Review (tentative title)

Soil Copper accumulation: rhizosphere processes and agronomic practices to limit its toxicity.

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1. Introduction

Among the processed crops, which include all agricultural and food products that are derived from their respective primary commodities, wine is one of the most important in the world (**FAOSTAT, 2012**). The world viticulture regions are generally located in climatic areas that are, however, favourable to the occurrence of fungal diseases, including downy mildew (*Plasmopara viticola*). The regular use of copper (Cu)-based fungicides (e.g., Cu sulfate, Cu oxychloride) to protect grapevine plants from these pests has led to a long-term accumulation of Cu in vineyard soils (particularly in the upper layers), reaching concentrations that are far higher as compared to the trace amounts that are required for healthy plant growth (Pietrzak and McPhail, 2004) and, in some circumstances, even exceeding the limits imposed in the EU for agricultural soils (**Komàrek et al., 2010**). Besides Cu-containing fungicidal and bactericidal sprays, substantial Cu addition to soils can occur from the addition of contaminated waste (**Liu et al., 2007**) and/or mineral- and organo-fertilizers (**Xiaorong et al., 2007**) including organic residues (e.g. pig and poultry manure and organic composts, **Brunetto et al., 2014; Couto et al., 2015**). It should be emphasized that Cu is an essential element for plants and, by definition, it is necessary for an organism to function properly, since it plays key roles in several physiological processes connected to plant growth and development **(Yruela, 2005).** Nonetheless, depending on the concentration and on the bioavailable fraction, it could also exert toxic effects on plants. In particular, when its bioavailable concentration is very high, the growth of plants (**Ambrosini et al., 2015a**) and their productivity could be severely impaired. For instance, in grapevine plants grown in acidic soils, Cu toxicity, besides affecting plants growth and productivity (**Gupta and Aten, 1993**), can also worsen the quality and the nutritional value of the products **(Komàrek et al., 2010, Tanyolaç et al., 2007; Toselli et al., 2009; Cambrollé et al. 2014; Juang et al. 2014**). For this reason, the deeper understanding of Cu dynamics in soil, particularly considering the complexity of rhizosphere processes driving the Cu acquisition mechanisms by plants, could represent a prerequisite for the development of agronomic strategies aimed at limiting soil Cu-availability. These aspects are even more relevant in relation to other common agricultural practices, such as herbicide applications for weed control in vineyards.

Therefore, this review will discuss the main Cu processes occurring at the soil-microbe-root interface (*i.e.* rhizosphere) that could play a role in the tolerance mechanisms adopted by plants when grown in soil characterized by high Cu availability. For this propose, Cu fractions in soils, mechanisms of root Cu uptake and its allocation in shoot as well root

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exudation phenomenon will be discussed in relation to the processes underlying the agronomic practices commonly adopted, also considering the role that herbicides or their residues in the soil could exert. Strategies to improve agronomic practices aimed at mitigating Cu toxic effects will also be discussed.

2. Copper in the rhizosphere

It is well known that the Cu-available levels strongly depend on mineral and organic forms in the soil and on biogeochemical cycles, which the different Cu sources undergo. Moreover, in the rhizosphere these cycles are strongly influenced by the intensive interactions between roots and microorganisms, which, in turn, shape the mineral and organic Cu fractions. In addition, the amount of Cu absorbed by plants depends on both its available concentration in the rhizosphere and the functionality of the protein systems committed to the nutrient acquisition at root level and, ultimately, to the allocation into shoot tissues. Moreover, Cu forms, that can be taken up, are closely dependent on the selectivity of those proteins, which mediate nutrients' transport across the plasma membrane.

Soil Cu forms and their biogeochemical cycles

Copper is a metallic element and its natural concentration in soil depends on its concentration in rocks. The average Cu concentration in soils registered worldwide ranges from 6 to 80 mg kg^{-1}. Soils with a Cu concentration lower than 8 mg kg^{-1} may be cause of the onset of Cu deficiency symptoms in crops **(McBride, 1994)**.

The distribution of Cu between solid phase and soil solution depends on precipitation/dissolution, adsorption/desorption and redox reactions. In the soil solution, Cu may be in free form as Cu^{2+} or, in most of the cases, complexed. The complexation may occur with either inorganic anionic binders or organic molecules. The formation of stable complexes in the soil solution can delay the adsorption of Cu with functional groups on the surface of reactive particles **(Sparks, 2003; Alleoni et al., 2005)**. In the solid phase, Cu can be sorbed through mechanisms such as ion exchange (non-specific adsorption), specific adsorption and complexation with soil organic matter **(Sposito, 1989; Alloway, 1995)**. Non-specific adsorption is weak and unstable. This type of adsorption weakly affects the availability of Cu. However, Cu can retain its internal hydration sphere and a high degree of rotational mobility on smectite exchange sites, showing that electrostatic forces may be involved in adsorption **(McBride, 1979; Alleoni et al., 2005)**. In specific adsorption on functional groups of inorganic particles, the metal partially or completely loses its hydration

water forming a inner sphere complex with either OH^- or a H_2O molecule bound to a metal ion of the crystalline lattice on the surface of Fe, Mn and Al oxides, non-crystallized aluminosilicates and edges of clay minerals **(Alleoni et al., 2005; Bradl, 2010; Ferreira et al., 2014**).

The nature of Cu interactions with soil components has been elucidated in the last years with the use of synchrotron X-ray techniques, especially X-ray absorption spectroscopy (XAS). With an X-ray multi-analytical approach, **Strawn and Baker (2009)** studied five Cu-contaminated soils with different characteristics and pHs (from 5.2 to 7.1). They found that in all soils, Cu exists predominantly as Cu-soluble organic matter complexes (Cu-SOM complexes) in the form of five membered ring chelates, despite differences in soil mineralogy, organic matter (OM) content, Cu sources, contamination date, and whether the soils were contaminated *in situ* or spiked in the laboratory. Therefore, especially in acid and neutral soils, the biogeochemistry of Cu is largely controlled by its interaction with natural OM, not so much because of OM abundance and polyfunctional character, as for its remarkable affinity towards Cu(II) in comparison to other divalent cations **(Manceau and Matynia, 2010)**.

