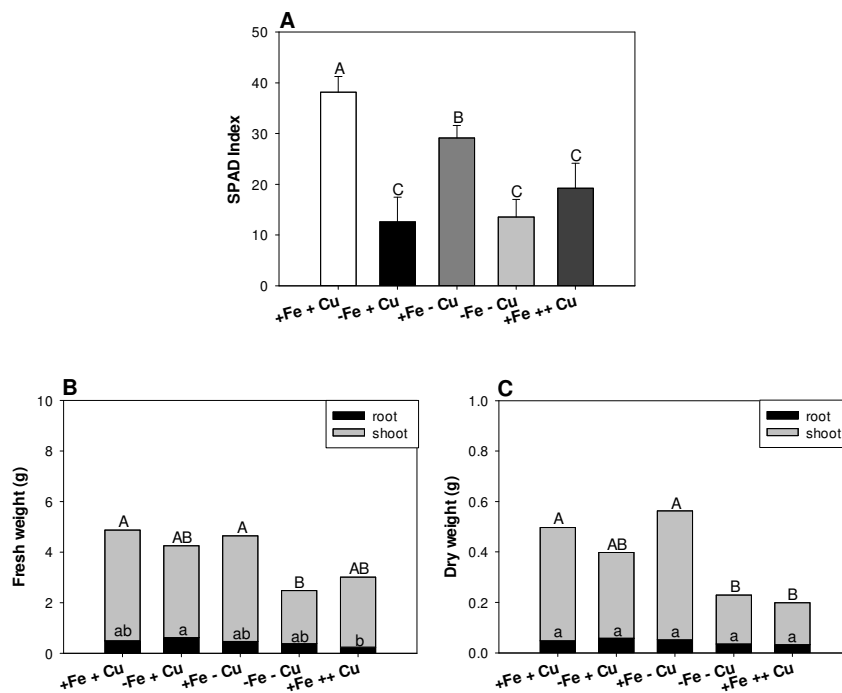
Figure 1.– SPAD index values of maize leaf(**A**) and fresh (**B**)and dry (**C**) weightsof root and leaf tissues were measured at the end of the growing period (21 days). Data are means +SD of three independent experiments (capital letters refer to statistically significant differences in shoots among the mean values,small letters refer to statistically significant differences in roots among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).
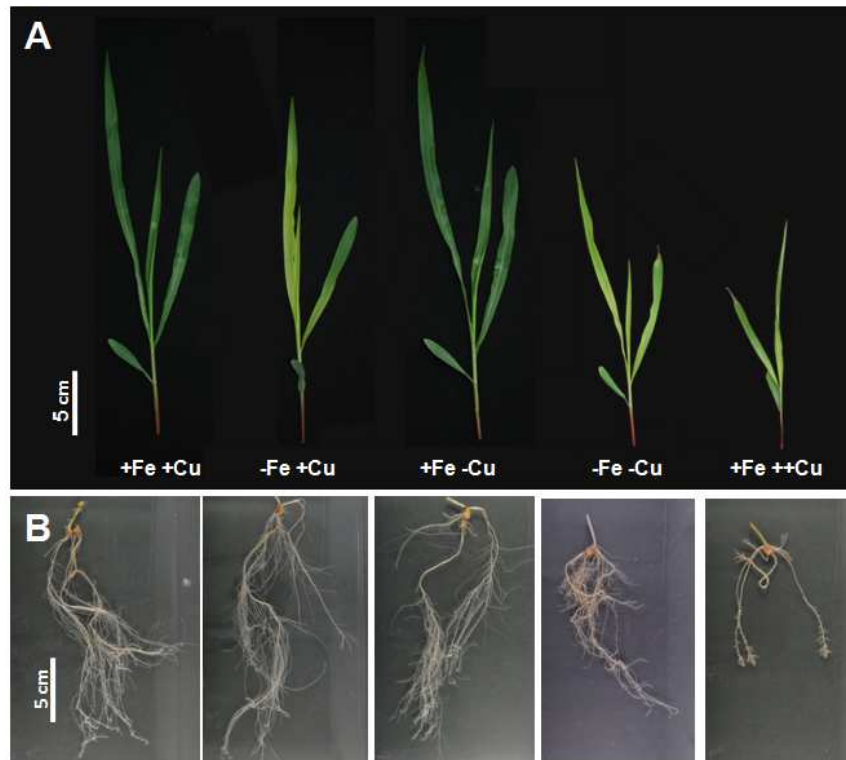
Figure2. Shoots(A) and root apparatus (B) of maize plants grown under different Cu-and Fe-nutritional conditions for 21 days. From the left to the right: +Fe +Cu (100 µM Fe-EDTA and 0.05 µM CuSO₄); -Fe +Cu (10 µM Fe-EDTA and 0.05 µM CuSO₄); +Fe -Cu (100 µM Fe-EDTA and 0 µM Cu); -Fe -Cu (10 µM Fe-EDTA and 0 µM Cu); +Fe ++Cu (100 µM Fe-EDTA and 100 µM CuSO₄).

The effect of treatments was also evaluated by elemental analyses of macro- and micro-nutrient concentrations in plants. In comparison with control condition, Fe-deficient plants showed a slight but significant increase of Cu content at the root level (a. +40%), while no difference was observed at the shoot level. Overall, there was a significant reduction of the content and translocation of Fe in comparison with the control and the Cu-deficient plants (respectively -48% and -63%) while Zn and Mn remainedunaltered (Figure 3).

In the -Fe -Cu plants, there was a marked decrease of Cu and Fe concentrations in shoots and roots in comparison with the control plants (approximately of three times for both). Likewise, a slight decreased of Zn and Mn levels were detected at the shoot level(Figure 3).

In comparison with the control plants, +Fe ++Cu plants exhibited a reduced level of Fe and Zn concentration in both shoots and roots, while Mn concentration was reduced only in shoots. As expected, in +Fe ++Cu plants, the Cu concentration drastically increased in roots in comparison with control plants, in shoots, the rise was more moderate (a. +50%).

There were only few differencesin macronutrient composition among the five nutritional conditions (Figure 4). In comparison to control plants, the most significant changes occurred for Cu-deficient plants, which showed a reduction in Ca concentration in the shoots. Moreover,-Fe-Cu plants showed a

reduction ofCa in both shoots and roots and Mg only in roots. The Cu toxicity led to a reduction of Ca in both shoots and roots and of P in shoots, while S concentration increased slightly in shoots.
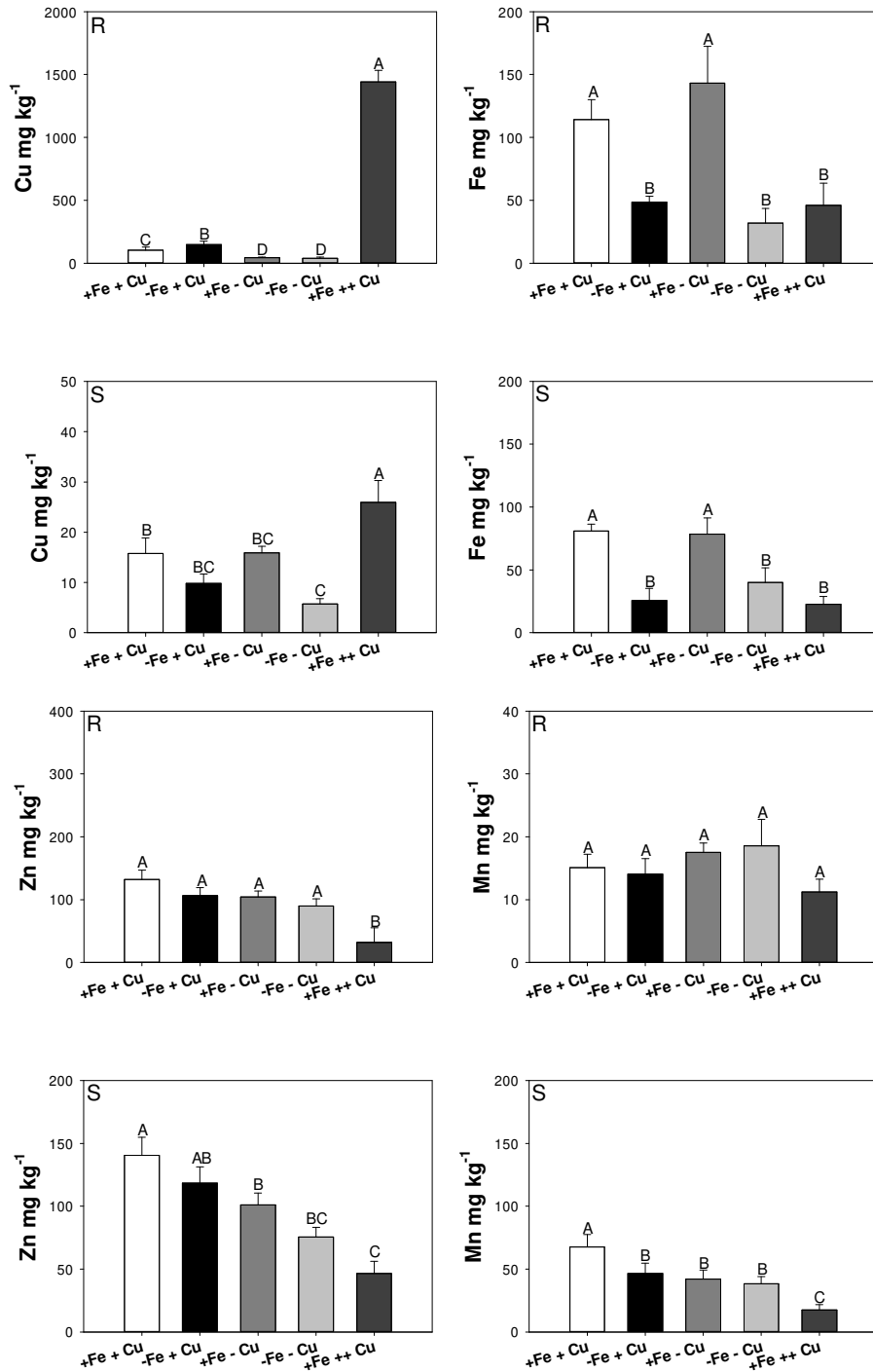
Figure 3.Concentration (mg kg^{-1} of dry weight) of micronutrient in shoots (**S**) and roots (**R**) of Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu-deficient (+Fe -Cu), Fe and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu) maize plants. For each element, letters indicate a significant difference among the mean (ANOVA Holm-Sidak; N=3, P<0.05).
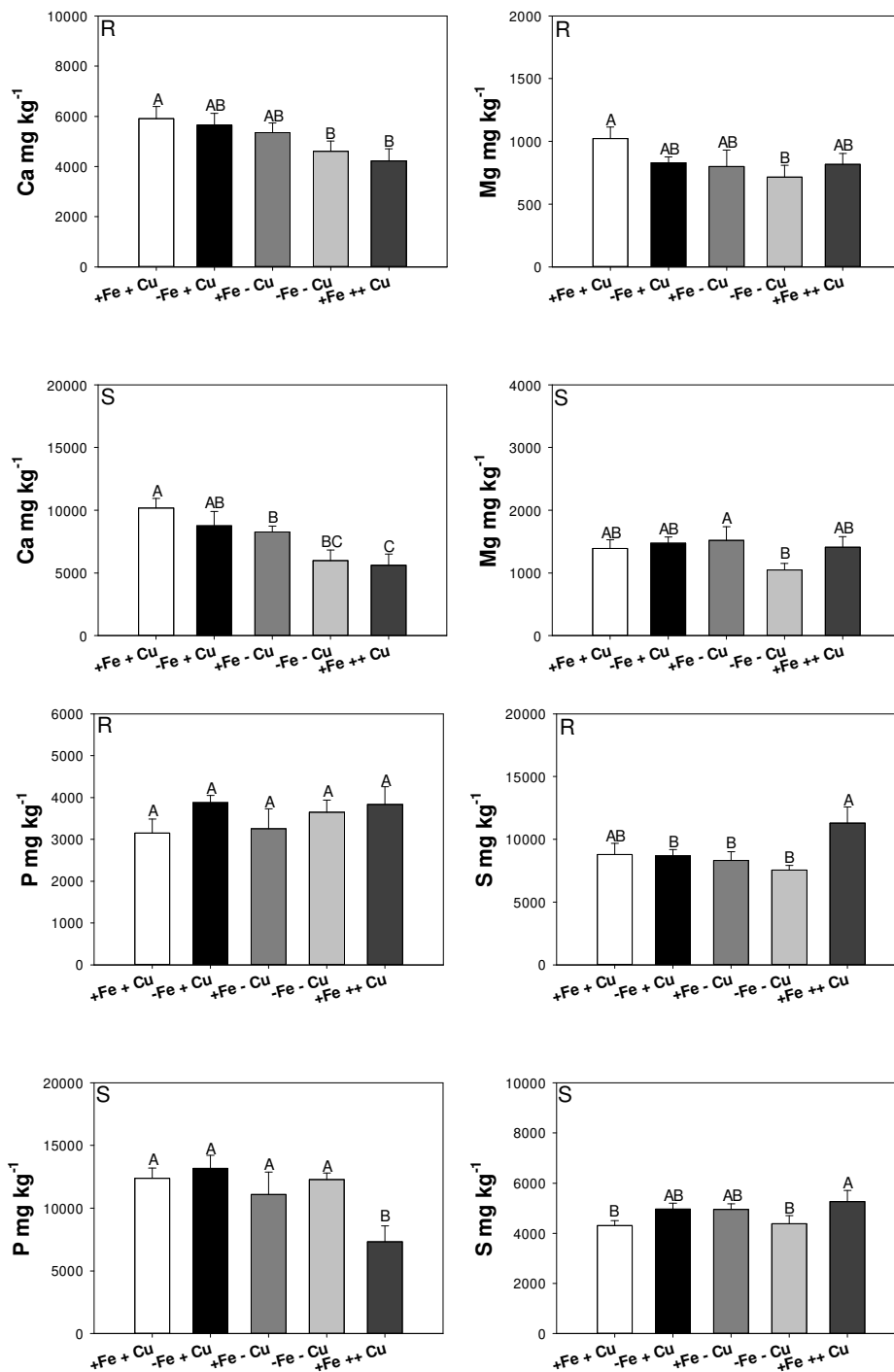
Figure 4. Concentration (mg kg^{-1} of dry weight) of macronutrient in shoots (**S**) and roots (**R**) of Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu-deficient (+Fe -Cu), Fe and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu) maize plants. For each element, letters indicate a significant difference among the mean (ANOVA Holm-Sidak; N=3, P<0.05).

Tomato

Plant growth was visibly reduced both inCu-deficient or Cu-excess conditions. However, only Cu-excess plants exhibited a visible chlorosis of leaves (Figure 5A, 6A). In Cu excess, the root development was dramatically alteredwith a strong limitation in root elongation and proliferation (Figure 5B, 6B). After 14 days, Cu-deficiency plants and Fe- and Cu-deficient plants exhibited a severe reduction of leaf dry weight compared with shoot of control plants (Figure 5), while plants grown in presence of a high Cu concentration showed a strong reduction of shoot and root weights. In Fe-deficiency plants, no significant changes in dry or fresh weights were detected in comparison to control plants.

Moreover, under Fe-limiting conditions there was, as expected, a reduction in the SPAD valuesin comparison with the control plants;interestingly, the reduction in the chlorophyll content was even more evident under Cu-excess treatment.
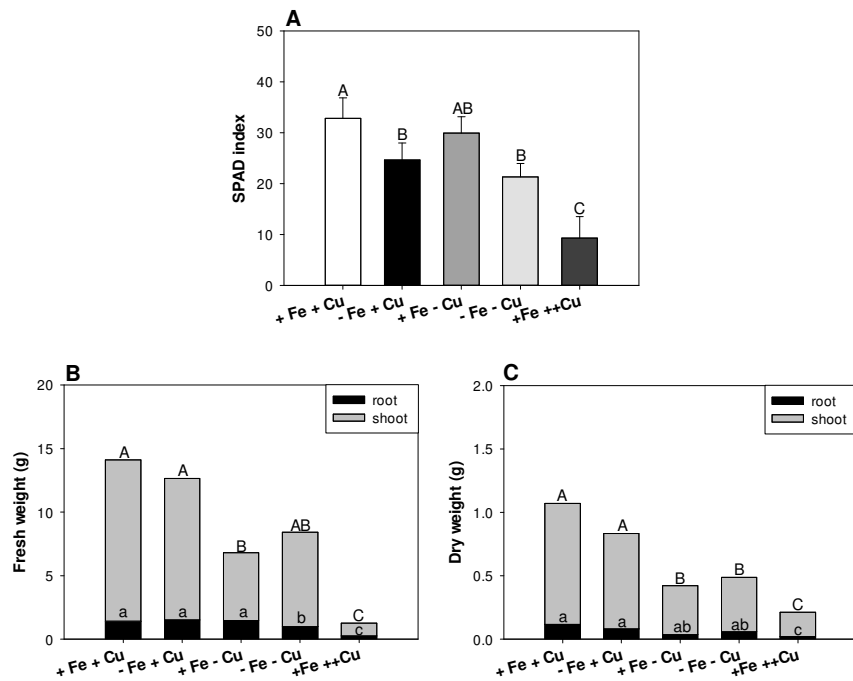
Figure 5. SPAD index values of tomato leaf (**A**) and fresh (**B**) and dry (**C**) weight were measured at the end of the growth period (35 days).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences in shoots among the mean values,small letters refer to statistically significant differences in roots among the mean values, ANOVA Holm–Sidak, N=3, P<0.05).
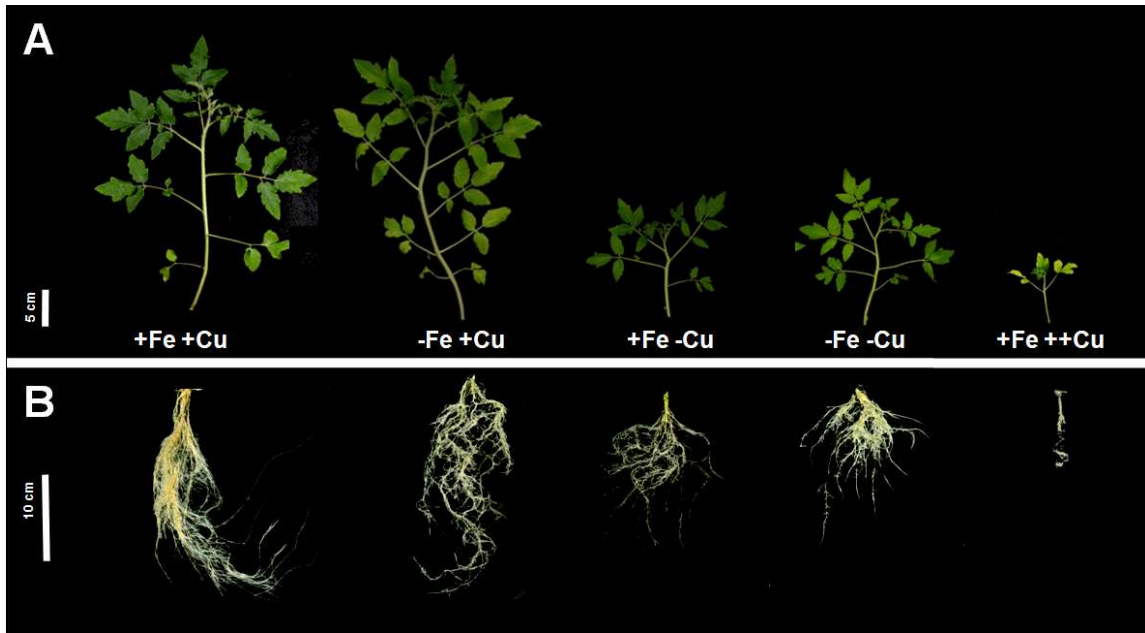
Figure 6. Shoots(A) and root apparatus (B) of tomato plants grown under different Cu- and Fe-supply conditions for 35 days . (A,B) from the left to the right: +Fe +Cu (100 μM Fe-EDTA and 0.2 μM CuSO$_4$); -Fe +Cu (10 μM Fe-EDTA and 0.2 μM CuSO$_4$); +Fe -Cu (100 μM Fe-EDTA and 0 μM Cu); -Fe -Cu (10 μM Fe-EDTA and 0 μM Cu); +Fe ++Cu (100 μM Fe-EDTA and 3 μM CuSO$_4$).

Concerning elemental analyses of micronutrients, Fe-deficient plants exhibited a significant increase of Cu and Zn concentration in the shoot in comparison with control plants (Fig 7). Moreover, there was a significant reduction of Mn content in the roots in comparison to Fe- and Cu-sufficient plants. In Cu-deficient plants, the Cu and Fe concentrations decreased in shoots; while Mn concentration increased in roots.

In comparison to control plants, Fe- and Cu-deficient plants showed a drastic reduction of Fe concentration, Zn and Mn translocation to the shoot increased significantly. About Cu-toxicity, +Fe ++Cu plants showed high levels of Cu concentration in shoots and roots, over 4 times than those of control plants, while the Fe accumulation was reduced down to fifteen times in shoot incomparisonto control plants; moreover, the amount of Fe in +Fe ++Cu roots was below detection limit. Zinc and Mn content were affected by high Cu level as they exhibited aconsiderably reduced concentration in comparison to +Fe +Cu plants.
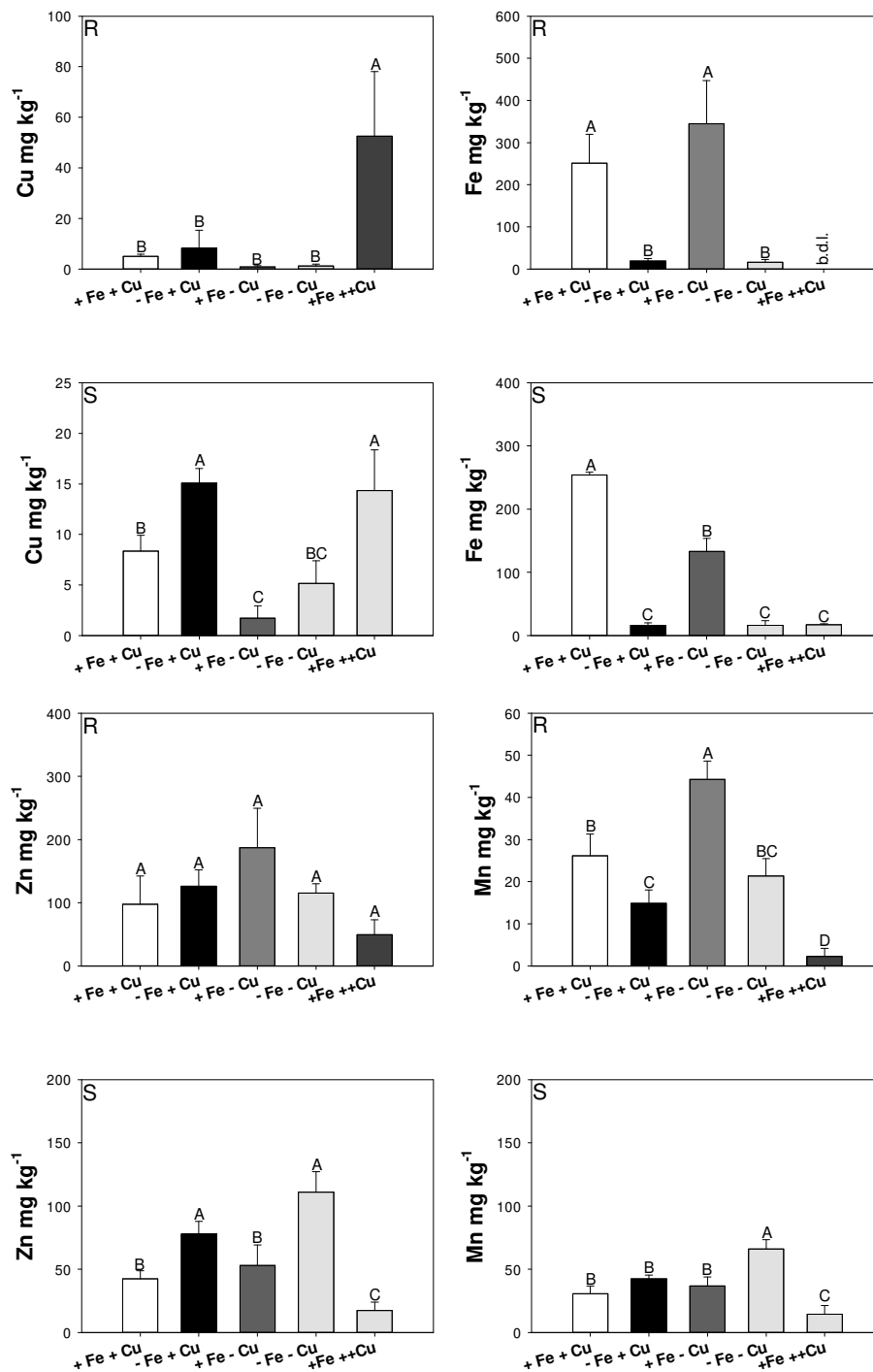
Figure 7. Concentration (mg kg^{-1} of dry weight) of micronutrient in shoots (**S**) and roots (**R**) of Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu- deficient (+Fe -Cu), Fe- and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu) tomato plants. Data are means +SD of three independent experiments. For each element, letters indicate a significant difference (ANOVA Holm-Sidak; N=3, P<0.05).

