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Review

Copper accumulation in vineyard soils: Rhizosphere processes and agronomic practices to limit its toxicity



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H I G H L I G H T S

- Speciation of Cu in soil solution is crucial to determine its toxicity to organisms.
- Root exudation is involved in both Cu exclusion and Cu accumulation in plants.
- Agronomic practices might either alleviate or exacerbate Cu toxicity

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A B S T R A C T

Viticulture represents an important agricultural practice in many countries worldwide. Yet, the continuous use of fungicides has caused copper (Cu) accumulation in soils, which represent a major environmental and toxicological concern. Despite being an important micronutrient, Cu can be a potential toxicant at high concentrations since it may cause morphological, anatomical and physiological changes in plants, decreasing both food productivity and quality. Rhizosphere processes can, however, actively control the uptake and translocation of Cu in plants. In particular, root exudates affecting the chemical, physical and biological characteristics of the rhizosphere, might reduce the availability of Cu in the soil and hence its absorption. In addition, this review will aim at discussing the advantages and disadvantages of agronomic practices, such as liming, the use of pesticides, the application of organic matter, biochar and coal fly ashes, the inoculation with bacteria and/or mycorrhizal fungi and the intercropping, in alleviating Cu toxicity symptoms.

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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 (Komárek et al., 2010). Besides Cu-containing fungicidal and bactericidal sprays, substantial Cu addition to soils can occur from the addition of contaminated wastes (Liu et al., 2007) and/or mineral- and organic-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 biochemical and physiological processes connected to plant growth and development (Yruela, 2005). Nonetheless, depending on the concentration and on the bioavailable fraction, Cu 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 (Cambrollé et al., 2013; Juang et al., 2014; Komárek et al., 2010; Tanyolaç et al., 2007; Toselli et al., 2009). For this reason, the deeper understanding of Cu dynamics in soil, particularly considering the complexity of rhizosphere processes driving 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 purpose, Cu fractions in soils, mechanisms of root Cu uptake and its allocation in shoot as well root 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 fraction for plants strongly depends 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 availability in the rhizosphere and the functionality of the protein systems committed to the nutrient acquisition at root level and, then to the allocation into shoot tissues. In other words, Cu forms, which can be taken up, are closely dependent on the selectivity of those proteins, which mediate nutrients' transport across the plasma membranes.

2.1. 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 because 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 (Fig. 1). In the soil solution, Cu may be in free form as Cu²⁺ or, in most 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 (Alleoni et al., 2005; Sparks, 2003). 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 (Alloway, 1995; Sposito, 1989). Non-specific adsorption is weak and unstable. This type of adsorption weakly affects the availability of Cu. In this type of sorption, Cu can retain its internal hydration sphere and a high degree of rotational mobility on exchange sites (Alleoni et al., 2005; McBride, 1979). In specific adsorption on functional groups of inorganic particles, the metal partially or completely loses its hydration water forming an inner sphere complex with either OH⁻ or a H₂O 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, 2004; Ferreira et al., 2014).

