

Potential for phytoextraction of copper by *Sinapis alba* and *Festuca rubra* cv. Merlin grown hydroponically and in vineyard soils

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Abstract The extensive use of copper-bearing fungicides in vineyards is responsible for the accumulation of copper (Cu) in soils. Grass species able to accumulate Cu could be cultivated in the vineyard inter-rows for copper phytoextraction. In this study, the capacity of *Festuca rubra* cv Merlin and *Sinapis alba* to tolerate and accumulate copper (Cu) was first investigated in a hydroponic system without the interference of soil chemical–physical properties. After the amendment of Cu (5 or 10 mg Cu l⁻¹) to nutrient solution, shoot Cu concentration in *F. rubra* increased up to 108.63 mg Cu kg⁻¹ DW, more than three times higher than in *S. alba* (31.56 mg Cu kg⁻¹ DW). The relationship between Cu concentration in plants and external Cu was dose-dependent and species specific. Results obtained from the hydroponic experiment were confirmed by growing plants in pots containing soil collected from six Italian vineyards. The content of soil organic matter was crucial to enhance Cu tolerance and accumulation in the shoot tissues of both plant species. Although *S. alba* produced more biomass than *F. rubra* in most soils, *F. rubra* accumulated significantly more Cu (up to threefold to fourfold) in the shoots. Given these results, we recommended that *F. rubra* cv Merlin could be cultivated in the vineyard rows to reduce excess Cu in vineyard soils.

Keywords Copper · Vineyard · *Festucarubra* · *Sinapisalba* · Phytoextraction

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Introduction

In Europe, copper-bearing fungicides such as copper sulfate (Bordeaux mixture) have been employed on vineyards since the end of the nineteenth century to control vine downy mildew. The intensive and prolonged use of such fungicides has resulted in Cu accumulation in the top layers of vineyard soils, at much greater concentration than the trace amounts that are normally required for healthy plant growth (Besnard et al. 2001; Brun et al. 2001; Cattani et al. 2006; Chaignon et al. 2003; Mirlean et al. 2007; Pietrzak and McPhail 2004; Ruyters et al. 2013). Indeed, while Cu concentration in arable lands generally ranges within 5–30 mg kg⁻¹, vineyard soils can contain amounts of Cu varying from 200 to 500 mg kg⁻¹ (Brun et al. 1998; Deluisa et al. 1996; Krishnamurti et al. 2007; Ruyters et al. 2013).

In Italy, vine-growing is one of the main agricultural activities and vineyards cover a wide area of arable land. Although studies have been conducted in some part of the country (Deluisa et al. 1996; Provenzano et al. 2010; Ruyters et al. 2013), the Cu content of many Italian vineyard soils is still largely unknown.

Copper is considered to be among the more mobile of the metals in surface environments. In both soil solution and solid phase, most of Cu is associated with organic matter by complexation or adsorption (Sauvé et al. 1997). Copper can also be adsorbed onto the surfaces of clay particles, and on Fe or Mn oxides, co-precipitate with carbonates and phosphates or be present in the lattice of primary silicate minerals (McBride 1981).

Bioavailable Cu, or the portion of Cu that can be transferred from the soil to the plant roots, can be significantly different from total Cu in soil. Indeed, copper mobility in

soil depends on Cu chemical speciation, and soil properties including pH, organic matter, cation exchange capacity (CEC), temperature, water content, texture, and Fe and Mn oxides (Marschner 1995; Hinojosa et al. 2010; Komárek et al. 2010). For instance, the soil solution of acidic soils (pH between 2.5 and 5) could contain up to tenfold more Cu than calcareous soils (pH between 7.5 and 7.8; Nachtigall et al. 2007).

Extractions with buffered (ammonium acetate) or unbuffered (e.g. CaCl_2 , $\text{Ca}(\text{NO}_3)_2$, NH_4NO_3) salt solutions, organic complexing agents (EDTA, DTPA), diluted acids (HCl, CH_3COOH), or a mixture of these reagents are routinely used as indicators of Cu bioavailability in the laboratory (Brun et al. 1998; Nogueirol et al. 2010; Schramel et al. 2000). The reliability of these extraction procedures is conventionally verified through comparison with the Cu content measured in plant tissues (Gunkel et al. 2004; Nogueirol et al. 2010).

In vineyards, copper accumulates in the topsoil and decreases with depth, eventually returning to natural levels between 40 and 60 cm (Komárek et al. 2010). Excess Cu in topsoil can affect the development of new vine shoots (Komárek et al. 2010; Romeu-Moreno and Mas 1999) and cause toxicity to young, newly planted vines with shallow root systems (Mackie et al. 2012). Also, since the major absorbing part of the root system may develop near the soil surface, depending on soil moisture conditions (Comas et al. 2010), considerable amount of Cu could be taken up even by older *Vitis* plants.

On this account, high levels of copper in soils may result in significant accumulation of Cu in roots of grapevines, thus affecting high quality viticulture. Indeed, excess Cu in roots can be translocated to the aerial part leading to high level of this element in grape and grape products (between 70 and 1,000 $\mu\text{g g}^{-1}$; Mirlean et al. 2005). Increased copper levels in berries were observed also in relation with copper applications in some Italian vineyards, but no phytotoxicity in grape leaves was detected (Provenzano et al. 2010).

To reduce Cu content in soils and decrease the risk of Cu accumulation in grapes, it is proposed that grass species able to phytoextract Cu in the shoot tissues should be planted in vineyards. A list of plant species tested for copper removal from vineyard soils and their Cu accumulation capacity can be found in Mackie et al. (2012).

