Iron fertilization to enhance tolerance mechanisms to copper toxicity of ryegrass plants used as cover crop in vineyards

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HIGHLIGHTS

- Heavy metal phytoremediation of Cu-contaminated soils by cover crops.
- Phenolic compounds are involved in alleviating Cu toxicity in Ryegrass.
- Cu toxicity promotes morphological changes and nutritional imbalances.

ARTICLE INFO

ABSTRACT

Ryegrass (*Lolium perenne* L.) is a plant species that can express mechanisms of tolerance to copper (Cu) toxicity. Therefore, the agronomical approach of intercropping system with ryegrass may represent a promising tool to limit the onset of Cu toxicity symptoms in the other intercropped plants species, particularly when an inadequate nutrient availability like iron (Fe) shortage is also concurrently present. This study aimed at assessing the mechanisms involved in the mitigation of Cu phytotoxicity and the stress effects on plant growth, root morphology and nutrition of ryegrass fertilized with two different Fe sources. To this purpose, seedlings of ryegrass were hydroponically grown for 14 days in controlled conditions with 4 different levels of Cu (0.2, 5.0, 25 and 50 μM) and with either 100 μM Fe-EDDHA or Fe-EDTA. Results show that high levels of Cu availability enhanced the root content of organic anions as well as the root exudation. Different Fe fertilizations at the condition of 50 μM Cu induced changes in root phenolic compounds, citrate and fumarate contents and the exudation pattern of phenolic compounds. Differences in plant growth were not observed between the two Fe sources, although Cu concentration in plant tissue fed with Fe-EDTA was lower in the condition of 50 μM Cu. The enhanced root exudation of Cu-complexing organic compounds (including phenolics) in ryegrass plants when exposed to excessive Cu availability could be at the basis of the ameliorated edaphic rhizosphere conditions (lower Cu availability). For this reason, from the agronomical point of view ryegrass plants used in intercropping systems with crops like vine plants could represent a promising strategy to control Cu toxicity in vineyard soils. Further studies under the field conditions must be taken to support present findings.

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1. Introduction

The intensification of grape production systems has led to an increase in the use of synthetic fertilizers and pesticides in order to guarantee an appropriate mineral acquisition of grapevines and an efficient control of the pathogen infections, respectively. However, the repeated applications of copper (Cu)-based fungicides to control the foliar diseases, as for instance Bordeaux mixture ($Ca(OH)_2 + CuSO_4$), have consequently resulted in the accumulation of the total Cu fraction in soils (Komárek et al., 2010; Mackie et al.,

2012; Miotto et al., 2014; Baldi et al., 2018). Although Cu is an essential element involved in important metabolic processes of plants, excessive acquisition of this nutrient by the roots can severely compromise plant growth and productivity by inducing changes in the root system architecture, nutritional imbalances, oxidative stress and the accumulation of reactive oxygen species - ROS (Kabata-Pendias, 2011; Cambrollé et al., 2015; De Conti et al., 2019; Marastoni et al., 2019a).

The adaptive mechanisms triggered by plants when exposed to nutritional stress (*i.e.* toxicity or deficiency) include, together with other molecular and physiological changes (Marschner, 2011), the increased root exudation of low-molecular-weight organic compounds (*i.e.* phenolic compounds, organic acids, amino acids and sugars, etc.) in the rhizosphere and the enhanced accumulation of organic anions in root tissues. For the metal-complexing properties of these organic compounds (Jones, 1998; Montiel-Rozas et al., 2016; Brunetto et al., 2016), their root release can consistently affect the bioavailable fraction of the metal in the rhizosphere and in the root apoplast as well as their accumulation inside the cells can considerably impact the translocation levels of these elements within the plant (Jones, 1998; Meier et al., 2012; Dresler et al., 2014; De Conti et al., 2018a). In fact, from the nutritional point of view, it is well known that both plants and microorganisms preferentially take up Cu^{2+} in its ionic free form (McBride, 1994; Kim et al., 2010; Meier et al., 2012). Therefore, the Cu-complexed forms with organic compounds can be less available and thus less toxic than the corresponding free metal (e.g. Cu^{2+}) and its complexation process could represent a strategy to hinder the transmembrane transport and the metal accumulation in the cells (*external metal exclusion mechanisms* - Leitenmaier and Küpper, 2013). It is clear that the chemical features of these root exudates (in particular the carboxyl and hydroxyl groups) are determinant for their effectiveness in the metal complexing properties and, in turn, in affecting the Cu bioavailable fraction in soil (Seshadri et al., 2015; Montiel-Rozas et al., 2016) thus limiting the onset of the toxicity symptoms (Montiel-Rozas et al., 2016; Zafari et al., 2016).

Beside their role in the external Cu^{2+} exclusion strategy, metal chelating compounds can also be involved in an *internal accumulation mechanism* (Leitenmaier and Küpper, 2013), in which the complexation of the heavy metals can promote the immobilization of metals, especially in organelles such as vacuoles. As a consequence, also their translocation through the xylem will be hindered (Dresler et al., 2014; Kısa et al., 2016). Furthermore, phenolic compounds have a role in ROS homeostasis in plant cells and can contribute in reducing the oxidative stress generated by excessive heavy metals availability (Michalak, 2006; Majdoub et al., 2017). However, both the type and the intensity of the abovementioned detoxification strategies (*external metal exclusion* and *internal accumulation mechanisms*) can vary among different plant species as well as they can also be dependent on i) the nutritional plant state, ii) the type and iii) the concentration of the contaminating metal (Meier et al., 2012; Dresler et al., 2014; Marastoni et al., 2019b). From an agronomical point of view, micronutrients like iron (Fe) may play a crucial role in guaranteeing the health status of crops, their productivity and the quality of the agricultural products. In this respect, it has been clearly demonstrated that the activation of the root mechanisms underlying the response to the Fe shortage may induce an accumulation of cations in plant tissues (Tomasi et al., 2014), being particularly dangerous when toxic elements like cadmium (Cd) are also available in the soil (Astolfi et al., 2003). In this regard, it appears clear that a limited availability of Fe in the growth medium can exacerbate the problem of Cu toxicity, when present at high levels in the soil/plant interface. With respect to Fe availability, it is well known that this nutritional disorder is particularly relevant for crops grown in alkaline soils, where the bioavailable fraction is often scarce (Mimmo et al., 2014). Therefore, to cope with this nutritional disorder, a fertilization program via either root or foliar applications is often recommended (López-Rayo et al., 2015). Traditionally, micronutrients fertilization is carried out using chelating compounds such as *ethylenediaminetetraacetic acid* - EDTA (Laurier et al., 1991), even though, at present, the most effective Fe fertilizer in neutral and alkaline soils represent the ferric *ethylenediamine-N,N′-bis(o-hydroxyphenylacetate)* (Fe(III)-EDDHA) (Nadal et al., 2012). When synthetic organic ligands (e.g. EDTA or EDDHA) are used to complex Fe, they may also interact with other ions concurrently present in the soil solution (e.g., Cu^{+2}), affecting therefore their availability to plants (Hernandez-Apaolaza, 2014). Thus, the chemical species distribution in the soil solution may change increasing complexation and reducing bioavailability or *vice versa* for several ions (Li et al., 2018). These aspects might be of paramount importance when field fertilization plans are implemented. In fact, when the dynamics of nutrients are studied in controlled experimental conditions (e.g. hydroponics), Fe is generally supplied as Fe(III)-EDTA (Hoagland and Arnon, 1950); the stability constant of the complex Fe(III)-EDTA (Log Kf = 25.10) is close to that of Cu(II)-EDTA (Log Kf = 18.80), suggesting that a metal ligand exchange reaction on the EDTA molecule may occur. This phenomenon may therefore reduce the bioavailable Cu fraction, leading to an overestimation of the possible Cu toxicity effect on plants. Indeed, it has been observed that plants treated with toxic concentrations of Cu accumulated higher amounts of Fe than control plants suggesting that Cu ions are able to displace Fe from Fe(III)-EDTA making Fe more available for plants (Lequeux et al., 2010). On the other hand, the stability constant of Fe(III)-EDDHA (LogKf = 35.09) demonstrate that the EDDHA ligand is highly selective for Fe, whereas the logKf of the complex Cu(II)-EDDHA (25.13) is several orders of magnitude lower, thus showing negligible competition between Fe and Cu for the ligand (Yunta et al., 2003). Therefore, depending on the chelating molecule chosen for the delivery of the micronutrient Fe, the bioavailable fraction of Cu might be influenced, either exacerbating or mitigating the effects of excessive Cu concentrations in the growth substrate.

