



Application of viticultural by-product biochar to alleviate soil copper toxicity in vineyard

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Abstract

The widespread application of copper (Cu) based fungicides in viticulture has led to significant copper accumulation in vineyard soils, threatening both ecosystem integrity and soil health. Reducing copper bioavailability in these soils is therefore crucial to promote environmental sustainability. Biochar, a porous carbon-rich amendment, offers a sustainable solution within a circular economy framework due to its well-documented sorptive properties, which allow biochar to immobilize copper, reducing its bioavailability in soils. This study evaluates the capacity of five biochars derived from vineyard by-products (grape marc, seeds, prunings) for retaining copper in soils through a percolation experiment. Results showed that copper concentrations in leachates were reduced by 20 to 98% in biochar-amended soils compared to unamended soils. Additionally, a phytotoxicity assay using *Phaseolus vulgaris* was conducted to assess biochar's impact on plant growth and copper accumulation. Biochar-amended soils showed a reduction in copper pore water concentration ranging from 61 to 91% compared to the Cu spiked soil, which corresponded to up to four times higher plant growth. Furthermore, biochar addition restored soil hydrolytic enzymatic activities, with values fourfold higher than those observed in the Cu spiked soil. These findings highlight the potential of vineyard by-products derived biochar, and especially grape marc biochar as an effective tool to mitigate copper toxicity in contaminated soils, thereby supporting sustainable agriculture and the preservation of ecosystems and biodiversity.

Highlights

- Biochars from vineyard by-products, especially from grape marc, reduced copper mobility and bioavailability in contaminated soils by up to 98%, demonstrating high immobilization efficiency.
- Copper immobilization was driven by strong sorptive interactions and a biochar-induced pH increase, which collectively altered copper speciation and reduced toxicity.
- Biochar amendments significantly improved *Phaseolus vulgaris* growth and restored key soil microbial enzymatic activities even under severe copper stress.
- Results highlight biochar's potential as a circular, sustainable remediation strategy for mitigating Cu toxicity.

Keywords Biochar · Copper Contamination · Vineyard Soils · Phytotoxicity · Circular Economy · Climate Action

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Introduction

Copper-based fungicides have been widely used in viticulture for decades, mainly to control the downy mildew causal agent, *Plasmopara viticola* a major constraint in grapevine culture (Pump et al. 2019). The antifungal properties of copper are largely attributed to its ability to degrade RNA and compromise cell membrane integrity (Garinie et al. 2024). Despite advancements in disease management and control, copper-based fungicides remain essential for vineyard protection. Moreover, their continuous application led to significant copper accumulation in soil (Gonzaga et al. 2020), particularly within the topsoil layers (Romi et al. 2014). Vineyard agroecosystems are among the most affected by copper inputs (Pietrzak and McPhail 2004; Ruyters et al. 2013), often exceeding the European Union's recommended thresholds for sustainable agriculture (Komárek et al. 2010). The average copper concentration in European agricultural soils is around 16.85 mg kg⁻¹, while vineyard soils show copper concentration levels three times higher reaching 49.26 mg kg⁻¹ (Ballabio et al. 2018).

Moreover, elevated copper levels pose serious ecological risks by disrupting soil microflora and microfauna, causing phytotoxicity, and contaminating aquatic ecosystems through runoff and leaching.

Although copper is an essential micronutrient, and serves as a cofactor for multiple plant enzymes, including the antioxidant Cu/Zn-Superoxide Dismutase (SOD), and plays a key role in photosynthesis via plastocyanin (Wang et al. 2024), elevated concentrations are toxic to plants. Symptoms of copper excess were observed in multiple plant organs; roots are reduced in size, showing abnormal morphology and darkening; furthermore root hair growth is inhibited (Wang et al. 2024). Root symptoms are especially important on young vines with shallower root systems (Ambrosini et al. 2015; Cesco et al. 2021), making root morphology a reliable indicator of copper stress. At the foliar level, copper photosynthetic inhibition leads to leaf chlorosis (Chen et al. 2022). Copper toxicity has also been documented in other plant species cultivated on former Cu-contaminated vineyard soils such as *Triticum turgidum durum* L. (Michaud et al. 2007).