In Italy, the sowing of grasses in the vineyard inter-rows is a common practice to improve soil fertility and grape quality. In the present study, the capacity of two grass species, *Festuca rubra* (L.) cv Merlin and *Sinapis alba* (L.) to tolerate and phytoextract Cu was assessed and compared first in a hydroponic experiment to avoid the interference of soil chemical-physical properties, and then in a pot study using soil from six Italian vineyards.

F. rubra cv Merlin was selected for this study as it is found in lead and zinc mining areas in the UK. It is known to

possess multi-metal tolerance (Wong et al. 1994) likely due to high production of metallothioneins (Ma et al. 2003). *S. alba* belongs to the *Brassicaceae* family, which includes many heavy metal accumulator species. *S. alba* was selected for this study as it has high tolerance to cadmium and lead (Evangelou et al. 2007). Due to these properties, both plant species could be included in phytoextraction programs, which comprise techniques for the removal of metals from soil by direct plant uptake and translocation to aboveground biomass.

Materials and methods

Plant growth

Seeds of *F. rubra* (L.) cv. Merlin and *S. alba* (L.) (Herbiseed, Twyford, UK) were imbibed in distilled water for 4 h and then surface-sterilized in 5 % (v/v) sodium hypochlorite for 10 min while shaking. Two experiments were performed.

Hydroponic experimental design

In the first experiment, sterilized seeds of both plant species were allowed to germinate on a mixture of loamy soil and sand (3:1). In order to reach the growth stage at which plants are commonly mowed from the vineyard, *F. rubra* and *S. alba* plants were grown in pots with the soil/sand mixture for 3 months inside a growth chamber with a day/night period of 13/11 h, air temperature of 20/15 °C, relative humidity of 70/85 %, and under a photon flux density (PFD) of 280 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The pots were watered every 3 days. Subsequently, plants were removed from the pots, gently washed with distilled water, and then transferred to 35 l aerated Hoagland (Hoagland and Arnon 1950) modified nutrient solution holding tanks. The nutrient solution was renewed every 48 h and its composition was the following (μM): KH_2PO_4 (40), $\text{Ca}(\text{NO}_3)_2$ (200), KNO_3 (200), MgSO_4 (200), FeNaEDTA (10), H_3BO_3 (4.6), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.036), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.9), ZnCl_2 (0.09), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.01). Eleven days after transferring the plants to tanks, three tanks per plant species were prepared with a density of 10 plants per tank. Copper amendments (0 Cu (control), 5 mg l^{-1} (78.5 μM) CuSO_4 , or 10 mg l^{-1} (157 μM) CuSO_4) were added to the nutrient solution. The experiment lasted 9 days, on the basis of results previously obtained with various plant species treated with the same Cu doses, and was conducted in triplicate at different times. For each replicate, six plants per treatment were randomly harvested, divided into roots and shoots, and roots were carefully rinsed with deionised water and 0.01 mM EDTA to remove not-absorbed Cu. Roots and shoots were blotted dry and weighted separately (fresh weight). The samples were next placed in a drying oven for 2 days at 80 °C and

then allowed to cool for 2 h inside a closed bell jar before measuring the dry weight.

Pot study experimental design

In the second experiment, *F. rubra* and *S. alba* seeds were allowed to germinate on a mixture of loamy soil and sand (3:1). Twenty days after sowing, seedlings were transplanted into 1.8 l pots at a density of three plants per pot. The weight of soil in each pot was about 2.2 kg. Soil was sampled from five vineyards located in the Veneto region (Lor, Ill, Spr, Sol, Tez) and one located in the Tuscany (Pan) region. In the vineyards “Pan” and “Spr”, a conventional pathogen defence practice was adopted since the planting of the vineyard (i.e. utilization of any type of fungicide or insecticide available), while in “Lor”, “Ill”, “Sol”, and “Tez” vineyards only biological or copper-based pathogen defence systems were used. In each vineyard, five soil samples were randomly collected (25 cm deep by 20 cm diameter). Soil sampled from the University of Padova experimental farm was used as a control (Contr), as it never received copper applications. Each soil sample was mixed and sieved, and then used to fill 4 pots per plant species. One pot filled with soil from each location was left without plants as a blank. All pots were watered every two days. Plant material was sampled 120 days after transplanting. Seed germination and plant growth occurred inside a growth chamber under the same conditions as for the hydroponic experiment.

The experiment was conducted in triplicate at different times. Six plants per treatment were randomly harvested, divided into roots and shoots, and roots were carefully rinsed with deionised water and 0.01 mM EDTA to remove not-absorbed Cu. The fresh and dry weight of roots and shoots were determined as described above.

Soil characterization

All soil samples were transferred to the soil chemistry laboratory, air-dried and ground to pass through a 2-mm sieve and then characterized according to the protocols given in Ministero delle Politiche Agricole e Forestali (M.I.P.A.F.; 2000). Soil pH was measured on 1:2.5 water-soluble extracts (w/v) and the soil texture was determined by using a densimeter method. Organic carbon was quantified through an oxidimetric method using 1 N $K_2Cr_2O_7$ and H_2SO_4 (98 %, v/v ; Walkey and Black 1934). Each soil subsample (1 g) was mixed with 10 ml $K_2Cr_2O_7$ and 20 ml H_2SO_4 . After boiling, 200 ml deionized H_2O , 5 ml H_3PO_4 (85 %, v/v) and 1 ml phenylalanine were added to each cooled reaction mixture. The amount of $K_2Cr_2O_7$ used in the reaction mixtures was evaluated by measuring the fraction of reduced $K_2Cr_2O_7$ by 1 N $FeSO_4$. To determine the quantity of organic carbon in each sample, the difference in volume between used $K_2Cr_2O_7$

and $FeSO_4$ was multiplied for the equivalent weight of carbon. The content of organic matter (OM) was calculated multiplying the amount of C for the empiric factor 1.72 (Baldock and Skjemstad 1999).