On the basis of these observations, the present work aimed at assessing the influence of the use of different Fe chelating compounds (*i.e.* EDTA and EDDHA) on the response to Cu stress in ryegrass plants. Indeed, ryegrass exhibits a good development in several types of soils and it could be potentially used in soils contaminated with heavy metals (Bai et al., 2015). In this sense, it could be especially used as cover crop in Cu contaminated vineyard soils, for both soil and plant protection, as well as to enhance nutrient cycling (Girotto et al., 2016; Montiel-Rozas et al., 2016; Tiecher et al., 2018).

2. Material and methods

2.1. Plant material, treatments and growing conditions

The study was carried out in hydroponics, under controlled conditions in a climatic chamber 14/10 h light/dark, 24/19 °C, 70% relative humidity and 250 mmol m^{-2} s^{-1} light intensity. Seeds of ryegrass (*Lolium perenne* L.) were germinated on filter paper moistened with 0.5 mM CaSO$_4$ solution in darkness for 5 days (Nikolic et al., 2012). After the germination period, ryegrass seedlings were transferred into a complete full-strength nutrient solution (NS). The composition of the hydroponic solution was as follows (mM): 2 Ca(NO$_3$)$_2$, 0.7 K$_2$SO$_4$, 0.1 KH$_2$PO$_4$, 0.1 KCl, 0.5 MgSO$_4$, and (μM): 1.0H$_3$BO$_3$, 0.5 MnSO$_4$, 0.2 CuSO$_4$, 0.5 ZnSO$_4$, 0.01 (NH$_4$)$_6$Mo$_7$O$_{24}$, 100 Fe(III)-EDTA. The solution was continuously aerated and changed every three days (Pii et al., 2015). The experimental units consisted of 2-L polyethylene containers with 1.5 L of

nutrient solution. Ten sets of seedlings (5 seedlings per set) were placed within each pot (ten sets of seedlings per container and 5 seedlings per set, which totaled 50 seedlings in each replicate).

After 13 days of cultivation in NS, plant roots were washed for 1 h in a 0.5 mM CaSO$_4$ solution; afterwards, plants were transferred to a new NS and were treated with different concentrations of Cu (*i.e.* 0.2, 5.0, 25 and 50 μM) added as CuSO$_4$, simulating the concentrations possibly found in vineyard soils contaminated with Cu (Komárek et al., 2010) and two different 100 μM Fe sources, Fe(III)-EDDHA or Fe(III)-EDTA. The solution was changed every three days. After applying the treatments, the plants were grown for further 14 days (27 days from germination) in the same climatic chamber and then harvested for further analyses. The experimental design was completely randomized with 3 replicates, thus it totaled 24 experimental units (containers). Ten (10) sets were cultivated in each replicate (5 seedlings per set).

2.2. Plant dry weight and nutrient analysis

At harvest, three sets of plants (15 plants) per treatment were sampled, washed three times in distilled water and then shoots and roots were separated. Afterwards, samples were dried at 65 °C until constant weight. Dry weight was recorded and, subsequently, plant material was ground for the analysis of nutrients calcium (Ca), magnesium (Mg), potassium (K), phosphorus (P), sulfur (S), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn). To determine total nutrient concentrations, samples were digested using a microwave-assisted procedure (SRC, UltraWAVE, Milestone Inc, Shelton, CT, USA), with 65% ultrapure HNO$_3$. Concentrations of nutrients were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian 720-ES; Varian, Mulgrave, Australia).Tomato leaves (standard reference material - SRM 1573a) and spinach leaves (standard reference material - SRM 1547) were used as external certified reference material and the obtained results showed an error < 5% (Marastoni et al., 2019b).

2.3. Collection and analysis of root exudates

During the growth period, root exudates were collected at two times: 3 days (1st sampling) and 14 days (2nd sampling) after treatment application (establishment of Cu levels and Fe sources). At each sampling, five sets of plants (25 plants) per treatment were removed from the nutrient solution and roots were washed three times in deionized water. Afterwards, the roots of each set were separately submerged into 20 mL of deionized water, which was continuously aerated for 4 h. The pots were wrapped with aluminum foil to keep the roots in the dark. Trap solutions were filtered at 0.20 μm, freeze-dried, resuspended in double-distilled water and methanol (60:40 v/v) and stored at −20 °C until further analysis. The roots were weighed after collecting exudates.

Total chelating compounds were colorimetrically determined using a modified procedure of the spectrophotometric Chrome Azurol S (CAS) method (Shenker et al., 1995). Absorbance was measured at 585 nm and the concentration of total chelating compounds was expressed as mmoles equivalent of EDTA per gram of root fresh weight. The content of total phenolic compounds in root exudates was determined using the Folin-Ciocalteu method (Folin and Ciocalteu, 1927). Absorbance was measured at 765 nm and the concentration of the total phenolic compounds was expressed as mmoles equivalent of Gallic acid per gram of root fresh weight.

2.4. Determination of the organic acids, amino acids and phenols content in root tissues

Roots collected 14 days after the application of treatments were washed three times in deionized water and instantly frozen in N$_2$, and then stored at −80 °C until the time of analysis. Subsequently, the roots were macerated with liquid N$_2$ and a sample of 0.2 g was extracted with 2 mL of 100% methanol (v/v) overnight (12 h) (Valentinuzzi et al., 2015). Then, the samples were centrifuged at 14,000 g for 15 min and the supernatant was filtered at 0.45 μm.

The determination of organic acids (OAs) was carried out by high performance liquid chromatography (HPLC) using a cation exchange column (Rezex ROA, Phenomenex; 300 × 7.8 mm), applying an isocratic elution with 10 mM H$_2$SO$_4$ as carrier solution at a flow rate of 0.6 mL min^{-1}. Organic acids were detected at 210 nm using a Waters Photodiode Array Detector (PDA 2998, Waters Spa, Italy). The organic acids were identified by comparing retention times of unknown compounds to those of pure organic acids and by standard additions (Sandnes et al., 2005).

Amino acids (AAs) were separated by HPLC prior to a precolumn derivatization with a commercial kit (AccQ:Tag, WAT052880-Waters Corporation, Italy), using a high-efficiency Nova-PakTMC18 silica based bonded column (4.6 × 250 mm, 4 μm, Waters Corporation, Italy) with a gradient elution with a flow rate of 1.0 mL min^{-1}: A = AccQ:Tag Eluent (WAT052890), B = 100% acetonitrile (HPLC grade), C = Milli-Q (waters application note for AA). The derivatized samples were detected using fluorescence detection (λex = 250 nm, λems = 395 nm, (Waters 2475, Italy) with the column condition set at 37 °C.

The concentration of total phenolic compounds in root tissues was determined using the Folin-Ciocalteu method (Folin and Ciocalteu, 1927), as described above.