No significant differenceswere detected for macronutrient content in plants grown under either Fedeficiency or Cu toxicity. In roots of +Fe -Cu plants, P, S and Ca levels increased, while, in shoots, the S decreased, while P concentration increased in comparison with control plants.Under both Fe and

Cudeficiencies, plants accumulated and translocated high amounts of Ca, both in shoot and roots, and there was a significant increase of P and Mg translocation to the shoot (+55% and +60%, respectively).
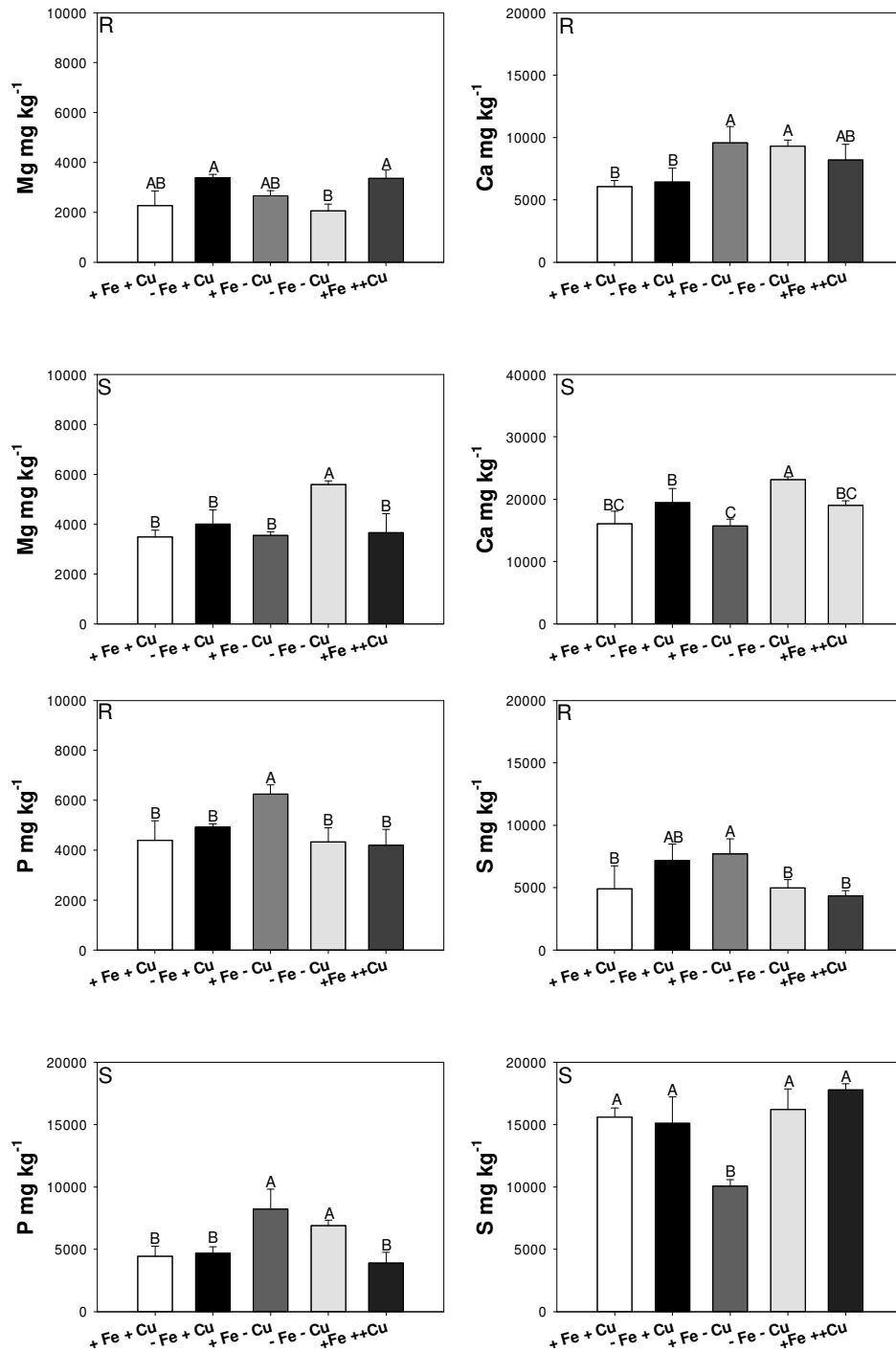

Figure 8. Concentration (mg kg^{-1} of dry weight) of macronutrient in shoots (**S**) and roots (**R**) of Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu-deficient (+Fe -Cu), Fe and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu) tomato plants. Data are means +SD of three independent experiments. For each element, letters indicate a significant difference (ANOVA Holm-Sidak; N=3, P<0.05).

Antioxidant enzyme activities on the leaves

Different isoenzymes exhibiting SOD activities were detected using SOD staining on native-PAGE (Supplementary Figure 1 and 2). In maize, SOD activity did not show any significant differences among nutritional treatments, except for Cu-excess plants which recorded the highest activity level (about three times more) and similar behavior was also observed for Cu-excess-treated tomato plants. A marked decreased of SOD activity in -Fe -Cu plants in comparison with the other treatments was also observed in tomato.

Concerning CAT activity, maize plants showed a marked limitation under Fe-deficiency or under both Fe- and Cu-deficiencies;in tomato, this behavior was evident only under both Fe and Cudeficiencies. The highest value of CAT activity was observed under Cu toxicity in both maize and tomato plants.

Confirming the behavior of the previous enzymes, also the activity of POX enzyme was strongly increased by the Cu toxicity in both species. However, in comparison to control plants (+Fe +Cu), the POX activity was reduced in-Fe + Cu (only in tomato) and -Fe –Cu treatments (in both species).
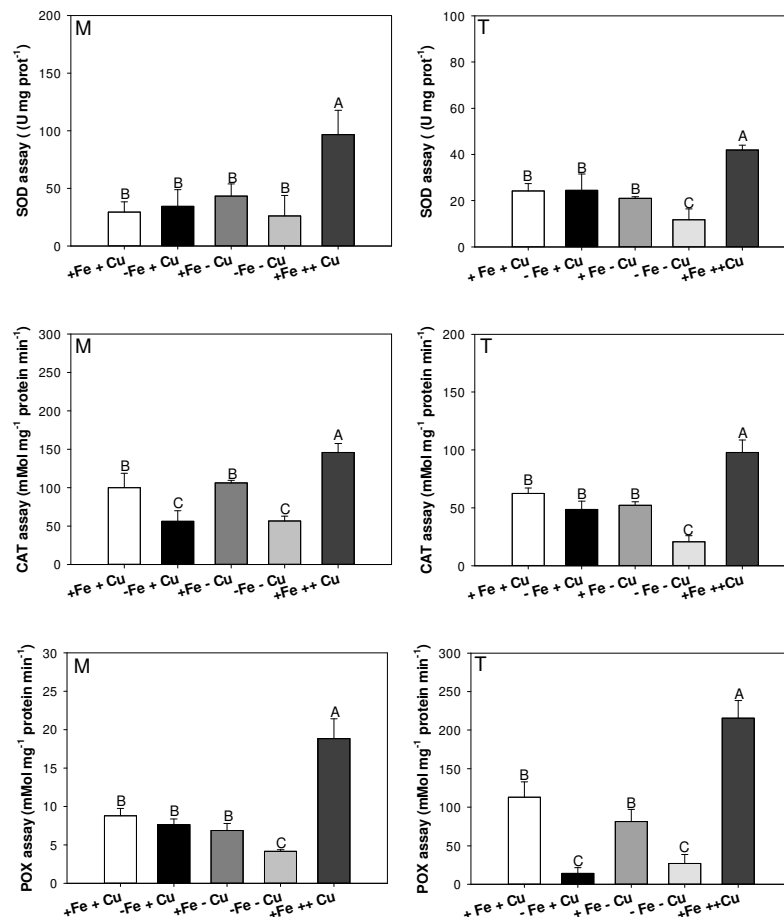
Figure 9. Effects of different nutritional conditions on the activity of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) in leaves of maize (**M**, in the left panels) and tomato (**T**, in the right panels). Data are means +SD of three independent experiments. For each element, letters indicate a significant difference (ANOVA Holm-Sidak; N=3, P<0.05).

Discussion

The purpose of this study was to increase our understanding on the effects of Cu availability on the growth and development of crops and correlate the plant nutritional status with the activity of ROS detoxifying proteins. For this reason, maize and tomato plants were grown under Cu deficiencyor under Cu excess and the plant responses were evaluated in comparison with plants grown under sufficient nutritional condition or under Fe deficiency. Availability of Fe was usedin a range from 10 to 100 µM which is similar to the general accessibility of Fe to the soil solution (Vangronsveld and Clijsters, 1994). Copper was supplied at concentrationsthatinduced clear symptoms on plant morphologyand physiology. 100 µM of $CuSO_4$ was found to be a proper concentration to causea drastic growth limitation and decrease of several physiological traits, but adequate to allowplants to survive.Aftersetting-up experiments, it was evident that it was not possible to use the same Cu concentration in both maize and tomato due to thehigh sensitivity of the last plant speciesto Cu(when suppliedas $CuSO_4$). For this reason, 3 µM of $CuSO_4$wereconsidered appropriate tostudy the toxicity in tomato.

In general, symptoms of Cu deficiency in maizeare related with leaves bluish-green which become chlorotic near the tips, chlorosis develops downward along both sides of the midrib (Alloway and Tills, 1984). Some Cu-deficiency symptoms were observed in tomato plants (Figure 6) while any visible symptoms were shown in maize (Figure 2).

The interveinal leaf chlorosis, typical symptoms of Fe deficiency, was observed in both crops (Figure 2 and 6) and it was in agreement with reduced chlorophyll content (SPAD index value, Figure 1 and figure 5) when grown in Fe-deficient conditions. However,only, in tomato, the root apparatus was strongly modified in agreement with Zamboni *et al.* (2012),witha high proliferation of root hairs in the subapical root zone (Figure 6).Furthermore, in maize, some analogies were shown in the development of the roots system of Fe-deficientplants, as previously described by Zanin *et al.* (2017).

Depending on the nutritional status, the ability of the crops to accumulate and allocate Cu and Fe was different and dependent on their availability in the root external solution. Under low-Fe availability (10 µM), obviously, both species accumulated less Fe than Fe-sufficient ones (Figure 3 and 7). In tomato, Fe deficiency induced an increase of Cu and Zn content in shoot in accordance with previously results obtained by Pineau *et al.*(2012); while, in maize, only a sharp decrease of Mn content in shoot was detected.The increase of Zn concentration in the tissues could be explained by the activation of transporters for Fe which arealso able to take up orpossibly translocatealso others metals such as Zn and Cu. Previous studies have shown that a low availability of Fe triggers also molecular responses linked to sulfur (S), Zn and phosphorous (P) metabolism (Zheng *et al.,* 2009; Ciaffi*et al.,* 2013).Few information about the double deficienciesare available in the literature, however,evidence showedthat in the absence of one of these micronutrients the uptake of the other elements usually increases (Chaignon*et al.,* 2002; Chen *et al.,* 2004; Ryan *et al.,* 2013). In general, both crops showed a great similarity in terms of SPAD index among the plants grown in Fedeficiency and in both Feand

Cudeficiencies, while fresh and dry weightmarkedly decreased (Figure 1 and 5).In tomato, Zn- and Mn-shoot content significantly increase under both Fe and Cu deficiencieswhile in maize any difference were shown (Figure 3 and 7). This behavior could be explained by the enhanced activity of the *Strategy I*mechanisms under Fe deficiency (*Strategy I plants*) which also tends toimprove the uptake of other micronutrients such as Zn, Mn and Cu (Eide *et al.,* 1996; Morrissey *et al.,* 2009).

Copper toxic effects on growth and development have been reported in several crops. Ouzounidou*et al.* (1998) have described that in spinach (*SpinaciaoleraceaL.*), 160 µM Cu in the solution culture decreased chlorophyll content by 45 % over control treatment.In the present study, both the crops have shown a strong reduction of growth and a chlorosis localized in the younger leaves which are in agreement with previously observations (Mocquot*et al.,* 1996; Adress*et al.,* 2015). Inboth the crops, shoots and roots system seemed to be strongly altered incomparison with their relative control plants (Figure 2 and 6). Moreover, a strong reduction of chlorophyll content and fresh and dry weight were detected (Figure 1 and 5). Micronutrientcontent wasstrongly affected by Cu toxicity as Fe, Zn and Mn content markedly decreased in both the species (Figure 3 and 7). Copper can compete with the acquisition of other micronutrients that aretaken up as bivalent cation, such as Fe^{2+} and Zn^{2+} (Michaud *et al.,* 2008; Keller *et al.,* 2014).Moreover,Azeez *et al.* (2015) have shown that Cu excess leads to a drastic reduction of Zn, P and Fe content in 5-week-old maize plantsgrown in soil under greenhouse conditions.In maize, Cu-excessplants showeda significant reduction of Ca and Pconcentration in accordance with previous observations(Ouzounidou*et al.*, 1995, Ali *et al.,* 2002). It was demonstrated that Cuions tend to displace Ca^{2+} ions from exchange sites and are strongly bound in root-free space (Jiang *et al.,* 2001). Moreover, the increase of S content and translocation to the shoot of maize (Figure 4) is in agreement with previous data, where the high S levels under Cu toxicity were associated to an upregulation of the sulfate transporters (Shahbaz *et al.,*2010).

Among the enzymes investigated, SOD showed a dose-response relationship between enzyme activity expressed per µg of protein and Cu concentration inboth plants. Under Cu toxicity, the increase in SOD activity for both the species under Cu toxicity (Figure 9) is likely to be related to an increase production of free radicals in plant cells due to the accumulation of this heavy metal. Thesecompounds initiate lipid peroxidation and destabilize the thylakoid membrane (Van Assche and Clijsters, 1990). Even if SOD activity can be induced by a variety of stress factors other than metals (Vangronsveld and Clijsters, 1994), in the conditions of this study, it seems that there isa link between Cu toxicity and the appearance of different isoenzymes(Supplementary data Figure 1 and 2).In general, spectrophotometric assay confirm the in-gel activity results as the SOD activity markedly increased in Cu-excessmaize leaves, while, in tomato, there was a slight increase after the same treatment. These behaviors are in agreement with previous studies which have shown an increase of SOD activity under Cu toxicity (Devi and Prasad, 1998; Martins and Mourato, 2006).

About the Fe and Cu deficiencies, it was reported by Yuand Rengel (1999) that the deprivation of some micronutrients (Cu, Zn or Mn) altered the activities of SOD forms in lupin and it seems to depend on the

kind and severity of the deficiency stress. In maize, noreduction of SOD activity was detected under the double starvation (-Fe -Cu) while in tomato there was a decreasein comparison with control plants(Supplementary data Figure 1,2 and Figure 9). The activity of CAT and POX showed a concomitant increase in Cu-toxicity plants and a sharply decreased under Fe and Cu-deficiency (Figure 9 and Supplementary data Figure 2). This behavior is due to the fact thatthe activity of these two enzymes are strongly connected to each other (Yruela 2009; Pantola and Shekhawat 2012; Adress*et al.,* 2015).

In summary, the Cu effect on plant mineral uptake and accumulation depends on the plant species, Cu concentration in the root medium, exposure duration, dose and growth conditions. In bothspecies, Cu concentration in root/shoot of plants increased with increasing Cu levels in the growth medium, and Cu was mainly accumulated in roots as compared to shoots. Copper presence has reduced Fe concentration in comparison with other nutrients and this behavior suggests a direct antagonistic relationship between Fe and Cu.

Even though particular attention has been paid to the preparation of nutrient solutions such as using analytical grade reagents, any symptoms of Cu deficiency was detected in maize while in tomato they appeared in both the treatments without Cu (+Fe -Cu and -Fe -Cu). This behavior could be explained by the high abundance of Cu as contaminant (Gries*et al.,* 1998).Furthermore, maize could have an endogen amount of Cu that derives from the seed and the growth period in maize was quite short (two weeks), so presumably Cu deficiency symptoms might become evident in a latter phonological stage (e.g. flowering and ripening of the seeds) (Graham, 1975; Dell, 1981; Plaza *et al.,* 2003).

Future investigations are needed to study the effects of different sources of Cu and Fe in excessconditions related with the growth and development and a better understanding of the physiological, transcriptional and metabolic profiles of Cu deficiency and Fe and Cu deficiency would be interesting.Coppertoxic threshold values in plant tissue and some easily measurable phenotype can give useful information to assess, as future perspective of this work, a soil quality evaluation related to Cu deficient or excess conditions.

Supplementary Material
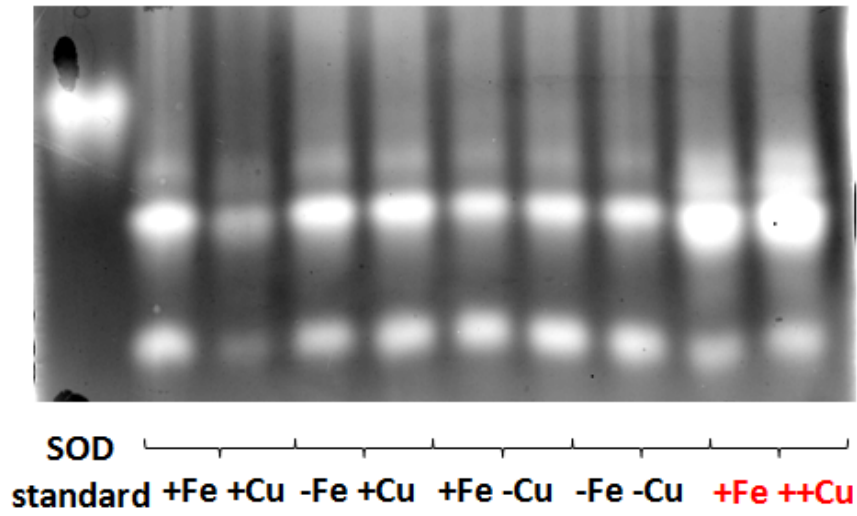
Figure 1. Activity staining of SOD isozymes after native-PAGE of maize leaves grown in a nutrient solution containing different concentrations of Fe and Cu (μM, two replicates per conditions): +Fe +Cu (100 Fe-EDTA; 0.05 CuSO$_4$); -Fe +Cu (10 Fe-EDTA; 0.05 CuSO$_4$); +Fe –Cu (100 Fe-EDTA; 0 CuSO$_4$); -Fe -Cu (10 Fe-EDTA; 0 CuSO$_4$); +Fe ++Cu (100 Fe-EDTA; 100 μM CuSO$_4$).
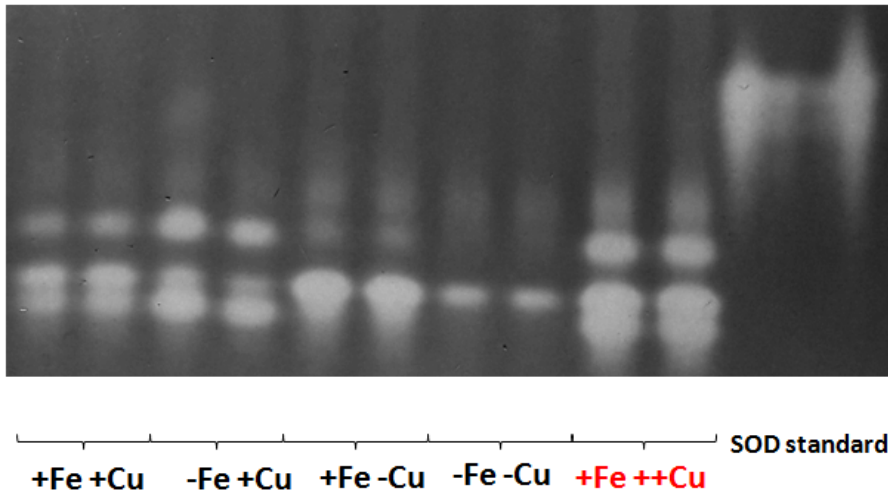
Figure 2. Activity staining of SOD isozymes after native-PAGE of tomato leaves grown in a nutrient solution containing different concentrations of Fe and Cu (μM, two replicates per conditions): +Fe +Cu (100 Fe-EDTA; 0.2 CuSO$_4$); -Fe +Cu (10 Fe-EDTA; 0.2 CuSO$_4$); +Fe –Cu (100 Fe-EDTA; 0 CuSO$_4$); -Fe -Cu (10 Fe-EDTA; 0 CuSO$_4$); +Fe ++Cu (100 Fe-EDTA; 3 CuSO$_4$).

		+Fe +Cu		-Fe +Cu		+Fe -Cu		-Fe -Cu		+Fe ++Cu	
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
Cu	s	15.80	3.09	9.84	1.87	15.88	1.31	5.74	1.01	25.95	4.31
	r	103.70	23.72	146.98	27.71	42.87	5.48	38.19	9.77	1442.67	91.43
Fe	s	80.96	5.44	25.55	9.69	78.56	12.81	40.01	11.65	22.71	6.21
	r	114.04	15.97	48.52	4.66	143.32	29.08	31.89	11.64	46.00	17.52
Zn	s	140.42	14.50	118.34	13.01	101.01	9.53	75.45	7.69	46.43	9.84
	r	131.78	15.13	106.19	12.94	104.27	9.29	89.63	11.34	31.96	23.05
Mn	s	67.51	10.01	46.51	8.22	42.01	7.29	38.26	5.83	17.39	4.18
	r	15.09	2.14	14.06	2.48	17.54	1.46	18.58	4.18	11.23	2.00
Mg	s	1389.24	139.32	1476.61	96.23	1519.05	220.38	1048.93	104.91	1411.13	167.58
	r	1022.99	90.44	828.28	48.61	799.34	132.38	715.91	94.37	817.39	86.94
Ca	s	10173.16	776.88	8766.22	1138.04	8260.91	487.72	5982.91	848.04	5604.58	893.03
	r	5907.62	479.40	5646.07	472.32	5344.54	392.39	4610.66	400.55	4219.25	487.23
P	s	12395.78	804.61	13168.71	1050.31	11091.13	1779.94	12277.11	538.36	7329.08	1271.79
	r	3152.13	331.04	3876.18	170.58	3258.10	474.43	3649.39	285.61	3832.26	426.73
S	s	4311.98	192.73	4967.16	232.05	4945.07	222.99	4379.91	321.34	5263.36	442.23
	r	8795.07	891.82	8680.24	507.62	8328.86	683.40	7547.13	373.93	11295.58	1283.08

Table 1. Elemental compositionof maize shoot (s) and root (r) samples of plant grown in the following conditions: Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu deficient (+Fe -Cu), Fe and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu)(mg kg^{-1} of dry weight). Means and standard deviations are reported. For each element, letters indicate a significant difference among the mean (ANOVA Holm-Sidak; N=3, P<0.05).

		+Fe +Cu		-Fe +Cu		+Fe -Cu		-Fe -Cu		+Fe ++Cu	
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
Cu	**s**	8.37	1.54	15.09	1.44	1.72	1.22	5.16	2.25	14.33	4.02
	r	5.00	0.87	8.17	7.16	0.78	0.47	1.18	0.72	52.57	25.50
Fe	**s**	254.03	4.42	16.31	3.82	133.23	20.46	16.06	7.61	17.06	1.38
	r	251.22	68.46	19.21	5.29	344.92	102.11	16.22	6.56	b.d.l.	b.d.l.
Zn	**s**	42.49	6.30	77.75	10.21	53.08	16.30	110.93	16.38	17.44	6.53
	r	97.57	44.55	126.06	26.33	186.86	62.59	115.14	15.02	49.37	23.68
Mn	**s**	30.69	6.05	42.48	2.99	36.71	7.18	66.15	7.30	14.61	6.65
	r	26.13	5.19	14.89	3.11	44.25	4.39	21.37	4.09	2.30	1.85
Mg	**s**	3486.30	268.66	4005.61	565.16	3543.45	146.54	5589.61	141.67	3665.26	759.25
	r	2268.81	589.26	3384.79	140.41	2664.08	207.82	2060.23	271.89	3364.16	336.11
Ca	**s**	16084.71	1977.29	19463.38	2231.07	15743.53	1074.82	23151.96	443.62	19027.27	707.60
	r	6065.20	492.55	6435.86	1093.06	9562.98	1302.28	9304.58	493.08	8195.91	1252.45
P	**s**	4456.86	794.64	4698.42	506.53	8238.11	1575.52	6901.45	449.75	3909.13	860.27
	r	4392.87	788.95	4915.08	144.64	6241.21	380.18	4330.37	570.07	4201.73	631.20
S	**s**	15595.12	748.56	15095.67	2119.69	10064.13	524.89	16209.61	1638.25	17778.29	483.93
	r	4902.51	1836.07	7158.54	1329.53	7714.79	1199.84	4985.91	643.35	4343.95	409.76

Table 2. Elemental composition of tomato shoot (s) and root (r) samples of plant grown in the following conditions: Fe-and Cu-sufficient (+Fe +Cu), Fe-deficient (-Fe +Cu), Cu deficient (+Fe -Cu), Fe and Cu-deficient (-Fe -Cu) and Cu excess (+Fe ++Cu) (mg kg^{-1} of dry weight). Means and standard deviations are reported. For each element, letters indicate a significant difference among the mean (ANOVA Holm-Sidak; N=3, P<0.05).

4.The effects of different sources of Cu and Fe on growth and metals' translocation in maize

Introduction

Copper (Cu) and iron (Fe) are two transition metals that play important roles as cofactors in several metabolic processes, including photosynthetic and mitochondrial electron transport, oxidative stress responses, and hormone perception (Himelblau *et al.,* 2000; Puig *et al.,* 2007). The intracellular levels of these micronutrients must be tightly regulated, since the same redox properties that make Cu and Fe essential elements may trigger the formation of reactive oxygen radicals (ROS) that damage cells by oxidizing membrane components, proteins and nucleic acids (Halliwell and Gutteridge, 1984; Yruela *et al.,* 2005).