Soil microbial communities are highly sensitive to copper contamination; high copper levels in soils are related to reduced diversity and abundance of fungi and bacteria, with fungi being more sensitive than bacteria thanks to bacterial ability to develop resistance (Ge and Zhang 2011; Altimira et al. 2012; Cavani et al. 2016). Copper-resistant bacteria are prevalent in contaminated soils (Dell'Amico et al. 2008); nonetheless, microbial sensitivity remains high, shown by high reduction in key soil enzymatic activities such as dehydrogenase, β -glucosidase, and phosphatase at 150–200

mg kg⁻¹ of copper concentration in soil (Fernández-Calviño et al. 2010). These microbial disruptions compromise soil health and fertility (Mackie et al. 2015; Leenhardt et al. 2023). Besides, copper also threatens aquatic ecosystems through leaching (Filipović et al. 2023) and surface runoff (Banas et al. 2010; Babcsányi et al. 2016), particularly in vineyards with non-grassed slopes (Droz et al. 2021; Leenhardt et al. 2023). This contributes to aquifer pollution (Arenas-Sánchez et al. 2019), with toxic implications for aquatic life (Husak 2015).

Copper mobility in soil is mainly influenced by pH and organic matter content (Cornu et al. 2017); high pH and content of organic matter generally reduce the amount of free, mobile copper (Cu²⁺) in soil solution. However, plants and microorganisms also have an effect on copper availability, for example root exudates can change copper speciation (Brunetto et al. 2016). Natural copper concentration in soil solution typically ranges from 0.001 to 1 mg.L⁻¹ (Cesco et al. 2021; Cornu 2023). Anthropogenic copper is generally more mobile—and therefore more bioavailable—than naturally occurring pedogenic copper (Duplay et al. 2014). As heavy metals, copper cannot be biologically degraded, requiring effective remediation strategies to reduce their levels and toxicity in soil (Qiu et al. 2022). Remediation approaches include phytoextraction (Melo et al. 2022; Pruteanu et al. 2026), bacterial bioremediation (Yu et al. 2025; Vasarevičius and Paliulienė 2025), and applications of symbiotic arbuscular mycorrhizal fungi (Brunetto et al. 2019; Banerjee et al. 2025). Additionally, soil amendments, such as lime, zeolite, and calcium silicate, have been used to stabilize copper and reduce its toxicity, by increasing soil pH (Ferreira et al. 2018; Rosa et al. 2020; Kumar et al. 2025).

Recently, biochar has gained increasing attention as a sustainable, cost-effective and multifunctional tool for trace elements remediation while enhancing soil health (Tan et al. 2023; Nguyen et al. 2023). Biochar is a carbon-rich and alkaline material, produced through high-temperature pyrolysis of organic materials, from manure to lignocellulosic biomass, under low or no oxygen conditions (Hui 2021), which improves soil properties, including pH enhancement, water retention, bulk density, cation exchange capacity, and nutrient retention (Semida et al. 2019; Hui 2021; Tan et al. 2023). Biochar also promotes microbial activity and diversity (Moore et al. 2018), by providing multiple habitats for microorganisms. It's also a promising amendment to reduce Cu runoff (Gholamhadi et al. 2023) and leaching (Zhang et al. 2013). Finally, biochar contributes to climate change mitigation by storing stable carbon in the soil (Shoudho et al. 2024).

Regarding copper, biochar effectively mitigates its toxicity in soil microorganisms and plants. This could be attributed to its soil pH alkalization (Simms et al. 2020)

and decrease in the exchangeable Cu fraction by increasing the amount of Cu bound to organic matter and residual fractions, thereby reducing Cu bioavailability (Gonzaga et al. 2020; Gholami and Rahimi 2021). Biochar application enhances microbial activity and basal respiration (Moore et al. 2018), while reducing Cu uptake by roots and its translocation to plant aerial parts (Emamverdian et al. 2024). At the same time, biochar improves plant metabolic parameters such as gas exchange (Abideen et al. 2023), chlorophyll content (Deng et al. 2025), and water use efficiency (Da Cruz Ferreira et al. 2024), ultimately leading to a better plant growth.

Viticulture generates large amounts of organic residues, including grape seeds, grape marc (Muhlack et al. 2018), and vine prunings, which are often discarded or incinerated (Sun et al. 2020; Wei et al. 2022). Transforming these by-products into biochar provides an environmentally sustainable alternative that aligns with circular economy practices. Recent studies (Li et al. 2018; Jin et al. 2020; Da Silva et al. 2024) highlight that biochars derived from vineyard by-products can effectively adsorb trace metals, including copper, making them a promising tool for the remediation of vineyard soils.