2.5. Root architecture

Root morphology was assessed at harvest (14 days after treatments application) in three sets of plants (15 plants) per treatment, using WhinRHIZO software (EPSON1680, WinRHIZO Pro2003b, Regent Instruments Inc., Quebec, Canada). The total root length (cm plant^{-1}), root surface area (cm^2 plant^{-1}), volume (cm^3 plant^{-1}) and average diameter (mm) were assessed.

2.6. Statistical analysis

The data are expressed as mean values ± SE. Analysis of variance was done with SISVAR software, version 4.0 (Ferreira, 2011). The parameters assessed in this study were compared among Cu levels within the same Fe source (EDDHA and EDTA) and between the two Fe sources within the same Cu level. The means were grouped by the Scott- Knott test at 5%. The principal component analysis (PCA) was performed using the CANOCO software product, version 4.5 (Ter Braak and Smilauer, 2002). The plant variables used in the analysis were the growth, nutrient contents, root morphology, total phenolic and chelating compounds exudates e accumulation of organic acids and total phenolic compounds in roots.

3. Results

3.1. Plant growth and root morphology

The ryegrass growth was impaired by Cu excess, which induced a significant decrease in shoot and root dry matter, especially in plants grown in 25 and 50 μM Cu (Fig. 1). There were no differences in shoot and root dry matter yield between Fe-EDDHA and Fe-EDTA-supplied plants. The roots displayed a higher sensitivity to
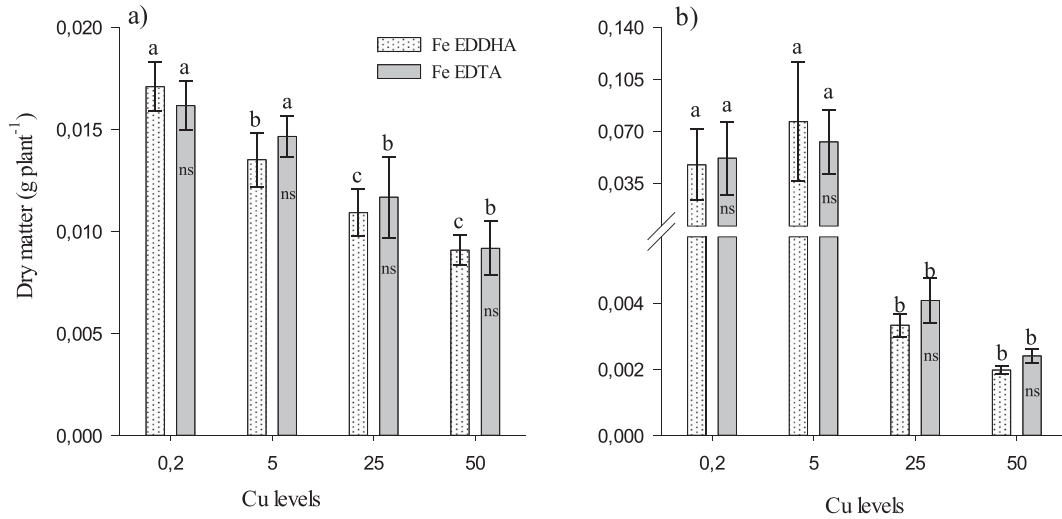
Fig. 1. Dry matter yield (a) shoots and (b) roots of ryegrass exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test (p < 0.05). ns non-significant; * significant (F test p < 0.05) difference between Fe source at the same Cu level.
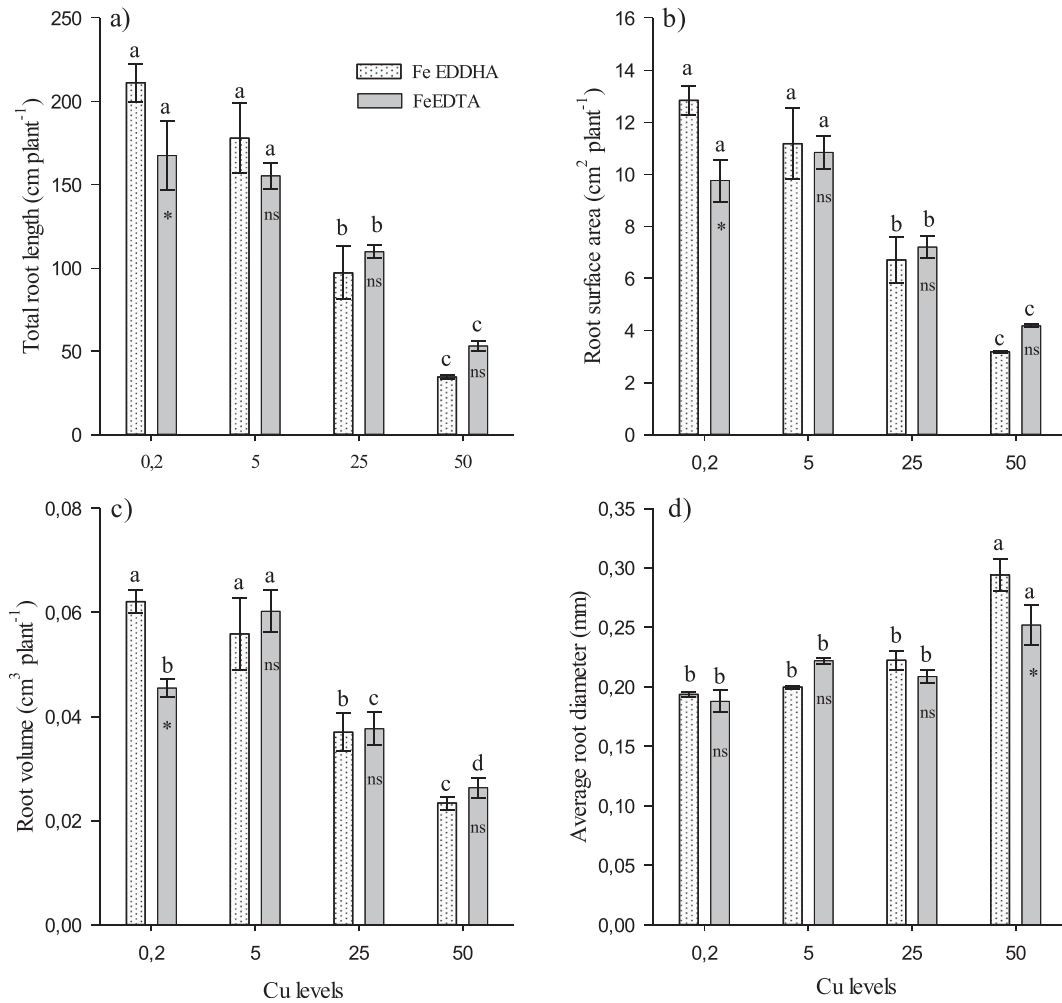

Fig. 2. Root morphological parameters of ryegrass exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test (p < 0.05). ns non-significant; * significant (F test p < 0.05) difference between Fe source at the same Cu level.

Cu toxicity, in fact the dry weight was reduced by about 2265 and 2058% in 50 μM Cu treated plants, supplied with Fe-EDDHA and Fe-EDTA, respectively, as compared to control plants (0.2 μM Cu) (Fig. 1b).

Plants exposed to 25 and 50 μM Cu also showed a significant reduction in total root length, root surface area and root volume (Fig. 2a, b, c). As expected, the lowest values of these root parameters were found in plants treated with 50 μM Cu, albeit no differences in these Cu concentrations were found between Fe-EDDHA and Fe-EDTA-supplied plants (Fig. 2a, b, c). Concerning the root diameter, plants grown in 50 μM Cu showed the highest values (Fig. 2d); interestingly, at this level, the Fe-EDDHA induced significant increase in root diameter in comparison to Fe-EDTA (Fig. 2d).