Using xxxxxxxxx (XANES) and xxxxxx (EXAFS) spectroscopy, and Cu–glutamate as the best-fit structural analog, **Karlsson and Skyllberg (2006)** showed that at pH 4.8–6.3 Cu(II) forms a five-membered chelate ring with one amino nitrogen (α-NH_2) or alcohol oxygen (α-OH) and one carboxylate oxygen from an α-substituted aliphatic carboxylic structure. **Manceau and Matynia (2010)**, exploiting XAS spectroscopy along with supporting thermodynamic equilibrium calculations and structural and steric considerations, showed evidence at pH 4.5 and 5.5 for a five-membered Cu(malate)$_2$-like ring chelate at 100–300 ppm Cu concentration, and a six-membered Cu(malonate)$_{1–2}$-like ring chelate at higher concentration. In addition, malate- and malonate-type structures are present only on aliphatic chains.

Because of the high affinity of Cu for soil colloids and especially SOM, Cu is rated a low-mobile element in near-neutral soils. For this reason, farmers have been able to apply large amounts of Cu salts to organic soils over time without, in general, causing toxicity to crops **(McBride 1994).**

In more alkaline soils, while free Cu^{2+} solubility is exceedingly low, soluble complexes of Cu (hydroxy-, carbonate-, and OM-complexes) forms increase the total soil fraction of soluble Cu. Consequently, mobility of this nutrient may be rather high in alkaline conditions **(McBride 1994).** To date, there are not detailed studies using XAS on Cu speciation in

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alkaline soils with a low content of OM, like those that can be often found in arid and semi-arid regions. A survey on 21 calcareous agricultural soils from Western Iran with a pH ranging from 6.8 to 7.9 showed that, on average, about 56% of Cu remained in the residual fraction after sequential extractions while about 20% was associated to SOM (**Khanlari and Jalali 2008**). Similarly, in a vineyard calcareous soil with pH 7.8, it has been observed that natural Cu is preferentially retained in the residual fraction, which is stable or unavailable (**Herrero-Hernández et al., 2011**). It is likely that (hydro)xides or hydroxy-carbonates Cu-forms may be the most abundant sources of Cu in these conditions (**McBride 1994).** Moreover, **Fernández-Calviño et al. (2009)** investigated soils from 170 vineyards in Spain treated for long time with Cu-based fungicides and possessing a pH between 4.9 and 6.6. From this survey they found that Cu was present mainly in less mobile fractions, with about 48% bound to soil organic matter, 15% associated to amorphous inorganic materials, 12% to crystalline Fe and Al oxides, and 23% as residual.

These pieces of information clearly show that Cu availability in soil depends on the nature of the binder (particularly OM, Fe, Al, and Mn minerals and (hydro)oxides) and their relative levels as well as on carbonates, soil pH and cation exchange capacity (CEC) (**Fernández-Calviño et al., 2010; Couto et al., 2014; Brunetto et al., 2014**). Copper adsorption in soil occurs primarily in the most avid binding sites, and the remaining elements are redistributed into fractions that are held together with less energy and that consequently exhibit a higher availability and mobility in the soil profile.

Root exudation process and its impact in Cu availability

The rhizosphere is characterized by radial and longitudinal fluxes and gradients of both organic and inorganic compounds that shape rhizosphere processes **(Mimmo et al., 2014).** These latter, in turn, are able to influence considerably the transformations and flows of nutrients from soil to plant. For these reasons, they are considered to be the bottleneck of nutrient mobilization in soil and subsequent acquisition by plants and, therefore, of crop yield. On the other hand, the ability of such processes to mobilize nutrients from insoluble sources in the soil might also be reflected in increased bioavailable fractions of toxic elements and/or of those nutrients capable to exert toxic effects on plants when occurring in a very high availability **(Valentinuzzi et al., 2015).** Rhizosphere processes and effects on plants are governed mainly by the release of root exudates, which comprise both low- and high-molecular weight inorganic and organic compounds, as for instance protons, carbohydrates, organic acids, amino acids, phytosiderophores (PS), phenolic compounds and

enzymes **(Dakora and Phillips, 2002).** Root exudates are fundamental for the induction of changes in the chemical, physical and biological characteristics of the rhizosphere, and are involved in paramount pedogenic and rhizosphere processes affecting functions as 1) the modulation of elements bioavailability in the rhizosphere (Fe, P, Zn; **Dakora and Phillips, 2002),** 2) the root protection against toxic metals (Al, Zn, Cd; **Jones, 1998)** and 3) the shaping of the rhizosphere microbiome **(Pii et al., 2015; Pii et al., 2016).**

Indeed, root exudates, especially those with a low molecular weight, can be used by microorganisms as easily accessible source of carbon on the rhizosphere, where the concentration of such compounds is usually higher as compared to bulk soil **(Hinsinger et al., 2009).** In addition, root exudates can also constitute powerful chemoattractors, stimulating the colonization of the rhizosphere by selected microbial strains, which might display plant growth-promoting traits. These properties are very often related to the ability of bacteria of either producing hormone-like compounds, which increase the growth of the root system, or protecting the host against pathogens attacks or enhancing the bioavailability of mineral macro- and micro-nutrients (e.g. N, P and Fe) **(Pii et al., 2015).**

With respect to the biogeochemical cycles of nutrients in soil, it is interesting to note that the bioavailable fractions of Cu are considerably tailored by the biological activities of root, *i.e.* release of exudates and in particular of low-molecular-weight organic compounds. This release is strictly dependent on plant species and environmental conditions, both in terms of concentration and quality **(Jones, 1998; Mimmo et al., 2011)**, and this phenomenon has been mainly studied for its potentialities to modulate microbial growth, to mobilize insoluble nutrients (e.g. P, Fe, Zn) and to detoxify harmful heavy metals **(Dakora and Phillips, 2002; Weisskopf et al., 2006; Pii et al., 2015).** In this latter case, the exudates, mainly organic and phenolic acids, are thought to chelate heavy metals either in the rhizosphere or in the apoplast, preventing the movement across the membranes to the root symplasts **(Kochian et al., 2004).** Therefore, the qualitative and quantitative pattern of root exudates may to play a fundamental role in alleviating Cu toxicity in plants. Low-molecular-weight organic compounds might be involved in both external and internal tolerance mechanisms. In fact, so-called excluder plants prevent the bioaccumulation of heavy metals in plant tissues by either blocking the uptake in the roots **(Lux et al., 2011)** or by active efflux pumps **(Van Hoof et al., 2001)**. Root exudates released by excluder 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. These plant species might thereby also act as phytostabilizator plants in contaminated soils **(Lorestani et al., 2013)**. On the other hand,

hyperaccumulator plants adopt an internal mechanism and can accumulate high concentrations of heavy metals in their shoot tissues (up to several per cent of the dry mass of their above-ground parts) without suffering any stresses **(Clemens, 2001).** In these species, the release of organic ligands may contribute to an enhanced mobility and replenishment of metals in the rhizosphere **(Wenzel et al., 2003)**.