Although there are many studies showing the general biochar effects in soil, most studies have focused on generic feedstocks, leaving limited knowledge on vineyard-specific biochars' efficiency in Cu immobilization and soil quality improvement. The mechanisms by which these biochars affect Cu speciation, bioavailability, and microbial dynamics, especially under high soil contamination, are poorly understood. Few studies have simultaneously assessed Cu leaching, plant responses, and microbial health, even if they are critical for sustainable remediation. Addressing these gaps is key to identify the most suitable vineyard by-product biochars and to develop viticulture-specific guidelines.

To ensure the effectiveness of any remediation approach, it is essential to implement a reliable evaluation tool. Given its sensitivity to environmental stress, French bean (*Phaseolus vulgaris*) serves as an effective bioindicator for assessing soil contamination (Dadasoglu et al. 2022). This study hypothesizes that biochar application can mitigate copper toxicity in *Phaseolus vulgaris* and soil microbial communities, while preventing Cu runoff. This study aims (1) to evaluate the ability of vineyard by-product biochars to immobilize copper in soil, prevent leaching and reduce bioavailable copper in soil pore water; and (2) to examine biochar effects on plant growth, plant copper uptake, and soil microbial health, under Cu stress. By comparing biochars produced from different vineyard by-products, the study aims to identify the most effective feedstock for Cu remediation, thereby promoting responsible viticulture and circular economy practices, while contributing to key development

goals such as supporting sustainable agriculture, ensuring responsible consumption and production systems, protecting and restoring terrestrial ecosystems, reversing land degradation, and halting biodiversity loss.

Materials and methods

Biochar production and characterization

Biochars were produced by slow pyrolysis (6 h) of several vineyard by-products (marc, prunings, seeds) at 700 °C with a heating rate of 5–10 °C/min, which favors the development of high surface area and aromatic structure compatible with Cu sorption. The biochar samples were labelled as follows: biochar from marc (**BM**); biochar from seed (**BS**); biochar from exhausted seed (**BES**); biochar from pruning (**BP1**); biochar from pruning following a double pyrolysis (**BP2**). Prior to analysis, all samples were crushed and sieved to 0.5–2-mm. Biochar pH, electrical conductivity (EC), SBET, elemental analysis and water holding capacity were determined according to Lebrun et al. (2018) and Cation exchange capacity (CEC) according to Munera-Echeverri et al. (2018).

Vineyard soil sampling

Inter-row top soil (0–20 cm) was collected in April 2023 in an old vineyard plot in Bourgueil, Centre-Val de Loire, France (47°16'48.4"N 0°11'10.8"E, altitude 40 m). The samples were air-dried and then sieved to 2 mm. In order to determine the profiles of copper and organic matter content, soil samples were collected every 10 cm to a final depth of 60 cm using a soil auger. Sampling beyond this depth was not possible due to the presence of coarse materials.

Vineyard soil proximate analysis

pH, electrical conductivity (EC), bulk density and water holding capacity of soil were measured according to methods previously described in Chafik et al. (2024). Soil texture, expressed as the percentage of clay, silt, and sand, was determined following *NF X31-107*. Soil organic matter (OM) content was estimated by measuring the mass loss of a soil sample after 24 h at 375 °C (Schulte 1995). Cation exchange capacity (CEC) was determined using the 0.05 M cobaltihexamine chloride method (*NF X 31-130*). Carbonate content in the soil was estimated by acidimetry (Pansu 2006). Dissolved organic carbon (DOC) and total nitrogen (TN) were quantified in soil pore water (SPW) extracted from the studied soil, acidified to pH 2 with phosphoric acid, and analyzed using a *Shimadzu TOC-L Total Organic*

Carbon Analyzer: Crystalline phases present in the soil were identified by X-ray diffraction (XRD) analysis performed on MINIFLEX 600 – Rigaku, over an angular range of 10° – 90° (2 θ).

Metal and ion contents determination

Ion concentrations in the studied soil samples (2 mL – 1:10 w/v) were determined by ionic chromatography (Thermo Scientific DIONEX AQUION Ion Chromatography System). Metal analysis was conducted using inductively coupled plasma atomic emission spectroscopy (ICP-AES), with a ULTIMA 2 instrument (HORIBA, Labcompare, San Francisco, USA). Standard copper stock solutions at various concentrations were used to generate calibration curves.