The cation exchange capacity (CEC) was estimated by extracting exchangeable cations from 2.5 g soil subsample using 50 ml $BaCl_2$ -thriethanolamine at pH 8.1. After 1 h of agitation, filtered samples (0.45 μm filter, Millipore) were diluted 1:10 with deionized water and analysed for the content of exchangeable bases via Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Spectro Ciros^{CCD}).

Elemental analysis

Dried plant material (300 mg) was digested with 5 ml of nitric acid 99 % (v/v) in a microwave mineralizer (Milestone Ethos model 1600), while dried soil samples (500 mg) were mixed with 28 ml of aqua regia and further mineralized in the microwave. The fraction of Cu adsorbed to the soil solid phase, i.e. extractable Cu (Cu_{EDTA}), was extracted with Na_2 -EDTA 0.01 M and CH_3COONH_4 1 M according to Brun et al. (1998). Copper concentration in plant tissues and soils was determined via Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Spectro Ciros^{CCD}).

The translocation factor (TF) and bioaccumulation factor (BAF) of copper were calculated as $[Cu]_{shoot}/[Cu]_{root}$ and $[Cu]_{shoot}/[Cu]_{soil}$, respectively, as reported for other metals by Rezvani and Zaefarian (2011).

Chlorophyll determination

The content of chlorophyll in leaves of *F. rubra* and *S. alba* was determined as a stress-indicator parameter. Fresh foliar tissue (300 mg) was ground in liquid nitrogen and then extracted with 15 ml ethanol (96 % v/v). The samples were kept in the dark for 2 days at 4 °C and the extracts were filtered and subsequently analysed via a spectrophotometer (Perkin–Elmer UV/VIS Lambda 1) at 665 and 649 nm. The $Chla$ and $Chlb$ concentrations were calculated using the Welburn and Lichtenthaler (1984) equation.

Statistical analysis

Data were analysed using one-way analysis of variance (one-way ANOVA). Statistical analysis was performed using SPSS 10.0. All probabilities were two-tailed. Data were checked for normality and homogeneity of variance (Levene test). Analysis of variance was followed by pairwise post hoc analyses (Student–Newman–Keuls test) to determine which means were significantly different. Statistically significant differences ($P < 0.05$) are reported in the text and shown in the figures.

Results

Hydroponic experiment

Effect of copper treatments on plant growth and chlorophyll contents

The tolerance index (TI) was calculated as the dry weight biomass of plants grown in Cu-amended nutrient solution (5 or 10 mg l⁻¹ Cu) divided by the dry weight biomass of plants grown on unamended nutrient solution. Results indicated that *F. rubra* was more sensitive to excess Cu than *S. alba* as the dry weight of *F. rubra* plants at both Cu concentration was less than the control plants (Table 1). Conversely, *S. alba* plants only had a decrease in biomass with the addition of 10 mg l⁻¹ Cu.

Under Cu treatment, the dry weight of *F. rubra* plants was less reduced than the fresh weight, while the values of dry weight of *S. alba* plants were not significantly different from the controls (data not shown). The shoot dry weight to fresh weight ratio significantly increased in both plant species exposed to 5 and 10 mg l⁻¹ Cu. On the contrary, the root dry weight/fresh weight ratio decreased in *F. rubra* plants after Cu addition, while it did not change in *S. alba* plants (Table 1). The presence of Cu in the solution affected the root growth more than the shoot growth as there was a decrease in root/shoot ratio (DW; Table 1).

The total chlorophyll (Chla + Chlb) content is considered to be an indicator of a plant stress (MacFarlane and Burchett 2001). In leaves of *F. rubra* and *S. alba*, the amount of total chlorophyll was affected at a similar degree (~30 % reduction) by 10 mg l⁻¹ Cu, while 5 mg l⁻¹ Cu significantly reduced the chlorophyll level only in *F. rubra* plants (Table 1).

Copper accumulation in plants

F. rubra and *S. alba* plants accumulated more Cu in the roots than in the above-ground tissues (Table 1). Cu accumulation in roots of both plant species and in the shoots of *F. rubra* increased with increasing Cu concentration. *F. rubra* accumulated approximately 70 % more Cu in roots than *S. alba* for both Cu concentration (5 or 10 mg l⁻¹ Cu). While no significant differences in shoot Cu concentration were recorded between the two plant species grown with 5 mg l⁻¹ Cu, the concentration of Cu in above-ground tissues of *F. rubra* was about threefold higher than that measured in *S. alba* when plants were supplied with 10 mg l⁻¹ Cu.

The total amount of Cu in *S. alba* was 23.62 µg Cu in shoots and 64.57 µg Cu in roots, which was higher than *F. rubra* (6.49 µg Cu in shoot and 17.99 µg Cu in roots) when plants were grown in solution with 5 mg l⁻¹ Cu. The opposite was found for shoot total Cu when plants were supplied with 10 mg l⁻¹ Cu (Table 2).