For instance, several studies have highlighted that citrate, oxalate and malate are the most common and effective organic acids in binding metals and metalloids, such as arsenic, chromium, cadmium and lead **(Jones and Darrah, 1994; Magdziak et al., 2011).** On the other hand, very little research has been carried out on the role of organic acid in alleviating Cu stress in plants. Early evidences showed that oxalate was exuded by coniferous trees *Pinus* and *Picea* as response to Cu stress **(Ahonen-jonnarth et al., 2000; Heim et al., 2001).** More recently, **Qin et al. (2007)** observed that, beside oxalate, poplar trees exuded formate and malate in a concentration that is directly dependent with the concentration of the Cu bioavailable fraction. In addition, it was also observed that the exudation of organic anions was accompanied by a release of inorganic cations (i.e. K^+, Ca^{2+} and Mg^{2+}) **(Qin et al., 2007).** Research carried out on metallophytes, namely *Oenothera picensis* and *Imperata condensata*, grown in culture media supplemented with toxic concentration of Cu, revealed that in these cases citrate and oxalate were predominantly released **(Meier et al., 2012)**. In addition, a large amount of succinic acid was also detected in the root exudates, suggesting that this could be an unspecific response towards Cu-induced stress, since the complex Cu-succinate has a much lower stability constant as compared to those of Cu-citrate and Cu-oxalate **(Borges et al., 2005).** Yet, the metallophytes were also shown to respond towards an excess of Cu by releasing phenolic compounds (*i.e.* cinnamic acid, coumaric acid and catechin), which can stabilize the heavy metal with high affinity **(Meier et al., 2012).** Furthermore, a strong release of tartaric acid as well as the amino acids valine and serine has been described form the Cu-tolerant species *Ricinus communis* L. **(Huang et al., 2015).** On the contrary, **Zhang et al., (1989)** discovered in Cu-deficient grasses an enhanced PS production and release, although up to date it is still uncertain whether PS release is a specific response to Cu deficiency or a consequence of a series of internal events linked to an impairment of Fe utilization that was observed in these plants **(Chaignon et al., 2002).**

However, most of these studies describing the different qualitative and quantitative patterns of root exudates triggered by Cu toxicity were carried out in hydroponic solutions, whilst it has been showed that the exudation processes might be considerably different in soil-grown plants **(Oburger et al., 2013).** Hence, future researches should focus on field-like conditions

bridging micro- and macro-scale studies considering also other aspects that are generally present in the field scale as the herbicides and/or their residues. In fact, it has already been shown that herbicides application can alter the profile of exudates released by roots in the rhizosphere **(Kremer et al., 2005**, **Damin, 2005; Mandersched 2005)** affecting considerably, thus, the capacity of roots to shape the rhizosphere. However, despite these evidences, a limited number of studies aiming at investigating the effect of herbicides at this level have been carried out. Therefore it is still an open question how this class of agrochemicals, and particularly those used in post-emergence or characterized by a long persistence in soils, can modify the characteristics of the rhizosphere soil and then interfere with Cu-acquisition process particularly in conditions where, for anthropic actions, a long-term accumulation of Cu in the upper layers occurred.

Copper uptake and translocation in plants

a) Mechanisms in relation to the Cu sources

As also stated before, the mechanisms of Cu trans-membrane transport in roots are the access path for the flow of the nutrient from the soil solution to the plant tissue in either growth conditions, low, equilibrate or excessive availability. However, the selectivity of these mechanisms, at least at some levels, can restrict the number of Cu forms that can be used by roots as Cu sources. Analyzing this aspect from the opposite point of view, it is possible to apprehend that there are therefore Cu forms in soil solution which cannot be used by these proteins and therefore they do not represent a risk when the crops are grown in conditions of very high abundance of these plant-unavailable Cu forms. For this reason, it is easy to comprehend that the understanding of these mechanisms of Cu acquisition and, in particular, of their functional characteristics can play a relevant role in order to define agronomic practices aimed at limiting the toxic effect of Cu. This aspect is particularly relevant when vineyard soils exposed for a long period to a regular deposition of Cu-based fungicides or of plant tissues treated with these agrochemicals, are considered. Similarly, mechanisms of Cu homeostasis within tissues and within cells can be relevant for the detoxification of Cu, once taken up by the plants.

The mechanisms of Cu transport in roots (micronutrient uptake) have been characterized in the last two decades **(Wintz et al., 2003; Sancenon et al., 2003)**. In particular, Cu transport protein 1 (COPT1) is thought to mediate most of the Cu uptake into cells, whereas other members of this protein family may mediate intracellular transport of Cu **(Sancenon et al., 2003).** However, other transporters seem to be involved in this process, as for instance

members of the Zn/Fe permeases (ZIPs) family, ZIP2 and ZIP4, have been shown to function as Cu-transporters in *Arabidopsis thaliana* **(Wintz et al., 2003)**; yet, it is well established that the main Fe transporter (IRT1) can also mediate the uptake of Cu, Cd, Co and Zn **(Korshunova et al., 1999)**. Moreover, Cu-PS might be taken up by grass roots, even though with a much lower affinity than Fe-PS, via yellow stripe-like (YSL) proteins **(Marchner, 1995; Wintz et al., 2003)**. This family of proteins seems to be also involved in the transport of Cu-nicotianamine **(Wintz et al., 2003)**, which is presumably involved in the translocation of this micronutrient (allocation to the shoot).