The nature of Cu interactions with soil components has been

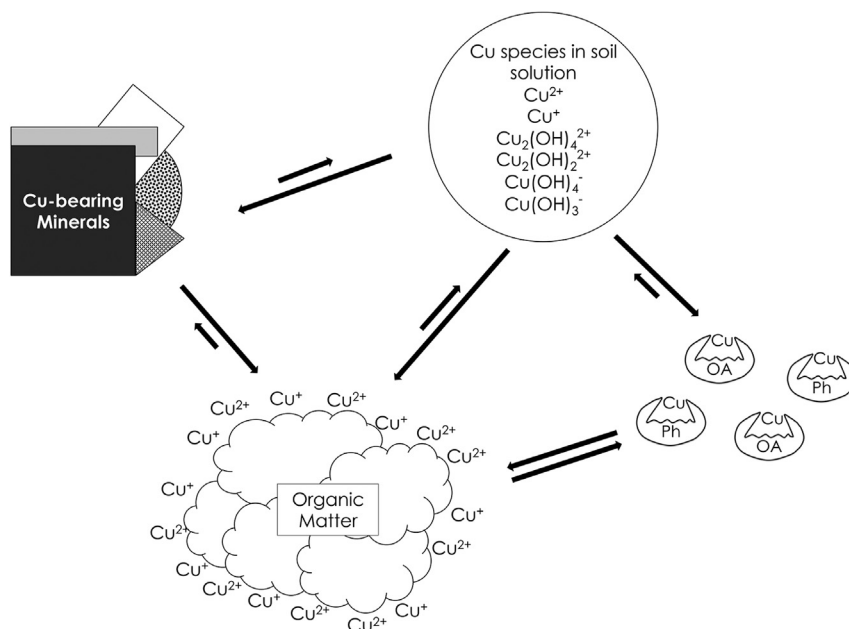


Fig. 1. Cu species in soil. Equilibria determining the Cu availability for living organisms. OA= Organic Acids; Ph = Phenolic Compounds.

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 pH (from 5.2 to 7.1). They found that in all soils, Cu exists predominantly as Cu-soil organic matter complexes (Cu-SOM) 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 X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) spectroscopy, and Cu–glutamate as the best-fit structural analog, Karlsson et al. (2006) showed that at pH 4.8–6.3 Cu(II) forms a five-membered chelate ring with one amino nitrogen ($\alpha\text{-NH}_2$) or alcohol oxygen ($\alpha\text{-OH}$) 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.

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) may form, thus increasing the total soil fraction of mobile 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 alkaline soils with a low content of OM, like those that can be 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 (hydr)oxides or hydroxy-carbonates Cu-forms may be the most abundant forms 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 is rather stable in soil and its availability depends on the nature of the binder (particularly OM, Fe, Al, and Mn minerals and (hydr)oxides) and its relative amount as well as on carbonates, soil pH and cation exchange capacity (CEC) (Brunetto et al., 2014; Couto et al., 2015; Fernández-Calviño et al., 2009). Copper adsorption in soil occurs primarily in the most avid binding sites, especially OM, and the remaining is redistributed into fractions that are held with less energy and that consequently exhibit a higher availability and mobility in the soil profile.

2.2. Cu concentration in vineyard soils

From a survey on the literature published worldwide about Cu concentration in the upper layers of vineyard soils, Komárek et al. (2010) observed that Cu concentrations in superficial horizons often exceed 200 mg kg^{-1} and that the highest Cu concentrations are present in the upper layers of soil profiles. Most of these values exceed the warning and critical legislative limits valid in most Countries around the World. For example, the predicted no effect concentration (PNEC) of Cu in soils in the EU (estimated in the EU Risk Assessment on Cu) ranges from 20 to $200 \text{ mg Cu kg}^{-1}$ depending on soil properties (Ruyters et al., 2013 and references

therein). In a recent paper by Tóth et al. (2016) on the concentration of heavy metals in agricultural soils of the European Union, a threshold of 100 mg kg⁻¹ was considered for Cu, with lower and higher guideline values set at 150 mg kg⁻¹ and 200 mg kg⁻¹, respectively. Exceptionally high concentrations of Cu in vineyard soils have been observed in France (above 1000 mg kg⁻¹, Flores-Vélez et al., 1996) and in Brazil (above 3000 mg kg⁻¹, Mirlean et al., 2007). Toxic effects of Cu on the microbial communities in vineyard soils have been observed above total concentrations of 150–200 mg Cu kg⁻¹ (Fernández-Calviño et al., 2010).

However, in a survey by Ruyters et al. (2013) on six established European vineyards, no Cu toxicity to plants, microbial processes and invertebrates was observed in vineyard soil samples at Cu concentrations well above European Union limits protecting the soil ecosystem. This is likely due to a decreased Cu bioavailability in vineyard soils compared to Cu-spiked soils. In fact, according to these authors, on average, 3.2 times larger Cu might be dosed in vineyards than in spiked soils to obtain a 10% inhibition, and is larger than the factor of 2.0, which is proposed in the generic risk assessment of Cu in soil. According to these data, in vineyard soils, Cu toxicity limits are not easily definable and, in principle, could be set at higher values than in other Cu-contaminated soils.

2.3. Root exudation process and its impact on 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 (Badalucco and Nannipieri, 2007). 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 at 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., 2016, 2015).