Table 1 Effect of Cu treatments on dry weight (DW)/fresh weight (FW) shoot and roots, root to shoot ratio, leaf chlorophyll *a* (Chla)+*b* (Chlb) content and tolerance index (TI=plant dry weight+Cu/control×100),

shoot, and root copper concentration in *F. rubra* and *S. alba* plants grown in complete nutrient solution (control) or in Cu-amended (5 or 10 mg l⁻¹) nutrient solution

Parameters	Control	5 mg l ⁻¹ Cu	10 mg l ⁻¹ Cu
<i>F. rubra</i>			
DW/FW (shoot)	0.16±0.02 b	0.23±0.02 a	0.27±0.04 a
DW/FW (root)	0.070±0.002 a	0.065±0.002 b	0.065±0.001 b
DW root/DW shoot	0.46±0.03 a	0.45±0.03 a	0.40±0.02 b
Chla+Chlb (mg g ⁻¹ FW)	2.55±0.21 a	2.04±0.12 b	1.74±0.33 b
TI (%)	–	48.16±3.37 a	22.35±2.98 b
Shoot Cu (mg kg ⁻¹ DW)	12.23±2.32 c	31.45±3.55 b*	108.63±12.51 a*
Root Cu mg kg ⁻¹ DW)	30.43±3.64 c	612.34b±23.68 b*	872.75±33.62 a*
<i>S. alba</i>			
DW/FW (shoot)	0.12±0.01 b	0.17±0.02 a	0.18±0.02 a
DW/FW (root)	0.08±0.02 a	0.10±0.02 a	0.10±0.02 a
DW root/DW shoot	0.34±0.04 a	0.21±0.02 b	0.25±0.03 b
Chla+Chlb (mg g ⁻¹ FW)	1.04±0.15 a	1.01±0.16 a	0.70±0.06 b
TI (%)	–	78.23±4.89 a	31.36±2.65 b
Shoot Cu (mg kg ⁻¹ DW)	18.76±5.87 a	20.09±5.45 a	31.56±10.26 a
Root Cu (mg kg ⁻¹ DW)	25.22±2.44 c	346.04±21.25 b	485.60±39.98 a

Data are the means of six (plant weight and chlorophyll content) and three (shoot and root Cu concentration) values (±STD) of three independent experiments. Values marked with different letters along rows are significantly different ($P<0.05$). Asterisks indicate significant differences ($P<0.05$) in Cu concentration between *F. rubra* and *S. alba* with respect to plant tissue and copper dose

Table 2 Total content of copper in shoot and root of *F. rubra* and *S. alba* grown in complete nutrient solution (control) or in Cu-added (5 mg l⁻¹ or 10 mg l⁻¹) nutrient solution

	Control	5 mg l ⁻¹ Cu	10 mg l ⁻¹ Cu
<i>F. rubra</i> total Cu (μg)			
Shoot	8.16±1.02 b	6.49±1.14 b	32.26±2.35 a
Root	2.22±0.23 c	17.99±3.22 b	29.31±2.58 a
<i>S. alba</i> total Cu (μg)			
Shoot	14.71±1.59 b	23.62±2.63 a	22.30±2.15 a
Root	4.37±0.85 b	64.57±7.22 a	50.80±6.25 a

Values are calculated on the basis of the dry weight (DW) and those marked with different letters along rows are significantly different ($P < 0.05$, ±STD)

Pot study experiment

Properties of vineyard soils

Soil properties, i.e. pH, organic carbon (OC), organic matter (OM), texture, and cation exchange capacity (CEC), for the six vineyard soils and the control soil, which might affect the bioavailability of Cu to plants are presented in Table 3.

All soil samples were slightly alkaline with a pH ranging from 7.3 to 7.95, except the “Sol” soil, which was acidic (pH 5.8). Organic matter (OM) ranged between 11.9 and 55.3 g OM kg⁻¹ (Table 3). The texture of soils was sandy loam (Contr, Spr, Lor, Sol), loam (Tez), sandy clay (Ill), and clayey (Pan; Table 3). The cation exchange capacity (CEC) was high in all the vineyard soil samples, varying from 24.04 to 33.39 cmol(+) kg⁻¹, and was greater than in the control soil (Table 3). Total Cu concentration varied greatly among soils. The control soil (Contr) had the lowest Cu concentration (36.7 mg kg⁻¹), while the vineyard soils ranged from 121 mg kg⁻¹ in the “Pan” soil to 868.5 mg kg⁻¹ in the “Sol” soil (Table 3).

Soil extractable Cu (Cu_{EDTA})

To estimate the concentration of bioavailable Cu, the amount of Cu extracted from soil with EDTA (Cu_{EDTA}) from soil was measured. The concentration of Cu_{EDTA} in control soil was 10.2 mg kg⁻¹, while it ranged from 17.6 mg kg⁻¹ for “Pan” soil to 534 mg kg⁻¹ for “Sol” soil (Table 4). The concentration of extractable Cu was highly correlated with the total Cu concentration in soils ($R = 0.98$, $P = 0.001$).

At the end of the growing period, the effect of plants on extractable Cu was recorded. The presence of *F. rubra* plants increased the amount of extractable Cu more than *S. alba* in most soils (Contr, Pan, Lor, Sol), reaching the maximum value in “Pan” soil (83.5 %). No significant differences in Cu_{EDTA} were observed for “Spr”, “Ill”, and “Tez” soils between the two plant species. The concentration of extractable Cu_{EDTA} was not significantly different for pots without plants between the start and the end of the experiment (Table 4).