Other proteins has been shown to be involved in the transport of Cu across membranes; some P1B-type ATPase (HMA) transporters can transport either Cu^{2+} or Cu^{+} **(Zimmermann et al., 2009)** playing a role mostly in the Cu-allocation into different organelles or translocation into other tissues **(DiDonato et al., 2004).** 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. In fact, Cu has a high affinity for peptide and sulfhydryl groups, as well as for carboxylic and phenolic groups. Therefore, in cells and in the xylem sap, almost 100% of the Cu is present in complexed/bound form and this high percentage of binding is also retained under conditions of excessive Cu supply **(Graham, 1979, White et al., 1981b; Pich and Scholz, 1996; Liao et al., 2000b; Liao et al., 2000a)**. 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, 1995)**.

On the basis of these studies it appears evident that in soil solution, where more than 98-99% of the Cu is present in complexed form **(Hodgson et al., 1966, Marschner, 2012),** the Cu sources usable by roots for the nutrient acquisition are mainly the ionic one (Cu^{2+} and/or Cu^{+}) and, limited to the grasses also the complexed form with PS, although it is still not clear the relative contribution between the different forms. In this latter group of plants, the utilization of two Cu forms (ionic and complexed with PS) could coexist, as documented for Zn (**VonWirén, 1996**). However, up to date the possibility that roots can absorb also other Cu sources (e.g. soluble Cu-complexes with low-molecular-weight organic molecules) could not be totally excluded, considering also the limited literature available on this aspect. Nonetheless, if this phenomenon happened, the idea is that its role in the root acquisition process of Cu would be rather limited. Among the different fractions of Cu in the soil, the soluble one is generally defined as being available, thus it is clear that this definition is not completely appropriate. In fact, it overestimates the Cu forms actually usable by the plants

and includes fractions (e.g. complexes with organic compounds of low-molecular-weight such as organic acids, amino acids, etc.) that could instead be the result of the operation of strategies aimed at excluding and/or limiting the Cu uptake process at the root level in Cu excess.

b) Herbicides effect on Cu uptake mechanisms and translocation

It is widely known that herbicides are commonly used in agricultural practices, in order to limit the competition for nutrients, water and light between weeds and crops. Such weeds control is often applied also in orchards, including vineyards. However, the interference of herbicides (or of their residues) in the functionality of nutrient uptake mechanisms in non-target plants (crops) has been recently demonstrated. A very clear example is the impairment on Fe-acquisition mechanism determined by *glyphosate* in soybean (**Bellaloui et al., 2009**) and in barley by *triazine* (**Del Buono et al. 2015**). In the first case, the impairment is on the root trans-membrane protein underlying the Fe^{III}-chelate reduction before its transporter-dependent flux across the membrane, while in the second the effect appear to be more focused on sulfur reductive assimilation pathway, prerequisite for the synthesis and release of PS. A similar depressive effect of *glyphosate* described for Fe in soybean plants has been shown by **Eker et al. (2009)** in sunflower plants, where Mn acquisition was impaired by the presence of this agrochemical.

With respect to Cu, it has been demonstrated that *chlorsulfuron* (a *sulfonylurea* used in pre- and post-emergence stages) depressed the Cu concentration in non-target wheat plants (**Tang and Robson, 2000**). This effect has been ascribed to an impact in the root capacity to take up Cu (**Robson and Snowball, 1990**) or, more in general, to an impairment in the root functionality as a consequence of the negative effect on the growth and the geometry of this tissue (**Dong et al., 1995**). A similar effect was also recorded in non-target *Triticum aestivum* and *T. rigidum* plants treated with *chlorsulfuron* with an impact not restricted only to Cu but also to Zn and Mn acquisition (**Rengel and Wheal, 1997**).

In this general context, an effect of herbicides on the trans-membrane electrochemical gradient governing the movements of solutes (including nutrients) across the membranes could be of relevance in the nutrient acquisition by roots. In this respect, **Shimabukuro and Hoffer (1994)** found in non-target oat plants that the herbicide *diclofop-methyl*, after the hydrolysis to its acidic active form, caused a membrane depolarization *via* an enhanced plasma membrane permeability to protons. Also other herbicides (*dictofop*, *hydroxydiclofop*, *CGA 82725*, *haloxyfop-methyl*, *haloxyfop*, *bentazon*, *dinoseb*, *4-hydroxy CIPC*, and *2-*

hydroxy CIPC) exhibited this mode of interference in oat coleoptiles (**Wright, 1994**). However, studies showed that *glyphosate* and *atrazine* applied as foliar sprays induced a higher Cu leaf accumulation than in untreated plants (**Correia and dos Santos, 2013**). This experience clearly indicates that herbicides might also influence the nutrients' allocation at the leaf level and/or involved in their homeostasis.

Cu toxicity in plants and soil microorganisms; effects and response strategies

As stated before, although Cu is an essential nutrient both in plants and in bacterial cells, when its concentration is too high, a series of toxic phenomena occurs and strategies aimed at limiting the harmful effects are adopted in both these organisms.

For what concerns the plants, root apices, the first target of this nutritional disorder in the soil, generally become shorter and thicker for changes in the cell wall formation (**cit**). Moreover, the number of lateral roots is often increased with also an evident plasmolysis of some epidermis cells. Taken together, these phenomena are able to cause a clear contraction of the root density **(Juang et al., 2012; 2014; Chen et al., 2013; Zhang et al., 2014)** which, in turn, has an impact in the root capacity to acquire nutrients and water; in these conditions also the root capability to take up other nutrients is impaired inducing, for example, symptoms of deficiency (**Cit**). Considering in particular the Cu acquisition process, it is interesting to note that living organisms have developed a complex homeostatic system to regulate the acquisition and use of Cu **(Sancenon et al., 2003).** In excess of Cu, the Cu uptake is downregulated and the synthesis of compounds, such as metallothioneins or phytochelatins, is induced **(Mukherjee et al., 2006)**. Both these polypeptides are able to bind Cu in order to reduce its availability in the cytosol and thus to alleviate its toxic effects. The compartmentalization of heavy metals in the apoplast of roots or in organelles such as the vacuole **(Chaignon and Hinsinger, 2003)** is also used for this purpose.