Indeed, root exudates, especially those with a low molecular weight, can be used by microorganisms as easily accessible source of carbon in 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 play a role as powerful chemo-attractors, 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 cycle of Cu 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, mobilize insoluble nutrients (e.g. P, Fe, Zn) and detoxify harmful heavy metals (Dakora and Phillips, 2002; Pii et al., 2015; Weisskopf et al., 2006). In this latter case, the exudates, mainly carboxylic and phenolic acids, are thought to chelate heavy metals, as for instance Cu, 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 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, the 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 phytostabilizers 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 shoot dry mass) without suffering any stress (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 acids in alleviating Cu stress in plants. Early evidences showed that oxalate was exuded by coniferous trees *Pinus* and *Picea* in response to Cu stress (Ahonen-Jonnarth et al., 2000; Heim et al., 2001). More recently, Qin et al. (2007) observed that, besides oxalate, poplar trees exuded formate and malate in a concentration that is directly dependent on 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⁺, Ca²⁺ and Mg²⁺) (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). Also, 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 (Table 1) (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 for the Cu-tolerant species *Ricinus communis* L. (Huang et al., 2015). On the contrary, Zhang et al. (1989) discovered an enhanced PS production and release in Cu-deficient grasses, 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

Table 1
Acid dissociation constants of organic compounds and stability constants of metal chelates.

	Acid dissociation constant (logK)	Stability constant (logK) of different metal chelate						
		Ca	Cu	Fe(III)	Mn	Ni	Sr	Zn
Citric acid	3.14 ^a	3.5 ^b	6.1 ^b	11.85 ^b	3.2 ^b	4.8 ^b	2.8 ^b	4.5 ^b
	4.77 ^a							
	6.39 ^a							
Formic acid	3.75 ^a	0.80 ^b	1.98 ^b	3.1 ^b			0.66 ^b	0.60 ^b
	Fumaric acid							
Malic acid	4.38 ^a	1.80 ^b	3.4 ^b		2.24 ^b		1.45 ^b	2.80 ^b
Malonic acid	5.11 ^a	1.8 ^c	5.04 ^c	8.12 ^c	2.5 ^c	3.24 ^c	1.34 ^c	2.91 ^c
Oxalic acid	5.69 ^a	3.0 ^b	6.3 ^b	9.4 ^b	3.9 ^b	5.16 ^b	2.54 ^b	4.9 ^b
Succinic acid	4.19 ^a	1.20 ^b	3.3 ^b	7.49 ^b	2.11 ^b	2.36 ^b	0.9 ^b	1.78 ^b
Tartaric acid	5.61 ^a	1.80 ^b	3.2 ^b	7.49 ^b		3.78 ^b	1.94 ^b	2.68 ^b
Glutamic acid	4.34 ^a	1.43 ^b	7.85 ^b		3.3 ^b	5.9 ^b	1.37 ^b	5.45 ^b
Glycine	4.31 ^a	1.43 ^b	8.22 ^b	10.0 ^b	3.2 ^b	6.1 ^b	0.91 ^b	5.16 ^b
Serine	9.78 ^a	1.43 ^b		9.2 ^b		5.44 ^b		
Valine	9.21 ^a		7.92 ^b	9.6 ^b	2.84 ^b	5.37 ^b		5.0 ^b
	9.74 ^a							

^a (Haynes, 2010).

^b http://www.coldcure.com/html/stability_constants.html.

^c (Martell et al., 2004).

(Chaignon et al., 2002).

By examining the stability constants of complexes formed between Cu and root exudates (Table 1), it is clear that, some of them (e.g. citrate, formate, oxalate, succinate) display a higher affinity for Fe(III) as compared to Cu. Therefore, the presence of Fe(III) in high concentrations might reduce the effectiveness of these compounds in alleviating Cu toxicity.

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., 2014). Hence, future researches should focus on field-like conditions, thus bridging micro- and macro-scale studies but considering also other aspects that are generally present in the field scale as the agrochemicals 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 (Damin et al., 2010; Kremer et al., 2005; Manderscheid et al., 2005) affecting considerably, thus, the capacity of roots to shape the rhizosphere. However, despite these evidences, a too limited number of studies aiming at investigating the effect of herbicides at this level have been carried out. Therefore, as it will be discussed later, 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.