Moreover, a strong overlap between Fe and Cu uptake mechanisms has been suggested (Ryan *et al.,* 2013) as the mechanisms of Cu- and Fe-transmembrane transport to root share the restriction of forms that can be used by roots (Adress *et al.,* 2015; Brunetto *et al.,* 2016). There are several hypotheses on how Cu is acquired from plants. Fe-chelate reductase proteins may be capable of donating electrons to other transition metals than Fe, including Cu (II, III, IV) (Marschner *et al.,* 1986; Uren, 1982; Bernal *et al.,* 2012). In *Arabidopsis,* FRO4 and FRO5 act redundantly to reduce Cu at the root surface (Bernal *et al.,* 2012). Proteins have been shown to be involved in the transport of Cu across membranes; Fe transporter (IRT1) can also mediate the uptake of Cu, Cd, Co and Zn (Korshunova *et al.,* 1999) and some Heavy Metal ATPases (HMA)can transport either Cu^{2+} or Cu^+ (Pilon and Tapken*,* 2009).

However,it is also possible that Cu uptake may proceed without prior reduction of Cu^{2+} to Cu^+,as free cation or complexed to substances released by plants(Wintz *et al.,* 2003).It was found that Cu was quantitatively the most important elements competing with Fe for complexation by the DMA (Schenkeveld *et al.,* 2014). Interestingly, Cu is transported in the xylem exclusively in complexed form (Graham, 1979), most likely with organic nitrogen ligands, as for instance amino acids (Kochian, 1991) and nicotianamine. This suggests that even under toxic conditions, plants have mechanisms to regulate complexation of Cu within the xylem sap and, hence, minimize potential damage caused from high concentrations of free-Cu ions (Welch and Schuman, 1995). This evidence suggests that in soil solution, where more than 98-99% of the Cu is present in complexed form(Marschner, 2011),the Cu sources usable for the nutrient acquisition may not be the freeionic one (Cu^{2+} and/or Cu^+) but from complexed form, such as the Cu-PS, in particular for *Strategy II* plants. In this latter group of plants, the utilization ionic and complexed forms could coexist, as documented for Zn (Von Wiren *et al.,* 1996). However, up to date there is a limited literature available on this problematic.

With this purpose,the aim of this work was to evaluate if different availabilities and sources of Cu and Fe can shed light on the mechanism(s) used by *graminaceous* plants, such as maize, to acquire Cu.

Materials and methods

Plant material and growth conditions

Maize seeds (*Zea mays* L., inbred line P0423, Pioneer Hybrid Italia S.p.A.) were germinated over aerated 0.5mM $CaSO_4$ solution in a dark growth chamber at 25°C. Six-day-old seedlings were then transferred for 15 days in a continuously aerated nutrient solution containing, μM: $Ca(NO_3)_2$ 1000; $CaSO_4$ 500; $MgSO_4$ 100; KH_2PO_4 175; KCl 5; H_3BO_3 2.5; $MnSO_4$ 0.2; $ZnSO_4$ 0.2; Na_2MoO_4 0.05; buffered to pH 6.0 with 2.5 mM MES-KOH). In different group of plants, Fe was supplied at 100 µM as Fe-EDTA or Fe-citrate while Cu was supplied at 0.05 or 100 µM as $CuSO_4$ or Cu-EDTA. Fe-citrate were prepared according to von Wirén *et al.* (1994) by mixing an aliquot of $FeCl_3$ with citrate (in 10 % chelate excess). Nutrient solutions were renewed every three days and the controlled climatic conditions of the growth chamber were the following: day/night photoperiod, 16/8 h; light intensity, 220 µE $m^{-2}s^{-1}$; temperature (day/night) 25/20°C; RH 70 to 80%.

Treatment	Symbol	µM Fe-EDTA	µM $CuSO_4$	µM Fe-citrate	µM Cu-EDTA
+Fe-EDTA; +$CuSO_4$	+FE +CS	100	0.05	-	-
+Fe-EDTA; ++$CuSO_4$	+FE ++CS	100	100	-	-
+Fe-EDTA; +Cu-EDTA	+FE +CE	100	-	-	0.05
+Fe-EDTA; ++Cu-EDTA	+FE ++CE	100	-	-	100
+Fe-citrate; +Cu-EDTA	+FC +CE	-	-	100	0.05
+Fe-citrate; ++Cu-EDTA	+FC ++CE	-	-	100	100
+Fe-citrate; +$CuSO_4$	+FC +CS	-	0.05	100	-
+Fe-EDTA; ++$CuSO_4$	+FC ++CS	-	100	100	-

Table 1. Composition of the nutrient solutions in maize in the last 15 days of growth.

Characterization of plant growth and element analysis

Plants were harvested separating root and shoot tissues and fresh weight (FW) were assessed. To evaluate chlorophyll content, light transmittance of fully expanded leaves was determined using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan) and presented as SPAD index values.

Subsets of samples (three replicates per treatments) were oven dried at 105°C and nitric acid-digested in a microwave oven (MARS Xpress, CEM, Matthews, NC, USA). Macro- and micro-nutrients (Cu, Fe; Zn, Mn; Ca, Mg, P and S) were measured by inductively coupled plasma optical emission spectroscopy analyses (ICP-OES, Varian Vista Pro axial, USA).

Protein content and enzyme activities

One gram of fine dry leaf powder was homogenized in 5.0 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 4%

(w/v) polyvinylpolypyrrolidone (PVPP) (Hippler *et al.,* 2016). The suspension was centrifuged at 12'000 g at 4 °C for 35 min, and the supernatant was stored at -80 °C for further analysis. The total protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

In-gel superoxide dismutase (SOD) activity was carried out as described by Dourado *et al.,* (2014). Electrophoresis was carried out under non-denaturing conditions in 12-% polyacrylamide gels loading 20 µg protein onto each lane. After non-denaturing PAGE separation, the gel was rinsed in distilled deionized water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium (NBT), and 0.3% N,N,N',N'-tetramethylethylene-diamine (TEMED). One unit of bovine liver SOD (Sigma Aldrich, Milan, Italy) was used as a positive control for activity. After 30 min, the gels were rinsed with distilled deionized water and then illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel.

Total SOD activity was assayed as described by Elavarthi and Martin (2010) in a 2-mL assay reaction mixture containing 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT and 0.025% Triton-X100. Forty microliters of diluted (2x) sample and 20 µL of 1-mM riboflavin were added and the reaction was initiated by illuminating the samples under a 15-W lamp.

During the 10-min exposure, the test tubes were placed in a box lined with aluminum foil. The box with the test tubes was placed on a slowly oscillating platform at a distance of approximately 12 cm from the light source. Duplicate tubes with the same reaction mixture were kept in the dark and used as blanks. Absorbance of the samples was measured immediately at 560 nm after the light exposure.

Catalase (CAT) activity was determined according to Kraus *et al.* (1995) with modifications (Azevedo *et al.,* 1998). The reaction was initiated by addition of 20 µL of plant extract in a reaction mixture containing 1.0 mL of 100-mM potassium phosphate buffer (pH 7.5) and 2.5 µL H_2O_2 (30% solution) at 25 °C. The enzyme activity was determined by following the decrease in absorbance at 240 nm, which is due to the disproportionation of H_2O_2, for 1 min against a plant extract-free blank. Catalase activity was calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

Peroxidase (POX) activity was determined following the method of Kar and Mishra (1976). The assay mixture contained 25 mM phosphate buffer (pH 6.8), 20 mM pyrogallol and 20 mM H_2O_2. The samples were incubated at room temperature for 1 min and 0.5% H_2SO_4 (v/v) was added to stop the reaction. The activity was estimated by measuring the absorbance at 420 nm for 1 min, and a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used in calculations. CAT and POX activities were expressed as µmol min^{-1} mg^{-1} protein.

Molecular analysis

Real-time RT-PCR experiments

Total RNA was isolated using the Invisorb Spin Plant RNA kit (Stratec Molecular, Berlin, Germany) and quantified by spectrophotometry using NanoDropTM 1000 (Thermo Scientific). A gel (1% agarose; 80mV) was utilized to check the quality of the RNA extraction. Five hundred nanograms of total RNAof each sample was retrotranscribedusing 1 pmol of Oligo d(T)23VN (New England Biolabs,Beverly, USA) and 10 U M-MulV RNase H⁻ for 1 h at 42 °C (Finnzymes, Helsinki, Finland) following the application protocol of the manufacturers. After RNA digestion with1 U RNase A (USB, Cleveland, USA) for 1 h at 37 °C, geneexpression analyses were performed by adding 0.16 μL ofthe cDNA to the realtime PCR complete mix, FluoCycle™sybr green (20 μL final volume; Euroclone, Pero,Italy), in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). Specific primers (Tm = 58 °C) were designed to generate 80–120 bp PCR products. *ZmGAPDH* and *ZmTUA*were used as housekeeping gene for relative quantification. Each Real-Time RT-PCR was performed in two technical replicates on the three biological replicates. Analyses of real-time result were performed using Opticon Monitor 2 software (Biorad, Hercules, USA). Sequences of forward and reverse primers are reported in Table 2.

		Primer Forward	Primer Revers
GRMZM2g046804	*ZmGAPDH*	CCTGCTTCTCATGGATGGTT	TGGTAGCAGGAAGGGAAGCA
GRMZM2g152466	*ZmTUA*	GGTCATCTCATCCCTGACG	TGAAGTGGATCCTCGGGTAG
GRMZM2G042412	*ZmCOPT1*	CCCACACACACACAGACAGT	CATGTTGTGCCCTCCTCTCA
GRMZM2G109354	*ZmSQU9*	GCATGATTGCTACCGTTTACCA	TCTGGTCTTGATTGAGTGATGTGA
GRMZM2G059991	*ZmSOD3*	CGATGAGGATTTTGGTTCGT	ATGCATGTTCCCAGACATCA
GRMZM5G864424	*ZmZn-Fe-SOD*	AGCTTTCGGTTGTTCATACACG	CGTGCTCCCACAAGTCTAGG
GRMZM2G156599	*ZmYS1*	AGGAGACAAGAACGCAAGGA	ACTGAACAAAGCCGCAAACT
GRMZM2G063306	*ZmTOM1*	AGGAGTTCTTCTTCGTCGCA	GCACCAAGAAAACCAGCGTA
GRMZM2G178190	*ZmNRAMP1*	GGAGAATTATGGCGTGAGGA	ACCACCAAACCGATCAGAAG
GRMZM2G325575	*ZmFER1*	GATGCTGCTTGAGGAAGAGG	CCGACCCAGAGTTGTCAGTT

Table 2. Primers used for quantitative RT-PCR analysis.

Statistical analyses

Statistical analyses were performed using SigmaPlot Version 12.0 software using one-way analysis of variance (ANOVA) with Holm–Sidak test (P <0.05, n = 3).

Results

At the end of the growing period, +FE++CS plantsexhibited visible interveinal yellowing of the young leaves (Figure 2) and a more than five-time reduction of the chlorophyll content (Figure 1) compared with control plants. +FE ++CEtreatment shown no significant differences in terms of developmentcomparedwith +FE +CE plants, except a slightly reduction of the fresh and dry weight of the shoot (-19% and -32% respectively). No differences in chlorophyll content weremeasuredin comparison with control treatment.

+FS++CE plants shown a significant reduction of chlorophyll content (-27%) and fresh and dry weight of the shoot (about -20%) compared with +FC ++CS plants (100 µM Fe-citrate and 100 µM CuSO$_4$), while no differences were found at the roots level in terms of biomass and morphology(Figure 1). +FC ++CS plants exhibited a decreased chlorophyll content compared with control plants (+FS +CE),this decrease was even more pronounced in +FE ++CSplants.Moreover, fresh and dry weight were significantly less than +Fe +CS plants.

In general, the nutritional status induced changes also at the root level, with both the treatments with CuSO$_4$in excess (+FE ++CS and +FC ++CS) that showed shorter roots and an increase in the diameter of the root tips (Figure 2).
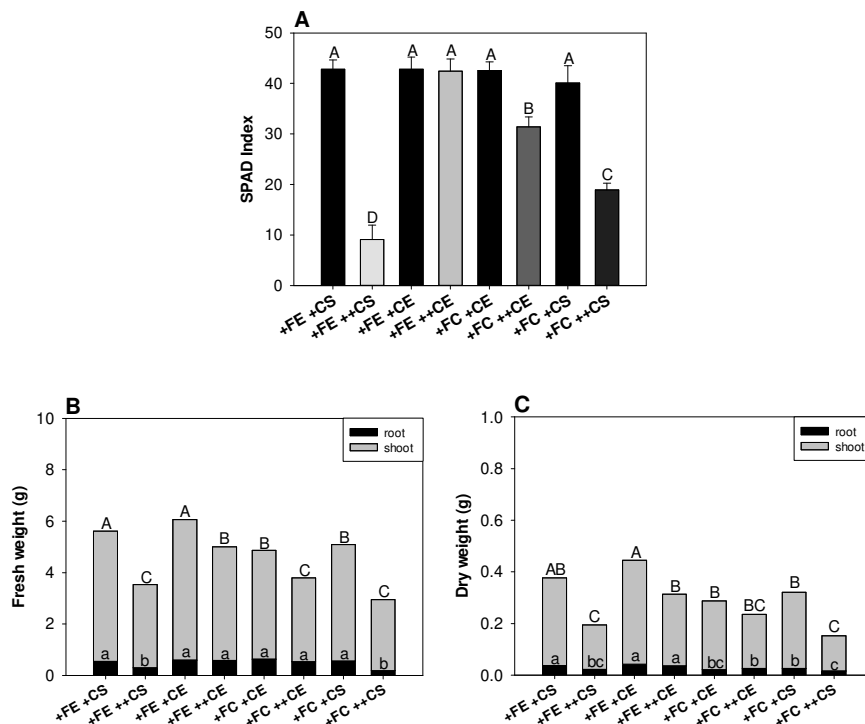
Figure 1.– SPAD index values of maize leaf (**A**) and fresh (**B**) and dry (**C**) weights were measured at the end of the growing period (21 days). The treatments were: +FE +CS (100 µM Fe-EDTA and 0.05 µM CuSO$_4$); +FE ++CS (100 µM Fe-EDTA and 100 µM CuSO$_4$); +FE +CE (100 µM Fe-EDTA and 0.05 µM Cu-EDTA); +FE ++CE (100 µM Fe-EDTA and 100 µM Cu-EDTA); +FC +CE (100 µM Fe-citrate and 0.05 µM Cu-EDTA); +FC ++CE (100 µM Fe-citrate and 100 µM Cu-EDTA); +FC +CS (100 µM Fe-citrate and 0.05 µM CuSO$_4$); +FC ++CS (100 µM Fe-citrate and 100 µM CuSO$_4$).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences in shoots among the mean values, small letters refer to statistically significant differences in roots among the mean values, ANOVA Holm–Sidak, N=3, P<0.05).

Figure2. Shoots(A) and root apparatus (B) of maize plants grown under different Cu and Fe nutritional conditions for 21 days. From the left to the right: +FE +CS (100 µM Fe-EDTA and 0.05 µM CuSO$_4$); +FE ++CS (100 µM Fe-EDTA and 100 µM CuSO$_4$); +FE +CE (100 µM Fe-EDTA and 0.05 µM Cu-EDTA); +FE ++CE (100 µM Fe-EDTA and 100 µM Cu-EDTA); +FC +CE (100 µM Fe-citrate and 0.05 µM Cu-EDTA); +FC ++CE (100 µM Fe-citrate and 100 µM Cu-EDTA); +FC +CS (100 µM Fe-citrate and 0.05 µM CuSO$_4$); +FC ++CS (100 µM Fe-citrate and 100 µM CuSO$_4$).

Generally, Cu-excesstreatments showed a sharp increase of Cu concentration in everytissues while Fe content slightly declined. In roots of +FE ++CS plants, Cu concentration increasedmore than twenty-five times while in +FC ++CS, it increased seven folds (Figure 3). Roots of +FE++CE plants exhibited a significant increase of Cu content compared with its control (+FE +CE), however, this rise was lower than in the other Cu-excesstreatments. The +FE ++CS and +FC ++CS have shown a drastic reduction of Fe and Zn content, in roots, approximately three times, while, in +FE ++CE and +FC ++CE,the reduction of content was not significant (Figure 3). There was a sharp increase of Mn content and translocation in all plants treated with Fe-citrate (approximately eighteen folds compared with +FE +CS) except for +FC ++CS in which Mn content in roots was similar to the control and the shoot content was even lower than in +FC +CS.
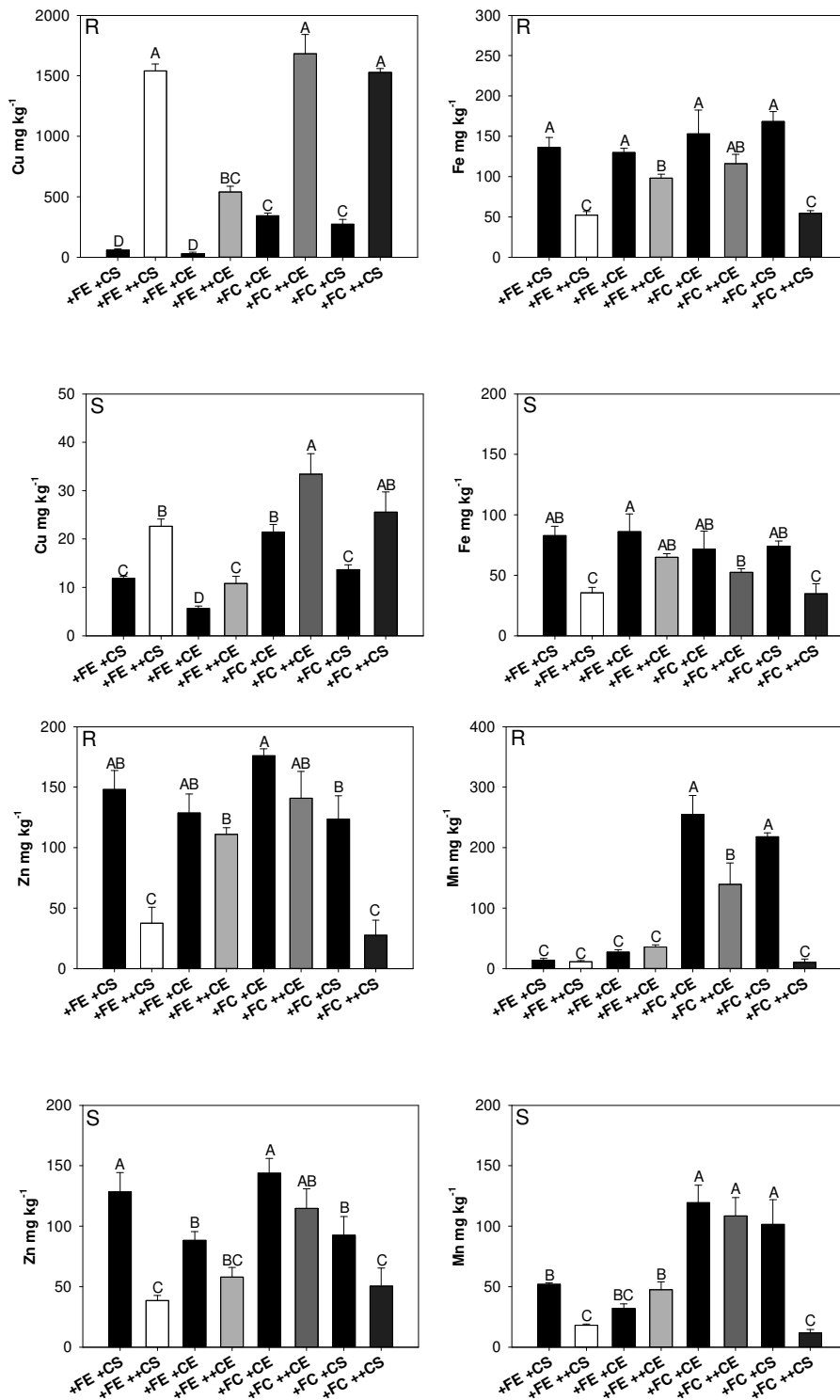
Figure 3. Concentration (mg kg^{-1} of dry weight) of micronutrients in shoots (**S**) and roots (**R**) of tomato plants grown in: +FE +CS (100 µM Fe-EDTA and 0.05 µM CuSO$_4$); +FE ++CS (100 µM Fe-EDTA and 100 µM CuSO$_4$); +FE +CE (100 µM Fe-EDTA and 0.05 µM Cu-EDTA); +FE ++CE (100 µM Fe-EDTA and 100 µM Cu-EDTA); +FC +CE (100 µM Fe-citrate and 0.05 µM Cu-EDTA); +FC ++CE (100 µM Fe-citrate and 100 µM Cu-EDTA); +FC +CS (100 µM Fe-citrate and 0.05 µM CuSO$_4$); +FC ++CS (100 µM Fe-citrate and 100 µM CuSO$_4$). For each element, letters indicate a significant difference (ANOVA Holm-Sidak; N=3, P<0.05).

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Calcium content in root increased approximately by one third in +FE +CE plants compared with the +FE +CS plants (Figure 4), while the content in +FE ++CS plants and +FC ++CS plants decreased significantly (by 30%). The translocation of Ca to the shoot seemed to be reduced in the same way for all the Cu-excess treatments except for +FC ++CE plants. Magnesium content remained steady in all the treatments in shoot and roots, while P translocation sharply decreased in +FE ++CS and +FC ++CS plants. Sulfur content in roots increased in +FE ++CS and +FC ++CS compare with +FE ++CS plats, while shoot contents were not significantly changed by either forms or availability of Cu or Fe.
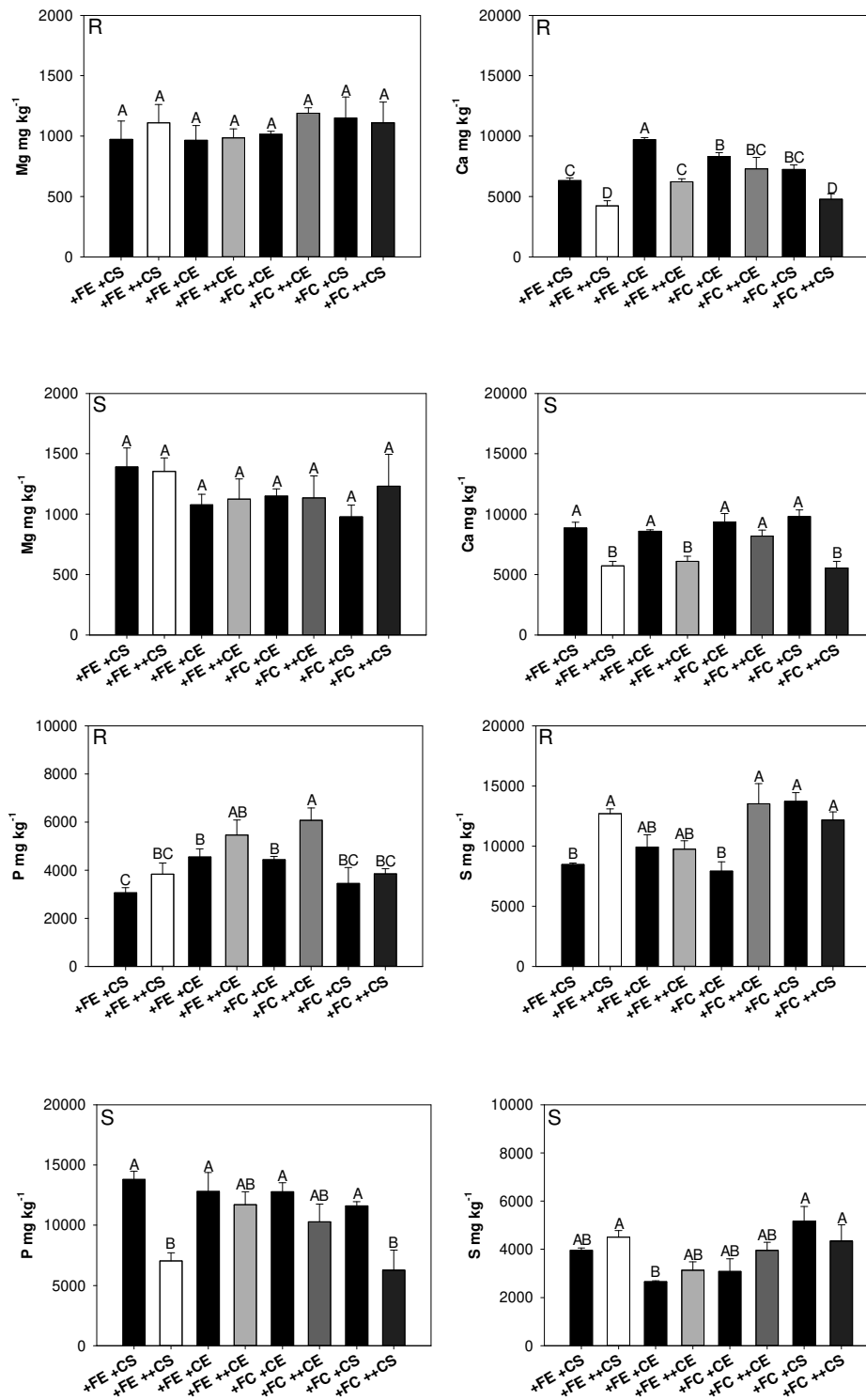
Figure 4. Total concentration (mg kg^{-1} of dry weight) of macronutrient in shoots (**S**) and roots (**R**) of +FE +CS (100 μM Fe-EDTA and 0.05 μM CuSO$_4$); +FE ++CS (100 μM Fe-EDTA and 100 μM CuSO$_4$); +FE +CE (100 μM Fe-EDTA and 0.05 μM Cu-EDTA); +FE ++CE (100 μM Fe-EDTA and 100 μM Cu-EDTA); +FC +CE (100 μM Fe-citrate and 0.05 μM Cu-EDTA); +FC ++CE (100 μM Fe-citrate and 100 μM Cu-EDTA); +FC +CS (100 μM Fe-citrate and 0.05 μM CuSO$_4$); +FC ++CS (100 μM Fe-citrate and 100 μM CuSO$_4$). For each element, letters indicate a significant difference (ANOVA Holm-Sidak; N=3, P<0.05).