3.2. Nutrient concentration

Plants treated with 50 μM Cu, regardless of Fe source, showed the lowest P concentrations in shoots, whilst the P content in roots was not influenced by the increasing Cu availability in the growth medium (Table 1). Contrarily, the Cu treatment induced the enhancement of K concentrations in both shoots and roots, to a similar extent independently from the form of Fe supplied (Table 1). The concentrations of Ca, Mg, P and S in roots were not influenced by both Cu treatments and Fe sources (Table 1).

Concerning micronutrients, the concentrations of Cu showed a step-wise increase in both shoots and roots according with the external concentration applied, albeit it did not statistically differ statistically between 25 and 50 μM Cu-treated and supplied with in Fe-EDTA (Fig. 3). Interestingly, when the external Cu concentration was 25 and 50 μM, the Cu content in both shoots and roots was higher in Fe-EDDHA-fed plants (Fig. 3). At shoot level, Fe concentration did not show any significant variation related to either Cu treatment or Fe source, except for 50 μM Cu-treated and Fe-EDDHA-supplied plants, which recorded the highest Cu levels (Fig. 3). In roots the Fe content was increased according to the increasing Cu treatments; only at the highest Cu concentration

(50 μM), the content of Fe resulted significantly lower in ryegrass plants fed with Fe-EDTA as compared to those supplied with Fe-EDDHA (Fig. 3). The highest concentrations of Mn and Zn in roots were detected in plants exposed to the highest Cu levels (25 and 50 μM). Interestingly, whilst at 25 μM Cu Fe-EDDHA plants showed a higher root Zn concentration as compared to Fe-EDTA supplied ones, at 50 μM Cu the opposite was observed, yet in the shoots (Fig. 3).

3.3. Characterization of root exudates

Root exudates released by ryegrass were collected during the cultivation period, at 3 and 14 days after the Cu levels e Fe sources treatments, and analyzed to determine total phenolic compounds, total chelating compounds and organic acids. Organic acids could not be detected, most likely because their concentrations were below the detection limit. Phenolic compounds showed an increasing trend with increasing Cu concentration in the nutrient solution; this was particularly evident at 3 days after treatment in plants exposed to phytotoxic levels of Cu (*i.e.* 25 and 50 μM Cu), albeit no significant difference was observed considering the different Fe sources (Fig. 4a). At 14 days after the treatment, only plants exposed of 50 μM Cu showed a significant increase in total phenolics exudation; in particular, Fe-EDDHA-fed plants released a higher concentration of phenolic compounds as compared to Fe-EDTA-fed ones (Fig. 4b). The total chelating compounds showed an exudation pattern similar to that observed for phenolic compounds (Fig. 4c and d); at 3 days after treatments, the higher exudation rate was observed in plants exposed to 50 μM Cu, independently from the Fe source supplied, and in plants treated with 25 μM Cu in the presence of Fe-EDTA (Fig. 4c). At 14 days after treatments, the exudation of chelating compounds was still enhanced at phytotoxic Cu concentrations (*i.e.* 25 and 50 μM) as compared to control plants, albeit the Fe source did not induce any significant alteration in the process (Fig. 4d).

Table 1
Macronutrient contents in shoot and root of ryegrass exposed to increasing Cu levels for 14 days in two Fe sources.

Element	Fe Source	Cu levels			
		0.2	5.0	25	50
		Shoot			
Ca (mg g^{-1})	EDDHA	6.13 ± 0.15 a[a]	5.62 ± 0.24 a	6.64 ± 0.26 a	5.74 ± 0.75 a
	EDTA	6.06 ± 0.44 a^{ns}	6.64 ± 0.15 a^{ns}	6.40 ± 0.82 a^{ns}	5.04 ± 0.81 a^{ns}
K (mg g^{-1})	EDDHA	8.54 ± 2.4 b	13.03 ± 3.2 b	17.02 ± 2.4 a	20.49 ± 1.3 a
	EDTA	9.18 ± 2.7 b^{ns}	11.66 ± 1.3 b^{ns}	16.20 ± 0.32 a^{ns}	20.93 ± 2.9 a^{ns}
Mg (mg g^{-1})	EDDHA	1.75 ± 0.04 b	1.74 ± 0.11 b	2.34 ± 0.09 a	2.05 ± 0.17 a
	EDTA	1.85 ± 0.11 a^{ns}	2.02 ± 0.04 a^{ns}	2.03 ± 0.13 a^{ns}	1.76 ± 0.15 a^{ns}
P (mg g^{-1})	EDDHA	3.72 ± 0.01 a	3.56 ± 0.10 a	3.28 ± 0.16 a	2.28 ± 0.23 b
	EDTA	3.44 ± 0.09 a^{ns}	3.44 ± 0.25 a^{ns}	3.37 ± 0.17 a^{ns}	2.12 ± 0.12 b^{ns}
S (mg g^{-1})	EDDHA	2.50 ± 1.7 a	2.36 ± 0.10 a	3.41 ± 0.44 a	3.03 ± 0.49 a
	EDTA	2.15 ± 0.08 b^{ns}	2.17 ± 0.04 b^{ns}	3.29 ± 0.82 a^{ns}	3.65 ± 0.30 a^{ns}
		Root			
Ca (mg g^{-1})	EDDHA	1.04 ± 1.75 a	0.46 ± 0.93 a	1.41 ± 0.12 a	1.51 ± 0.07 a
	EDTA	0.88 ± 0.65 a^{ns}	0.24 ± 0.6 a^{ns}	1.27 ± 1.1 a^{ns}	1.82 ± 0.58 a^{ns}
K (mg g^{-1})	EDDHA	9.61 ± 8.1 b	3.73 ± 1.9 b	22.81 ± 0.75 a	16.39 ± 0.59 a
	EDTA	10.07 ± 8.2 b^{ns}	2.70 ± 8.83 b^{ns}	22.63 ± 0.98 a^{ns}	21.68 ± 1.48 a^{ns}
Mg (mg g^{-1})	EDDHA	0.48 ± 0.37 a	0.16 ± 0.09 a	0.76 ± 0.06 a	0.64 ± 0.02 a
	EDTA	0.41 ± 0.32 a^{ns}	0.11 ± 0.03 a^{ns}	0.68 ± 0.06 a^{ns}	0.59 ± 0.07 a^{ns}
P (mg g^{-1})	EDDHA	1.64 ± 1.2 a	0.57 ± 0.29 a	2.44 ± 0.19 a	2.07 ± 0.23 a
	EDTA	1.59 ± 1.3 a^{ns}	0.40 ± 0.11 a^{ns}	2.84 ± 0.14 a^{ns}	1.97 ± 0.11 a^{ns}
S (mg g^{-1})	EDDHA	6.35 ± 6.0 a	6.63 ± 5.0 a	2.44 ± 0.04 a	2.24 ± 1.5 a
	EDTA	3.19 ± 1.2 a^{ns}	1.12 ± 0.90 a^{ns}	1.79 ± 0.02 a^{ns}	2.47 ± 0.21 a^{ns}