2.4. Copper uptake and translocation in plants

2.4.1. Mechanisms in relation to the Cu sources

As also stated before, the mechanisms of Cu trans-membrane transport in root share the access path for the flow of the

nutrient from the soil solution to the plant tissue in either growth conditions of low, balanced or excessive availability. However, the selectivity of these mechanisms, at least at some levels, can restrict

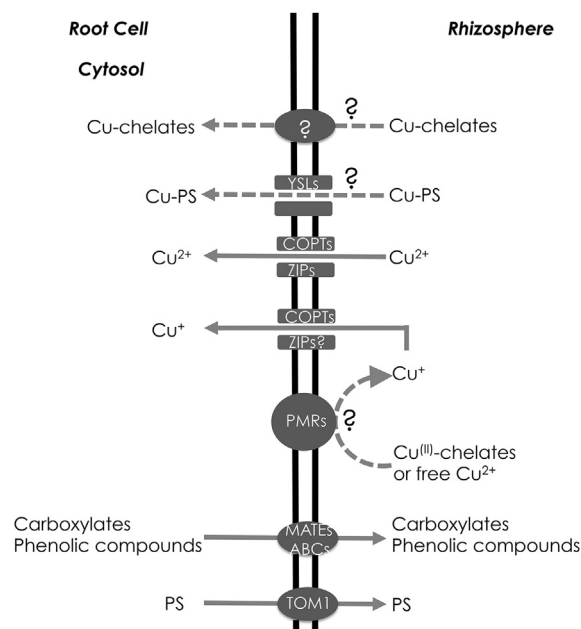


Fig. 2. Schematic representation of the putative mechanisms involved in Cu acquisition in plants. Arrows with continuous lines stand for characterized Cu-uptake systems. Dashed lines and question marks highlight putative processes. PS = phytosiderophores; YSLs = yellow stripe-like proteins; COPTs = copper transporters, e.g. COPT1; ZIP = Zn/Fe permease proteins, e.g. IRT1; PMRs = plasma membrane-bound reductases, e.g. Fe-chelate reductases; MATEs = multidrug and toxic compound extrusion proteins, e.g. AtMATE1, PEZ1; ABCs = ATP-binding cassette transporters, e.g. ABCG37/PDR9; TOM1 = protein involved in PS released, limited to grass species.

the number of Cu forms that can be used by roots as Cu sources (Fig. 2). Analyzing this aspect from an opposite point of view, it is established that there are Cu forms in soil solution which cannot be used by transporter 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. 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 deriving from plant tissues treated with these agrochemicals, are considered. Similarly, mechanisms of Cu homeostasis within tissues and within cells can be relevant for the detoxification from Cu, once taken up by the plants.

The mechanisms of Cu transport in roots (micronutrient uptake) have been characterized in the last two decades (Sancenón et al., 2003; Wintz 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 (Sancenón 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 (Marschner, 2011; Wintz et al., 2003). This family of proteins seems to be also implicated in the transport of the chelate Cu-nicotianamine (Wintz et al., 2003), which is presumably involved in the translocation of this micronutrient (allocation to the shoot). A schematic representation of the putative mechanisms involved in Cu acquisition is summarized in Fig. 2.

Other proteins have 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 (Di Donato 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; Liao et al., 2000a, 2000b; Pich and Scholz, 1996; White et al., 1981). This suggests that even under toxic conditions, plants have mechanisms to regulate complexation of Cu within the xylem sap and, hence, minimize potential damage caused from high concentrations of free Cu ions (Welch and Shuman, 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 (Marschner, 2011), 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 (Von Wiren et al., 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) should not be totally excluded, considering also the limited literature available on

this aspect. Nonetheless, if this phenomenon occurs, 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 with Cu excess.

2.4.2. 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 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 by *terbutylazine* in barley (Del Buono et al., 2015). In the first case, the impairment is on the root transmembrane 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* as that described for Fe in soybean plants has been shown by Eker et al. (2006) 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 only restricted to Cu but also to Zn and Mn acquisition (Rengel and Wheal, 1997).

In this general context, an effect of herbicides on the transmembrane 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 (*diclofop*, *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, some 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 report clearly indicates that herbicides might also influence Cu allocation at the leaf level and/or be involved in their homeostasis.

2.4.2.1. 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 microbial cells, when its concentration is too high, a series of toxic phenomena occurs and strategies aimed at limiting its harmful effects are adopted.