At the rhizosphere level, roots can modify the pH value of the external soil environment as a consequence of a preferential uptake of cations or anions. When anions prevail (as after nitrate fertilization), there is an increase in pH values favoring, for example, Cu-complexing processes. As a consequence, the proportion of free-metal species of Cu in the solution is restricted and, then, also its toxicity to plants is reduced **(Brimecombe et al., 2001).** Also the organic acids (such as citric acid, malic acid, fumaric acid, oxalic acids, among others) released by roots, as described also for vine plants **(Covarrubias et al., 2012),** or derived from OM decomposition, promoted by soil microorganisms **(Oburger et al., 2009; Pii et al., 2015**), can bind the Cu thanks to their high affinity binding with heavy metals **(Jones &**

Darrah, 1994; De conti et al., 2016). This latter phenomenon, together with the rhizosphere alkalinisation, is able to alter the Cu solubility (modifying the distribution of Cu chemical species) reducing the toxic potential of the heavy metal to crops **(Meier et al., 2012; Pérez-Esteban et al., 2014)**. However, when the availability of Cu is even higher and the strategies adopted by the plants are unable to enough control Cu availability, this disorder is able also to cause tissue necrosis and, if the stress is prolonged, plant death **(Marschner, 1995; Toselli et al., 2009; Miotto et al., 2014)**.

At the leaf level, this nutritional disorder causes a decrease in the pigment concentration with a subsequent drop of the photosynthetic rate (**Cambrollé et al., 2013; 2015**). A typical leaf symptom of this toxicity is the interveinal foliar chlorosis, partially ascribed to the negative effect on Fe acquisition exerted by the excess Cu (**Yau et al., 1991; Ouzounidou, 1995**) As a consequence, plants subjected to Cu toxicity are generally smaller with also an accumulation of carbohydrates and starch at the leaf level for the lower demand of energy for the biomass production (**Alaoui-Sosse et al., 2004**). Furthermore, the high levels of Cu in these tissues induce oxidative stress as a consequence of an increased concentration of reactive oxygen species (ROS). Among the damages caused by ROS, peroxidation of membrane lipids is one of the worst for cell survival **(Apel and Hirt, 2004; Yruela, 2005; Miotto et al., 2014**). Moreover, in addition to its capability to bind to proteins and amino acids, Cu can also replace other metals from their natural binding sites, which increase Cu toxicity **(Halliwell and Gutteridge, 1990)**. As described before, also at the leaf level the synthesis of metallothioneins or phytochelatins is induced by the plant exposure to Cu excess **(Mukherjee et al., 2006)**. It is interesting to note that under low Cu availability, intracellular Cu distribution is performed by metallochaperones, which are soluble Cu-binding proteins (for a review see **Huffman and O'Halloran, 2001**; **Puig and Thiele, 2002)**.

A further aspect that could be of interest in relation to the plant responses to the excess of Cu is its interaction with other nutrients/elements, which may involve complex responses at the metabolic level. In this respect, it is well demonstrated that Fe acquisition in maize **(Astolfi et al., 2003),** barley **(Astolfi et al., 2006)**, tomato **(Zuchi et al., 2009)** and wheat **(Zuchi et al., 2012)** plants and of nitrate in spinach and cucumber plants (**Prosser et al., 2001 J Exp Bot 52:113–121; Nikolic et al., l2007 Funct Plant Bio**) as well as the response to Cd toxicity in barley (**Astolfi et al. 2012**) are all linked to sulfur (S) availability. It should be remembered that plants require a relevant consume of thiols (and mainly glutathione) to overcome the oxidative stress (**Foyer and Noctor, 2011**; **Del Buono et al., 2011**), thus also the exposure to Cu toxicity could result in higher S nutritional requirement in these plants. As a consequence,

an inadequate S availability could impair the plant capability not only to cope with the excess of Cu, but also to acquire other nutrients. This aspect is even more serious considering that, over recent years, decrease of atmospheric SO_2 emissions and lower S supply through mineral fertilisation have led to the increase occurrence of S deficiency in many agricultural soils and physiological responses of plants to S deficiency have been recorded (**McGrath and Zhao 1995, McGrath et al., 2002**). In this context and to make it even more complicated there is the evidence that also herbicide detoxification requires S, being the conjugating of these molecules with glutathione one of the main steps for the detoxification of the herbicide itself (**Del Buono & Ioli, 2011**). Moreover, it has been also shown that herbicides can have negative effects on Fe-acquisition processes exacerbating the problems of Fe deficiency (for review see **Mimmo et al., 2014**). All these evidences taken together suggest that the crop response to Cu toxicity, at least for the large request of S needed for coping with the stress, can interfere both with the acquisition processes of nutrients (with deleterious impacts for yield and its quality), and with the crop capacity to detoxify herbicides, which are commonly used in order to optimize crop productivity.

With respect to soil microorganisms, it is well demonstrated that heavy metal ions, when present above a given threshold level, can form nonspecific complexes within the cells thereby exerting toxic effects **(Dupont et al., 2011)**. In both *Escherichia coli* and *Bacillus subtilis*, the excess of Cu have been shown to cause an increased uptake of Fe and S due to a decreased stability of Fe-S clusters during biogenesis or when bound to their target proteins **(Chillappagari et al., 2010; Macomber and Imlay, 2009)**. For instance, several enzymes containing Fe-S clusters involved in the biosynthesis of branched amino acids, in the cycle of tricarboxylic acids and in the pentose phosphate pathway resulted strongly impaired in their functionality by high levels of Cu in both *E. coli* and *B. subtilis* **(Macomber and Imlay, 2009).** Since these enzymes play pivotal roles in the cell metabolism, Cu concentration in the cytosol is kept to minimum values through the activity of (Cu-extruding) P-type ATPase which allows only extremely low concentrations of free Cu within the cells **(Changela et al., 2003).** In aerobic conditions, Cu catalyzes reactions that result in the production of hydroxyl radicals through the Fenton and Haber–Weiss reactions **(Halliwell and Gutteridge, 1984).** These highly reactive ROS thereby formed are responsible for lipid peroxidation, oxidation of proteins and damage to nucleic acids **(Halliwell and Gutteridge, 1990; Stadtman, 1992).** The majority of bacteria circumvent these issues by targeting Cu-containing enzymes towards the periplasm or exposing them onto the outer face of the plasma membrane **(Dupont et al., 2011),** thus adopting an exclusion mechanism.