Cu concentration in plants versus soil properties

F. rubra and *S. alba* plants preferentially accumulated Cu in the roots (Fig. 1). Copper concentrations observed in *F. rubra* roots varied from 77 (Contr) to 847 mg kg⁻¹ (Tez), while they ranged from 25 (Contr) to 58 mg kg⁻¹ (Ill) in the shoots. In *S. alba*, the accumulation of Cu was lower and varied from 95 (Contr) to 443 mg kg⁻¹ (Tez) in the roots, and from 17 (Contr) to 38 (Sol) mg kg⁻¹ in the shoots. The greatest Cu concentration was recorded in roots of plants grown on soils containing the maximum levels of Cu, i.e. “Sol” and “Tez”. Both plant species showed the highest TF and BAF values when cultivated in control soil (Table 5). However, these values were lower than 1 suggesting that only part of the high amount of Cu accumulated in roots was effectively translocated to the shoot. This is typical of the ability of Cu to accumulate in roots without a proportional transport into aerial parts (Kidd et al. 2007). When cultivated in vineyard soils, *F. rubra* plants

Table 3 Selected soil properties of the six vineyard soils (Pan, Spr, Lor, Ill, Sol, Tez) and of the control soil (Contr)

Parameters	Control	Pan	Spr	Lor	Ill	Sol	Tez
pH (H ₂ O)	7.92±0.05 a	7.95±0.08 a	7.41±0.08 c	7.40±0.13 c	7.68±0.05 b	5.80±0.09 d	7.30±0.05 c
OM (g kg ⁻¹)	16.14±1.69 d	11.89±2.16 d	23.92±2.24 c	16.31±2.97 d	32.60±2.98 b	55.32±4.22 a	33.4±2.65 b
Texture	Sandy loam	Clay	Sandy loam	Sandy loam	Sandy clay loam	Sandy loam	Loam
Clay (%)	14.02±2.16 c	41.98±3.32 a	19.96±2.65 c	16.34±1.99 c	31.53±2.46 b	10.11±1.10 d	18.31±2.19 c
Silt (%)	26.11±2.28 a	22.23±1.87 c	26.00±1.39 b	21.80±2.04 c	18.04±2.95 c	15.89±1.24 d	32.11±2.54 a
Sand (%)	59.87±4.51 c	35.79±2.88 d	54.04±3.97 c	61.86±4.56 b	50.43±3.22 c	74.00±3.29 a	50.38±4.22 c
CEC (cmol(+) kg ⁻¹)	17.92±1.23 c	25.82±2.13 b	32.77±2.87 a	24.04±2.21 b	33.33±3.65 a	30.03±3.04 a	33.39±3.12 a
Cu (mg kg ⁻¹ DW)	36.74±3.25 f	121.05±8.15 e	276.04±15.32 d	306.32±10.78 c	234.80±25.36 d	868.50±26.66 a	719.12±24.05 b

OC organic carbon, OM organic matter, CEC cation exchange capacity

Each value was the mean of three replicates. Values marked with different letters along rows are significantly different ($P < 0.05$, ±STD)

Table 4 Extractable soil Cu (Cu_{EDTA}) at the start of the experiment, and Cu_{EDTA} at the end of the experiment following *Festuca rubra* and *Sinapis alba* plant growth, or no plant growth

	Extractable Cu (Cu_{EDTA}) mg kg^{-1}	Cu_{EDTA} (mg kg^{-1}) no plants	Cu_{EDTA} (mg kg^{-1}) post <i>F. rubra</i>	Cu_{EDTA} increase (%) post <i>F. rubra</i>	Cu_{EDTA} (mg kg^{-1}) post <i>S. alba</i>	Cu_{EDTA} increase (%) post <i>S. alba</i>
Contr	10.25±0.43 b	10.21±0.21	12.71±0.58 a	24.0 %	10.75±0.52 b	4.9 %
Pan	17.64±0.67 c	16.03±1.64	32.32±1.01 a	83.2 %	29.04±0.68 b	64.6 %
Spr	135.18±3.59 b	134.32±4.66	151.89±2.13 a	12.4 %	149.05±3.21 a	10.3 %
Lor	206.53±5.88 b	205.91±3.84	215.10±2.69 a	4.1 %	206.92±3.45 b	0.2 %
Ill	149.04±4.12 b	144.34±2.13	164.54±4.00 a	10.4 %	165.93±4.12 a	11.3 %
Sol	534.60±8.95 b	529.35±6.99	570.63±6.01 a	6.7 %	549.33±5.02 b	2.7 %
Tez	373.10±5.89 b	364.00±6.45	401.80±5.33 a	7.7 %	394.20±6.54 b	5.7 %

Values are calculated on a dry weight basis and each one is the mean of three replicates. The percentage of Cu_{EDTA} increase in soil pots by plants is also reported. Different letters indicate significant differences along rows, i.e. differences between the concentration of Cu_{EDTA} at the start of the experiment and after plant growth ($P < 0.05$, $\pm \text{STD}$)

showed higher potential to copper bioaccumulation than *S. alba*, as confirmed by greater BAF values.

Cu concentration in the roots positively correlated with the shoot Cu concentration ($R = 0.76$, $P = 0.05$ for *F. rubra*, $R = 0.83$, $P = 0.023$ for *S. alba*). The root Cu concentration of both plant species also significantly correlated with soil total Cu

concentrations and Cu_{EDTA} concentrations. A higher correlation was recorded in *S. alba*, with $R^2 = 0.78$ ($P = 0.007$) for total Cu and $R^2 = 0.68$ ($P = 0.022$) for Cu_{EDTA} (Fig. 2a, b), compared with *F. rubra* ($R^2 = 0.62$, $P = 0.036$ for total Cu and $R^2 = 0.56$, $P = 0.05$ for Cu_{EDTA} ; Fig. 2c, d).