Concerning, root apices, the first target of Cu nutritional disorder in the soil, generally become shorter and thicker for changes in

the cell wall formation (Ambrosini et al., 2015a). Moreover, the number of lateral roots often increases with also an evident plasmolysis of some epidermis cells. Taken together, these phenomena are able to cause a clear contraction of the root density (Chen et al., 2013; Juang et al., 2014, 2011; 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 (Ambrosini et al., 2015b). 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 (Sancenón et al., 2003). In excess of Cu, the Cu uptake is down regulated and the synthesis of compounds, such as metallothioneins or phytochelatin, 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 favouring, 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, 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 (De Conti et al., 2016; Jones and Darrah, 1994). This latter phenomenon, together with the rhizosphere alkalization, is able to alter the Cu solubility (modifying the distribution of Cu chemical species) thus 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, Cu can cause necrosis and, if the stress is prolonged, plant death (Marschner, 2011; Miotto et al., 2013; Toselli et al., 2009).

At the leaf level, Cu disorder causes a decrease in the pigment concentration with a subsequent drop of the photosynthetic rate (Cambrollé et al., 2015, 2013). A typical leaf symptom of this toxicity is the interveinal foliar chlorosis, partially ascribed to the negative effect on Fe acquisition exerted by Cu excess (Ouzounidou, 1995; Yau et al., 1991). 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-Sossé 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; Miotto et al., 2013; Yruela, 2005). 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 phytochelatin 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 specific review see Huffman and O'Halloran, 2000; 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 has been demonstrated that acquisition of Fe 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 (Nikolic et al., 2007; Prosser et al., 2001) as well as the response to Cd toxicity in barley (Astolfi et al., 2012) are all linked to sulfur (S) availability. In fact, plants produce a large amount of thiols (mainly glutathione) to overcome oxidative stress (Del Buono et al., 2011; Foyer and Noctor, 2011) and for synthesis of metal-binding S compounds (Shahbaz et al., 2010), 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, the decrease of atmospheric SO₂ emissions and lower S supply through mineral fertilization have caused an increased occurrence of S deficiency in many agricultural soils, with physiological responses of plants to S deficiency (McGrath and Zhao, 1995; McGrath et al., 2002). In addition, this picture is further complicated by the fact that also herbicide detoxification requires S, being the conjugating of these chemicals with glutathione one of the main steps for the detoxification of the herbicide itself (Del Buono and 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 more details see Del Buono et al., 2015; 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 quality) and the crop capacity to detoxify herbicides, which are commonly used 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 non specific complexes within the cells thereby exerting toxic effects (Dupont et al., 2011). In both *Escherichia coli* and *Bacillus subtilis*, the excess of Cu has 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 possible alternative approaches to those Cu-based and/or limiting the distribution in soils of Cu-containing amendments/products. In this way, it will be reduced 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 diminishing considerably and worsening in its quality (Cambrollé et al., 2015; Juang et al., 2014; Tanyolac et al., 2007; Toselli et al., 2009), 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 compounds, such as limestone, phosphates, clays, zeolites, Fe oxides, OM, and biochar (Ambrosini et al., 2015a; Arunakumara et al., 2013; Kumpiemi et al., 2008; Terzano et al., 2005b). 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. 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., 2001). 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. Advantages and disadvantages of the different agricultural practices discussed are also summarized in Table 2.

3.1. Lime application to soil

This practice, promoting the alkalization of soil solution and, thus, inducing the de-protonation of acidic functional groups on the surface of soil particles, 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 solubilization, 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 by limiting the replacement of Mg in chlorophyll molecules by Cu (Yruela, 2009). Moreover, both cations, favouring a site-specific competition for ions, could prevent the Cu excess within cell (Arunakumara et al., 2013). Generally, the lime application to acidic soils, through alkalization, can increase 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. However, it is important to note that liming 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 alternatives 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).

3.2. Organic matter (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; Shaheen et al., 2015), mainly for the high affinity for Cu of its carboxylic functional groups (see Section *Soil Cu forms and their biogeochemical cycles*). 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 molecular weight 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) favouring 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 application. 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), thus deteriorating 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 on its surfaces (Beesley et al., 2011). Uchimiya et al. (2011) showed that CEC was the primary mechanism by which biochar enhanced Cu retention, whereas 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 tool for controlling Cu mobility is the application of treated coal fly ash to soil with the formation of zeolites or aluminosilicate geopolymer (Terzano et al., 2005a). 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).