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Antioxidant enzyme activities on the leaves

Different isoenzymes of SOD were detected using native-PAGE and SOD staining (Supplementary Figure 1). No significant difference was shown in the SOD activity except for +FE ++CS and +FC ++CS plants which showed a massive increase (more than three times) compared with the +FE +CS plants. At the same time, also CAT activity markedly increased in +FE ++CS and +FC ++CS (38% and 44% respectively). Peroxidaseactivity exhibited a slightly reduction in several treatments (48% in +FE ++CS plants) while in +FE ++CS and +FC ++CSplants it increased respectively by 46 and 35% (Figure 9).
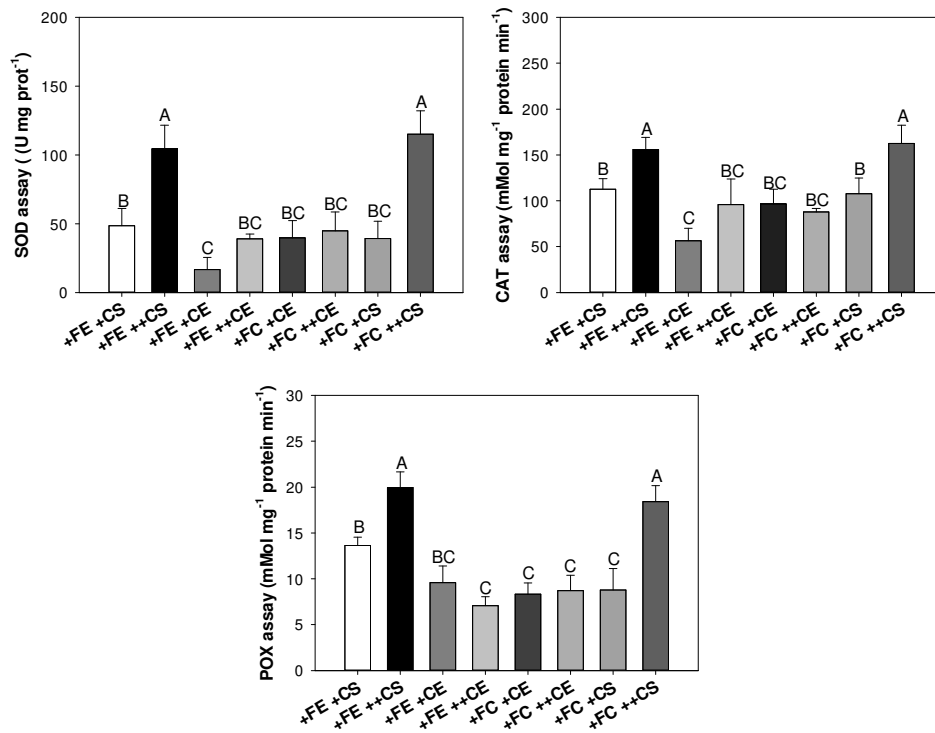
Figure 9. Effect of different nutritional conditions on the activity of superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POX) in leaves of maize plants. Data are reported as mean + standard deviation. For each element, letters indicate a significant difference among the means (ANOVA Holm-Sidak; N=3, P<0.05).

Expression analyses

The gene expression analyses were performed on genes involved in either Fe orCu acquisition. Membrane transporters, transcription factors and proteins involved in solubilization/acquisition mechanisms and response to the oxidative stress were investigated.

Taken together, the data obtained about the expression of genes involved in the Fe and Cu availability did not show any differences (data not shown).

Discussion

In the present study, different Cu sources were supplied at two concentrations previously tested to supply a normal or excessive amount of Cu (as $CuSO_4$) in combination with different Fe complexes to maize seedling with the purpose to evaluate if they affected growth and development of maize plants. Regardless of the source of Fe used, the excessive supply of $CuSO_4$ in the nutrient solution influenced the growth of maize plants while the same concentration of Cu-EDTA had no morphological effects (Figure 3). Shootand rootarchitectures were altered by the treatments, as shown in Figure 2, $CuSO_4$ excess induced a sharp reduction of fresh and dry weight of both shoots and roots, with symptoms such as stunted growth, leaf deformation, necrosis of apical meristems and also a strong effect to the root architectures which confirmed previous observation (Adress *et al.,* 2015; Ali *et al.,* 2015) along with a steady decline in chlorophyll content when Cu was supplied as $CuSO_4$ at high concentration (approximately 80% of the value found in Cu-EDTA treated plants; Figure 3).On the other hand, metal content and translocation seem to be scarcely affected by the sources of Cu supplied. Interesting, all the treatments with Cu excessrecorded an increaseof Cu content in both leaves and roots (e.g. about 27 times in the roots of +FE ++CS), while, the content and translocation of Fe and Zn decreased significantly only in treatments with excess of $CuSO_4$ (approximately 60%).This might be due to the fact that the root apoplast is a major metal accumulation compartment in plants(Krzeslowska, 2001). Allan and Jarrell (1989) explained that the adsorption of Cu on the root surface could take place in cationic form with negative cell-wall charges due to the network of cellulose, pectins and glycoproteins and act as specific ion exchangers. Regarding Fe, several studies showed that increasing Cu concentration in soil and nutrient solution resulted in reduced mobilization and uptake of Fe presumably due to a competition between Cu and Fe for complexation by phytosiderophores (PS) and/or the inhibitory effect of Cu-PScomplexes on Fe uptake (Zhang *et al.,*1991; Ma and Nomoto, 1996). In addition, based on model computations, Reichman and Parker (2005) reported that at neutral to alkaline pH values, Fe would be unable to outcompete with Cu for complexation by PS, so that soil Cu contamination in calcareous soils may impair Fe uptake and enhances the risks of Cu-induced Fe deficiency.Plants treated with Fe-citrate exhibited an altered content of Mn, with a marked increase, except inplants treated withthe highest amount of $CuSO_4$ which induced a sharp decreased in both shoot and roots (approximately-80%).This last behavior is in accordance with previous studies conducted in wheat which found out that Mn andalso Zn concentration significantly decreased in shoots of plant grown with 30 μM of $CuSO_4$(Keller *et al.,* 2014).

Ca content was reducedin roots and shootswhen plants were treated with $CuSO_4$ excess (+FE ++CS and +FC ++CS), this result could be explained by the high concentration of Cu^{2+} in the nutrient solution which tend to displace bivalent cations as $Ca^{2+}$from exchange sites and are strongly bound in root-free space (Jensèn and Adelsteinsson, 1989; Dronnet*et al.,* 1996).

When CuSO$_4$was supplied at high concentration,the activities of SOD, CAT and POX increased as also reported inChen *et al.* (2000), while all the Cu-EDTA treatments showedno differences in SODand CATactivity. Generation of ROS and antioxidant response in plants depend upon plant species, severity and the duration of Cu stress. Adress*et al.* (2015) reported that the increase of Cu levels in the growth medium causes a dose-dependent increase in ROS generation. Interestingly, in the condition used in this research, all the Cu-EDTA treatments didnot shownany increase in activity of ROS detoxicating enzymes. Generally, EDTA chelate has been used for chelate-enhanced phytoextraction of various heavy metals such as Pb, Cd, Zn or Cu. At the same time, EDTA may enhance the transport of elements from roots to shoots, because it may increase translocation. EDTA was found to induce the accumulation of Zn and Cu from heavy metal polluted soil in *Brassica juncea* (Baylock*et al.,* 1997).However, ÁrpádSzékely *et al.,* (2011) questioned whether EDTA really facilitates the translocation of Cufrom root to shoot. Isermann (1979) placed the chelates in the following order of chelate stability for EDTA: Fe^{3+}>Cu^{2+}>Zn^{2+}>Fe^{2+}>Mn^{2+}>Ca^{2+}>Mg^{2+}, this means that Cu^{2+}-EDTA chelate is among the stablemetal-EDTA complexes. It was found that Cu^{2+} can also form stable complexes and can be transported in the xylem sap bound to citrate (Mullins *et al.,* 1986), asparagine and histidine in soybean (White *et al.,* 1981) or nicotianamine in tomato (Pich and Scholz, 1996). In *Brassica carinata,* histidine and proline were the most important candidates for Cu binding at supraoptimal Cu concentrations, while the concentration of nicotianamine, a non-proteinogenic amino acid, increased in the xylem sap under Cu deficiency (Irtelli*et al.,* 2009).Metal-chelate complexesareexpectedtobelessphytotoxicthanfree-metal ions themselves, and synthetic chelate-metal complexes can be transported in the xylem sap as well as they can increase shoot metal concentration.When plants are supplied with Fe-PS,Fe concentration in the xylem sapis higher than when supplied with Fe-EDTA(Kawai *et al.,* 2001) demonstratingthat Fe-PS is more effective Fe sourcethan Fe-EDTA for grasses. On the base of our results, we suppose that when Cuis supplied as Cu-EDTA, Cu is scarcelyavailable, and it is much less transported into plant cells and most of it probably bind to the apoplast of root cells, thereforeit does not become phytotoxic. Instead, when Cu is supplied in the form of CuSO$_4$ it can be taken upprobably in the free-Cu ionor Cu-PS formbytransport systems occurring on the plasma membrane of root cells. Hence, when supplied as CuSO$_4$, Cu might compete with other cations for their carriers/transporters and also for the cell wall cation binding sites.

Based on our results and those from several studies,ascheme of describing the transport and accumulation of Cu in maize plants can be proposed as follows:Cu in the environment may bind to PSor maybe other exudates, like organic acids (citrate, malate, oxalate), and the Cu-complexes would be absorbed through membranes into root cytosolby a Cu-chelate transporter, which has not yet been identified,probably a yellow stripe-like transporter. In alternative, Cu could be directly taken up as free-Cu^{2+} by some unknown transporters. Or Cu might be taken up as Cu$^+$by transporter such as COPT1, which would require the reduction of Cu^{2+}bya plasma membrane reductase, as described in Arabidopsis (Bernal *et al.*, 2012) or by some root exudates that are able to reduce Cu. However, there are scarce evidence in the literature of any Cu reductase activity in (Fe) *Strategy II* plants (Babalakova and

Traykova, 2001). We tried to measure it on maize roots and we were unable to detect any (data not shown), therefore we are not expecting that a reductive based acquisition of Cu occurs in maize plants. Under high concentration of Cu-EDTA and with Fe-citrate as source of Fe, the higher affinity of EDTA for Fe might induce the exchange of chelator between the two complexes: Cu is then bound with citrate or become free of any chelator as Cu^{2+} and Fe will be chelated by EDTA. Copper could be bound to citrate and then taken up by some unknown membrane transporters or acquire as described previously in the ionic form(s). Conversely, in presence of Fe-citrate and high level of $CuSO_4$, Cu toxicity may be further increased due to a higher availability of Cu when citrate is present in the media.
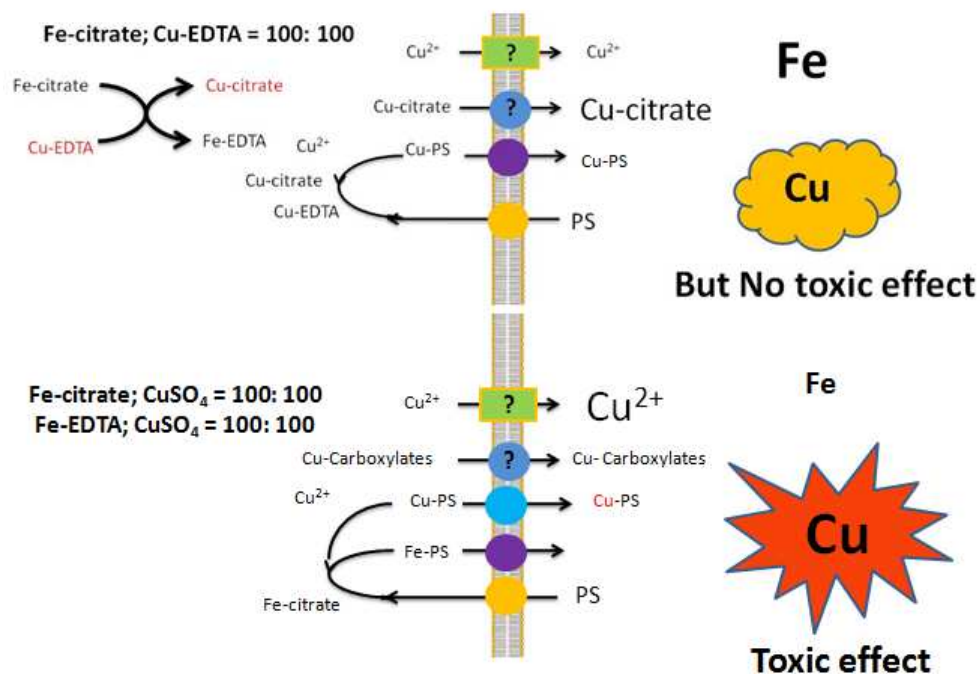
Figure10. Conceptual model of the putative mechanisms involved in the acquisition of Cu and Fe under Cu excess sources. PS= phytosiderophores.

The present study highlights new questions regarding the forms of Cu that can be acquired by *graminaceous* plants in function of the Fe sources supplied to the plants and presents a hypothesis regarding membrane-localized transporters involved in the uptake of Cu-complexed by citrate.

Further research on Cu acquisition and homeostasis are needed to improve the understanding of in which form this metal is available and how it is taken up and translocated by crops and its interaction with the acquisition of other micronutrients.

Supplementary Material
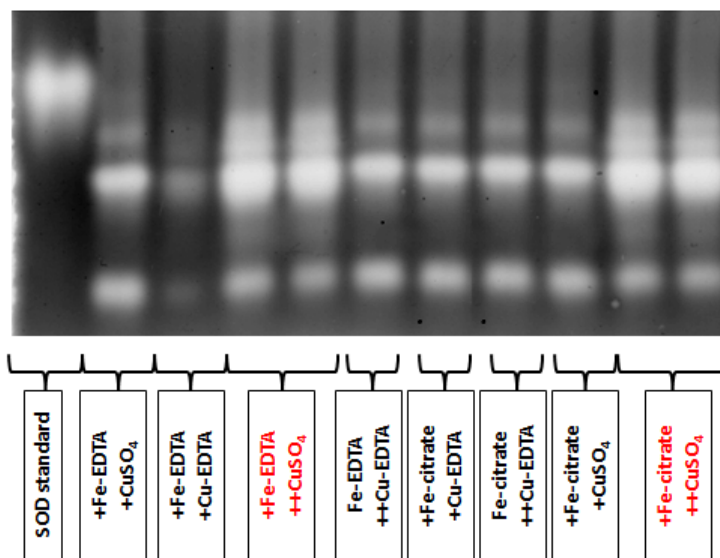
Supplementary Figure. In-gel activity of SOD isozymes of tomato leaf extracts from plants grown in a nutrient solution containing different concentrations and sources of Fe and Cu (μM): +Fe-EDTA +$CuSO_4$ (100 μM Fe-EDTA and 0.05 μM $CuSO_4$); +Fe-EDTA ++Cu (100 μM Fe-EDTA and 100 μM $CuSO_4$); +Fe-EDTA +Cu-EDTA (100 μM Fe-EDTA and 0.05 μM Cu-EDTA); +Fe-EDTA ++Cu-EDTA (100 μM Fe-EDTA and 100 μM Cu-EDTA); +Fe-citrate +Cu-EDTA (100 μM Fe-citrate and 0.05 μM Cu-EDTA); +Fe-citrate ++Cu-EDTA (100 μM Fe-citrate and 100 μM Cu-EDTA); +Fe-citrate ++$CuSO_4$ (100 μM Fe-citrate and 0.05 μM $CuSO_4$); +Fe-citrate ++$CuSO_4$ (100 μM Fe-citrate and 100 μM $CuSO_4$).

		Fe-EDTA CuSO4		Fe-EDTA ++CuSO4		Fe-EDTA Cu-EDTA		Fe-EDTA ++Cu-EDTA		Fe-citrate Cu-EDTA		Fe-citrate ++Cu-EDTA		Fe-citrate CuSO4		Fe-citrate ++CuSO4	
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
Cu	s	11.84	0.47	22.61	1.52	5.66	0.50	10.79	1.49	21.46	1.58	33.40	4.28	13.71	0.99	25.60	4.12
	r	57.74	10.22	1539.52	58.91	30.23	10.16	540.27	47.29	343.65	22.56	1682.40	158.55	272.71	41.54	1527.81	33.14
Fe	s	82.95	7.64	35.58	4.49	86.01	14.77	64.93	3.05	71.59	14.96	52.51	2.92	74.15	4.13	34.78	8.27
	r	136.40	12.00	52.03	4.60	130.02	5.28	98.16	4.69	153.16	29.27	116.06	11.42	168.24	12.49	54.20	3.65
Zn	s	128.49	15.99	38.48	4.19	88.25	7.38	57.88	8.08	143.84	12.18	114.79	16.24	92.60	15.37	50.58	14.74
	r	148.13	15.54	37.59	13.05	128.55	15.89	111.08	5.37	176.11	5.63	140.91	22.17	123.70	19.17	27.45	12.57
Mn	s	51.92	1.25	18.23	1.00	32.08	3.75	47.43	6.53	119.62	14.43	108.41	15.40	101.63	20.35	11.77	2.86
	r	13.75	3.10	11.16	2.12	27.14	4.27	35.48	3.76	254.82	31.40	139.35	35.30	217.98	6.30	10.31	5.40
Mg	s	1391.75	155.64	1353.91	111.65	1076.64	88.46	1125.68	166.45	1150.17	60.06	1136.05	181.39	976.59	98.86	1233.31	263.37
	r	972.09	152.18	1110.61	152.60	965.92	120.43	985.51	74.10	1016.34	23.73	1188.71	46.84	1149.88	174.38	1109.53	170.94
Ca	s	8858.08	462.00	5723.48	374.25	8590.46	118.35	6105.07	402.29	9358.00	703.92	8184.56	505.25	9797.83	550.00	5539.58	542.28
	r	6318.17	231.84	4228.02	409.83	9693.77	177.22	6221.06	257.26	8308.08	316.87	7291.08	945.99	7238.73	375.58	4766.31	445.74
P	s	13802.60	684.28	7026.10	686.13	12818.83	1567.54	11711.17	1069.55	12779.59	760.14	10280.95	1483.20	11595.59	356.71	6269.75	1663.62
	r	3067.08	211.66	3840.48	456.71	4542.32	343.55	5464.29	616.81	4435.32	135.36	6071.17	508.89	3448.22	658.66	3848.72	211.23
S	s	3956.25	99.50	4500.12	279.27	2653.71	44.89	3131.03	353.11	3074.54	533.39	3950.91	339.85	5166.45	612.65	4345.82	670.61
	r	8462.91	123.62	12681.90	412.98	9918.95	1022.19	9738.39	702.91	7916.26	787.76	13520.09	1663.08	13721.83	723.99	12161.39	685.86

Table. Elemental composition of maize shoot (s) and root (r) samples from plants grown in presence of:+Fe-EDTA +CuSO$_4$ (100 µM Fe-EDTA and 0.05 µM CuSO$_4$); +Fe-EDTA ++Cu (100 µM Fe-EDTA and 100 µM CuSO$_4$); +Fe-EDTA +Cu-EDTA (100 µM Fe-EDTA and 0.05 µM Cu-EDTA); +Fe-EDTA ++Cu-EDTA (100 µM Fe-EDTA and 100 µM Cu-EDTA); +Fe-citrate +Cu-EDTA (100 µM Fe-citrate and 0.05 µM Cu-EDTA); +Fe-citrate ++Cu-EDTA (100 µM Fe-citrate and 100 µM Cu-EDTA); +Fe-citrate ++CuSO$_4$ (100 µM Fe-citrate and 0.05 µM CuSO$_4$); +Fe-citrate ++CuSO$_4$ (100 µM Fe-citrate and 100 µM CuSO$_4$).Data are reported as means and standard deviations of 3 replicates per samples. For each element, letters indicate a significant difference among the means (ANOVA Holm-Sidak; N=3, P<0.05).

5. Implication of copper and iron availability on tomato growth and development

Introduction

Copper (Cu) is an essential mineral nutrient that participates in the plant metabolism mediating oxido-reductive reactions of considerable biological importance such as photosynthesis and mitochondrial respiration(Festa and Thiele, 2011). This heavy metal plays an important role as a cofactor in several enzymes, such as in the superoxide dismutase, cytochrome c oxidase, amino oxidase, laccase and polyphenol oxidase (Yruela, 2005; Krämer and Clemens, 2006). Copper also interacts with the metabolism of carbon and nitrogen and allows the diffusion of hormonal signals (Hansch and Mendel, 2009). At the cellular level, it has an essential role in cell wall metabolism, signaling to the transcription protein trafficking machinery, oxidative phosphorylation, iron mobilization and the biogenesis of molybdenum cofactor (Raven *et al.,* 1999; Gratão *et al.,* 2005). Copper is highly reactive and can be toxic for the plant *via*Fenton reaction. Thus, the intracellular Cu level must be tightly regulated, since at high concentrations, it can cause morphological, anatomical and physiological changes in plants, resulting in a decrease of both food productivity and quality (Waters, 2013). Typical symptoms of Cu deficiency include stunted growth, leaf deformation, necrosis of apical meristems and chlorosis of young leaves (Rahimi and Bussler, 1978).

As Cu, iron (Fe) is anothertransitionmetal that is required by plants for itsroles in redox chemistry and for this reason both Fe and Cu homeostasis are deeply linked in the cell metabolism. Based on the capability of Fe and Cu to change their redox status, these two nutrients mediate several oxidation/reduction processes in plants (Hänsch *et al.,* 2009; Marschner, 2012; Welch, 1995).

In order to overcome Fe limitation, plants have evolved different mechanisms to acquire Fe from sparingly availablesources from soils (Giehl *et al.,*2009). The Fe acquisitionmechanism in non-graminaceous plants occurs through a reduction-based mechanism (*Strategy I*), while grasses (*Strategy II*) rely on the biosynthesis and release of phytosiderophores (PS) and direct uptake of Fe(III)–PScomplex(Hördt*et al.,*2000).

Despite few information are available about the mechanisms of Cu acquisition (Sancenón *et al.,* 2003; Wintz *et al.,* 2003; Ryan *et al.,* 2013), it seems that some features of Fe acquisition processes are similar to those activated by roots to take up Cu. Copper mightbe taken up by eithermechanisms: reduction-based strategy or through a direct acquisition ofCu-PS complexes (Chaignon *et al.,* 2002; Ryan *et al.,* 2013).Therefore, it is plausible suppose that, due to thesimilar molecular functions and mechanism of absorption, some crosstalk between the Cu and Fe nutritional acquisition pathways and their regulation might exist. Moreover, due to the wide use in agriculture of Cu-based phytosanitary products, it is necessary to develop new strategies to improve Cu-excess tolerance and iron acquisitionin Cu-contaminated soils.

Tomato (*Solanumlycopersicum* L.) is a crop widely cultivated in the word and it has been extensively used for Fe deficiency studies, tomato beinga model plant for studying the response of*Strategy I* plants.The better comprehension of the Cu-acquisition mechanism and the cross connection between

Fe and Cu nutritional pathways in tomato can improve the knowledge on plant nutrition and contribute to define new guidelines for an environmental sustainability of agriculture.

Materials and methods

Plant material and growth conditions

Tomato seedling (*Solanum lycopersicum* L., cv. 'Marmande superprecoce' from DOTTO Spa, Italy), germinated for 6 days on filter paper moistened with 0.5 mM CaSO$_4$, were grown for 14 days in a continuously aerated nutrient solution:(mM): K$_2$SO$_4$ 0.7, KCl 0.1, Ca(NO$_3$)$_2$, MgSO$_4$ 0.5, KH$_2$PO$_4$ 0.1;(μM): H$_3$BO$_3$ 10, MnSO$_4$ 0.5, ZnSO$_4$ 0.5, CuSO$_4$ 0.2, (NH$_4$)$_6$Mo$_7$O$_{24}$ 0.01. pH of the solution was adjusted at 6.0 with 0.6 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH.Iron and Cu were provided in forms ofFe-ethylenediaminetetraacetic acid(EDTA), 10μMFe-EDTA for the Fe limitingcondition or 100 μM Fe-EDTA for the Fe sufficient conditions, respectively, and in form of CuSO$_4$0.2μMCuSO$_4$ for the Cu sufficient condition; thereafter, some plants were transferred for two weeks to a Fe-limiting and Cu-free nutrient solution (-Fe -Cu, 10 μMFe-EDTA and 0 μM CuSO$_4$), to a Fe-limiting nutrient solution (-Fe +Cu, 10 μMFe-EDTA and 0.2μM CuSO$_4$), to a Cu-free nutrient solution (+Fe -Cu, 100 μMFe-EDTA and 0 μM CuSO$_4$), and some tomato plants were transferred for two weeks to a complete nutrient solution containing both Fe and Cu as control (+Fe+Cu, 100 μMFe-EDTA and 0.2μM CuSO$_4$). Nutrient solutions were renewed every three days. The controlled climatic conditions were the following: day/night photoperiod:16/8 h; light intensity:220 μE m^{-2}s^{-1}; temperature (day/night): 25/20°C; relative humidity:70 to 80%.

Treatment	Symbol	μM Fe-EDTA	μM CuSO$_4$
Control	+Fe +Cu	100	0.2
low-Fe	-Fe +Cu	10	0.2
Cu deficiency	+Fe -Cu	100	0
low-Fe and Cu deficiency	-Fe -Cu	10	0

Table 1. Composition of the nutrient solutions in tomatoin the last two weeks of growth.