Plant growth and Cu phytoextraction

Shoot biomass for *F. rubra* and *S. alba* plants was affected by the vineyard soil in which they were grown. *S. alba* produced more shoot biomass when cultivated in the vineyard soils than in the control soil, except when plants were grown in “Pan” soil (Table 6). Similarly, the biomass of *F. rubra* plants was also reduced in “Pan” soil and only plants grown in the “Ill” and “Sol” soils showed greater shoot biomass than plants of the control soil (Table 6).

To evaluate the phytoextraction potential of the two plant species, total Cu accumulation in the plant shoot was calculated (Table 6). *F. rubra* plants contained more Cu in the shoot than *S. alba* plants. Maximum values of Cu accumulation were observed in both plant species grown in “Sol” soil (323 and 108 mg Cu per shoot for *F. rubra* and *S. alba* plants, respectively). Interestingly, despite “Pan” soil having fourfold higher Cu amounts than the control soil, shoot Cu accumulation in both plant species cultivated in Pan soil was significantly lower than in control plants.

Discussion

Hydroponic experiment

To assay whether hydroponic systems could represent an effective screening tool for Cu extracting plant species, two grasses, *F. rubra* and *S. alba*, were grown in nutrient solution in the presence of different copper dosages. This method allowed to evaluating differences in Cu tolerance and

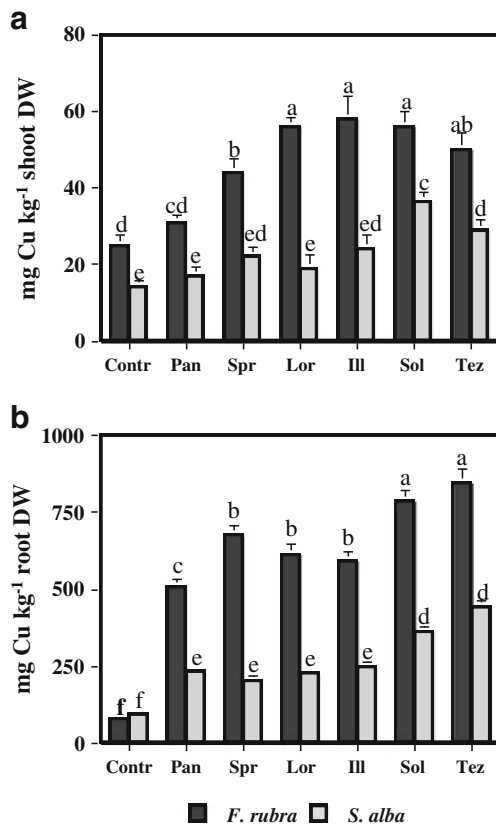


Fig. 1 Concentration of copper in the shoot (a) and roots (b) of *F. rubra* cv Merlin and *S. alba* plants grown for 120 days in the control soil (Contr) or one of the six vineyard soils. Data are the means of six replicates of three independent experiments ($\pm \text{STD}$). Letters above bars for shoots or roots indicate significant differences between *F. rubra* and *S. alba* plants, and between soils ($P < 0.05$)

Table 5 Translocation and bioconcentration factors for *F. rubra* and *S. alba* plants

	Control	Pan	Spr	Lor	Ill	Sol	Tez
<i>F. rubra</i>							
TF	0.23±0.04 a	0.06±0.01 b	0.07±0.01 b	0.09±0.01 b	0.09±0.02 b	0.07±0.01 b	0.06±0.01 b
BAF	0.68±0.11 a	0.26±0.04 b	0.16±0.03 c	0.19±0.02 c	0.25±0.04 b	0.06±0.01 d	0.07±0.01 d
<i>S. alba</i>							
TF	0.15±0.02 a	0.08±0.01 b	0.09±0.01 b	0.08±0.01 b	0.09±0.01 b	0.10±0.01 b	0.07±0.01 b
BAF	0.46±0.09 a	0.15±0.03 b	0.08±0.02 cd	0.06±0.01 d	0.09±0.02 c	0.04±0.00 e	0.04±0.00 e

Translocation factor (TF) is the Cu concentration in the shoots divided by the Cu concentration in the roots, Bioaccumulation factor (BAF) is the Cu concentration in the shoot divided by the Cu concentration in the soil. Different letters indicate significant differences along rows ($P < 0.05$, $\pm$ STD)

accumulation between the two plant species without the interference of the physical and chemical properties of soil.

In this study, excess of Cu increased the dry weight to fresh weight ratio as Cu, similarly to other heavy metals, can disturb the plant water status, which eventually results in osmotic stress and growth reduction (Poschenrieder and Barcelo 2004).

F. rubra cv Merlin displayed a significant decrease in tolerance to Cu when exposed to concentration as low as 5 and 10 mg l⁻¹ Cu. In comparison, a work by Wong et al. (1994) reported plants were able to tolerate up to 20 mg l⁻¹ Cu without any physiological symptoms. It must be considered that Cu concentration we detected in tissues of *F. rubra* plants

was higher, likely because Wong et al. (1994) used different experimental conditions, such as the iron concentration in the nutrient solution that was 10 times higher. As Cu can compete with Fe for uptake by plants (Chaignon et al. 2002; Michaud et al. 2007), high Cu accumulation we observed in plant tissues could be due to the lower Fe/Cu ratio and to the competitive advantage of Cu over iron. As a consequence, excess Cu can induce Fe-deficiency and thereby leaf chlorosis (Lanars et al. 1993). In our study, *F. rubra* showed a decrease in the content of chlorophyll when supplied with 5 and 10 mg l⁻¹ Cu, while only in *S. alba* plants exposed to 10 mg l⁻¹ Cu the chlorophyll content decreased significantly. It has been reported that chlorophyll accumulation in Cu-