3.3. 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 this metal. For example, in vineyards with a long-term foliar application of Cu-based fungicides, some AMF species, in order to ensure their own survival and also of associated plants, have developed strategies to tolerate high Cu contents in the soil, such as the release of organic substances (e.g. glomalin), which may form complexes with Cu, thus restricting its bioavailability (Ambrosini et al., 2015b; Bedini et al., 2010). 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). Generally, 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

Table 2
Advantages and disadvantages of agronomic practices in reducing Cu toxicity to crops.

Agronomic practice	Advantages	Disadvantages
Liming	Enhanced Cu sorption on soil Fast Cu-precipitates formation Increased concentrations of Ca and Mg in soil Increased P availability Cheap and easy application	At higher pH, increased mobilization of Cu through formation of soluble complexes with dissolved organic matter (DOM) and OH ⁻
Organic matter (OM) addition	Formation of strong complexes with high molecular weight insoluble organic molecules Organic matter from waste composting can be used Cheap and easy application	In the presence of DOM, soluble Cu complexes may form Long term stabilization is limited by OM turnover
Biochar	Cu sorption on biochar especially through cation exchange reactions Increased soil fertility Cheap amendment also solving problems related to its disposal	Reduced effectiveness of herbicides Potential introduction of other contaminants Potential negative impact on soil bacteria and fauna Long term stabilization is limited by OM turnover
Coal fly ash	Fast soil alkalization and Cu sorption on aluminosilicate phases Cheap amendment also solving problems related to coal fly ash disposal	Mobilization of other potentially toxic elements incorporated in coal fly ash
Clays and zeolites	Cu stabilization on the mineral surfaces mainly by cation exchange processes Improvement of soil properties related to soil fertility (CEC, water retention, etc.) Easy application	Problems related to the exploitation of mineral natural resources. For zeolites, synthetic minerals can be easily synthesized from waste materials Remobilization of Cu if other cations displace it from exchange sites
Arbuscular Mycorrhizal Fungi	Release of organic substances which may form complexes with Cu Cu cellular compartmentalization in vesicles and spores of AMF Increased interface between roots and soil, facilitating nutrient uptake	Cu uptake can be increased as well by the increased root biomass
Bacteria inoculation	Bacterial detoxification of Cu through hyperaccumulation or release of strong metal chelating molecules Reduction of metal uptake by plants Reduction of ethylene-dependent detrimental stress	Competition with plants for water and nutrients
Intercropping	Cu immobilization in the root apoplast of grasses Cu uptake and accumulation in grasses Cu phytostabilization by release of Cu-complexing exudates Easy application	Competition with crops for water and nutrients Higher supply of N needed
N fertilization	NO ₃ ⁻ application favors soil alkalization around roots thus reducing Cu availability	NH ₄ ⁺ application causes acidification of the rhizosphere thus increasing Cu availability Costly
Fe fertilization	Adequate Fe supply reduces rhizosphere acidification by roots thus limiting Cu availability	High Fe concentrations can displace Cu from organic complexes thus increasing Cu availability Costly
S fertilization	Increased Fe and N assimilation Increased capacity to respond to Cu stress, and to detoxify herbicides and xenobiotics	Costly

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 potential exploitation at the field level as a promising agricultural practice to guarantee crop yield.

3.4. 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., 2010, 2009). *Elsholtzia splendens* inoculated with the 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 plants to metals, 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 metals, 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 amounts of Cu in a produced exopolymer 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 cloacae* 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, besides the ability of several bacterial strains to hyperaccumulate and/or sequester 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., 2002, 2005; 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 plants 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 (Blaha et al., 2006; Glick et al., 2007; Honma and Shimomura, 1978), which interferes with the biosynthesis of ethylene. In this way, PGPR can prevent plant ethylene levels from becoming growth inhibitory (Burd et al., 1998; Glick, 1995). 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., 2000, 1998; Cheng et al., 2009; Nie et al., 2002; Reed and Glick, 2005; Reed et al., 2005; Safronova et al., 2006).