Characterization of plant growth and element analysis

Plants were harvested separating roots and shoots and Fresh weight (FW) of roots and shoots were assessed. Chlorophyll content was evaluated measuring light transmittance of fully expanded leaves using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan) and reportedas SPAD index values. Measurements were carried out weekly on young leaves (at least 2 young leaves per plant) and twelve SPAD measurements were taken per each leaf, five plants were measured per each treatment.

The elemental analysis was conducted on shoot and root samples which were oven-dried at 105°C and nitric acid-digested in a microwave oven (MARS Xpress, CEM, Matthews, NC, USA).Macro-andmicro-nutrients (Cu, Fe; Zn, Mn; Ca, Mg, P and S) were measured by inductively coupled plasma optical emission spectroscopy analyses (ICP-OES; Varian Vista Pro axial, USA).

Acidification capability of the whole root system

Root acidification induced bytomato plants grown in the different conditions (control, CTR, -Fe +Cu, +Fe -Cu, -Fe -Cu) were visualized on agar gel containing a pH indicator, bromocresol purple. Intact plants were taken from the pots and the roots were rinsed in distilled water. Afterward, for each plant, the whole root systemwasplaced on a 3-mm-thick agar gel layercontaining 0.1 gl^{-1} pH indicator (bromocresol purple).The agar gel film was wrapped with an aluminum foil to avoid light expositiononto the root zone and placed in the growth chamber for 4 hours before visualization.

Visualization of root reduction of Fe^{3+} and Cu^{2+}

Roots were embedded in a gel containing of 0.9% (w/v) agar, 5 mM MESbuffer (pH 5.5), 0.1 mM Fe(III)-ETDA and a colorimetric indicator for Fe reduction(0.3 mM Na$_2$-bathophenanthrolinedisulfonic acid(BPDS)). For the visualization of Cu^{2+} reduction, the gelcomposition was: 0.9% (w/v) agar, 5 mM MES buffer (pH 5.5), 0.2 mM CuSO$_4$, 0.6 mM Na-citrate, and 0.4 mM Na$_2$-2,9-dimethyl-4,7-diphenyl-l,10-phenanthrolinedisulfonic acid (BCDS). The visualization reagents were added to agar after dissolving the agar in a heated MES buffer and after that the gel wascooleddown to 45°C. The liquefied agar mixture was poured into glass trays, and when cooled to 30°C the roots were submerged in the viscous solution. Upon further cooling, the agar solidified around the roots. Reddish-color of Fe(II)-BPDS complex staining patterns developed around roots thatwere actively reducing Fe^{3+} to Fe^{2+}. Orange-color of Cu(I)-BCDS complex staining patterns developed around roots which were actively reducing Cu^{2+} to Cu^+.

Assay of Fe^{3+} and Cu^{2+}reductases

Rates of root-associated Fe^{3+} and Cu^{2+} reduction were quantified via the spectrophotometric measurement of Fe(II)-BPDS or Cu(I)-BCDS production. The assay solution for the quantification of Fe^{3+}reductioncontained 0.2 mM CaSO$_4$, 5 mM MES buffer (pH 5.5),0.1 mM Fe(III)-ETDA and 0.3 mM Na$_2$-BPDS; while for the Cu^{2+} reduction assay, the solution contained 0.2 mM CuSO$_4$, 0.6 mM Na$_3$citrate, and 0.4 mM Na$_2$BCDS. Just prior to initiation of the reductase assays, the roots were rinsed for 5 min in 0.2 mM CaSO$_4$ solution. The roots of intact plants were used for the reductase assays. The absorbance(535 nm for Fe^{2+}-BPDS; 483 nm for Cu^+-BCDS) of the assay solutions were determined spectrophotometrically after10,20,30, 40 and50 minutes, an aliquot of identical solution from assay medium containing no roots was used as blank. The concentration of Fe(II)-BPDS was calculated using a molar extinction coefficient of 22.14. mM cm^{-1}; while the molar extinction coefficient used for Cu(I)-BCDS was 12.25 mM cm^{-1}. Results were expressed by μmol g^{-1}FWh^{-1}.

Protein content and antioxidant enzyme activities

One gram of fine leaf powder was homogenized in 5.0 mL of 100-mM potassium phosphatebuffer (pH 7.5) containing 3 mM dithiothreitol (DTT), 1 mM EDTA and 4% (w/v) polyvinylpolypyrrolidone (PVPP) (Hippler et al., 2016). The suspension wascentrifuged at 12,100 × g at 4 °C for 35 min, and the supernatant was stored at -80 °C for furtheranalysis. The total protein content was determined according to Bradford (1976) using bovineserum albumin (BSA) as a standard.

The superoxide dismutase (SOD) activity staining was carried out as described by Sathya and Bjorn (2014).Two-mL assay reaction mixture contained 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT, and 0.025% Triton-X100. Forty microliters of diluted (2x) sample and 20 µL of 1-mM riboflavin were added and the reaction was initiated by illuminating the samples under a 15-W lamp.

During the 10min exposure, the test tubes were placed in a box lined with aluminum foil. The box with the test tubes was placed on a slowly oscillating platform at an approximatively distance of 12 cm from the light source. Duplicate tubes with the same reaction mixture were kept in the dark and used as blanks. Absorbance of the samples was measured immediately after the reaction was stopped at 560 nm. The enzyme activity (grams per fresh weight) of a sample was determined from a standard curve obtained by using a pure SOD.

Catalase activity was determined according to Kraus *et al.* (1995) with modifications (Azevedo *et al.,* 1998). The reaction started by addition of 20 µL of plant extract in a reaction mixture containing 1.0 mL of 100-mM potassium phosphate buffer (pH 7.5) and 2.5 µL H_2O_2 (30% solution) at 25 °C. The enzyme activity was determined by following the decrease in absorbance at 240 nm, which represents the disproportionation of H_2O_2, for 1 min against a plant extract-free blank. Catalase activity was calculated using an extinction coefficient of 39.4 M^{-1} cm^{-1}.

Peroxidase (POX) activity was determined following the method of Kar and Mishra (1976). The assay mixture contained 25 mM phosphate buffer (pH 6.8), 20 mM pyrogallol and 20 mM H_2O_2. The samples were incubated at room temperature for 1 min and 0.5% H_2SO_4 (v/v) was added to stop the reaction. The activity was estimated by measuring the absorbance at 420 nm for 1 min, and a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} was used in calculations. CAT and POX activities were expressed as µmol min^{-1} mg^{-1} protein.

Real-time RT-PCR experiments

Total RNA was isolated using the Spectrum™ PlantTotal RNA kit (Sigma-Aldrich) and quantified by spectrophotometry using NanoDrop™ 1000 (Thermo Scientific).A running gel (1% agarose; 80mV) was usedto check for the quality of the RNA extraction. Five hundred nanograms of total RNAof each sample was retrotranscribedusing 1 pmol of Oligo d(T)23VN (New England Biolabs,Beverly, USA) and 10 U M-MulV RNase H[-] for 1 h at 42 °C (Finnzymes, Helsinki, Finland) following the applicationprotocol of the manufacturers. After RNA digestion with1 U RNase A (USB, Cleveland, USA) for 1 h at 37 °C, geneexpression analyses were performed by adding 0.16 µL ofthe cDNA to the realtime PCR complete mix, FluoCycle™sybr green (20 µL final volume; Euroclone, Pero,Italy), in a DNA Engine Opticon Real-Time PCR Detection (Biorad, Hercules, USA). Specific primers (Tm = 58 °C) were designed to generate 80–150 bp PCR products. *LeH1*, coding for histone protein, was used as housekeeping gene for relative quantification.Each Real-Time RT-PCR was performed in 2 technical replicates on each of the three biological replicates foreach treatment.Analyses of real-time result wereperformed using Opticon Monitor 2 software (Biorad,Hercules, USA). Sequences of forward and reverseprimers are reported to Table 2.

		Primer Forward	Primer Revers
Solyc06g084020.2.1	LeHL1	CAAAGGCCAAAACTGCTACC	AGGCTTTACAGCTGCTTTCG
Solyc03g032090	LeANTL1	GTCCTGTTGCATCCCTCATT	CGAACAACCATAATGCACGA
Solyc06g005620	LeZIP	AGACAACTTCACTTGGGGATACA	GGTTTCTCCATGCCTCTCCC
Solyc02g069200	LeIRT1	CTGGCTACGGGGTTTATGCA	GCGGACAACATTGCCACAAA
Solyc11g012700	LeOPT3	GAAGCTCTTCATCCGGACAG	AATCCTCCGGGACCAAATAC
Solyc11g007130	LeTCR	GAGGCACCACGAGAGAGAAC	TGGTCACCAACGTCTCAAAA
Solyc11g018530	LeNRAMP1	ATTGGCCTGCAGAGATATGG	TTCCCCAAAGAAACAAGCTG
Solyc01g006150	LeMn-CDF	AAGGCAATGCAAATCTCCAA	CCAACGTAGATGCAGCGATA
Solyc09g072620.2	LeGLT	GCATTTGTTGCCAAGGAGCA	CACATGTTTGGCGACAGCAA
Solyc06g048410.2	LeFe-SOD	ACCTGAAGACAAAAAGCTTGCTC	CCAAACGTCGATGGTGAGGA
Solyc06g049080.2.1	LeMn-SOD	TCTGGGTATAGACGTTTGGGA	TTCTTCAGGTAATCTGGTCTTACA
Solyc00g026160	LeFRO4	AACGAACCACAACAACACGA	AATTATCGCTCCAAGCCAGA
Solyc01g094910	LeFRO1	ACTGGGGCTACAAATCGAGG	TCAGATGGGTTGGGCTTGAA
Solyc01g080670.2.1	LeSPL7	TGCATCACCTGGAAACATGC	CTGGTCCCTTCAGCTTGACT
Solyc06g051550.2.1	LeFER	ACATTGCCAGATCCTATTTCGC	TTTTGGTGGTAGCCGTTGTG
Solyc07g064040.2.1	LeILR3	CAGCAATTGGAAACCCTCAT	GGTACTCTCCGGTGAAACCA

Table 2. Primer used for quantitative RT-PCR analysis.

Statistical analyses

Analyses were performed on three independent biological replicates obtained from independent experiments and a pool of three plants was used for each sample. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test ($P<0.05$, n = 3). Statistical analyses were performed using SigmaPlot Version 12.0 software.

Results

Morphological modifications

In this study, tomato plants were grown in four different conditions: complete nutrient solution, Fe deficiency, Cu deficiency,both Fe and Cu deficiencies. After 37 days, plant morphology and size were affected in different manners by the four treatments. Different symptoms were observed in plantsdepending on the type of nutrient deficiency that was induced (Figure 1). Observing the leaf modification, Fe deficientplants showed the typical symptoms of interveinal chlorosis in the young leaves (Alloway *et al.,* 2008). The symptoms induced by Cu starvation are in agreement with those previously described by other works, such as symptomsin leaves of wilting, melanism and twisted tips (Sommer, 1931; Bailey and McHargue, 1943; Alloway *et al.,* 2008). Plant grown with both Fe and Cu deficiencyshowed attenuated symptoms, the main visible phenotypewas a limited shoot development in -Fe-Cu tomato plants.

Comparing the control with the other three treatments, some changes in the root morphology and quantity occurred (Figure 2). Plants grown in Fe deficiencyshowed a high proliferation of lateral roots and root hairs. In Cu-deficient plants,a much smaller root apparatus was observed, with less root hairs and shorter lateral roots. In general, plants grown in double deficiencies (-Fe-Cu) have a high proliferation of lateral roots but with scarce density of root hairs.

Biomass accumulation

Concerning the biomass accumulation, tomato plants showedsome differences in the fresh and dry weight (Figure 3) with significant differences between plants grown under Cu deficiencyor in the double deficiency. This latter condition (-Fe-Cu) showed a decreased of 50 % of leaf dry weight in comparison with the control condition (+Fe+Cu), although no changes occurred for root dry weight.
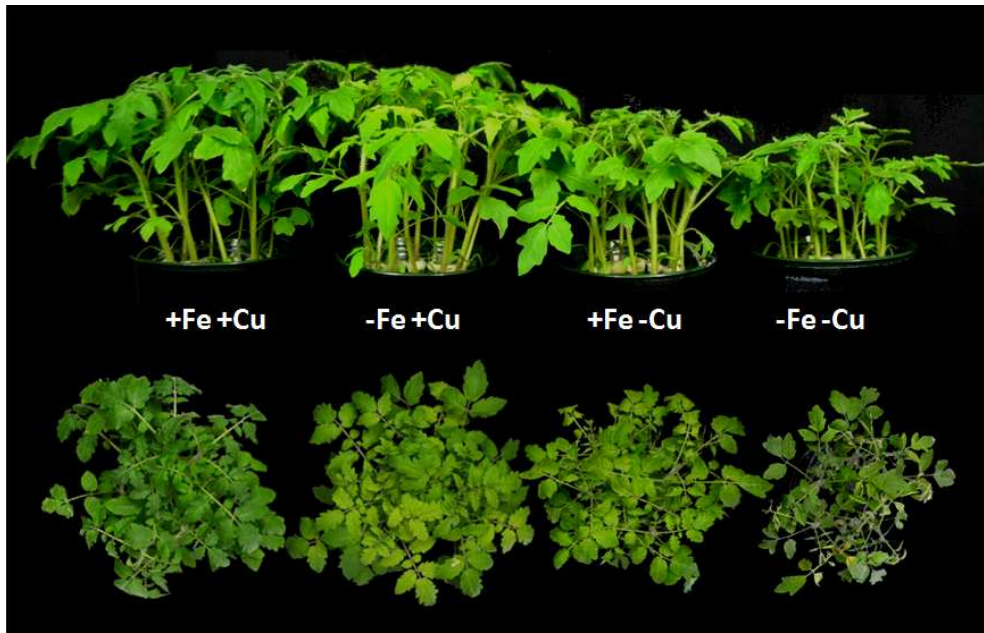
Figure 1. Tomato plants at 37 days of hydroponic cultivation in conditions of complete nutrient solution (+Fe+Cu), Fe deficiency (-Fe+Cu), Cu deficiency (+Fe-Cu), both Fe and Cudeficiencies(-Fe-Cu).
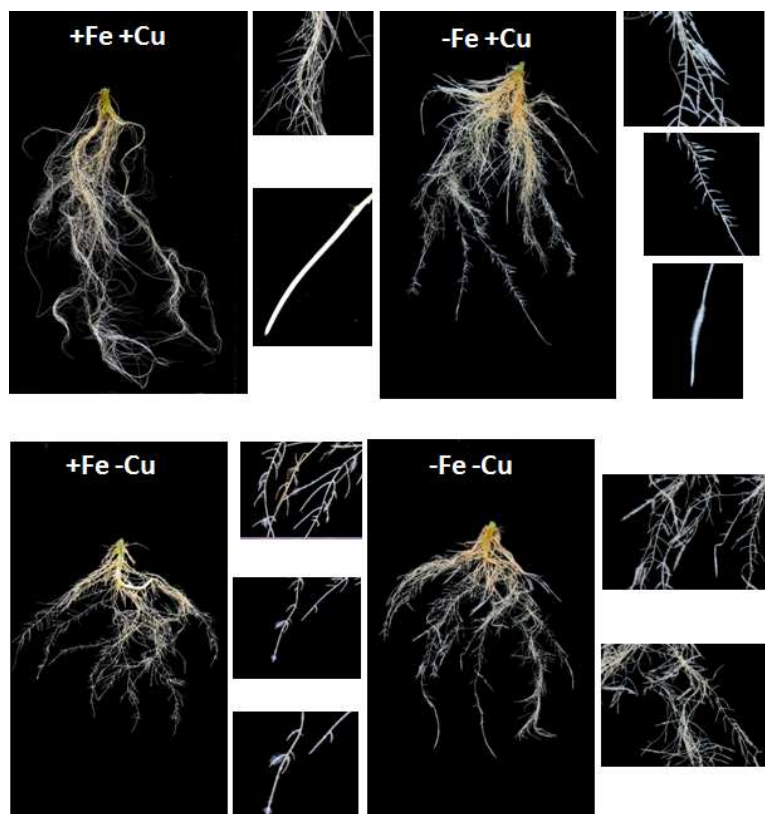
Figure 2. Tomato root development at 37 days of growth under complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe+Cu), Cu deficiency (+Fe-Cu), both Fe and Cu deficiencies (-Fe-Cu).
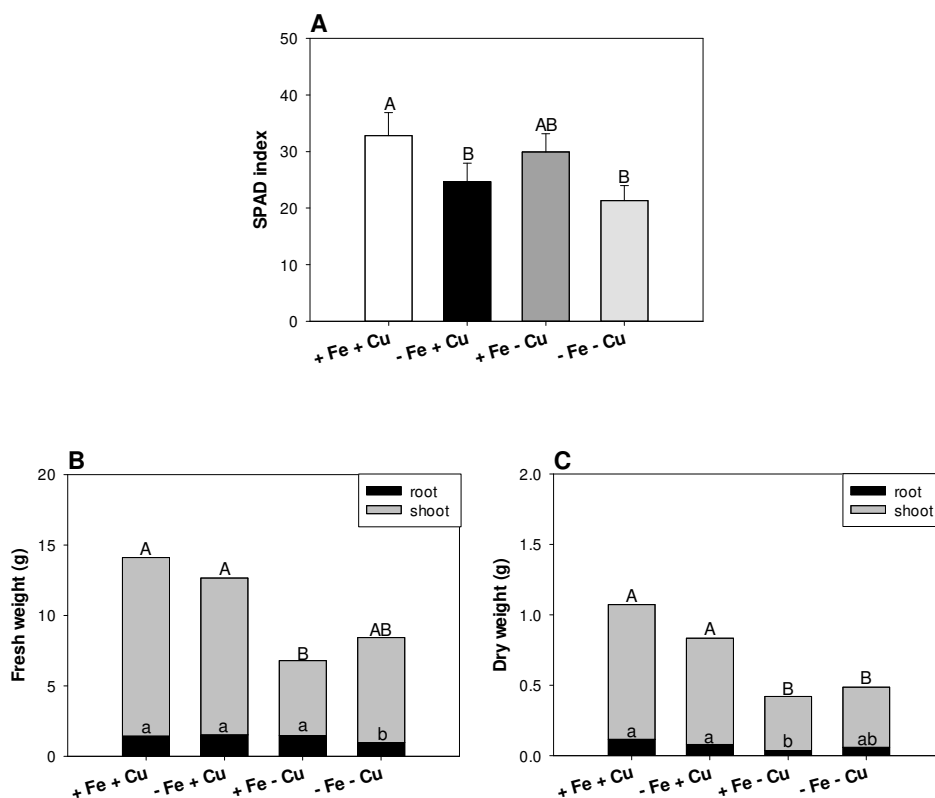
Figure 3. SPAD index values of tomato leaf (**A**) and fresh (**B**) and dry (**C**) weight were measured at the end of the growth period (35 days).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences in shoots among the mean values, small letters refer to statistically significant differences in roots among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).

The SPAD index determination is a quick and frequent analysis that allows to evaluatethe chlorophyll content in leaves. SPAD index values of plants grown under Fe deficiency (-Fe +Cu or –Fe -Cu) showed a significant reduction in comparison to Fe and Cu sufficient plants (+Fe+Cu), while leaves of Cu deficient plants (+Fe-Cu) showed intermediate values that were not significantly different from those induced by the other nutritional conditions.

Elemental composition of plants

Besides Fe and Cu, ICP-OES analysis allowed the quantification of other plant nutrients, such as Zn, Mn, Ca, Mg, S, and P (Figure 4 and 5). There were similar trends in metal contents both in shoots and roots. In general, roots have a lower concentration of these minerals than shoots. As expected, Fe or Cu deficiencyinduced a lower content of either metal when plants were grown under this specific metal starvation. In comparison to control plants, Fe-deficienttreatments (-Fe +Cu and -Fe -Cu) showed a drastic reduction of Fe content in both shoots and roots. Same behavior was observed concerning the Cu content in shoots and roots of Cu deficient plants (+Fe -Cu and -Fe -Cu). Moreover, it was observed that only -Fe +Cu leaves showed an increase of Cu content, while +Fe -Cu plants showed an increase of Fe content in the roots and a concomitant decrease of Fe in the shoots.

Under Fe deficiency, both -Fe +Cu and -Fe -Cu plants showed a significant increase of Zn content in shoots and a decrease of Mn in roots in comparison to control plants (+Fe +Cu). Instead, Mn shoot content is similar for all treatments, except for Fe and Cu deficiency condition that showed a significantly increase of Mn content (double level than in control plants).

Contentof macronutrients (Figure 5) showedsome significant differences; Mg content in root was higher under Fe deficiency, while in shoot no significant changes occurred in Fe- and/or Cu-deficient plants in comparison to control. Calcium was significantly more concentratedin roots of Cu-deficiency treatments (+Fe -Cu, -Fe -Cu) than those ofcontrol plants, while, in shoots, Ca was much more abundant in Fe-deficient plants (-Fe +Cu, -Fe -Cu). Compared to control plants, P content increased in roots under +Fe -Cu while it increased in shoot under both Cu-deficient conditions (+Fe-Cu, -Fe-Cu). Finally, Cu-deficienttreatment (+Fe -Cu) showed a drastic reduction in shoots of S content compared with control.
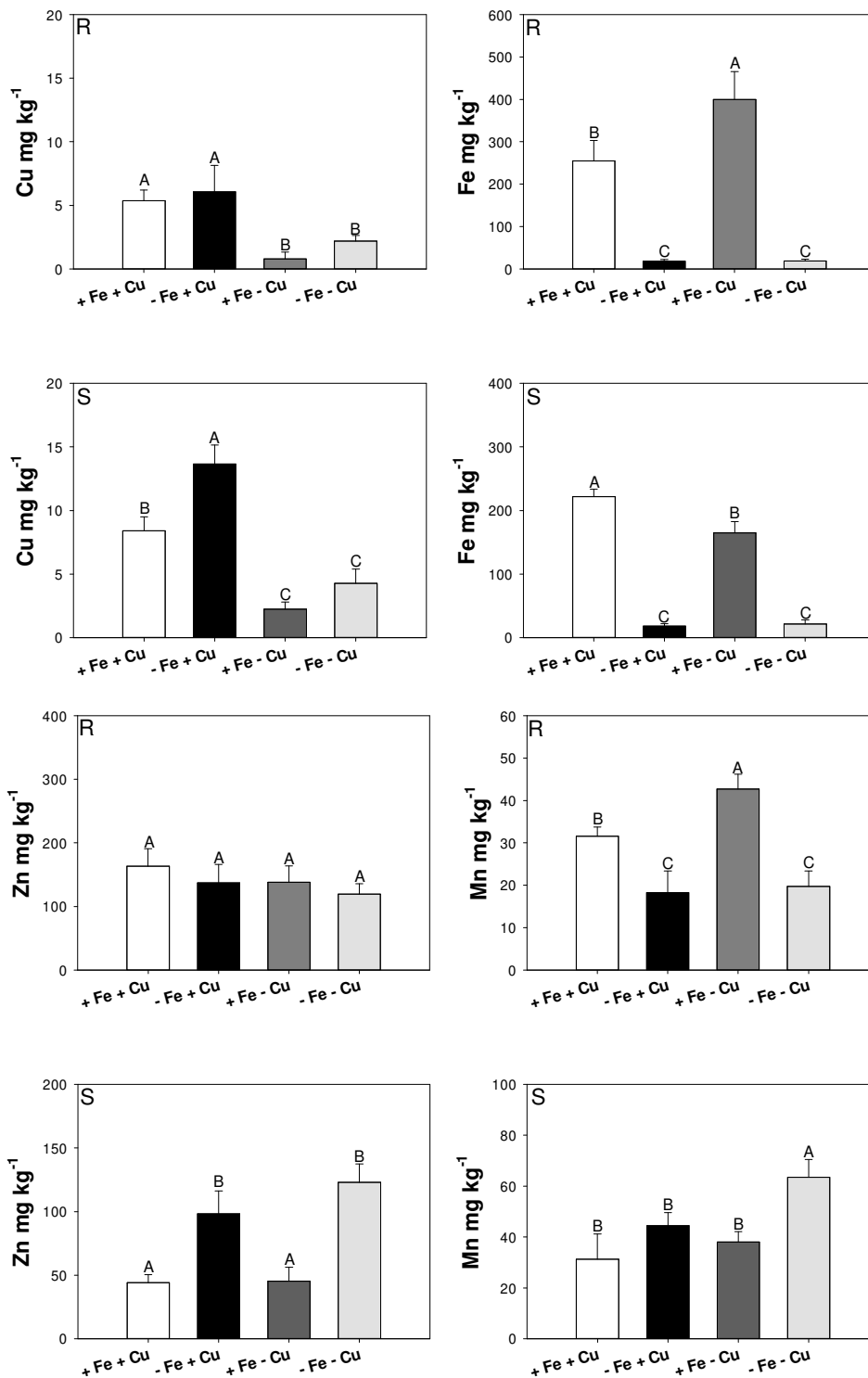
Figure 4. Copper, Fe, Zn and Mncontent(mg kg^{-1} of dry weight) in shoots (**S**) and roots (**R**) of plants grown under different mineral nutritional status: complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) or both Fe and Cu deficiencies(-Fe -Cu).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).
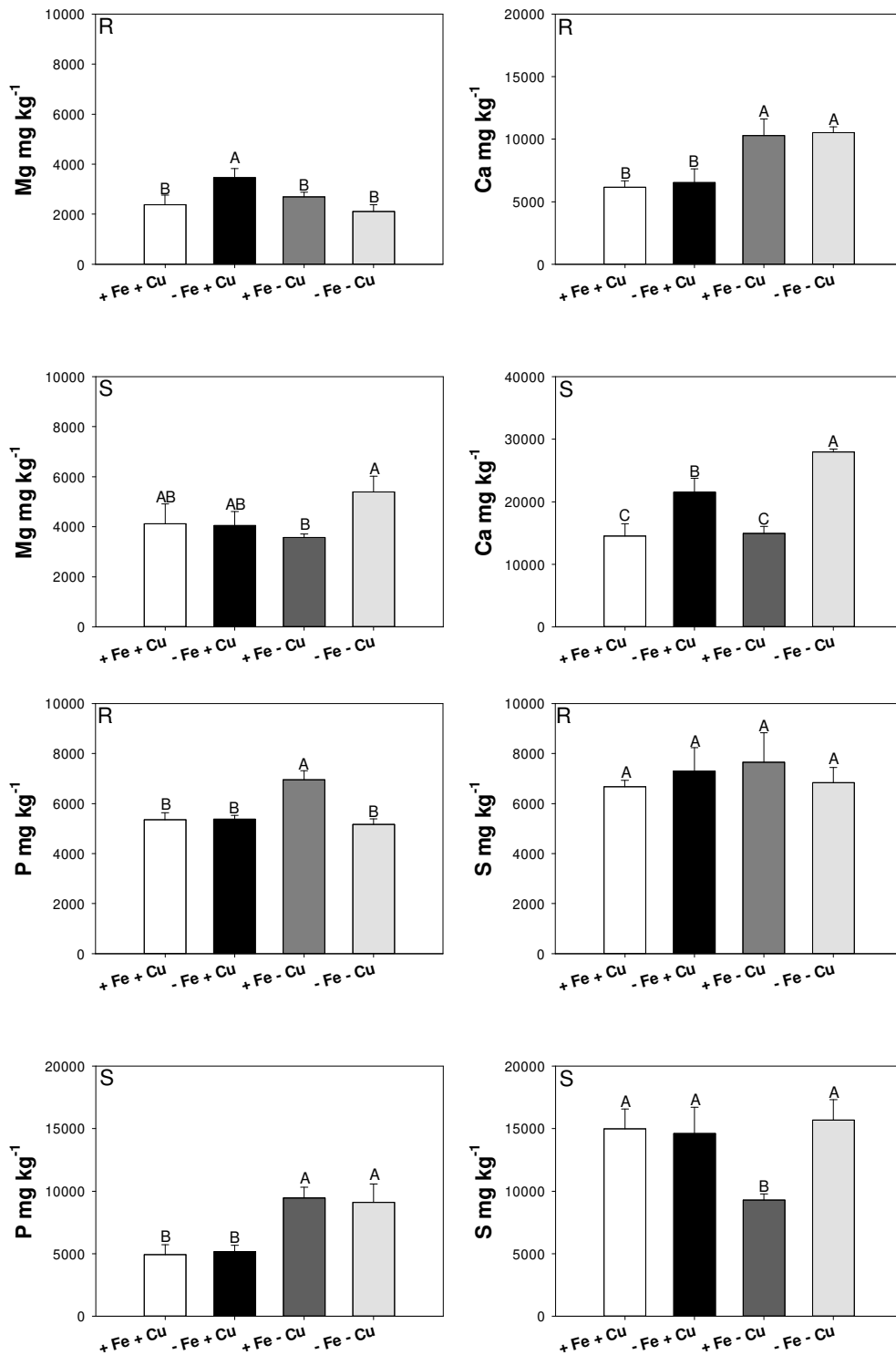
Figure 5. Magnesium, Ca, P and S content (mg kg^{-1} of dry weight) in shoots (S) and roots (R) of plants grown under different mineral nutritional status: complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) or both Fe and Cu deficiencies (-Fe -Cu). Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).