Fig. 2 Linear correlation of root copper concentration with total soil copper for *S. alba* (a) and *F. rubra* cv Merlin (c). Linear correlation of root copper concentration with extractable copper (Cu_{EDTA}) for *S. alba* (b) and *F. rubra* cv Merlin (d) plants ($n=3$) were grown for 120 days in the control soil (Contr) or one of the six vineyard soils

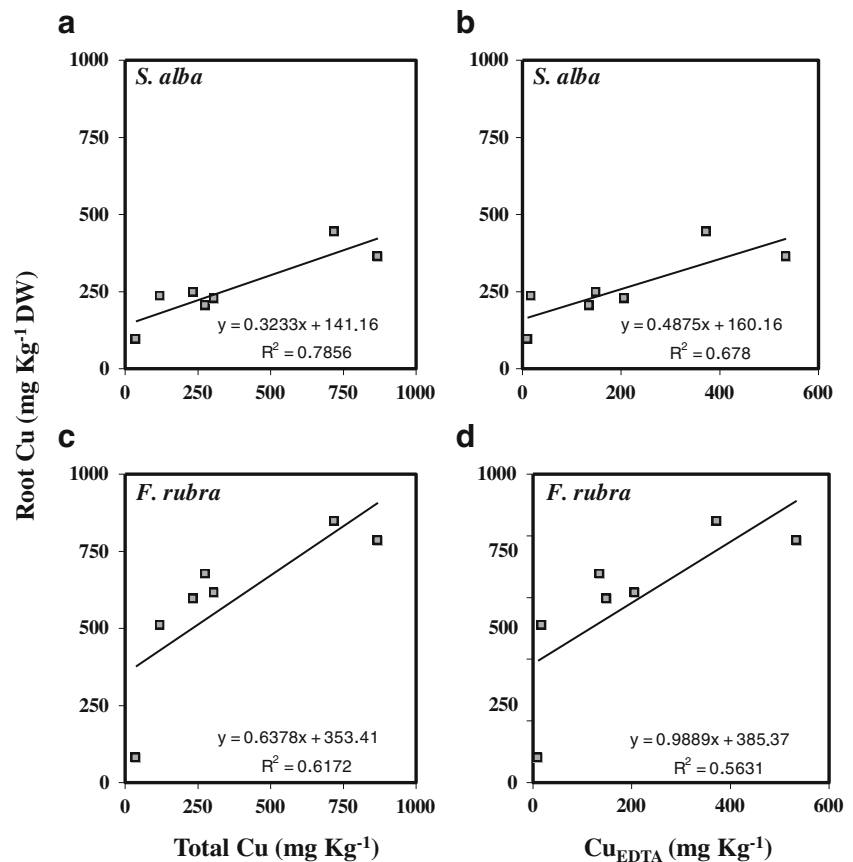


Table 6 Dry weight and total Cu of the shoot biomass for *F. rubra* and *S. alba* plants grown in the different soils

	Shoot biomass DW(g)		Shoot Cu content (μg)	
	<i>F. rubra</i>	<i>S. alba</i>	<i>F. rubra</i>	<i>S. alba</i>
Contr	2.17 $\pm$ 0.52 c	1.27 $\pm$ 0.10 c	82.39 $\pm$ 12.03 d	21.53 $\pm$ 2.56 c
Pan	1.65 $\pm$ 0.12 d	0.76 $\pm$ 0.06 d	52.67 $\pm$ 10.43 e	13.59 $\pm$ 1.97 d
Spr	3.11 $\pm$ 0.11 b	3.14 $\pm$ 0.31 a	170.66 $\pm$ 8.16 c	69.13 $\pm$ 4.56 b
Lor	3.16 $\pm$ 0.13 b	2.32 $\pm$ 0.21 b	185.36 $\pm$ 9.99 c	44.03 $\pm$ 5.09 c
Ill	3.74 $\pm$ 0.48 b	2.73 $\pm$ 0.19 ab	224.38 $\pm$ 14.71 b	65.41 $\pm$ 4.67 b
Sol	5.67 $\pm$ 0.98 a	2.85 $\pm$ 0.25 ab	323.09 $\pm$ 22.12 a	108.42 $\pm$ 8.22 a
Tez	3.37 $\pm$ 0.29 b	3.32 $\pm$ 0.28 a	178.61 $\pm$ 14.42 c	96.15 $\pm$ 4.21 a

Shoot Cu contents are calculated on the basis of the dry weight. Different letters along columns indicate significant differences ($P < 0.05$, $\pm$ STD)

treated plants depends on differences in tolerance of plant species to Cu toxicity (Mysliwa-Kurdziel et al. 2004).

In comparison to *F. rubra*, *S. alba* showed higher tolerance to Cu and lower Cu concentrations in tissues. Differences in Cu tolerance and concentration between *F. rubra* and *S. alba* could be due to the mechanisms that control metal absorption and accumulation, such as the regulation of specific plasma membrane Cu transporters (Sancenón et al. 2003) and/or the synthesis of intracellular chelators (i.e. metallothioneins, Ma et al. 2003).

It is noteworthy that when both plant species were grown under adequate Cu levels, the Cu concentration in shoots and roots was comparable. On the other hand, when there was excess of Cu in the nutrient solution, the element was more concentrated at the root level. A possible explanation is that plants can reduce Cu translocation to the shoot to limit potential phytotoxic effects (Brun et al. 2001; Michaud et al. 2007; Schiavon et al. 2007). This aspect must be taken into account in programmes of Cu phytoextraction as high level of copper in the growth medium can affect plant biomass.