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.

3.5. 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 protection from erosion. However, in this particular crop system vine plants, when grown in Cu-contaminated soils, do not exhibit any symptoms at lower concentrations (Miotto et al., 2013). 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 contemporary 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 prerequisite 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 (Ojeda-Real et al., 2009; Parisi et al., 2006), aspect of paramount importance for example for the grapevine (Bell et al., 1979; Ough and Bell, 1980). 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), prerequisite to restrict the fraction of available Cu in soil solution and to make this crop practice really effective for the purpose.

In this regard, also the use of transgenic plants able to extract and accumulate high amounts of Cu from soil could be foreseen. Several pieces of research have been focused on the phytoextraction of metals, Cu included (Cherian and Oliveira, 2005; Eapen and D'Souza, 2005; Kotrba et al., 2009; Krämer and Chardonnens, 2001; Papoyan and Kochian, 2004), demonstrating that the cloning and the overexpression of the gene encoding for the glutathione synthetase in *Brassica juncea* determined both a higher tolerance and a higher accumulation of Cd, Zn, Cr, Cu and Pb in this specie (Bennett et al., 2003; Zhu et al., 1999). However, despite these very promising results, reports about field trials involving transgenic hyperaccumulating plants are still missing. Moreover, the impact of these genetically modified plants in the environment is still matter of debate limiting their application in a real productive context in several countries.

3.6. 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 NO₃⁻, an alkalization of the soil surrounding the roots occurs as a consequence of the nitrate acquisition by roots (Britto and Kronzucker, 2013); this effect can limit the availability of Cu. This aspect is particularly relevant in the case of acidic soils. In fact, high concentrations of protons can prevent acidic functional groups of organic acids from dissociation and thus from complexing Cu, thereby reducing their ability to alleviate Cu toxicity in plants. For this reason, also the N-fertilization with ammonium, which uptake induces the acidification of the rhizosphere, might exacerbate Cu toxicity.

A further relevant aspect 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 (Brancadoro et al., 1995); this action is ascribed to the roots effort to mobilize the nutrient from its barely available forms in the soil (Marschner, 2011). 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, concomitantly, to prevent Cu toxicity for crops. On the other hand, this practice should be used carefully in order to avoid an excessive availability of Fe. In fact, organic acids display a higher affinity for Fe than Cu; therefore, in the presence of high Fe concentrations, metal exchange reactions can occur (Fe vs. Cu) thus limiting the effectiveness of these compounds in contrasting Cu toxicity.

The interactions between mechanisms underlying, at the root level, the acquisition of nutrients could be even more relevant 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 (Astolfi et al., 2006; Kuwajima and Kawai, 1997), 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., 2011; Nikolic et al., 2007; Prosser et al., 2001). These aspects are of relevance in relation to Cu toxicity; in fact, an effective response to the oxidative stress (Del Buono et al., 2011; Foyer and Noctor, 2011), as also induced by Cu excess, as well as an efficient detoxification from xenobiotics like herbicides (Del Buono and Ioli, 2011), strictly depend on an adequate availability of S. Considering that agricultural soils are becoming depleted in 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 cope with the stress induced by an excess of Cu, as suggested for example for the management of Cu contaminated paddy soils (Sun et al., 2016).

These evidences taken together highlight the need 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 those soils where the Cu accumulation and toxicity are already present thus limiting agricultural production, it is essential to define appropriate strategies aimed at restoring the fertility and future usability of these soils.

Some strategies are already available and frequently utilized (e.g. lime and OM applications). Some of the mechanisms underlying these strategies are 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 at field conditions. For other strategies (e.g. intercropping, fertilization with interactions among nutrients, etc.), experience and knowledge are either still scarce at the field scale or, on the contrary, 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, as in the case of dicot/grass intercropping. In fact, through the awareness of what is occurring in the rhizosphere of these soils (i.e. soil processes of elements/nutrients solubilization/mobilization, 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 able to both a) cope with the problem of guarantying the yield and b) avoid environmental worsening, as it might occur through an uncontrolled mobilization of Cu accumulated in the soil with its leaching along the profile. Facing these challenges is even more relevant considering that the food production to fulfil the demand of the progressively increasing world population will need a deeper exploitation of all agricultural surfaces, including the contaminated ones.

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