		+Fe +Cu		-Fe Cu		+Fe -Cu		-Fe -Cu	
		Average	*St.Dev.*	*Average*	*St.Dev.*	*Average*	*St.Dev.*	*Average*	*St.Dev.*
Cu	*s*	8.40b	1.11	13.99a	1.57	2.23c	0.56	4.27c	1.14
	r	5.37a	0.83	6.07a	2.08	0.80b	0.53	2.18b	0.45
Fe	*s*	221.76a	11.66	18.21c	3.86	164.80b	17.92	21.45c	6.62
	r	254.98b	48.26	18.41c	3.46	399.75a	65.57	18.82c	3.99
Zn	*s*	44.07a	6.48	98.36b	17.97	45.23a	11.00	123.06b	14.47
	r	163.27a	27.32	136.92a	29.30	138.21a	25.49	119.41a	16.19
Mn	*s*	31.25b	9.94	44.51b	5.16	38.04b	4.11	63.55a	6.98
	r	31.55b	2.20	18.23c	5.17	42.73a	3.48	19.72c	3.67
Mg	*s*	4115.22ab	796.89	4048.47ab	562.81	3578.81b	130.67	5392.96a	626.02
	r	2372.50b	388.55	3465.34a	354.49	2691.94b	190.58	2110.59b	267.09
Ca	*s*	14538.21c	1951.16	21538.13b	2241.88	14961.28c	1085.58	27972.21a	462.80
	r	6148.45b	517.15	6529.86b	1095.68	10291.23a	1315.80	10517.58a	458.09
P	*s*	4920.42b	794.64	5161.98b	506.53	9451.67a	875.51	9102.51a	1466.87
	r	5356.44b	273.36	5378.64b	144.64	6954.77a	354.84	5168.93b	218.10
S	*s*	14992.76a	1574.18	14599.99a	2115.80	9297.48b	496.05	15694.67a	1632.11
	r	6667.79a	265.44	7302.07a	933.14	7650.54a	1184.36	6837.04a	598.14

Table 2. Elemental composition (mg kg^{-1} of dry weight) of shoots (S) and roots (R) of plants grown under different mineral nutritional status: complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) or both Fe and Cu deficiencies (-Fe -Cu).Results are expressed in mg Kg^{-1} with mean value and standard deviation (St. Dev) for each treatment. Different letter mean significantly differences between treatments (ANOVA Holm–Sidak, N=3, P <0.05).

Reductases' activity: qualitative evaluation

To evaluate the activities of Fe- and Cu-reductases, two "indicator" molecules were used. These molecules are able tochange the color depending on the oxidative states of the complexed metal. In this study, the Fe reduction (from Fe^{3+} to Fe^{2+}) was visualized using the Na$_2$- bathophenanthrolinedisulfonic acid (BPDS), while the Cu reduction (from Cu^{2+} to Cu^+) was visualized through Na$_2$-2,9-dimethyl-4,7-diphenyl-l,10- phenanthrolinedisulfonic acid (BCDS).

Iron-reduction activity showed by reddish color of Fe (II)-BPDS complex was located near the root zones and was observed for all treatments (Figure 6). Observing the four gels, the reduction activity of Fe-deficient roots (-Fe +Cu, -Fe -Cu) was visibly higher compared with that of treatments where Fe was supplied (+Fe +Cu, +Fe -Cu). Iron deficient (-Fe +Cu) tomato plantshad anincreased reduction activity in all root apparatus, some spots of high reduction activity were visible in the middle part of some secondary roots. Interesting to note the difference between -Fe +Cu and -Fe-Cu treatment, where a decrease in the reduction activity was evident around the roots of the double deficient plants.

BCDS is used to detect Cu reduction activity of roots indicated by a yellowing color of the Cu (I)-BCDS complex. In this case, differences among treatments are smaller (Figure 7). Considering root size and

architecture is difficult to evaluate much difference between treatments. Plants kept in Fe deficiencyseems to show a higher Cu-reduction activity than the others.Gels highlighted that Cu-reduction occurs but a difference in the activity among Cu treatments was not evident.
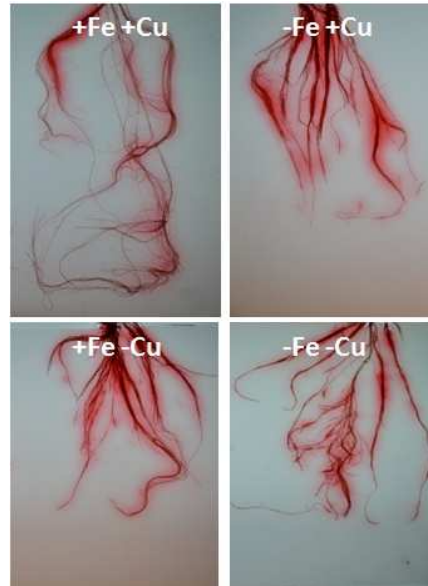
Figure6.Iron reductase activity(red color) of root apparatus for the four growth conditions of complete nutrient solution (+Fe+Cu), Fe deficiency (-Fe+Cu), Cu deficiency (+Fe-Cu) and both Fe and Cudeficiencies(-Fe-Cu)
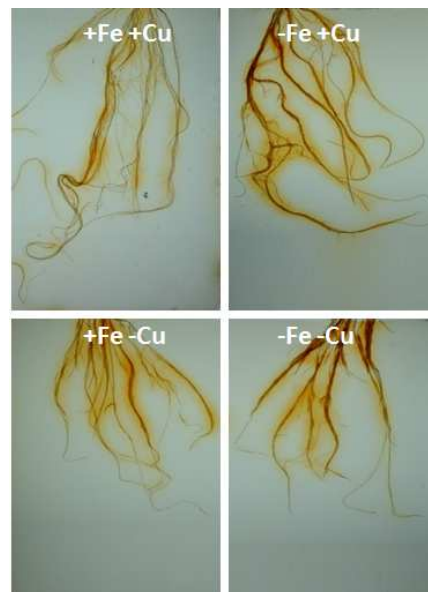
Figure 7. Copper reductase activity (yellow color) of root apparatus for the four growth conditions of complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and both Fe and Cu deficiencies (-Fe-Cu)

Quantification of Fe and Cu reduction activities and expression of *LeFRO1 and LeFRO4*genes

BPDS and BCDS were used to quantifythe enzymatic activity depending on the color intensity of the root-bathing solution. The quantification of Fe-orCu-reductase activities was evaluated by measuring the production rate of Fe^{2+} or Cu^+, respectively,in the root external solution (Figure 8).
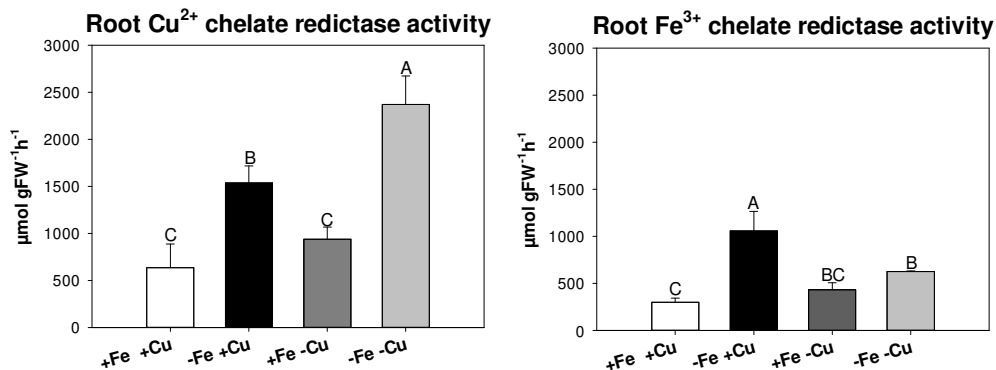
Figure 8.Quantification of Cu-reductase activity (**left**) and Fe-reductase activity (**right**) in roots of tomato plants grown under different nutritional conditions: complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu), and both Fe and Cu deficiencies(-Fe -Cu).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).

The quantification of Fe reduction activity showed the highest intensity in the roots of Fe-deficient plants. Plants treated with double shortage hadan enzymaticactivity comparable with those treated with Cudeficiencyand significantly higher than those with the complete nutrient solution.

Activity ofCu reduction werehigher than those measured for Fe reduction. Roots from plants grown with the complete nutrient solution hadthe lowest activity, while the plants kept in double nutrient deficiencyshowedthe highest. There was no increase activity of Cu reductase between treatments of Cu deficiencyand the control condition. Both Fe deficienttreatments (-Fe +Cu and -Fe -Cu) showed a markedly higher activity than in the other treatments.

Realtime RT-PCR of *LeFRO1* gene revealed that it is highly expressed in Fe-deficient roots and even more inducedin double deficientroots (-Fe -Cu, Figure 9). The highest expression of *LeFRO4*was observed in control roots (+Fe +Cu). In comparison to control plants (+Fe +Cu), plants grown under Cudeficiency (+Fe -Cu, -Fe -Cu) showed low levels of gene expression, whileeven lower values were observed under Fe deficiency(-Fe +Cu).
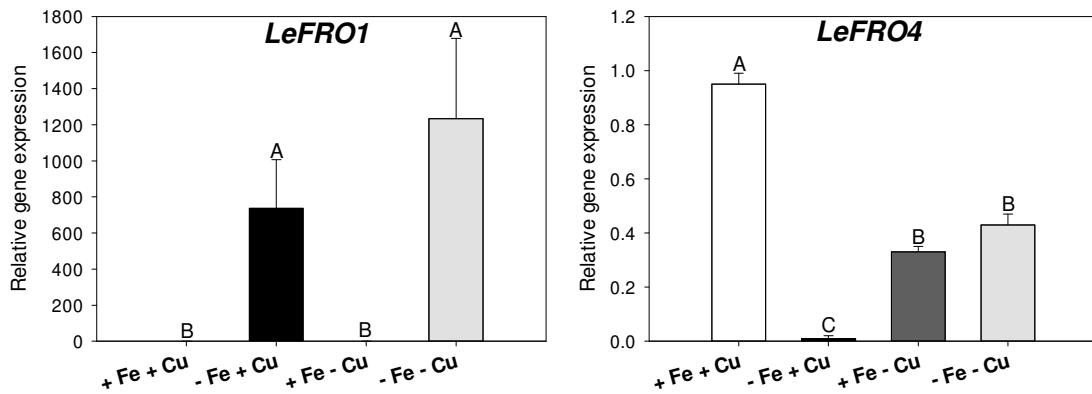
Figure 9. Relative gene expression of *LeFRO1* and *LeFRO4* genes putatively encoding for the principal Fe and Cu reductases, respectively in roots of plants treated as followed: complete nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu), and both Fe and Cu deficiencies (-Fe -Cu).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).

Quantification of SOD, CAT and POX activities and root gene expression of *LeFe-SOD and LeMn-SOD*

Activity of SOD didnot showany alterations in condition of Cu deficiency(Figure 10). SOD activity was affected by Fe deficiency, since both -Fe+Cu and -Fe-Cu roots showed a significant reduction of this enzymatic activity.Moreover,gene expression analyses oftwo SOD isoforms (*LeFe-SOD* and *LeMn-SOD*genes)were performed (Figure 11). Expression analysis revealed an important downregulation of *LeFe-SOD* gene expression in Fe- and/or Cu-deficient plants (-Fe +Cu, +Fe -Cu, -Fe -Cu) while nodifference wasmeasured for *LeMn-SOD*expression.
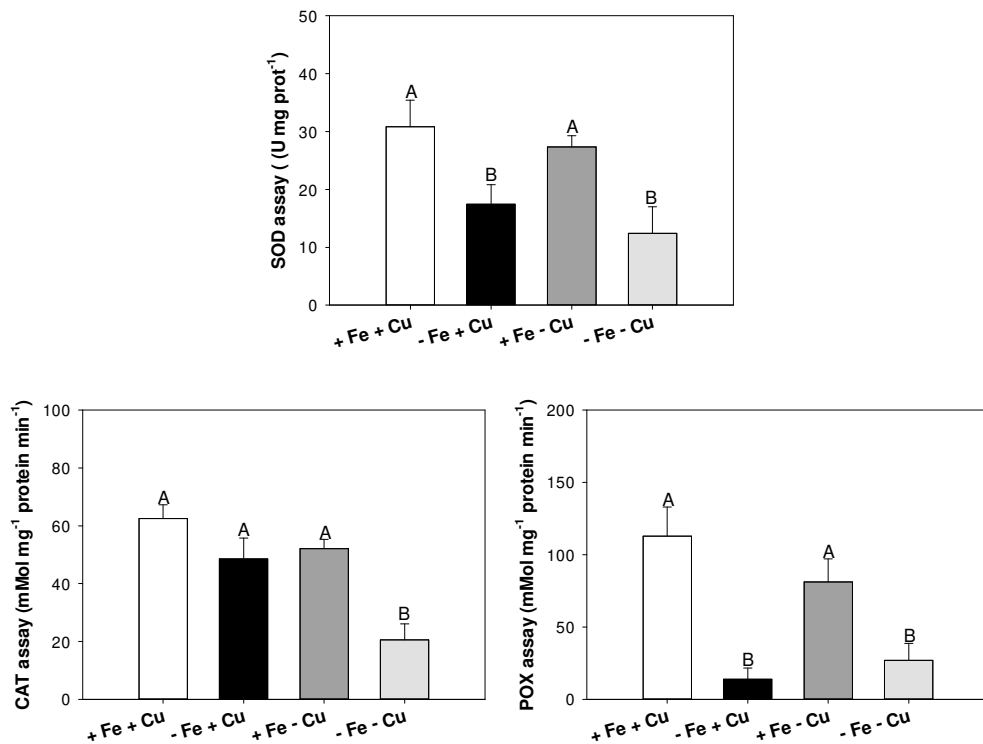
Figure10. SOD, CAT and POX activities quantification in leaves of tomato plants grownin complete mineral nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and bothFe and Cu deficiencies (-Fe -Cu).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P<0.05).
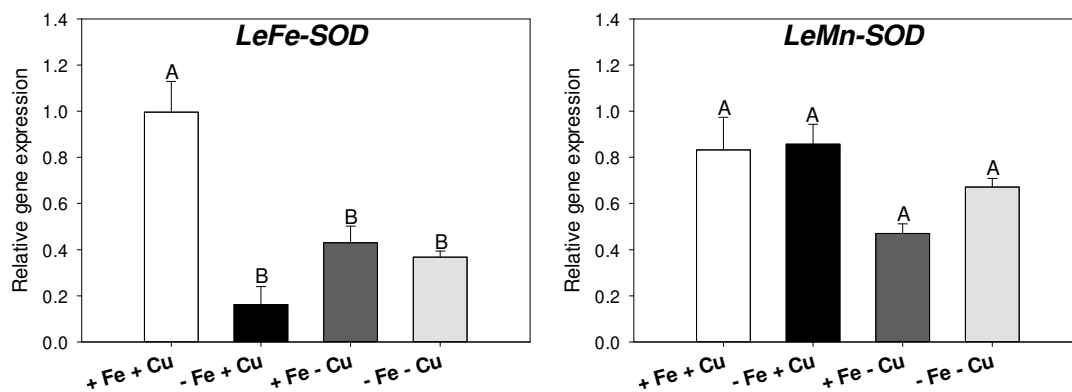
Figure 11.Relative gene expression value measured by quantitative RT-PCR of LeMn-*SOD* and *LeFe-SOD* in four different nutrient solutions. Complete mineral nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and both Fe and Cu deficiencies (-Fe -Cu).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences among the mean values, ANOVA Holm–Sidak, N=3, P<0.05).

In the -Fe -Cu treatment, CAT activity decreased significantly with a level that was3-fold lowerthan that of control plants. About POX activity, it follows the same trend of SOD assay as Fe-deficient plants and

Fe- and Cu-deficientplants shown a drastic reduction of the enzymatic activity compared with the control condition (Figure 10).

Expression analysis

Gene expression analyses were performed on three categories of genes involved in Fe and Cu acquisition or regulation of theresponse to these nutrients. Membrane transporters, transcription factors and proteins involved in solubilization/acquisition mechanisms were investigated. An upregulation of metal related transporters *LeNRAMP1*, *LeOPT3*, *LeIRT1* was measured in Fe deficientconditions. Under Fe deficiency and double deficiency (-Fe-Cu), these three genes are upregulated while conditions of Cu deficiency didnot show any significant difference in comparison to that present in control condition(Figure 12).

Changes in expression of gene encoding fortranscription factors are fundamental to understand if there are some mechanisms of crosstalk between Fe and Cu. The mRNA quantification of *LeFER*, *LeILR3* and *LeSPL7* were performed to detectdifferences in the gene expression of these transcription factors (Figure 13).
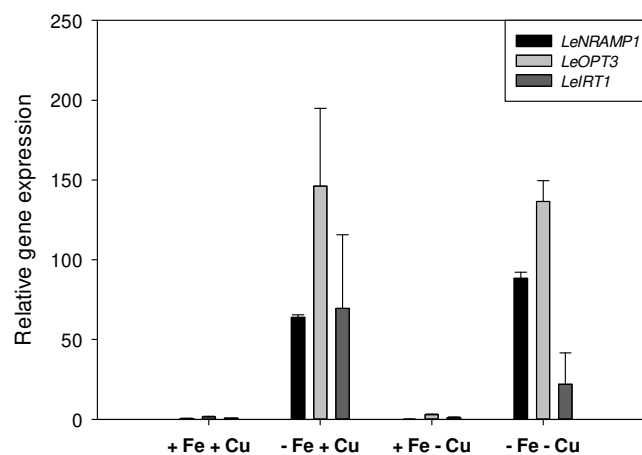
Figure 12. Relative gene expression values of *NRAMP1*, *OPT3* and *IRT1*in roots of plants grown in four different nutrient solutions: complete mineral nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and both Fe and Cu deficiencies (-Fe -Cu). Data are means +SD of three independent experiments (ANOVA Holm–Sidak, N=3, P<0.05).

LeFER transcription factor gene was upregulated in Fe deficientconditions, while Cu deficientdoes not affect *LeFER* expression levels in comparison with the control condition. *LeSPL7* and *LeILR3* exhibited a similar pattern of gene expression level with a downregulationin conditions of Cu deficiencyand double deficiencies. Data suggest that Cu deficiencydid not influence the expression levels of *LeSPL7* related transcription factor both in Cu- and double-deficient conditions.
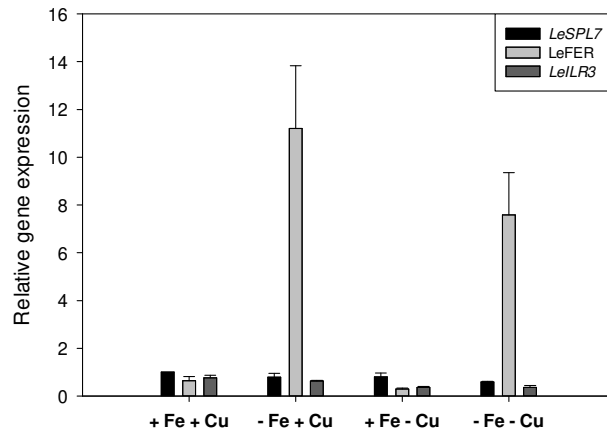
Figure 13.Relative gene expression values of *LeSPL7*, *LeFER* and *LeILR3* in roots of plants grown in four different nutrient solutions: complete mineral nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and both Fe and Cu deficiencies (-Fe -Cu). Data are means +SD of three independent experiments (ANOVA Holm–Sidak, N=3, P<0.05).
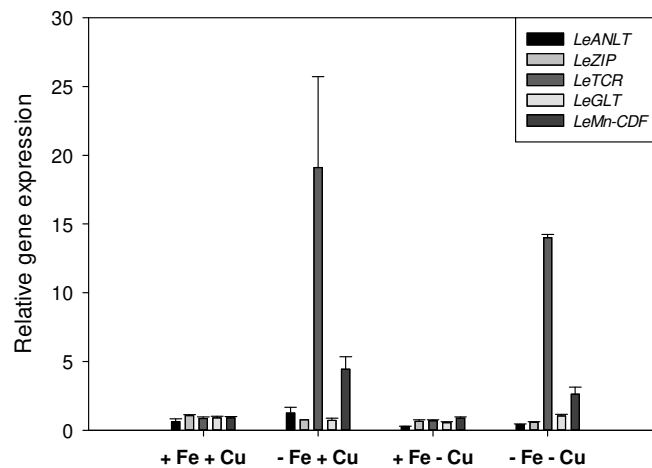
Figure 14.Relative gene expression values of *LeANTL1, LeZIP, LeTCR, LeGLT, LeMn-CDF* in roots of plants grown in four different nutrient solutions: complete mineral nutrient solution (+Fe +Cu), Fe deficiency (-Fe +Cu), Cu deficiency (+Fe -Cu) and both Fe and Cu deficiencies (-Fe -Cu). Data are means +SD of three independent experiments (ANOVA Holm–Sidak, N=3, P<0.05).

In Figure 14 isreported the gene expression analysesof those genes involved in the plasma membrane transport: *LeANTL1* (glycine transmembrane transporter activity), *LeZIP* (The Zinc-Iron Permease Family, ZIP), *LeTCR* (TCR-type metal cation transporter), *LeGLT* (glutathione gamma-glutamylcysteinyltransferase) and *LeMn-CDF* (manganese cation transporter).These genes did not change their expression level in conditions of Cu deficiency compared to control one. They are more responsive to Fe deficientconditions, as indicated by an overexpression of *LeTCR* and *LeMn-CDF*under both Fe-and double-deficiency treatments.