No differences in total Cu accumulation were detected between *S. alba* plants cultivated with 5 mg Cu l⁻¹ and 10 mg Cu l⁻¹ despite the differences in Cu tissue concentration. This was due to the reduction of biomass recorded at the maximum Cu dose, as deduced by the TI. On the contrary, *F. rubra* plants accumulated more Cu at the highest dose of Cu, regardless of the strong reduction in plant growth. These results indicate that although *S. alba* was more tolerant of excess Cu than *F. rubra*, the latter accumulated more Cu in the shoot when exposed to high Cu and, for this reason, may be more feasible for phytoextraction of Cu from Cu contaminated soils such as in vineyards.

Pot study experiment

To confirm the results obtained from the hydroponic experiment, *F. rubra* and *S. alba* were grown in pots filled with Cu

contaminated soils from six vineyards. Similar to the hydroponic experiment, there was a strong correlation between either soil total Cu or Cu_{EDTA} and Cu concentration in roots for both *F. rubra* and *S. alba*. The preferential concentration of Cu in root tissues was also observed in *F. rubra* and *S. alba* plants cultivated in soil. Furthermore, the higher BAF values showed by *F. rubra* cultivated in vineyard soils indicated that this plants species is more suitable for the remediation of copper-contaminated sites than *S. alba*. Higher BAF values can be obtained in hyperaccumulator plants, such as *Thlaspi caerulescens*, where copper accumulates more than 1,000 mg Cu kg⁻¹ in shoots (McGrath et al. 2001). Unfortunately, hyperaccumulators often have low biomass and slow growth, and they are not suitable for Cu phytoextraction in vineyards, consequently. It is noteworthy that BAF values for *F. rubra* and *S. alba* strongly decreased when grown in vineyard soils with Cu concentration higher than 700 mg Cu kg⁻¹. The same trend was observed in peanut plants grown in soils with greater than 500 mg Cu kg⁻¹ (Andreazza et al. 2011).

In this study the level of organic matter influenced the growth of both plant species more than the amount of total and bioavailable copper. When plants were grown in the vineyard soil with the lowest Cu quantity and the lowest OM content (Pan), a significant reduction ($P < 0.05$) of biomass was observed compared to plants cultivated in the control soil and in the other vineyard soils. In “Ill”, “Tez”, and “Sol” soils, characterized by high OM content (over 32 g kg⁻¹), plants produced more biomass than the control despite the high levels of soil total and extractable Cu. In comparison, organic carbon concentration and clay content were not correlated to Cu concentrations in wheat plants cultivated in Cu-contaminated soils (Michaud et al. 2007).

S. alba plants were likely able to better use soil OM than *F. rubra* as these plants produced more biomass compared to the controls when cultivated in “Spr” soil, which had an OM content of about 24 g kg⁻¹. The high level of CEC in “Spr”, “Ill”, “Sol”, and “Tez” soils could also have influenced the high plant biomass production, as it is known that Cu phytotoxicity decreases with increasing soil CEC (Ruyters et al. 2013).

Both *F. rubra* and *S. alba* plants enhanced the mobility of Cu in soil by increasing the amount of Cu_{EDTA} and, in most of the soils investigated, *F. rubra* significantly increased it ($P < 0.05$) compared to *S. alba*. It is known that plants can alter the bioavailability of copper in the rhizosphere by modifying pH (Chaignon et al. 2002; Hinsinger 2001) or by releasing organic compounds into the rhizosphere (Chaignon et al. 2002; Cattani et al. 2006). Michaud et al. (2007) found that in calcareous soils the release of phytosiderophores might explain the greater extractability of Cu observed in the rhizosphere, and the increased uptake of Cu by plants. Thus it is possible that *F. rubra* could have exuded more phytosiderophores than *S. alba*, but this still requires further study to confirm.

Larger field scale studies will also be required to determine if the increase in Cu observed in this study has the potential to significantly increase Cu leaching, which could affect the quality of groundwater near vineyards.

Conclusions

The results of this study indicate that *F. rubra* cv Merlin is able to accumulate more Cu in plant tissues than *S. alba*, regardless of substrate and Cu availability. Therefore, it could be taken into consideration for future phytoextraction studies. In vineyards, *F. rubra* could be periodically mowed from the inter rows during the growing season and the shoot tissues removed to decrease the Cu content of the soil. The harvested shoot biomass could be diverted into specific feeding programs, where copper must be added to the animal diet, or disposed of in confined areas. Considering a density of 200 plants per m² (density usually found in grassy area) in the vineyard inter rows (covering about two thirds of a hectare), three shoot cuttings per year (common practice in Italian vineyards), a soil OM higher than 24 g kg⁻¹ and a soil Cu content within the range 200–800 mg kg⁻¹, the amount of Cu that can be potentially removed by *F. rubra* cv Merlin could vary between 0.6 kg and 1.3 kg per hectare per year. As the concentration of Cu in soil is variable, Cu phytoextraction will vary with location and time, which can increase the overall time required to decrease Cu in soils in vineyards.

F. rubra and *S. alba* might be employed in vineyard soils with characteristics comparable to those described in this study. Otherwise, the effective capacity of these plant species to remove copper must be evaluated.

The first step for the identification of other plant species suitable for the remediation of copper from vineyard soils could be the realization of simple hydroponic tests, given the correlation between the results from hydroponic and soil experiments.

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