Two different sets of samples were collected: soil liquid fractions such as soil pore water (SPW) or soil extracts – to assess copper biodisponibility – obtained by different extraction methods – aqueous extraction (1:10 w/v ratio), 1.0 M EDTA extraction (1:10 w/v ratio), 1.0 M DTPA extraction (1:10 w/v ratio), 0.1 M CaCl_2 extraction (1:10 w/v ratio). Each different fraction and SPW were then acidified to a final concentration of 0.15 M HNO_3 . For solid samples, including plant (*Phaseolus vulgaris*) aerial parts and roots as well as the soil itself, 200 mg of each powdered, dried sample were digested in aqua regia (1:2 v/v) using a microwave digestion system (Multiwave 3000; Anton Paar GmbH, Germany) at 180 °C for 45 min and then filtered. Finally, all samples were passed through ICP-AES measurement to estimate their Cu concentration.

Table 1 Abbreviations and descriptions of treatment mixtures applied to vineyard soil doped with Cu ($1452 \pm 99 \text{ mg} \cdot \text{kg}^{-1}$) and amended with different types and rates of biochar (% w/w)

Abbreviation	Copper addition	Biochar type	Application dose (%)	Replicates (n)
C (control)	X	-	-	3
SPK (Cu spiked)	✓	-	-	3
BP1 2%	✓	Pruning 1 pyrolysis	2%	3
BP1 4%	✓	Pruning 1 pyrolysis	4%	3
BP2 2%	✓	Pruning 2 pyrolysis	2%	3
BP2 4%	✓	Pruning 2 pyrolysis	4%	3
BS 2%	✓	Grape Seed	2%	3
BS 4%	✓	Grape Seed	4%	3
BES 2%	✓	Exhausted Grape Seed	2%	3
BES 4%	✓	Exhausted Grape Seed	4%	3
BM 2%	✓	Grape Marc	2%	3
BM 4%	✓	Grape Marc	4%	3

Sequential extraction

To determine the proportion of mobile copper in the soil, a sequential extraction was performed using a method adapted from Pérez-Cid et al. (1998) and modified by Pascaud et al. (2014). Copper distribution across various soil fractions (F1, F2, F3, and F4) was thus assessed. The mobility factor (MF) was then calculated from the contents obtained in the different fractions using Eq. (1).

$$\text{Mobility Factor (MF)} = \frac{[Cu]_{F1}}{[Cu]_{F1} + [Cu]_{F2} + [Cu]_{F3} + [Cu]_{F4}} \% \quad (1)$$

Percolation experiment

A percolation experiment was designed to evaluate the ability of biochar to retain copper in this specific vineyard soil. Pots ($6 \times 6 \times 6 \text{ cm}$), lined with a paper filter, were filled with 200 g of dry soil (sieved to 2 mm) mixed with 2% or 4% (w/w) of different types of biochar ($n=3$). The soil was spiked with dry CuCl_2 powder to achieve a final Cu concentration of $1452 \pm 99 \text{ mg} \cdot \text{kg}^{-1}$, allowing the assessment of biochar effects under high copper contamination and ensuring Cu availability in the soil pore water (SPW) above reference values ($0.001\text{--}1 \text{ mg} \cdot \text{L}^{-1}$ (Cesco et al. 2021; Cornu 2023)). Such high spiking ($>1000\text{--}1500 \text{ mg} \cdot \text{kg}^{-1}$), corresponding to the most adverse contamination conditions reported in vineyard soils (Komárek et al. 2010; Romi et al. 2014; Brunetto et al. 2016; Cesco et al. 2021), ensured a sufficient response, and had already been evaluated in controlled experimental systems (Fontanive et al. 2023; Lv et al. 2023; Sheng et al. 2024). Table 1 summarizes the different soil mixtures, treatments abbreviations, proportions of biochar (w/w) and copper addition ($\text{mg} \cdot \text{kg}^{-1}$). Then the pots were saturated with deionized water overnight via capillary action. Then they were subsequently drained for several hours, and the leachates were analyzed for metal concentration to determine the exact Cu content in soils.

Subsequently, 25 mL of deionized water was added to the top of each pot, and the percolate was collected after 1 h of drainage. This procedure was repeated three more times. The pH, electrical conductivity (EC), and copper concentration of each percolate were measured; however, only the pH and EC data from the final percolate were presented. The percentage of copper recovered in the percolates after four water additions relative to the total copper initially present in the pots was determined for both control and biochar-amended treatments using ICP-AES. These measurements were used to assess the effectiveness of the biochars in retaining copper in the soil. In addition, copper sorption per

gram of biochar within the pots was calculated by comparing with the Cu-control (SPK).