		+Fe +Cu		-Fe Cu		+Fe -Cu		-Fe -Cu	
		Average	*St.Dev.*	*Average*	*St.Dev.*	*Average*	*St.Dev.*	*Average*	*St.Dev.*
Solyc03g032090	*LeANT L1*	0.61 a	0.22	1.24 a	0.43	0.26 a	0.04	0.40 a	0.06
Solyc06g005620	*LeZIP*	1.06 a	0.06	0.77 b	-	0.63 ab	0.12	0.58 ab	0.04
Solyc02g069200	*LeIRT1*	0.66 a	0.17	69.46 a	46.21	1.18 a	0.38	21.95 a	19.65
Solyc11g012700	*LeOPT*	1.51 b	0.51	146.18 a	48.73	3.03 b	0.32	136.41 a	13.20
Solyc11g007130	*LeTCR*	0.84 b	0.13	19.11 a	6.61	0.68 b	0.09	14.00 a	0.25
Solyc11g018530	*LeNRA MP1*	0.37 c	0.32	63.91 b	1.59	0.23 c	0.12	88.47 a	3.63
Solyc01g006150	*LeMn-CDF*	0.89 b	0.10	4.43 a	0.90	0.87 b	0.09	2.63 ab	0.51
Solyc09g072620. 2	*LeGLT*	0.89 a	0.11	0.70 a	0.17	0.55 a	0.07	1.04 a	0.12
Solyc06g048410. 2	*LeFe-SOD*	0.81 a	0.19	0.12 b	0.11	0.35 b	0.06	0.30 b	0.02
Solyc06g049080. 2.1	*LeMn-SOD*	0.79 a	0.21	0.84 a	0.10	0.50 a	0.04	0.71 a	0.04
Solyc00g026160	*LeFRO 4*	0.95 a	0.04	0.01 c	0.01	0.33 b	0.02	0.43 b	0.04
Solyc01g094910	*LeFRO 1*	0.56 a	0.22	736.29 a	270.19	0.81 b	0.61	1232.92 a	445.56
Solyc01g080670. 2.1	*LeSPL 7*	1.00 a	0.00	0.79 a	0.16	0.80 a	0.16	0.59 a	0.03
Solyc06g051550. 2.1	*LeFER*	0.64 b	0.18	11.21 a	2.63	0.29 b	0.05	7.59 a	1.76
Solyc07g064040. 2.1	*LeILR3*	0.76 a	0.12	0.63 ab	0.03	0.37 b	0.03	0.37 b	0.07

Table 3. Relative gene expression value measured by quantitative RT-PCR, housekeeping gene used was *LeH1*. Means that are significantly different between treatments are marked with different letters.

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Discussion

Plants have evolved different mechanisms to cope with conditions of low nutrient availability, including modifications at morphological, physiological and molecular levels(Amtmann and Armengaud, 2009; Tejada-Jiménez *et al.,* 2009).Under Fe orCu deficiency, dicot plants (e.g. tomato)activatesome strategies to increase the solubility of these nutrients in the rhizosphere, such asincrease in root hair proliferation, root exudation, external acidification, reductionactivities (of Fe^{3+} and of Cu^{2+}) and successive intake into the root cells (Kobayashi and Nishizawa, 2012;Ryan *et al.,*2013).

Tomato plants grownin Fe and Cu deficientconditions showed changes inmorphology, physiology and transcription of genes encoding for proteins involved in Fe and Cu acquisition. After 37 days of hydroponic cultivation, Cu-deficient and both Fe-and Cu-deficient plants decreasedsignificantly their leaf biomass accumulation comparedwiththat of control and Fe-deficient plants (Figure 3). Copperdeficient plants (+Fe -Cu; -Fe -Cu) showedstunted growth and leaf curling (Figure 1), which are referred as typical symptoms of Cu deficiency (Follet*et. al.,* 1981, Bailey and McHargue, 1943). Moreover, the shortage of Fe was confirmed in the Fe-deficient plants due to the chlorosis of the young leaves (Zamboni *et al.,* 2016).

Changes in elemental compositions confirmed thenutrient deficientconditions for both root and shoot, where deficiency status of Fe and Cu was induced during cultivation(Figure 4 and 5). In general, growing plants in deficiencyof one metal induced an increase in accumulationof otherdetermining an imbalance in cell metabolism (Erenoglu *et al.,*2000; Miltra, 2015; Dotaniya and Meena, 2015).Copper and Fe content were strongly reduced in the treatments where Cu or Fe, respectively,were not provided (Figure 5) while root of +Fe -Cu and in shoot of -Fe +Cu have shown an increase in acquisition of the element supplied. Moreover, the increase of cations, as Zn and Mn in Fe-deficient plants (-Fe+Cu and -Fe-Cu) could be partially explainedby the role of Fe transporters, such as *LeIRT* and *LeNRAMP*, which can transport other divalent metals than Fe such as Zn and Mn (Vert *et al.,*2002). The increase of Ca and P content in the shoot of Fe-deficient plants and double deficientplantswere shown for the first time.

To better understand the mechanisms underlying Fe and Cu acquisition, the Fe- and Cu-reductase activities and mRNA expression analyses were performed. *LeFRO1*(coding for Fe-chelate reductase 1) is highly expressed in roots under Fe deficiency and in doubledeficiencies. This data is in agreement with physiological evidence, since the Fe^{3+} reductase activity was higher in Fe-deficient plants. Root Fe reductases are enzymes known to be responsive to Fe deficiency at both transcriptional and also post-transcriptional level (Li *et al.,* 2016). Moreover, it is also known that this enzyme is under transcriptional regulation byFER, a transcription factor that was highly induced inFe deficiency in both-Fe +Cu and -Fe-Cu tomato roots (Figure 13) (Ling *et al.,*2002). *FRO4*, a putative Cu(II) reductase oxidase, was shown to be overexpressedin

arabidopsis plants under Cu deficiency (Bernal *et al.,* 2012). In the present work,inabsence of Cu (+Fe -Cu and -Fe -Cu) the expression of this gene was downregulated, and it was even more repressed under Fe

deficiency. This evidence did not fit with the physiological pattern, since the Cu reduction was highly induced by Fe deficiency. A reasonable explanation is that other gene encoding reductasescould be better expressed than *FRO4* under Cudeficiency conditions (Jain *et al.,* 2014).

In the present work, the expression of three metal transporters were analyzed, as *LeIRT1, LeNRAMP1* and*LeOPT3*. These transporters are known to mediate the Fe acquisition (Kim and Guerinot, 2017;Kobayashi and Nishizawa, 2012), and are possible candidates to mediate also Cu transport. Their expression valuesrevealed that none of them were responsive to Cu deficiency in tomato roots. Furthermore, this data does not exclude their involvement inthe Cu transport mechanism;but it is clear that they were not regulatedat transcriptional level in Cu deficiency.Concerning*LeIRT1,*it has been shown that this transporter shows higher selectivity for Fe orZn than for Cu(Trivedi, 2016).Although, evidence of *NRAMP*involvement in Cu uptake are not available in plants. Liu et al. (1997) and Chen *et al.*(1999) have shown that, in *Saccharomyces cerevisiae*, two *NRAMP* homologues, *SmF1* and *SmF2*mediated the transport of Fe^{2+}, $Mn^{2+}$and also Cu^{2+}. The upregulation of *LeOPT3*under low-Fe deficiency and low-Fe and Cu deficiency could be explain as the *LeOPT3* transporters could be involved in the uptake and remobilization of Zn, Fe and maybe Cu in both roots and shoot (Lubkowitz *et al.,* 2006).

Moreover, in both Fe deficienttreatments, there were an upregulation of *LeTCR*and*LeMn-CDF*.*LeTCR*belong to the MFS group (Major Facilitator Superfamily) andhave a key role in iron and Zn homeostasis in arabidopsis plants (Haydon *et al.,*2007; Krämer *et al.,* 2007). Homolog of *LeMnCDF*in rice is required for Mn translocation to the root stele, and thereby Mn uptake (Ueno *et al.,*2015). For both thesetwo genes noreferences areavailable in tomato but the increase of their expression under Fe and Cu deficiency could have caused the accumulation of Mn and Zn in shoots in this condition (Figure 4).

Since Fe and Cu are cofactorsof antioxidant enzymes and can also induce the production of ROS, the activity of several proteins involved in the ROS detoxification processes (SOD, Cat and POX enzymes) were studied(Corpas*et al.,* 2006, Heck *et al.,* 2010, Pandey *et al.,* 2017). In general, enzymatic activity and expression analysis have showncontrasting resultsas there was a marked reduction of the SOD activityunder Fe and Cu deficiency (Figure 10), which confirmed previously observation of Agarwal et al. (2006), however this trendwas not confirmed at the molecular level (Figure 11). The same reduction of SOD activity under Cu deficiency was confirmed in lupin by Yu et al. (1999) which demonstrated that especially Mn and CuZnSOD activity were affected.

CAT and POX assay showed a different limitationof the activity of these enzymes under Fe deficiency and both nutrient deficiencieswhich could be explainedby the fact that these enzymes are hemoprotein and the decrease in theiractivity wasreflecting a depletion of the available Fe pool in the root cells. Indeed, it has been reported that the activity of these enzymesdecreased under Fe-deficiency in sugar beet (Zaharieva and Abadia, 2003).

Littleinformationis available about Cu deficiency in plantsand even lower is available on the bothFe and Cu deficiencies. In the present study, a wide characterization of morphology, ionomic and physiological changes occurring in Cu deficient and in -Fe-Cu plants has been provided. However, evidences about the antioxidant activity and the relative gene expression require to be investigated deeply.

Present data and literature evidence could allow hypothesizinga conceptual model regarding the plant response to Cu and Fe starvation (Figure 15). Under Fe deficiency,increase of ATPaseactivity andreleaseof $H^+$and organic exudates into the rhizosphere is well known(Dell'Orto *et al.,* 2000). Therefore, in the rhizosphere next to a plant in this nutritional stress,the solubility of Fe species increases (Guerinot and Yi, 1994) and maybealso Cu species. Moreover, roots increase the release of organic compounds (organic acids, phenolic compounds) which could chelate,and possibly reduce, either Cu^{2+}orFe^{3+}. A step of reduction by plasma membrane reductases, $Cu^+$and Fe^{2+} could be takenup by different transporters (e.g. *COPT, IRT, ZIP, NRAMP*). Transcriptionfactors such as *SPL7* and *LeFIT* could regulate the expression of some transporters involved in the uptake of these metals.
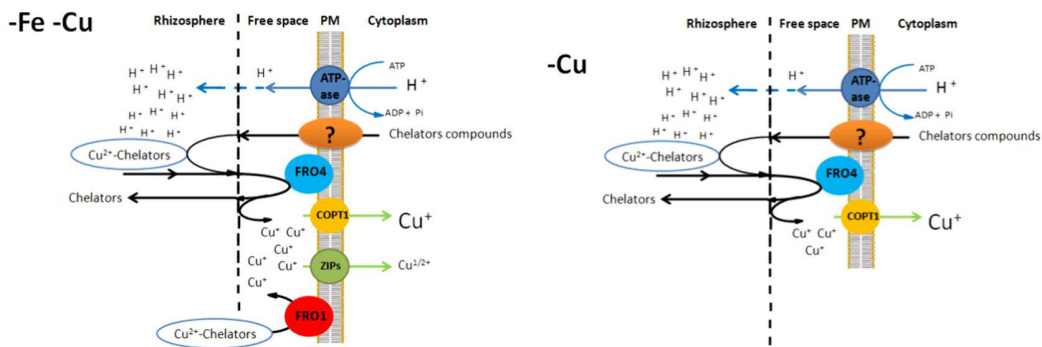
Figure 15. Conceptual model of putative mechanisms involved in the acquisition of Cu and Fe under Cu deficiency.
ATPase= proton pump,*COPTs*= Copper transporters, *ZIPs*= Zn/Fe permease proteins; *FROs*= Plasma membrane-bound reductases.

In conclusion, this study provides new insights about Fe and Cu acquisition in plants. Molecular analyses are ongoing in order to check critical aspects of this model.

6.Cu and Fe status in melon plants grown with split root technique

Introduction

Copper (Cu) and iron (Fe) are essential mineral nutrients for plant growth and development. These metals are present in the active site of numerous enzymes involved in metabolic processes (Schulten *et al.,* 2017). Iron and Cu homeostasis are deeply linked in the cell metabolism and they mutually influence each other acquisition and transport (Ryan *et al.,* 2013 ; Adress *et al.,* 2015). The cupric- and ferric-chelatereductaseactivityisneededforuptakeofeitherFe or Cuindicots, such as *Arabidopsis thaliana* and melon (*Cucumis melo*), and these plants use a reduction-based strategy to take upFe (Welch *et al.,* 1993; Waters *et al.,* 2012).

The *fefe* mutation originated spontaneously in melon (*Cucumismelo*)and was crossed into the variety Mainstream to generate the C940-fe germplasm (Nugent and Bhella, 1988; Nugent, 1994). The *fefe*mutantlacks the induction of ferric-chelate reductase activity and of H$^+$-ATPase activity in Fe deficiency (Jolley *et al.,* 1991), two important mechanisms for the reductive-based strategy for Fe acquisition in *Strategy I* plants. The *fefe* mutant phenotype was rescued by applying either high Fe supplied or by inducing the Cu deficiency response in these plants. The latter condition stimulates ferric-chelate reductase activity, *FRO2* expression, and thus Fe acquisition. Moreover, accumulation of Fe in Cu-deficientplants indicates that simultaneous Fe and Cu deficiency synergistically up-regulated gene expression of components of the Fe-uptakemechanisms(Waters *et al.,* 2014).

Split-root techniques have been used to study plant nutrition by dividing the root system of a plant and placing each segment into adjoining containers containing different solutions(Sherif *et al.,* 1994).De Nisi *et al.* (2012) used split-root technique under hydroponic condition to study the regulation of Fe-deficiency responses in cucumber roots and discriminating the roles of the systemic and localized signals involved in this regulation.

The aim of this work was to identify the coordination of biochemical and molecular responses to the Fe deficiency and Cu deficiency in two melon genotypes (Edisto and *fefe*).Understanding the crosstalk between the regulationof the response to Fe or Cu deficiency.

Material and methods

Seeds of melon (*Cucumis melo* L.) cv Edisto (Victory Seed Company, Molalla, OR, USA) and seeds of C940-fe (*fefe*) melon (Nugent, 1994; which were generously given byMichael A. Grusak, USDA-ARS Children's Nutrition Research Center, Houston, TX, USA) were sprouted on germination paper in a 30-°C incubator until transplanting to hydroponics after 4 days. After this period, seedlings were placed in sponge holders in lids of black plastic pots containing a Cu-free nutrient solution with the following composition: 1.5 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $NH_4H_2PO_4$, 20 µM Fe(III)-EDDHA, 0.2 mM $MgSO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 2 µM $MnCl_2$, 2 µM $ZnSO_4$, 0.5 µM Na_2MoO_4 and 1 mM MES buffer (pH 5.5). Plants were grown in a growth chamber with a mix of incandescent and fluorescent light at 300 µmol m^{-2} s^{-1}. After 2 days, the primaryroot of the seedlings wascut, and plants were left in the pots for another 9 days (Figure 1 A). After that, root system was split into two parts and kept for three days in separated compartments (Figure B and C) filled with a medium in which Fe was omitted or supplied as 20 µM Fe(III)-EDDHA and Cu was omitted or supplied as 0.1 µM $CuSO_4$(Table 1).

Light transmittance of young fully expanded leaves was determined using a portable chlorophyll meter SPAD-502 (Minolta, Osaka, Japan) and presented as SPAD index values. SPAD index measurements, ferric and cupric reductase activity and collection of samples for gene expression were performed on18-days plants.

Treatment	Symbol	µM Fe-EDDHA	µM CuSO₄
Control	-Fe -Cu	0	0
	+Fe +Cu	20	0.1
Fe deficiency	-Fe -Cu	0	0
	-Fe +Cu	0	0.1
Cu deficiency	-Fe -Cu	0	0
	+Fe -Cu	20	0
Fe and Cu deficiency	-Fe -Cu	0	0
	-Fe -Cu	0	0

Table 1. Composition of the nutrient solutions in melon in the last 3 days of growth.
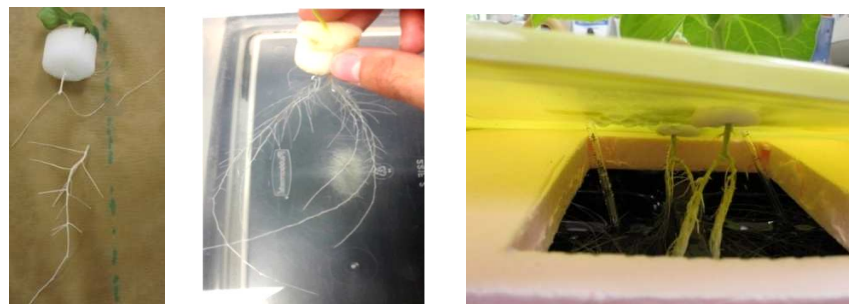
Figure. 1. Split root treatment: (**A**): cutting, (**B**): splitting and (**C**): transferring in compartments containing differentnutrient solutions. The medium of the two compartments had the same nutrient composition (pH 5.5) in presence or absence of 20 µM Fe-EDDHA and 0.1 µM CuSO$_4$.

Assay of Fe^{3+} and Cu^{2+} reductase activity.

Rates of root-associated Fe^{3+} and Cu^{2+} reduction were quantified via the spectrophotometric measurement of Fe(II)-BPDS or Cu(I)-BCDS production. The assay solution for the quantification of Fe^{3+}reduction contained 1 mM MES buffer (pH 5.5), 0.1 mM Fe (III)-ETDA and 0.1 mM ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulfonic acid sodium salt, Sigma-Aldrich, St Louis, MO, USA); while for the Cu^{2+} reduction assay the solution contained 0.2 mM CuSO$_4$, 0.6 mM Na$_3$citrate, and 0.4 mM Na$_2$-2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (BCDS). Three 18-day-old plants for each treatment were collected and root ferric reductase assays were performed for 30 to60 min on each split roots. Just prior to initiation of the reductase assays, the roots were rinsed for 5 min in 0.2 mM CaSO$_4$ solution. The absorbance (562 nm for Fe^{2+}-ferrozine; 483 nm for Cu$^+$-BCDS) of the assay solutions were determined spectrophotometrically, an aliquot of identical solution from assay medium containing no roots was used as blank. The concentration of Fe(II)-ferrozinewas calculated using a molar extinction coefficient of 28.6mM cm^{-1}; while the molar extinction coefficient used for Cu(I)-BCDS was 12.25 mM cm^{-1}. Results were expressed in µmol g^{-1}FWh^1.

Real time RT-PCR

Total RNA was extracted from roots using the Plant RNeasy kit (Qiagen, Hilden, Germany). RNA quality and concentration were determined by UV spectrophotometry. A gel (1% agarose; 80mV) was utilized to check the quality and presence of RNA degradation. 0.5 µg of DNase-treated RNA (RNase-free DNase I, New England Biolabs, Ipswich, MA, USA) was used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription kit (ABI, Foster City, CA, USA) with random hexamers at 2.5 µM final concentration. cDNA corresponding to 1.5–2.5 ng of total RNA was used in a 15 µl real-time PCR reaction performed in a MyIQ (Bio-Rad, Hercules, CA, USA) thermal cycler using GoTaq qPCR MasterMix (Promega, Madison, WI, USA) and 0.2 µM gene-specific primers (Sequences of forward and reverse primers were reported to Table 2). Specific primers were designed to generate 80–120 bp PCR products. *CmAPL2*, coding for clathrin adaptor complex

subunit, was used as housekeeping gene for relative quantification. The following standard thermal profile was used for PCRs: 50°C for 2 min, 95°C for 8 min; 40 cycles of 95°C for 15 s, 56°C or 65°C for 15 s, and 72°C for 15 s. The Ct values for all genes were calculated using LightCycler 480 Real-Time PCR Software version 2.0 (Roche 480, Hercules, Germany). Gene expression was determined by normalizing to the Ct value of AP-2 complex using the Livak method (Livak & Schmittgen, 2001), with the equation Relative Expression= $2^{-\Delta\Delta Ct}$, where ΔΔCt = ($Ct_{target\ gene}$ (treatment 1) - $Ct_{target\ gene}$ (controltreatment)) - (Ct_{UBQ} (treatment 1) - Ct_{UBQ} (control treatment)).

		Primer Forward	Primer Revers
Melo3c014042.2	*CmAPL2*	TGGACAAACTGAACCAACCA	TGTGAACATGGGAACCTGAA
Melo3c017424	*CmFIT*	AGAAACAGCCATTGCAGGTC	CTAGTTTCCAGCCGTCCGAT
Melo3c011744	*CmFRO1*	GGCAGTGGAATCACCCCATT	GATCGGCGGTGGTTTTGAAG
Melo3c019493	*CmFRO2*	GGGCCTCCTTCTACTCACTT	GTGCTTCGAACAGTCGTCTC
Melo3c007271.2	*CmIRT1*	TGCGCTGTGCTTTCATCAAC	GGCGTCGTAACCGAGAAGAA
Melo3c003197.2	*CmCOPT2*	ATCGCTTCCTTACAGGCGTT	CCTGCCACAGCCGCTATAAA
Melo3c020592	*CmCOPT-type*	TGGGGTTCTTGGTTTATGGGA	TAAGTGGTGGAAGATCAGACAAA
Melo3c005259	*CmVIT1*	CAACAAACCCTTCACCAGCG	AGCTAGGGTTGACGTGGTTG
Melo3c004185	*CmNAS4*	TGGAGCAAGAGCATTTTTGTACC	AATAACTTCATCGGTGGGATG
Melo3c013263	*CmMATE*	GGTCCTTGCTTGGAGATTGA	TAACTCTTGCCAGCAGCAGA

Table 2. Primer used for quantitative RT-PCR analysis.

Statistical analyses

Analyses were performed on three independent biological replicates obtained from independent experiments and a pool of three plants was used for each sample. Statistical significance was determined by one-way analysis of variance (ANOVA) using Holm–Sidak test (P <0.05, n = 3). Statistical analyses were performed using SigmaPlot Version 12.0 software.

Results

Plant morphometrics

In general, the 3-day treatments in split root condition did not cause any significant changein plant fresh weight (Figure 2). On the other hand, some changes in the chlorophyll content were observed, -Fe -Cu and -Fe +Cu plants of both genotypesshowed chlorosis in the young leaves and the values of SPAD index confirmed a decrease of chlorophyll content in leaves.
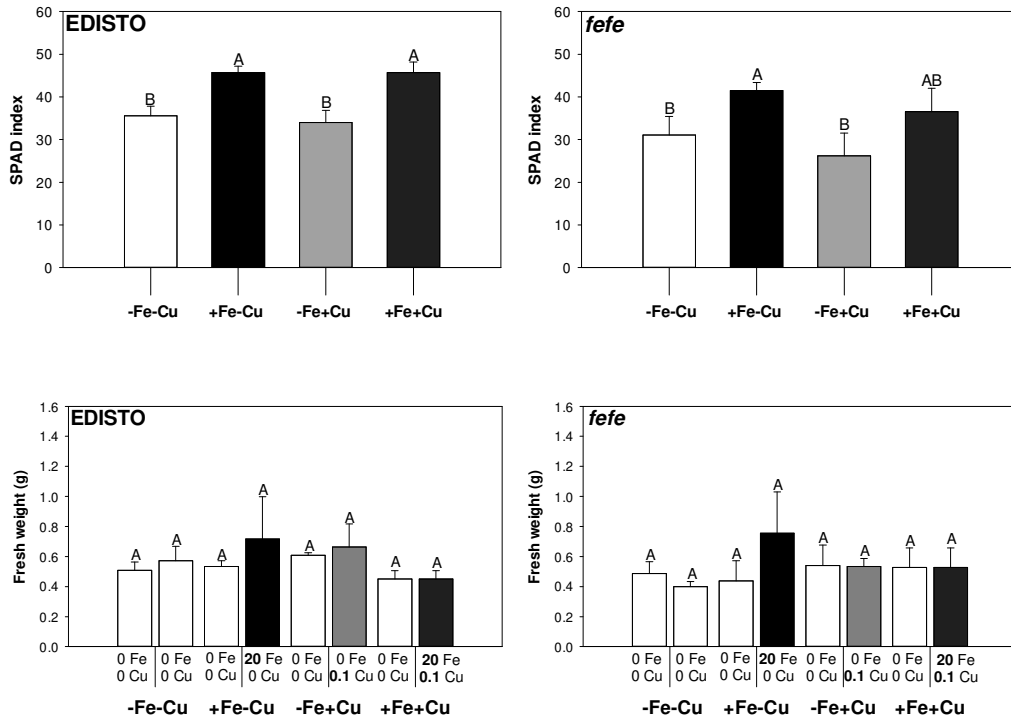
Figure 2. SPAD index value and root fresh weight of wild-type(EDISTO) and *fefe*melon were measured at the end of the growth period (18 days).Data are means +SD of three independent experiments (capital letters refer to statistically significant differences in shoots and roots among the mean values, ANOVA Holm–Sidak, N=3, P <0.05).
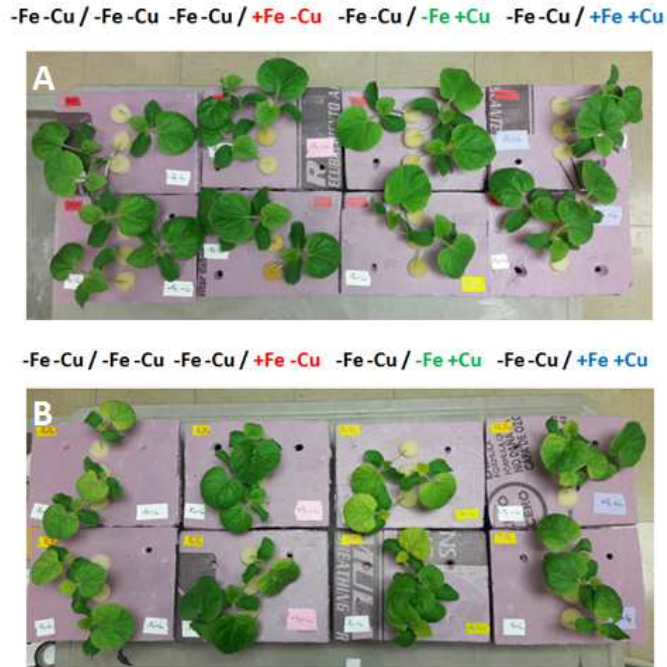
Figure 3. Picture of EDISTO (A) and *fefe* (B) melon plants at the end of the treatments in hydroponic cultivation (18-day-old plants) in split root and in Cu-free nutrient solution (+Fe -Cu). One side of the root system wasplaced in both Fe and Cu deficiencies(-Fe -Cu) while the other side was placed in: complete nutrient solution (+Fe +Cu), Cu deficiency (+Fe -Cu), Fe deficiency (-Fe +Cu), double deficiencies (-Fe -Cu).