[a] Means followed by the same letter do not differ between Cu levels within the same Fe source by the Scott-Knott test ($p < 0.05$). ^{ns} non-significant; * significant (F test $p < 0.05$) difference between Fe source at the same Cu level. The data represent the mean ± SE of three replicates.
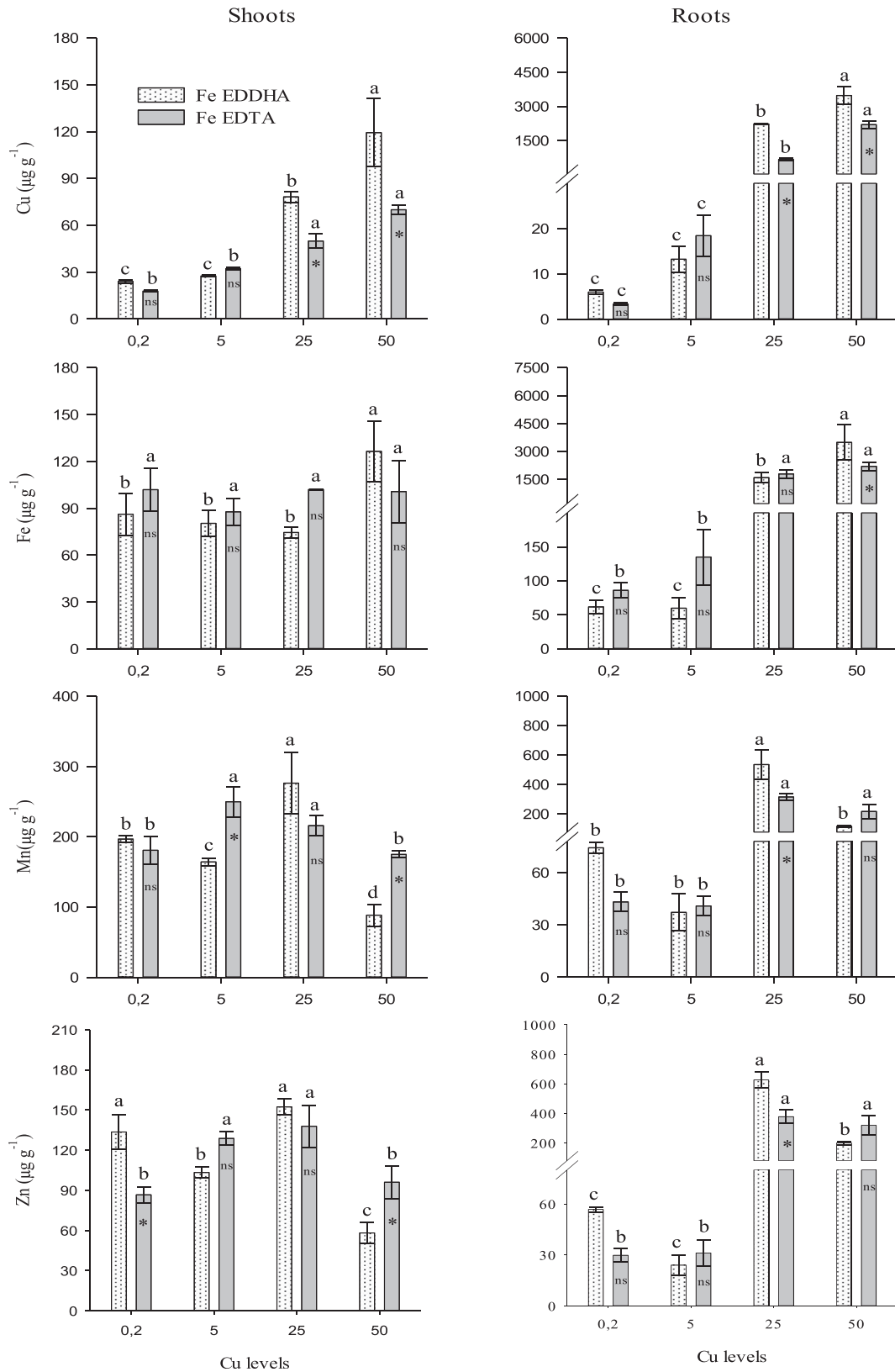
Fig. 3. Micronutrient contents in shoot and root of ryegrass exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test (p < 0.05). [ns] non-significant; [*] significant (F test p < 0.05) difference between Fe source at the same Cu level.
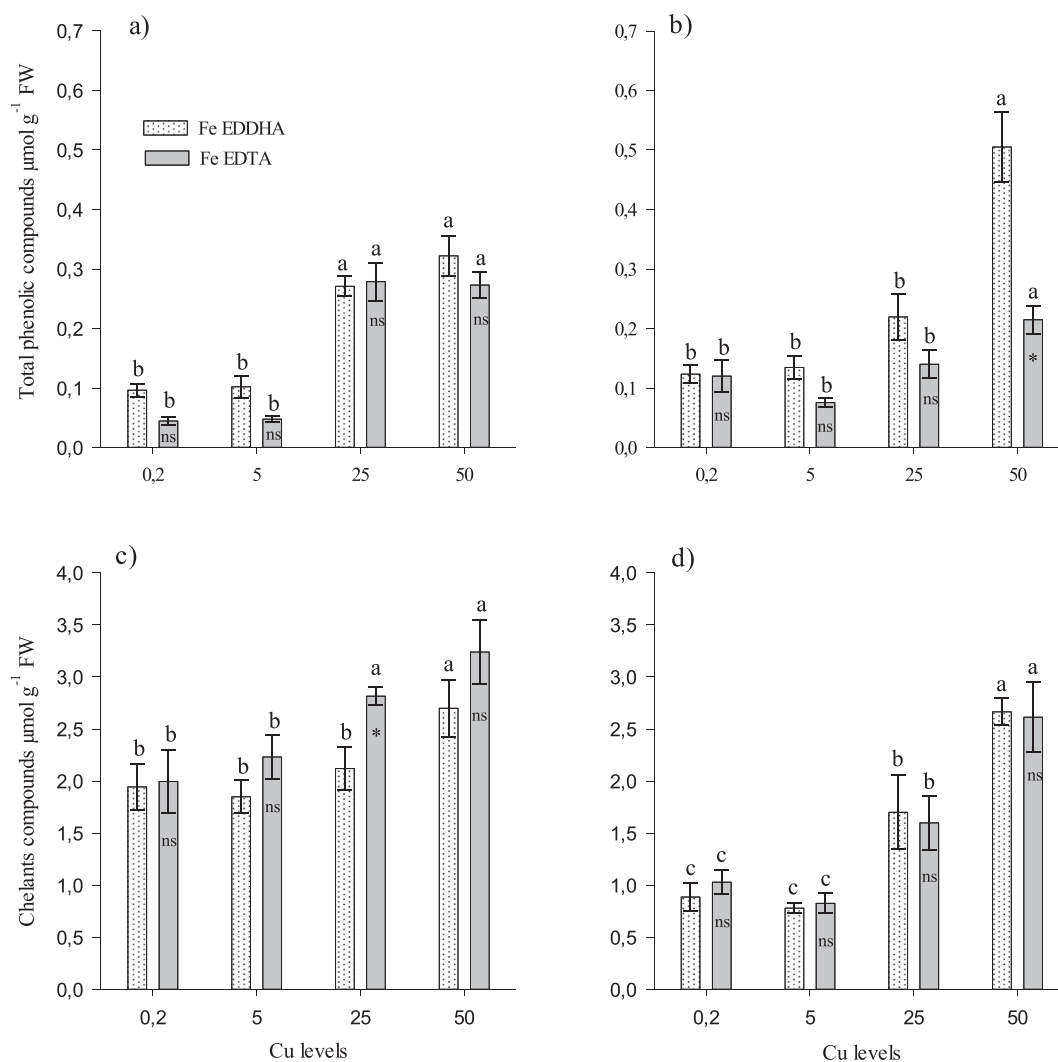
Fig. 4. Concentration of total phenolic compounds and chelating compounds 3 days (a and c) and 14 days (b and d) after exposure of ryegrass to increasing Cu levels, in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test (p < 0.05). ns non-significant; * significant (F test p < 0.05) difference between Fe source at the same Cu level.