Taken together, these pieces of evidence point out that the presence of high Cu availability can consistently affect soil microbial population having then, as a consequence, also an impact in the soil biogeochemical cycles of the other essential elements **(Mackie et al., 2013).**

3. Agronomic practices impacting the rhizosphere and aimed at limiting Cu toxicity

It is evident that, for fields where anthropic events have caused Cu accumulation in the upper layers, the most appropriate agronomic practices in the short- and long-term are those based on protecting plants against pathogens using alternative approaches to those Cu-based (where and when it is possible) and/or limiting the distribution in soils of Cu-containing amendments/products. In this way, it will be possible to limit the continuous supply of Cu to soil. However, where the problem of Cu toxicity is already present and must be faced anyhow, the only alternative available for farmers in order to ensure crop yield, otherwise diminished considerably and worsened in its quality (**Tanyolaç et al., 2007; Toselli et al., 2009; Cambrollé et al. 2014; Juang et al. 2014**), is the application of agricultural practices aimed at restricting the plant-available Cu fractions in the soil. To this purpose, it is possible to use organic and inorganic substances such as nitric oxide, limestone, phosphates, clays, zeolites, iron oxides, OM, and biochar (**Terzano et al., 2005a; Kumpiene et al., 2008; Fan et al., 2011; Arunakumara et al., 2013**). Their use is based on the capability to induce various sorption processes (adsorption to mineral surfaces, formation of stable complexes with organic ligands, surface precipitation, and ion exchange) limiting the Cu bioavailability. Precipitation as salts and co-precipitation can also contribute to reducing contaminant mobility, a serious issue; in fact, Cu leaching can be a further problem of these soils and, for this reason, it should be limited or avoided. In general, soil amendments (as adding OM, lime, phosphates, etc.) are not a novelty, and they have been used for many years both to limit the Cu phytotoxicity and to improve crop growth and yield **(Bolan et al., 2003).** In the following chapters, lime and OM applications are discussed in relation to the most recent literature. In addition, the possibility to inoculate crops with arbuscular mycorrhizal fungi (AMF) and bacteria strains, to adopt different systems of cultivation and plans of fertilization, are also discussed.

Lime application to soil

This practice, promoting the alkalinisation of soil solution and, thus, inducing the de-protonation of functional groups (quali?), increases the soil cation exchange capacity (CEC) and, concurrently, enhances the adsorption of Cu (**Joris et al., 2012**). In addition, the CO_3^{2-} and OH^- species, derived from limestone dissociation, can react with Cu forming Cu-hydroxides and/or Cu-carbonates, which subsequently precipitate. So, by means of these two phenomena, it is possible to achieve a significant decrease of the free-Cu activity in soil solution. A further advantage of soil lime application is the increase of Ca and Mg in the soil. While at the root level the increase of Ca limits the anatomy changes caused by Cu (**Chen et al., 2013**), the enhanced content of Mg in shoots guarantees the maintenance of the photosynthetic activity limiting the replacement of Mg in chlorophyll molecules by Cu (**Yruela, 2009**). Moreover, both cations, favoring a site-specific competition for ions, could prevent the Cu excess in cell (**Arunakumara et al., 2013**). More in general, the lime application to acidic soils, alkalinizing the pH, can favor phosphorus availability for plants. In this case, the interaction of Cu with phosphorous can reduce the mobility of the metal and then Cu availability for roots (**Smith and Smith, 2008**). However, it is important to note that this practice should be used carefully for its effect on soil pH: in fact, Cu mobility is actually the lowest at slightly alkaline pH but, due to the formation of OH^- complexes, it could definitely be higher at more alkaline conditions (>10) (**van der Sloot et al., 1997**). In this respect, an increased Cu mobility may represent a risk for the environment due to the possibility of Cu leaching along the soil profile. It is interesting to note that by-products like dolomitic residue and, to a lesser extent, gypsum and phosphogypsum are valid alternative for limiting the mobility and availability of Cu in soil (**Garrido et al., 2005**). In general, lime and organic amendments (described in the following chapter), are the most used to decrease Cu availability in agricultural soils (**Pietrzak and Uren, 2011**).

OM, Biochar and coal fly ash applications

It is well demonstrated that the mobility and availability of Cu can be consistently limited by OM applications (**Bes and Mench, 2008; Khan et al., 2015; Shaheen et al., 2015**), mainly for the high affinity for Cu of its acidic functional groups. However, for the coexistence in OM of compounds with different molecular weight and water solubility, there may be two distinct phenomena: 1) high molecular weight and insoluble fractions of OM can retain significant concentrations of Cu, restricting the fraction of Cu in soil solution (**Chirenje and Ma, 1999**); 2) in the presence of low and soluble compounds (dissolved OM), the Cu complexes formed could maintain the solubility. In this latter case, the Cu mobility could also

increase (**Hsu and Lo, 2000**) favoring leaching of the metal. In this respect, Ruttens et al. (**2006**) showed that Cu leaching can increase up to 30 times as a consequence of organic compost distribution. These evidences highlight that, in the management of this agronomic practice, particular attention should be paid in order to not transform a potential risk (Cu not soluble and not mobile) in a real risk (Cu with higher mobility) deteriorating in this way the already altered soil conditions. In this respect, an advantage could derive from the utilization of biochar, which has been considered for heavy metals immobilization *via* complexing the metal on its surfaces (**Beesley et al. 2011**). **Uchimiya et al. (2011**) showed that CEC was the primary mechanism by which biochar enhanced Cu retention, differently, in a clay-rich, alkaline soil with higher intrinsic Cu retention capability, the sorption to mineral (ash) components of the char assisted the retention (precipitation). A further efficient measure controlling Cu mobility is the application of treated coal fly ash to soil with the formation of zeolites or aluminosilicatic geopolymers (**Terzano et al., 2005b**). Mixing sources of OM with fly ash can be of benefit for offsetting a decrease in soil pH due to the decomposition of OM (**Jackson and Miller, 2000**). The application of fly ash-stabilized sewage sludge also reduced Cu leaching and availability to crops (**Su and Wong, 2004**). To this purpose, by-products like sugar foam can also be used (**Garrido et al., 2005**) .