Reduction activity

In Edisto, ferric–chelate reductase activity showeda marked increase in roots placed in condition of Fe deficiency and double deficiency (-Fe +Cu and -Fe -Cu) while the +Fe -Cu roots showedquite the same trend as the +Fe +Cu ones. Moreover, Figure 4 showedthat in *fefe*only in +Fe -Cu roots there was a significant increase of the ferric-reduction activity, and this increase occurred in the roots exposed to Fe-containing solution; for all the other treatments the ferric chelate reductase activity was comparable withthe control (+Fe +Cu).

TheCu-reduction activity was higher than that measured for Fe reduction. In Edisto, the Cu-reduction patternwas quite similar to that described before for the Fe-reductase activity, with -Fe -Cu and -Fe +Cu roots having a higher Cu-reductase activity than that of thecontrol roots (+Fe +Cu) or of theCu-deficient roots (+Fe -Cu). In *fefe,* only -Fe -Cu roots showeda strong increase of the Cu reduction activity in comparison to the other conditions (+Fe-Cu, -Fe+Cu, +Fe+Cu, Figure 4).
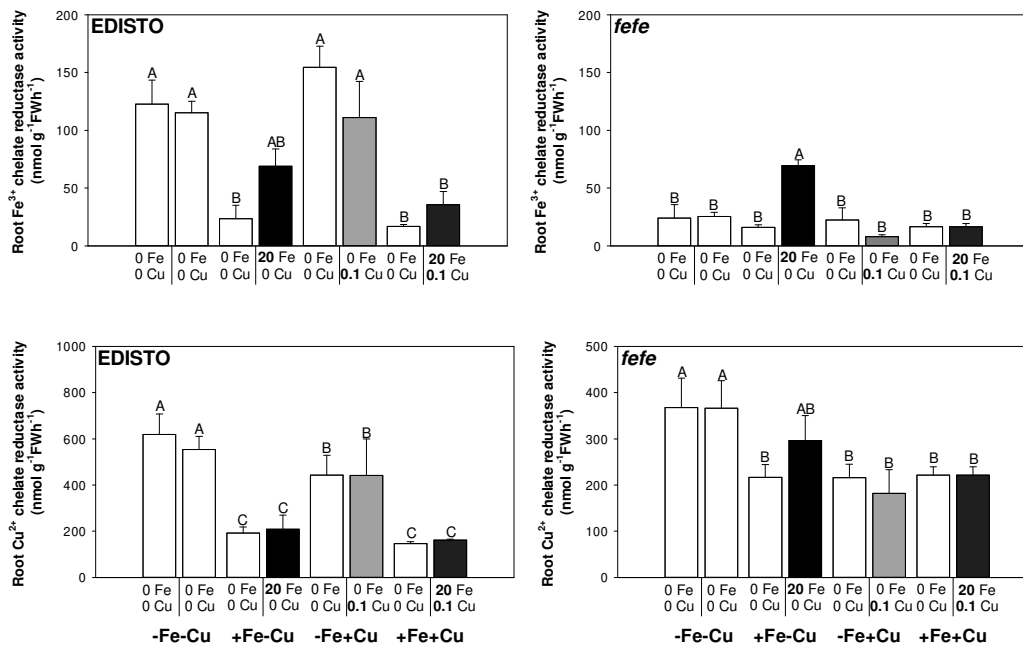
Figure 4. Evaluation of Fe-reductase activity (above) and Cu-reductases activity (below) in roots of wild type(EDISTO) and *fefe*melon plants grown under split roots . One side of the root system was placed double deficiencies(-Fe -Cu) while the other side was placed in: complete nutrient solution (+Fe +Cu), Cu deficiency (+Fe -Cu), Fe deficiency (-Fe +Cu), double deficiencies (-Fe -Cu).

Gene expression analysis

Gene expression analysis was focus on the +Fe -Cu treatments on roots of both genotypes: EDISTO and *fefe*. The reason of this choice was due to the interesting results on the Cu and Fe reductase activities of thistreatment and all the expression data are shown relativized to the level of gene expression measured in roots of EDISTO plants grown under complete nutrient solution (+Fe +Cu, Figure 5). In general, gene expression of *CmFIT* increased in both EDISTO and *fefe* mutant while only the split root in both deficiency (-Fe -Cu) of *fefe*-Fe-Cu/+Fe-Cu plants shown an upregulation of *CmFRO1*. At the same time, in EDISTO and *fefe*-Fe-Cu/+Fe-Cu plants,the expression of*CmFRO2* increased in roots bathing in the Fe-containingsolution. Moreover, *CmIRT1*gene was upregulated only in Cu-deficientside of -Fe-Cu/+Fe-Cu *fefe* plants. Instead, *CmCOPT2* was upregulated inboth sides of -Fe-Cu/+Fe-Cu EDISTO and *fefe* while *CmCOPT-type* were upregulated only in *fefe*in both sides of the split-root. *CmVIT1* was upregulated only in the deficient side of both the genotype while *CmNAS4* was upregulated in Fe sufficiency side of EDISTO. Finally, no significant difference was shown in any roots for *CmMATE* expression*.*
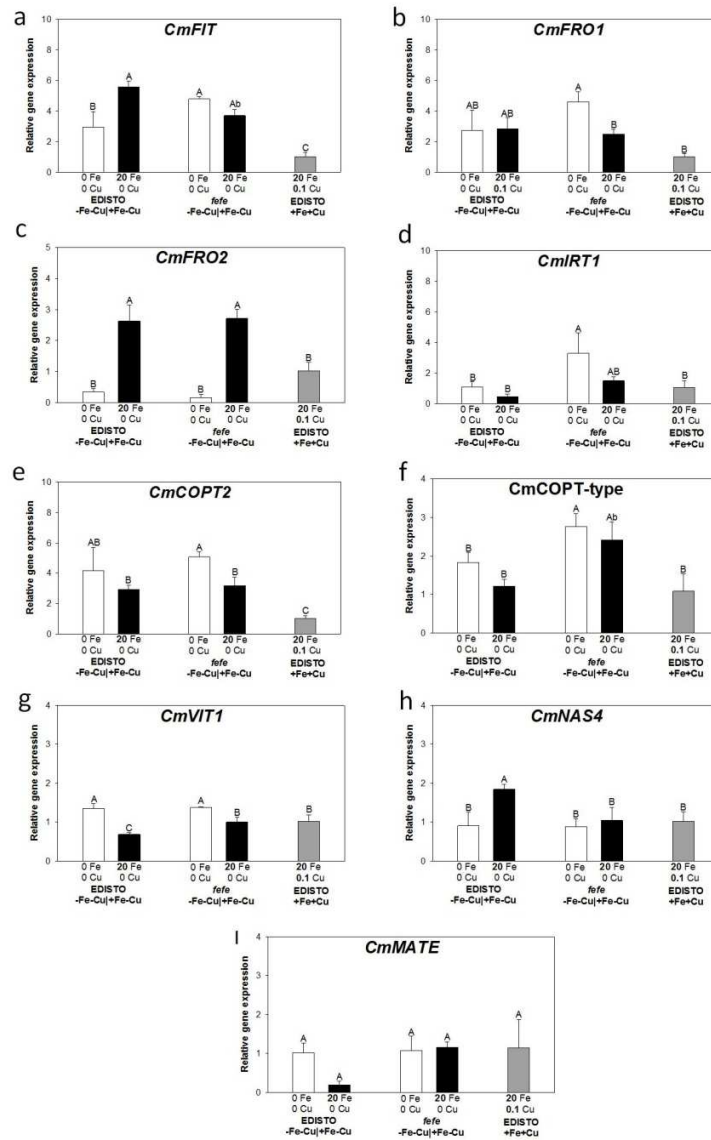
Figure 5. Gene expression in roots EDISTO and *fefe* after 3-day treatment in split roots with one side without both Fe and Cu (-Fe -Cu, white bars) and the other side with 20 μM Fe-EDDHA and 0μM Cu (black bars). Reference is EDISTO 20 μM Fe-EDDHA and 0.1 μM CuSO$_4$ (grey bars). Genes are (a) *CmFIT*, (b) *CmFRO1*, (c) *CmFRO2*, (d) *CmIRT1*, (e) *CmCOPT2*, (f) *CmCOPT-type*, (g) *CmVIT1*, (h) *CmNAS4*, (I) *CmMATE*. Significant differences between control (+Fe+Cu EDISTO) and treatments are shown with capital letters that refer to statistically significant differences among the mean values of the different root samples, ANOVA Holm–Sidak, N=3, P <0.05).

| | | EDISTO | | | | fefe | | | | EDISTO | |
| | | +Fe +Cu | | -Fe Cu | | +Fe +Cu | | -Fe Cu | | +Fe +Cu | |
		Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.	Average	St.Dev.
Melo3c014042.2	*CmAPL2*	0.74a	0.1933a	0.62a	0.12	0.94a	0.11	0.74a	0.12	1.01a	0.23
Melo3c017424	*CmFIT*	2.93b	1.01	5.56a	0.38	4.78a	0.15	3.69ab	0.40	1.0216c	0.26
Melo3c011744	*CmFRO1*	2.73ab	1.31	2.84ab	0.71	4.59a	0.67	2.48b	0.31	1.01b	0.20
Melo3c019493	*CmFRO2*	0.34b	0.11	2.62a	0.53	0.16b	0.10	2.7a	0.29	1.02b	0.28
Melo3c007271.2	*CmIRT1*	1.11b	0.32	0.46b	0.17	3.29a	1.33	1.50ab	0.26	1.07b	0.46
Melo3c003197.2	*CmCOPT2*	4.12a	1.59	2.91b	0.28	5.09a	0.33	3.14b	0.55	1.01c	0.18
Melo3c020592	*CmCOPT-type*	1.84b	0.26	1.2b	0.17	2.76a	0.33	2.42ab	0.46	1.07b	0.44
Melo3c005259	*CmVIT1*	1.34a	0.12	0.67c	0.05	1.37a	0.02	0.99b	0.13	1.01b	0.18
Melo3c004185	*CmNAS4*	0.9b	0.35	1.84a	0.13	0.87b	0.21	1.04b	0.34	1.01b	0.25
Melo3c013263	*CmMATE*	1.01a	0.25	0.19a	0.09	1.06a	0.38	1.15a	0.13	1.14a	0.74

Table 6. Relative quantification of gene expression, housekeeping gene used is *CmAPL2*.Means and standard deviations are reported. Means that are significantly different between treatments are marked with different letters.

Discussion

In this work, using asplit-root system, we highlighted mechanisms which control Fe uptake in response to the Fe and Cu status of plants using the combination of the split root techniqueand the use of amutant that does not respond to Fe deficiency.

Despite the incapacityof the *fefe* plantsto induce ferric-chelate reductase activity and H$^+$-ATPase activity in Fe-deficiency (Jolley et al., 1991), when plants are grown in Cu-deficiency,anincrease of the Fe reductase activity in the split-root side where Fe was supplied,could be measured (Figure 4). In wild type plants (Edisto), we observed a link between the chlorosis in the leaves and the activity of the reductases as -Fe -Cu and -Fe +Cu plants shown symptoms of Fe deficiency and a marked increase of the Fe and Cu reduction (Figure 3 and Figure 4).

The FRO/IRT/NAS pathways of Fe homeostasis are fundamental for the managing of these micronutrients in the plants (Willey, 2015) and it is well documented that nicotianamineis an intracellular metal chelator that has been implicated in homeostasis of Fe and Cu (Takahashi *et al.,* 2003; Curie *et al.,* 2009; Klatte *et al.,* 2009). So, the upregulation of *CmNAS4* (*nicotianamine synthase 4*)only in wild type (Edisto) plants could be explained by the apparent lack of FRO activity in *fefe* (Jolley *et al.,* 1991) which may not lead to a direct response to the deficiency. The slight upregulation of *CmVIT1* in the side without Fe and Cu inboth the genotypes contradict previous observation that shown the implication of this transporter in the vacuolar Fe storage (Kim *et al.,* 2006;Zhang *et al.,* 2012), hence it is expected that in Fe deficiency the storage of Fe is inhibited and the mobilization of Fe from the vacuole is increased *via* NRAMP3/4..

Molecular analyses of the bHLH transcription factors FER in tomato (Ling *et al.,* 2002) and FIT in Arabidopsis (Colangelo and Guerinot, 2004) have shown that this protein is required for up-regulation of genes encoding for critical members of the Fe acquisition mechanisms in *Strategy I* plants. FIT protein forms heterodimers with one of the subgroup-Ib bHLH proteins (bHLH38, bHLH39, bHLH100, bHLH101) (Wang *et al.,* 2013; Yuan *et al.,* 2008) and this heteroduplex regulates the expression of several Fe-regulated genes in roots. These genes include*FRO2*, which encodes a primary root ferric-chelate reductase gene (Robinson *et al.,* 1999), and *IRT1*, which encodes a root iron transporter (Eide *et al.,* 1996; Vert *et al.,* 2002). Also, in our study, *CmFIT*gene expression increased in both genotypes (Figure 5) but the genes that should be controlled by this transcription factor were not always upregulated, e.g. *CmFRO1*, *CmFRO2, CmIRT*and *CmCOPT2* (Figure 5). It has been reported that FIT regulation activity is not entirely dependent on transcriptional control, as short-lived 'active' forms of FIT protein have been described, and this post-translational control is dependent on Fe status (Meiser *et al.,* 2011; Sivitz *et al.,* 2011). Moreover, some Cu responsive genes had altered expression under Fe deficiency in roots and shoots of *Arabidopsis thaliana,* included the transcriptionfactors*spl7* (Sitain and Waters, 2012; Waters *et al.,* 2012; Waters *et al.,* 2013) which is known to

be regulated by the microRNA miR398s (Yamasaki *et al.,* 2007; Yamasaki *et al.,* 2009). Others molecules, such as IAA , ethylene, sugar, Fe complexed by a ligand and nitric oxide have been proposed as inhibitory signal (Römheld *et al.,* 1986; Landsberg, 1984; Bienfait *et al.,* 1987; Garcia *et al.* 2011). Moreover, FIT is known to interact with other transcription factors (bHLH38, bHLH39, bHLH100, bHLH101, POPEYE, BRUTUS, WRKY) in order to regulate the gene expression of proteins involved in Fe acquisition (Long*et al.,* 2010; Hindt *et al.,* 2017; Rodriguez-Celma *et al.,* 2017). Thus, it can be speculated that in split roots and / or in Cu-deficiency that FIT partners change and this causes a fine tuning of the gene regulationin order to managethe homeostasis of both Cu and Fe.

In conclusion, this study provides new insights about Cu and Fe uptake under split roots technique. The ferric –chelate reductase activity in the condition of -Fe-Cu/+Fe-Cuwas similar for both genotypes. It was difficult to correlate the expression of *CmFIT* with most of the genes involved in the pathways of Fe homeostasis, indicating that a post-transcriptional regulation occurs or that Cu regulation system acts independently of FIT or downstream of *FIT* gene expression and alter the expression level of gene encoding for components of the Fe acquisition mechanism.

7. FINAL CONCLUSION

The aim of this thesis was to improve the knowledge regarding the interactions between Fe and Cu acquisition in crops. Since Fe deficiency is easily obtainable under hydroponic conditions, in the first period of my research I focused my attention on setting up experiments to find conditions that induced Cu deficiency and toxicity in both tomato and maize. We observed that Cu affected plant mineral uptake and accumulation depends on the plant species and although Cu toxicity has been relatively easy to induce, Cu deficiency was hard to obtain and it has required various precautions to avoid Cu contamination of the nutrient solution.

The next step was to focus on the conditions of toxicity and deficiency separately. For this reason, in maize it was useful to highlight the different response of the excess of Cu depending on the source of Fe used. Ionomic data and morphometric evidences help us to propose a conceptual model of the putative mechanisms involved in the uptake of either Fe and Cu under the different growth conditions. On the other hand, the integration of these results with the molecular aspects was not performed due to the difficulty encountered in finding reference genes that were stable in the different conditions, in particular,when Cu toxicity was considered.

In tomato, the studies of the shortage of Cu alone and in combined deficiencies of Fe and Cu confirmed previous observations and at the same time provide morphological, physiological and molecular information that were not known before. In particular, the elemental analyses showed that the Cu deficiency influences the absorption of other micronutrients such as Zn and Mn. Moreover, the activity of Cu-chelate reductase shown to be much higher than its homologue for Fe, so this could mean that, despite the prolonged deficiency of this element, these proteins are able to remain highly active under long period of deficiency. The ionomic results suggested that the acquisition mechanisms, and possibly thesereductases,are not very selective and can participate to the (over)accumulation of other metals in plants. However, in these conditions of growth, the molecular and physiological results takentogether did not provide a complete interpretation of the Cu and Fe interactions. On the other hand, on the basis of the results obtained and from several the evidence found in the literature, it was possible to present a conceptual model of the plant's response to the condition of a combined deficiency of Fe and Cu.

Finally, during a short period of research conducted abroad, it was possible to approach the acquisition dynamics of Fe and Cu with using the split root technique and a Fe-unresponsive mutant. This study provides new insights and interesting evidences about the local and systemic signals that regulate the acquisition of these two micronutrients.

Future perspectives of this work concern the deepening of molecular studies of these model plants under the different growth conditions. For this reason, as Cu deficiency in tomato has not yet been characterized,

it might be interesting to set up a metabolomic study that can allow a more complete evaluation of the effects of the shortage of this element in the metabolism of plants. This could be a fundamental step to be performed in order to better understand the Fe and Cu interactions and crosstalk.

Finally, as Cu deficiency showed interesting response in terms of Fe, Zn and Mn accumulation in shoot and roots in different crops, isotopic studies could be set up with the purpose to characterize transport systems and their substrate specificities.

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8. ACKNOWLEDGEMENTS

9.ANNEXES

Paper

Physiological and transcriptomic data highlight common features between iron and phosphorus acquisition mechanisms in white lupin roots

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ABSTRACT

In agricultural soil, the bioavailability of iron (Fe) and phosphorus (P) is often below the plant's requirement causing nutritional deficiency in crops. Under P-limiting conditions, white lupin (Lupinus albus L.) activates mechanisms that promote P solubility in the soil through morphological, physiological and molecular adaptations. Similar changes occur also in Fe- deficient white lupin roots; however, no information is available on the molecular bases of the response. In the present work, responses to Fe and P deficiency and their reciprocal interactions were studied. Transcriptomic analyses indicated that white lupin roots upregulated Fe-responsive genes ascribable to Strategy-I response, this behaviour was mainly evident in cluster roots. The upregulation of some components of Fe acquisition mechanism occurred also in P-deficient clusters roots. Concerning P acquisition, some P-responsive genes (as phosphate transporters and transcription factors) were upregulated by P deficiency as well by Fe deficiency. These data indicate a strong cross-connection between the responses activated under Fe or P deficiency in white lupin. The activation of Fe and P acquisition mechanisms might play a crucial role to enhance the plant's capability to mobilize both nutrients in the rhizosphere, especially P from its associated metal cations.

In the present work I was involved on the plant breeding, collection of the samples and in the physiological assays (e.g. ferric reduction assay, visualization of root reduction and acidification).

Posters

Physiological and transcriptomic characterization of white lupin response to Fe deficiency

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ABSTRACT

In agricultural soil, the bioavailability of iron (Fe) and phosphorus (P) is often below the plant requirement causing nutritional deficiency in crops. To cope with P limitation, white lupin (*Lupinus albus L.*) activates morphological, physiological and molecular modifications aiming at increasing the P solubility in the soil. In particular, white lupin plants develop cluster roots that release large amounts of carboxylates, phenolic compounds and protons to counteract low P availability in the soil (Neumann and Martinoia, 2000; Lambers *et al.,* 2015). Morphological, physiological and transcriptional profiling of cluster roots in P-deficient lupin plants has been performed (O'Rourke *et al.,* 2013; Secco *et al.,*2014; Wang *et al.,* 2015). Similar morphological and physiological adaptations occur also in Fe-deficient lupin roots, however no information is available on the molecular bases of the response. Therefore, aim of the present work was to investigate the adaptive mechanisms developed by white lupin when Fe is limiting, and to highlight common features between P and Fe acquisition processes.Transcriptomic analyses allow the characterization of four transcriptomic profiles of lupin roots: +P+Fe, -Fe apex, -Fe cluster and -P cluster; the thesis +P+Fe was used as control referring to plants grown under complete nutrient solution. Under Fe-deficient lupin roots modulated more than 5000 transcripts with different expression respectively in cluster or apex root tissues; while cluster roots of P-deficient plants modulated around 2000 transcripts in comparison to control (in -Fe apex vs +P+Fe: +916 and -707 modulated transcripts; in –Fe cluster vs +P+Fe: +2066 and -3350 modulated transcripts; in –P cluster vs +P+Fe: +1118 and -917 modulated transcripts). RNAseq data indicate that white lupin roots upregulate Fe-responsive genes ascribable to Strategy I response, this behaviour being mainly evident in cluster roots. The upregulation of some components of Fe-acquisition mechanism occurs also in P-deficient clusters roots. Concerning P acquisition, some P-responsive genes (as phosphate transporters and transcription factors) were upregulated by P deficiency as well by Fe deficiency.These data indicate a strong cross-connection between the responses activated under Fe or P deficiency in white lupin, including the activation of sensing and signalling networks, of Strategy Icomponents and of P-Starvation Responsegenes. This behaviour could be functional to the mobilization of both nutrients from poorly soluble P-Fe sources naturally occurring in the soil and increasing their availability for root uptake. The activation of

common feature between Fe and P nutrient pathways might play a crucial role to enhance the nutrient uptake efficiency by cultivated plants.

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Implication of copper and iron sources and availability for plant growth and development

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Copper (Cu) is an essential mineral nutrient for plant growth and development. This metal is present in the active site of numerous enzymes involved in metabolic processes, such as mitochondrial respiration, photosynthesis, lignin synthesis and oxidative stress tolerance. Copper is highly reactive and can be toxic for the plant *via* the Fenton reaction. Thus, the intracellular Cu level must be tightly regulated, since at high concentrations it can cause morphological, anatomical and physiological changes in plants, resulting in the decrease of both food productivity and quality (Yruela, 2005).

Iron (Fe) and Cu homeostasis are deeply linked in the cell metabolism. Iron-Cu crosstalk may influence mineral acquisition and transport (Hördt*et al.,* 2000). Non *graminaceous* plants have a reduction-based mechanism to acquire Fe, while grasses rely on the biosynthesis and release of phytosiderophores (PS) and uptake of ferric–phytosiderophore complex (Waters, 2013). Copper should be taken up by both mechanisms: reduction-based and, probably, Cu-PS complex transport.

Understanding crosstalk between Fe and Cu nutrition has become a topic of great interest, in terms of crop yield and due to the accumulation of Cu in some agricultural soils, which could lead to develop novel strategies to improve growth on soils with low or excess metals.

Based on these considerations, this work investigates the interaction between Cu and Fe in maize (Cu-tolerant species, *Strategy II*) and tomato (Cu-sensitive species, *Strategy I*) with the purpose to understand if their status in the plants and supply may reciprocally affect their acquisition. Therefore maize and tomato plants were grown under complete nutrient solution (+Fe +Cu) or in Fe and/or in Cu deficiency (-Fe +Cu; +Fe -Cu; -Fe -Cu) or under Cu excess (++Cu +Fe; ++Cu -Fe).

Elemental analyses (ICP-OES/MS) on shoots and roots of maize revealed that excess of Cu may interfere with the translocation of Fe from roots to shoots, possibly explaining the chlorotic symptoms in leaves of ++Cu plants. In tomato Fe deficiency increased the translocation of Cu and Zn into the leaves, while when Cu was not supplied, the translocation of Mn and Zn to the shoot increased. Several studies have indicated that Fe deficiency is associated with an increase in micronutrient concentration, except for Cu.

Root-associated Fe(III) and Cu(II) reductions by Fe(II)-BPDS or Cu(I)-BCDS assays (Welch *et al.*, 1993) and the activity of enzymes related to the antioxidant system in shoot and roots (SODs, CAT, APX and POX)were also tested.

In tomato biochemical analyses showed an increase of the ferric-chelate reductase activity in -Fe +Cu, while there was no increase in the enzyme activity in -Fe -Cu plants. At the same time, Cu reductase activity was significantly increased in -Fe-Cu plants.

Our results show that Fe and Cu interact each other affecting their uptake. Further understanding on the mechanisms involved in Cu and Fe homeostasis are still required to better evaluate the possible interactions between these two micronutrients.

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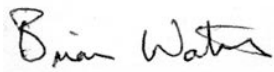385 Plant Sciences Hall

October 16, 2018

To: AlessandroFranco,
        Department of Agricultural and EnvironmentalSciences
        University of Udine, Udine,Italy

Dear Alessandro,

This letter is to acknowledge that you have completed your visit my laboratory for the period from August to 15th October, 2018, as a visiting scholar.

During your visit, you learned skills in plant molecular physiology using melon wild-type and *fefe* mutant plants, and performed experiments that can be related to your PhD project on iron and copper deficiencies in tomato, and how these deficiencies interact. Specifically, you learned to grow plants in a split-root system to apply different iron and copper treatments to each root half to study root-to-shoot and shoot-to-root communication, to perform root ferric-chelate reductase assays, and to measure gene expression by reverse transcriptase-quantitative PCR. You also worked with Dr. Jaekwon Lee to learn to measure SOD protein activity with native gels and spectrophotometric assay.

Sincerely,

Brian Waters
Associate Professor