3.4. Organic acid, total phenolic compound and amino acid contents in roots

At harvest (14 days after treatments), metabolites were extracted from root tissues. The analyses of organic acids in root extracts of ryegrass revealed the presence of four different carboxylates, namely citrate, malate, succinate and fumarate (Fig. 5). Citrate was the only carboxylate showing an induction in Fe-EDTA-fed plants exposed to 50 μM Cu as compared to control plants (Fig. 5a). The other acids detected were either not modulated (*i.e.* malate in Fe-EDTA-supplied plants) or reduced in response to the increasing Cu concentration in the growth medium (Fig. 5bB, c, d). Indeed, succinate, which showed the highest concentration in the roots, was also the most responsive to Cu treatments: in fact, in 50 μM Cu-treated plants it was reduced by about 262 and 315% in Fe-EDDHA and Fe-EDTA-fed, respectively, as compared to the control plants (Fig. 5c). Interestingly, also fumarate concentration roots showed a decreasing trend according to the increasing Cu concentration in the external medium (Fig. 5d); furthermore, the supplementation of Fe-EDTA as Fe source caused a significant decrease in fumarate concentration in roots (Fig. 5d).

The concentration of total phenolic compounds determined in the root tissues (Fig. 5e) showed a similar trend as those determined in the root exudates (Fig. 4a and b). The highest contents of root phenolic compounds were found in plants grown in the presence of 25 and 50 μM Cu (Fig. 5e); Furthermore, when exposed to 50 μM Cu, plants fed with Fe-EDTA displayed a significant decrease in the phenolic compounds concentration of roots (Fig. 5e).

The physiological changes caused by excess Cu also affected the concentration of amino acids in roots of ryegrass. Remarkably, an increase in the concentration of serine and glycine was detected in plants grown in the presence of 50 μM Cu, independently from the Fe source (Supplement 1). Interestingly, in plants treated with 5 and 25 μM Cu, a higher concentration of serine was detected in Fe-EDDHA plants as compared to those supplied with Fe-EDTA (Supplement 1). Glycine showed the same behavior when plants were treated with the highest Cu levels (Supplement 1). At 50 μM Cu, both alanine and leucine concentrations were significantly increased, albeit only in Fe-EDTA supplied plants, whereas plants supplied with Fe-EDDHA did not record difference between Cu levels (Supplement 2). Contrarily, the concentration of both cysteine and valine were reduced when plants were treated with the highest Cu levels, independently from the Fe sources
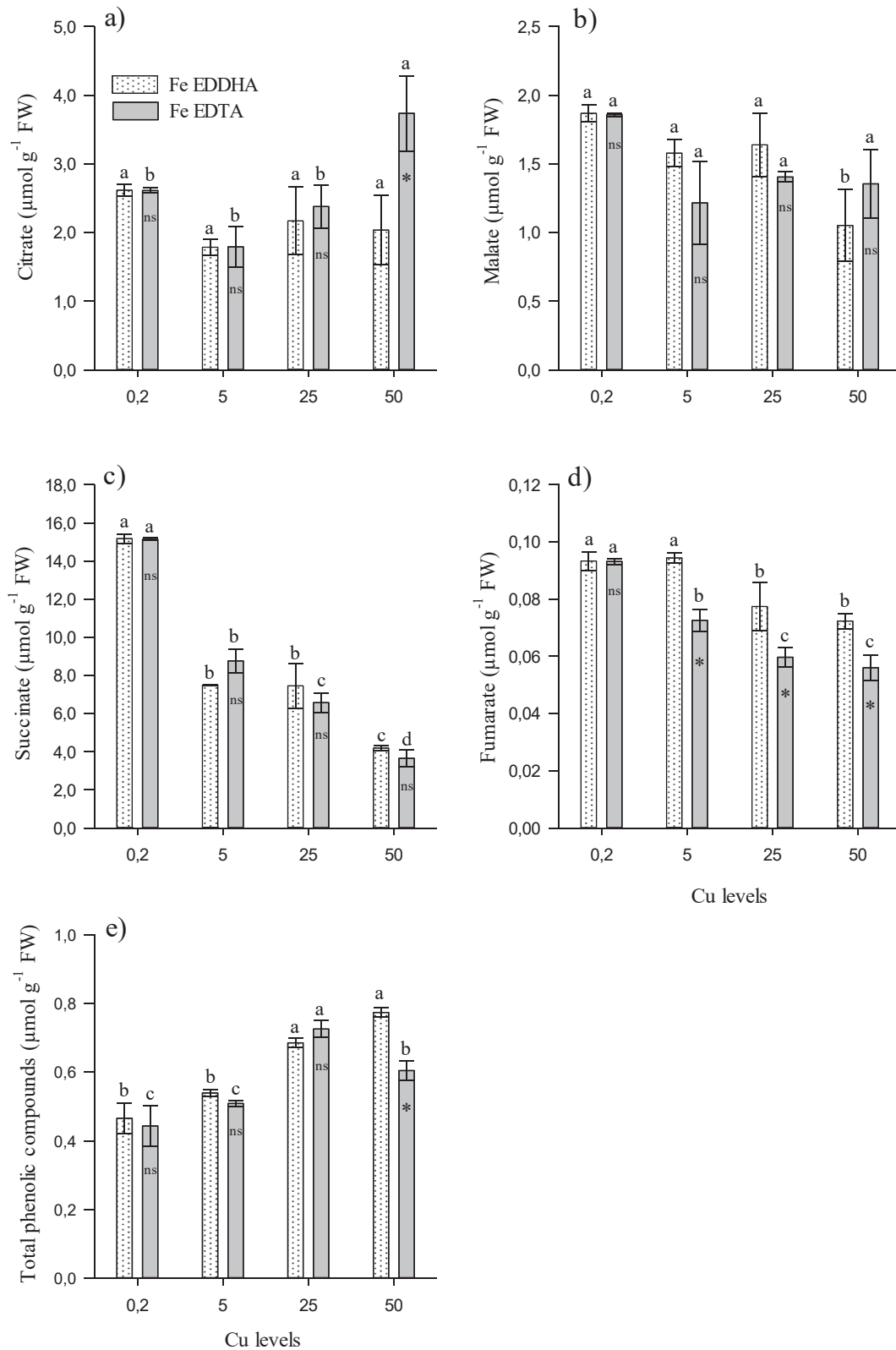
Fig. 5. Accumulation of organic acids and total phenolic compounds in ryegrass roots exposed to increasing Cu levels for 14 days in two Fe sources. Different letters indicate difference between Cu levels within the same Fe source by the Scott-Knott test ($p < 0.05$). [ns] non-significant; * significant (F test $p < 0.05$) difference between Fe source at the same Cu level.

(Supplement 2).

3.5. Principal component analysis

In order to further investigate possible differences and communalities among the treatments imposed, an unsupervised pattern recognition analysis (Principal Component Analysis − PCA) was applied to the whole dataset referred to plants at harvest (14 days after treatments). The scatterplot obtained by combining the Principal Component 1 (PC1) and the PC2 accounted for a total variance of about 70.1% (Fig. 6). The PC1, explaining 53.43% of the total variance, showed separation of samples according the Cu treatments into two main clusters: one formed by plants exposed to 0.2 and 5.0 μM Cu and another encompassing those treated with 25 and 50 μM Cu. The distribution along the PC1 was driven in the positive direction by the concentration of phenolic compounds, in both root exudates and root extracts, the concentration of chelating compounds in released by roots, the Cu concentration in roots and the average root diameter and chelating compound exudates (Fig. 6). Plants dry weight, the concentration of macronutrients, as well as that of specific micronutrients (i.e. Zn and Mn) determined the separation of samples towards the negative direction of PC1 (Fig. 6). Although PC2 explained 16.67% of the total variance, no clear separation of samples was highlighted along this component, except for plants exposed to the highest Cu concentration that clustered according with the Fe source (Fig. 6). The distribution of samples along PC2 was mainly determined, in the negative direction, by the accumulation of Fe and K and, in the positive direction, by the organic acid concentration in root extracts (Fig. 6).