AMF inoculation

The mutualistic association between arbuscular mycorrhizal fungi (AMF) and plant roots can also be a way of minimizing the toxic effects of Cu in plants grown in soil contaminated with the metal. For example, in vineyards with a long history of foliar application with Cu-based fungicides, AMF species, in order to ensure their own survival and also of plants to which they are associated, have strategies to tolerate high Cu contents in the soil, such as release of organic substances (e.g. glomalin) to the soil, which may form complexes with Cu, thus restricting its bioavailability **(Bedini et al., 2010; Ambrosini et al., 2015b).** Additionally, AMF can store Cu in cellular compartments such as vesicles and spores, where the metabolic rate is reduced; in this way the toxic effects on plant metabolism can be reduced with benefits for the plant growth and hence for themselves **(Cornejo et al., 2013).** More in general, the colonization of the root cortex of higher plants by AMF provides an increased interface between roots and soil, functioning as an exchange site of nutrients between the plant and AMF. Thus, nutrients with low mobility in soil, such as P, can be absorbed by roots in greater quantity with a positive impact in the nutritional status of plants **(Ambrosini et al., 2015b)**.

The positive advantages of this root colonization support the hypothesis of its really exploitation at the field level as a promising agricultural practice to guarantee crop yield.

Bacteria inoculation

Beside AMF, also bacteria can be used for the limitation of Cu-induced toxicity. In general, the mechanisms used by microorganisms to detoxify heavy metals include the active transport (through the activity of efflux pumps), intra- and extracellular sequestration and transformation to other less toxic compounds *via* enzymatic reactions **(Colin et al., 2012)**. Studies have indicated that the interactions between bacteria and plant roots can greatly enhance the ability of plants to take up metals **(Dary et al., 2010; Glick, 2010; He et al., 2009. 2010).** *Elsholtzia splendens* inoculated bacterial strain MS2 had for instance significantly greater concentrations of Cu in shoots and roots than un-inoculated plants **(Chen et al., 2005)**. Furthermore, microorganisms can also increase the tolerance of metals in plants, which can occur by a) transformation of the metals into less toxic forms **(Carlot et al., 2002)**, b) reduction of metal uptake by plants **(Vivas et al., 2006)** and/or c) reduction of ethylene-dependent detrimental stress caused by heavy metal, without affecting their uptake **(Rajkumar and Freitas, 2008).** For instance, the Cu-resistant actinobacteria *Amycolatopsis tucumanensis* was able to alleviate Cu toxicity symptoms in the indicator species *Zea mays* **(Albarracín et al., 2010).** *Amycolatopsis tucumanensis* has been shown to accumulate high amount of Cu in a produced exopolymer **(Albarracín et al., 2008)** that is mainly stored within the cell **(Albarracín et al., 2008).** Such phenotype of Cu-bioaccumulation is related to the expression of a Cu P-Type ATPase, also found in other resistant microorganisms **(Solioz and Vulpe, 1996).** Similarly, the co-inoculation of *Vicia faba* grown under Cu-stress with *Rhizobium* and a mixture of PGPR, namely *Enterobacter clocae* and *Pseudomonas* sp. determined an increase in the plant biomass production and a strong reduction in Cu taken up by plants, demonstrating that Cu-accumulating PGPR can alleviate Cu stress **(Fatnassi et al., 2015)**. Nonetheless, beside the ability of several bacterial strains to hyper-accumulate and/or sequestrate Cu, many microorganisms inhabiting the rhizosphere might release metal-chelating molecules, such as siderophores (MS), in the close proximity of the root **(Dimkpa et al., 2009).** These MS can, on one hand, influence the plant uptake of various metals, as for instance Fe, Zn and Cu **(Carrillo-Castañeda et al., 2005; Carrillo Castañeda et al., 2002; Egamberdiyeva, 2007)** whilst, on the other hand, they can most likely chelate and reduce the toxic metal concentration in the rhizosphere, exerting a bioprotection effect. Indeed, the

ability of some bacteria to protect plant against either Ni, Pb or Zn toxicity has previously been shown related to the production of MS (Burd et al., 2000; Dimkpa et al., 2008).

In addition, the majority of the environmental stresses result in the production of inhibitory levels of "stress ethylene" in plants **(Abeles et al., 1992)**. Several PGPR can express the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase **(Honma and Shimomura, 1978; Blaha et al., 2006; Glick et al., 2007)**, which interferes with the biosynthesis of ethylene. In this way, PGPR can prevent plant ethylene levels from becoming growth inhibitory **(Glick, 1995; Burd et al., 1998)**. Several examples of bacteria ameliorating heavy metal stress in plants by using this strategy have already been reported for Ni, Cd and Cu **(Burd et al., 1998, 2000; Nie et al., 2002; Reed and Glick, 2005; Reed et al., 2005; Safronova et al., 2006; Cheng et al., 2009).**

Also in this case there are positive experiences supporting the idea that bacterial inoculation could be a promising strategy to cope with the excess of Cu in the soil.

Intercropping strategy

It has been clearly demonstrated in Fe-deficiency that dicots, as for instance citrus and peanut plants, when intercropped with cover crops, can benefit of the PS released by the roots of grasses with an improvement in the acquisition of Fe (**Cesco et al., 2006; Xiong et al., 2013**). In this regard, it is interesting the finding made in large areas of Brazil where vine plants were co-cultivated with grasses. The main reason of this choice was related to the soil conservation and/or to protect from soil erosion. However, in this particular crop system vine plants, when grown in Cu-contaminated soils, do not exhibit any symptoms at all or much lower ones (**cit**). This evidence seems to indicate that also in this case, where Cu toxicity is the critical point, a beneficial interaction in the intermingling root occurred. At the moment the phenomenon is still not clarified, however it could be hypothesized that the presence in the same volume of soil (rhizosphere) of grasses' roots and those of the other crop species (in this specific case vine plants) could reduce the magnitude of the available fraction of Cu *via* different mechanisms as i) immobilization of Cu in the root apoplast of grasses, ii) competition in its uptake (**Terzano et al., 2014**) and accumulation and compartmentalization of the metal at the shoot level, iii) phytostabilization as a consequence of the release of Cu-complexing exudates. A deeper understanding of this phenomenon is a perquisite for a better exploitation of the potential aspects and advantages of this practice. In this regard, it should be noted that the co-presence of two plant species in the same field, while on one side allows to limit the impact of excess Cu (although *via* processes yet to be clarified), on the other side