4. Discussion

The set-up of agronomic practices to limit the onset of Cu toxicity symptoms in vine plants grown in soils with high Cu availability is particularly required and urgent in order to guarantee the vine cultivation in the vocated areas for viticulture. The use of ryegrass as a cover crop, for both soil and plant protection, as well as to enhance nutrient cycling (Girotto et al., 2016; Montiel-Rozas et al., 2016), is showing promising results. In fact, the capability

of this plant species to have an equilibrated growth and development in different types of soils, also contaminated, makes ryegrass plants particularly suitable for this purpose (Bai et al., 2015). In this work, in order to improve the efficiency of this agronomical practice aimed at limiting the ionic free form of Cu available in the soil solution, the effect of Fe-fertilization with two synthetic sources (i.e. EDTA and EDDHA) commonly used at the field scale has been evaluated.

Results here presented show (Fig. 1a and b) a clear decrease of the dry matter accumulation in ryegrass plants treated with 25 and 50 μM Cu. This is a clear confirmation of the onset also in the experimental conditions here used of the typical toxic effects on plant development ascribed to an excessive Cu availability in the growth medium (Cambrollé et al., 2015; Baldi et al., 2018). This phenomenon is referred to physiological and biochemical Cu-induced changes impacting cell elongation and division (Kabata-Pendias, 2011; Ambrosini et al., 2015). Furthermore, the compromised functionality of the photosynthetic system and the consequently limited CO_2 assimilation due, at least in part, to the increased levels of reactive species of oxygen, as well as to the pronounced lipid peroxidation and chlorophyll degradation, contribute to exacerbate the phenomenon (Girotto et al., 2016; De Conti et al., 2018b). Moreover, results here presented show that high levels of Cu availability limited the length-, surface area- and volume-development of the root system, whilst the average root diameter of plants exposed to 50 μM Cu was enhanced, regardless of the Fe source supplied (Fig. 2). These morphological root changes may be part of a hormonal response/imbalance induced by excessive Cu levels of availability modifying or inhibiting cell multiplication, especially in the meristematic regions. Responses induced by abiotic stresses, including metals, are mediated by the synthesis of molecules with signal function, mainly of auxin, which plays a key role in reorienting and redistributing root growth (Potters et al., 2009; Bochicchio et al., 2015). Sofo et al. (2013) observed significant association between auxin/cytokinin ratio and morphological root changes in *Arabidopsis thaliana* subjected to heavy metal toxicity, a fact that indicated integrated hormonal response under heavy metal toxicity conditions. In addition, the ionic free form of the metal (i.e. Cu^{2+}) might bind to the carboxylic groups (-COO⁻) of the
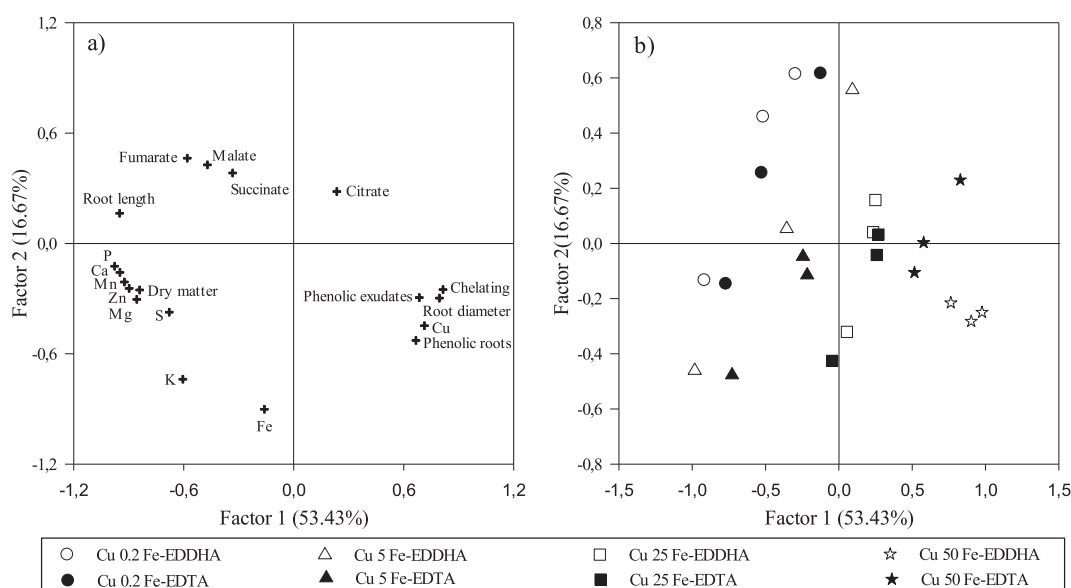

Fig. 6. Scatter plot of principal component analysis (PCA) of the total dry matter (shoot + root), root morphological parameters, accumulation of nutrients, phenolic compounds and chelating compounds in exudates and accumulation of organic acids and phenolic compounds in root tissue of ryegrass exposed to increasing Cu levels for 14 days, in two Fe sources.

cell wall affecting the division and organization of cortical cells. For this reason the area of the cortex and its diameter can be increased (Ambrosini et al., 2015) leading, in turn, to a decreased root length. Furthermore, the accumulation of phenolic compounds in the endodermal cells can contribute to reinforce the cell wall structure thus helping the formation of a physical barrier against both the transmembrane transport and the metals distribution in the vascular cylinder (and then the allocation to the shoot level) where severe damages can occur (Michalak, 2006; Ambrosini et al., 2015). In general, it has been demonstrated that at the root level the changes in the morphological and anatomical structure compromise the growth of the cover crops in grapevine plants by limiting water and nutrients uptake (De Conti et al., 2018a; Marastoni et al., 2019b). This phenomenon is particularly relevant in nutrient-poor soils and in regions with low rainfall regime (Bai et al., 2015; Girotto et al., 2016).

Shoot Cu concentration of plants grown in high Cu levels (Fig. 3) was, as expected, above the range considered adequate for most plant species (5–15 mg kg^{-1} of DM - Adrees et al., 2015) showing an inverse relationship between its values and the dry matter yield (Fig. 6a). In this respect it has been recently demonstrated that an excessive Cu availability can not only affect the functionality of transmembrane carriers and ion-channels (Marastoni et al., 2019a) but also alter considerably the membrane permeability inducing nutritional imbalance in environments contaminated with heavy metals (Cambrollé et al., 2015; De Conti et al., 2019; Marastoni et al., 2019b). Most of the Cu accumulated in plants is found at root level, a fact that reduces the fraction allocated in the shoot (Fig. 3). This phenomenon is described in several plant species (Cambrollé et al., 2015; Girotto et al., 2016) as a tolerance mechanism triggered to prevent, or limit, the onset of the toxic symptoms at the shoot level. It can be the result, at least in part, of a strong interaction between Cu and the sulfhydryl groups of enzymes and proteins present in root cell apoplast, limiting thus the magnitude of the Cu fraction available for the transmembrane fluxes to the symplast (Yruela, 2005). Moreover, once inside the root cells, its complexation with organic compounds or phosphate ions (Dresler et al., 2014; Baldi et al., 2018) and then the compartmentalization of these complexes in organelles with low metabolic activity, such as vacuoles, can surely contribute to the extent of Cu tolerance. Indeed, the Cu complexation with P at the root level could have an impact on the extent of the metabolically active P pool, affecting the nutritional balance among plant tissues (Marastoni et al., 2019b). The limited P allocation at the shoot level measured in the present work in plants fed with 50 μM Cu (Table 1) can further corroborate this hypothesis.