this cropping system requires higher availability of water and nutrients to limit the onset of nutrient competitions between the two plant species. This aspect is of considerable relevance in particular in relation to the nitrogen nutrition and the role of amino acid metabolism in the levels of aromas in fruits (**cit**), aspect of paramount importance for example for the grapevine (**cit**). Moreover, an adequate supply of nitrogen is essential also for grasses in order to ensure an adequate production and release of PS (and organic acids) in the rhizosphere (**Aciksoz et al., 2011 doi:10.1111/j.1399-3054.2011.01460.x**), prerequisite to restrict the fraction of available Cu in soil solution and to make this crop practice really effective for the purpose.

Fertilization

Fertilization is a very old practice aimed at maintaining the adequate levels of soil fertility at the beginning of a new crop cycle and specifically at satisfying the needs of the just-planted crop. Moreover, with respect to the nutrients considered in the classic plans of fertilizations, farmers have the possibility to use, for the same nutrient, different forms and sources. This is an important aspect also in relation to the excess of Cu; in fact, different sources/forms of fertilizers can have a totally different impact in the rhizosphere and, then, with respect to Cu, in its levels of toxicity. An example can be nitrogen fertilization: when planned with NO_3^-, an alkalinization of the soil surrounding the roots occurs as a consequence of the nitrate acquistion by roots (**cit**); this effect can limit, for what described in the previous chapter, the availability of Cu. This aspect is particularly relevant in the case of acidic soils.

A further aspect of relevance is the nutritional status of crops. In fact, it is well known that Fe deficiency induces in several dicots (like also rootstocks of grafted vine plants) an intense acidification of the rhizosphere (**Zocchi**); this action is ascribed to the roots effort to mobilize the nutrient from its barely available forms in the soil (**Marschner, 2012**). This phenomenon occurs often in calcareous soils where the availability of Fe could be consistently limited (**Mimmo et al., 2014**). It is clear that, if this nutritional disorder (Fe deficiency) occurred also in soils contaminated by Cu, this acidification would also increase Cu availability. For this reason, the maintenance of an adequate Fe availability, also through targeted fertilizations, could be a valid strategy both to limit the onset of Fe deficiency and, concurrently, to prevent Cu toxicity for crops.

The interactions between mechanisms underlying, at the root level, the acquisition of nutrients could be an additional element of relevance in relation to the definition of agronomical practices aimed at coping with the Cu toxicity. In fact, as stated before, it has been clearly demonstrated that S deficiency can limit the capability of plants to acquire Fe in

maize (Astolfi et al. 2003; Bouranis et al. 2003), barley (Kuwajima and Kawai 1997; Astolfi et al. 2006), tomato (Zuchi et al. 2009) and durum wheat (Zuchi et al. 2012). In these studies it has been suggested that in grasses ("Strategy II" plants) this effect could be ascribed to a decrease in the production and release of PS induced by S deficiency, whereas in tomato (a "Strategy I" plant) the effect was rather due to a reduced nicotianamine and ethylene production, which could impair both the activity of the Ferric chelate reductase and the capacity of the Fe transporter IRT1 to mediate the Fe^{II} flux across the plasma membrane. It has been also demonstrated that the acquisition of nitrate and its assimilative metabolism is impaired by inadequate availability of S (**Iacuzzo et al. 2012**, **Prosser et al., 2001 J Exp Bot 52:113–121; Nikolic et al., 2007 Funct Plant Bio**). These aspects are of relevance in relation of Cu toxicity; in fact an effective response to the oxidative stress (**Foyer and Noctor, 2011**; **Del Buono et al., 2011**), as also induced by Cu excess, as well as an efficient detoxification from xenobiotics like herbicides (**Del Buono & Ioli, 2011**) strictly depend on an adequate availability of S. Considering that agricultural soils are becoming depleted for S (**McGrath et al., 2002**), the strategy to ensure a stable input of this nutrient *via* specific plans of fertilization could ensure both the efficiency of the processes for the acquisition and assimilation of other nutrients and an effective capacity to respond to the stress induced by an excess of Cu, as suggested for example for the management of Cu contaminated paddy soils (Sun et al., 2016, DOI 10.1007/s00374-015-1050-z).

These evidences taken together highlight the availability of practices to deal with Cu toxicity. It is therefore presumable that the simultaneous use of more than one practice at a time may allow dealing more effectively these critical issues.

4. Conclusions and future perspectives

Considered that limiting the Cu distribution in the field is a priority to face the problem of the progressive Cu accumulation in the soils, there is no doubt that for soils, where the Cu accumulation and toxicity are already present limiting agricultural production, it is essential to define appropriate strategies to deal with the problem aimed at restoring the fertility and the usability of these soils.

Some strategies are already available and frequently utilized (e.g. lime and OM applications). There are some properties of them already known; however, there are processes, in particular those occurring at the rhizosphere level, still not totally clarified. Only their comprehension can guarantee a better exploitation of these practices in field conditions. For other strategies (e.g. intercropping, fertilization considering interactions between nutrients, etc.), experience

and knowledge are still scarce at the field scale or, for some of them, yet only limited at field observations. It is clear that their effective exploitation requires a wider understanding of the processes and mechanisms occurring at the rhizosphere and underlying the effects recorded, as in the case of dicot/grass intercropping. In fact, through the awareness of what is occurring in the rhizosphere of these soils (which involves soil processes of elements/nutrients stabilization, mechanisms of nutrients acquisition and allocation, release of exudates, competitions/interactions between nutrients, root/bacterium/fungus interaction), it is possible to define the applicability details of agricultural practices that are able to both a) cope with the problem guarantying the yield and b) to avoid environmental worsening, as it might take place through an uncontrolled mobilization of Cu accumulated in the soil and its leaching along the profile. The meeting of this challenge is even more relevant considering that the food production to fulfil the demand of the progressive increasing of world population will need the exploitation of all agricultural surface also including the contaminated one.

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