When ryegrass plants were fertilized with Fe-EDTA, lower Cu accumulation both at the root and shoot level has been recorded, particularly in plants treated with 50 μM Cu (Fig. 3). The formation of Cu-EDTA complex via a metal exchange between Fe and Cu with the ligand EDTA could be at the basis of this result, being the ionic and free form of Cu (Cu^{2+}), among all those present in soil solution, that preferentially taken up by plants and microorganisms (McBride, 1994). In this respect the similar stability constant of the complexes Cu(II)-EDTA and Fe(III)-EDTA (Log Kf = 18.80 and 25.10, respectively) indicates that a metal-ligand exchange reaction can occur. Furthermore, the free-EDTA fraction released in the rhizosphere once the roots have taken up Fe from the Fe-EDTA source, can surely contribute to the formation of the Cu-EDTA complex. In this respect it should be noted that a similar phenomenon has been also reported by Li et al. (2018) in soybean plants supplied with Fe-EDTA. This hypothesis is further supported by the more pronounced uptake and accumulation of Cu measured in plants supplied with Fe-EDDHA (Fig. 3). In fact, the high stability constant of this Fe-EDDHA complex (Hernandez-Apaolaza, 2014) excludes any impact of this synthetic ligand on the free Cu^{2+} in the soil solution

and thus on its plant availability. In addition, with respect to the Fe fertilization with Fe-EDTA or Fe-EDDHA, a sort of Cu/Fe competition for the uptake process cannot be excluded, at least in part. However, also in this context, the high stability constant of the complex Fe-EDDHA could have limited the efficiency of the Fe acquisition process in a grass species like ryegrass (Cesco et al., 2002), thus limiting the beneficial effect of the Fe fertilization with the Fe-EDDHA source in Cu toxicity prevention.

The increased root exudation of total phenols and chelating compounds measured in ryegrass plants fed with high Cu levels (Fig. 4) could be part of a more complex detoxification mechanism operating at the external level of roots. In fact, thanks to the hydroxyl and carboxyl groups of these organic compounds, their exudation promotes the formation of complexes with the free metal ions, reducing thus, in the case of Cu, its ionic free form concentration and, thus, its bioavailability (Seshadri et al., 2015). This phenomenon is more pronounced in the rhizosphere soil (Hinsinger et al., 2009), as also described in black oat plants by De Conti et al. (2018a). As expected, the increased exudation of these phenolics is associated with an enhanced content of these compounds in the root tissues (Figs. 5e and 6). This phenomenon was described also by Kısa et al. (2016) for maize plants exposed to high levels of Cu availability highlighting the pivotal role of these organic molecules in the plant adaptation to the environment conditions. However, their function is not limited to the exudation in the rhizosphere. In fact, these compounds can also take part in the Cu stabilization within the plant tissues (Cu chelation and accumulation in root cells/cell organelles) limiting its translocation to shoots and thus its toxic effects at this level (Michalak, 2006; Cambrollé et al., 2015; De Conti et al., 2018b).

It is well known that organic acids, besides their role at the metabolism level, take part also in the cell homeostasis of metals (Jones, 1998; Dresler et al., 2014) thanks to their cation complexing properties (Jones, 1998). Fig. 5 shows that in our experimental conditions citrate, malate, succinate and fumarate were all detectable in the root tissues. In this respect it is widely demonstrated that several factors, including abiotic stresses, are able to affect the Calvin cycle impacting the quali-quantitative pattern of organic acids accumulated in the plant tissues (Montiel-Rozas et al., 2016). Moreover, the different stability constant of the Cu complexes that can be formed with these organic acids could play a decisive role in the extent of Cu tolerance. In fact, the higher stability constant of the Cu-citrate complex with respect to that of Cu-fumarate (Borges et al., 2005) and the highest citrate content in Fe-EDTA-fed plants exposed to 50 μM Cu probably could be at the basis of a lower Cu accumulation in the shoots of these plants with respect to those of plants fertilized with Fe-EDDHA. It is interesting to note that increased citrate contents in roots of plants exposed to high Cu levels have been also found in young grapevine (Baldi et al., 2018), maize (Dresler et al., 2014) and Imperata condensata (Meier et al., 2012) plants. Although organic acids could not be detected through the adopted technique, most likely because their concentrations were below the detection limit, once exuded these acids are also considered of pivotal relevance in determining the extent of the different soil Cu-fractions in the rhizosphere and, thus, in impacting the transmembrane transport into the root cells of the metal (Montiel-Rozas et al., 2016; De Conti et al., 2019). In fact, it is well known that an enhanced exudation of these organic compounds can consistently promote the solubilization and chelation of chemical elements, thus increasing/decreasing their bioavailability for the root acquisition process (Pii et al., 2015; Marastoni et al., 2019b).

The accumulation and exudation of amino acids is also a protective mechanism that can be triggered by some plant species in adverse environmental conditions. Indeed, they are able to behave

as ligands for elements/nutrient in complexation processes is at the base of this phenomenon (Pii et al., 2015). In addition, their synthesis and accumulation under heavy metal stress conditions can contribute both to the enzyme stabilization and to the energy storage in conditions of limited growth and reduced photosynthesis activity (Zafari et al., 2016). Results here presented show that the contents of cysteine and glycine are consistently modified by a high availability of Cu (Supplement 1 and 2). In particular, being cysteine involved in the synthesis of phenolic compounds for a prompt antioxidant action (Ali et al., 2006), it is reasonable that the enhanced contents of this amino acid in ryegrass plants fed with high levels of Cu are tightly related to the increased root content and to the more pronounced root exudation of phenolic compounds (Figs. 4 and 5). With respect to the increased contents of glycine, its involvement in the synthesis of glutathione and phytochelatins could explain, at least in part, the phenomenon, analogously to what described by Zafari et al. (2016) in Fe toxicity conditions. It is interesting to note that also in Fe-deficiency an increase in glycine content in cucumber plants has been described (Pii et al., 2015). These evidences seem to indicate a direct role of glycine in the adaptive responses to adverse nutritional conditions, irrespectively of toxicity or deficiency. Considering the changes in plant accumulation and root exudation patterns of phenols, organic acids and amino acids in response to the high availability of Cu, it is clear that this adaptation process (regardless of guided or suffered by the plants) should have a metabolic cost for the plant and should have induced a consistent change in the carbon metabolism. For this reason, it can be reasonable to think that the enhanced biosynthesis of these organic compounds could have had a negative impact on the carbon pool to be assigned to the biomass growth of the ryegrass plants (Figs. 4 and 5).

5. Conclusions

In conclusion, results here presented showed that the Fe fertilization based on Fe-EDTA can affect the levels of Cu availability in the rhizosphere via a Cu−Fe exchange with the ligand (i.e. EDTA) and/or via a complexation process of the free fraction EDTA resulting after the acquisition of Fe by the roots. A competition between the two nutrients (Cu and Fe) for the cation transmembrane transporters cannot be excluded, particularly when the availability of Fe is not limited (e.g. after the application of Fe-EDTA). Being the stability of the Fe-complex of pivotal role for its fate in the soil and for its interaction with Cu, this approach does not seem to be appropriate for all types of Fe-fertilizers (i.e. at least for FeEDDHA). Furthermore, the increased synthesis/accumulation at the root level of Cu-complexing compounds (phenols, organic acids, amino acids) as well as their enhanced root exudation in the rhizosphere confirm their crucial role in Cu homeostasis (once inside the cells/plant tissues) and/or impact on the geobiochemical cycle of Cu in the soil/plant interface (in particular with respect to the ionic free fraction of this nutrient), respectively. Thus, ryegrass had the potential to be used in intercropping programs in order to limit the onset of the symptoms of Cu toxicity in vine plants and its negative effects on the yields. It is important to proceed with studies in the topic under the field conditions to support present findings since in soil other soil properties may affect differently Cu and Fe availability and plant roots development. As well as, the dynamics of ryegrass growth during the whole cycle may affect differently the response of the grass to excess of Cu in soil.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125298.

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