Moreover, in order to deeply understand marc biochar (BM) dynamics, a supplementary experiment was performed using only BM at different concentration: 0, 0.5, 1, 2, 3 and 4% (w/w, $n=4$).

Toxicity assessment using *Phaseolus vulgaris* seedlings

Three days before planting, *Phaseolus vulgaris* seeds were pre-germinated on moistened paper using deionized water, and incubated in the dark at a constant temperature of 22 ± 2 °C and 65% relative humidity. Seedlings were then sown in each pot prepared as explained above (Table 1) and grown for 21 days in a phytotronic chamber under controlled conditions (16-hour photoperiod, 8-hours of darkness, 20 ± 2 °C, and 65% relative humidity).

Pots were placed in individual cups according to each treatment ($n=3$) and watered 3 times a week with tap water. After the growth period, plant height was recorded. The plants were then harvested and leaves, stems, and roots were separated. Roots were thoroughly washed with tap water to remove all soil particles while preserving the entire root biomass. The dry weight of each organ was measured after drying overnight at 100 °C in an oven. Copper concentrations in *Phaseolus vulgaris* organs were subsequently analyzed following the acid digestion procedure described above for soils. Copper Mobility Index (MI) was calculated using Eqs. (2–4) to determine copper mobility through the soil–plant system (Chafik et al. 2025a).

$$\begin{aligned} \text{Soil-Roots Mobility Index (MI}_{\text{Soil-Roots}}) \\ = \frac{[\text{Mean Cu in roots}] \text{ mg.kg}^{-1}}{[\text{Mean Cu in soil}] \text{ mg.kg}^{-1}} \end{aligned} \quad (2)$$

$$\begin{aligned} \text{Root-Stems Mobility Index (MI}_{\text{Roots-Stems}}) \\ = \frac{[\text{Mean Cu in stems}] \text{ mg.kg}^{-1}}{[\text{Mean Cu in roots}] \text{ mg.kg}^{-1}} \end{aligned} \quad (3)$$

$$\begin{aligned} \text{Stems-Leaves Mobility Index (MI}_{\text{Stems-Leaves}}) \\ = \frac{[\text{Mean Cu in leaves}] \text{ mg.kg}^{-1}}{[\text{Mean Cu in stems}] \text{ mg.kg}^{-1}} \end{aligned} \quad (4)$$

At the same time, soil pore water (SPW) was recovered from each pot using soil moisture samplers (Rhizon™, Model MOM, Rhizosphere Research Products, Wageningen, The Netherlands) after plant growth. Then, pH, EC, ions and copper concentration were determined, following methods previously mentioned. To complement the ICP metal estimation, toxicity tests on *Lepidium sativum* we carried out on

SPW samples according to procedures outlined in Bożym et al. (2021) and normalized as a percentage of deionized water control.

Enzymatic activity test and Biolog™ plates

To evaluate soil health, four enzymatic activities (i.e., alkaline phosphatase, intracellular enzyme activity, β -glucosidase, and urease) of each treatment were assessed. In addition, the functional diversity and metabolic activity of microbial communities were evaluated using Biolog™ EcoPlates, which assess the ability of microbes to utilize a range of carbon sources (detailed protocols available in supplementary data).

Data analysis

Data processing was conducted using *Microsoft Excel 2024*. Results are expressed as mean $\pm$ standard deviation. For each variable, normality of residuals was assessed using the Shapiro-Wilk test, where $p > 0.05$ indicates that residuals do not significantly deviate from a normal distribution. Homogeneity of variances across treatments was assessed using Levene's test, with $p > 0.05$ indicating homogeneity of variances across treatments. When both assumptions were met, ANOVA followed by Tukey's post-hoc test was applied to identify significant differences ($p < 0.05$). For not normally distributed data, the Kruskal-Wallis test was applied. Effect sizes were quantified using omega squared (ω^2) for ANOVA and eta squared (η^2) for Kruskal-Wallis tests to indicate practical significance. Pearson's correlation and Principal Component Analysis were also performed – using R (v. 4.3.2).

Results and discussion

Soil and biochars characterization

Biochars properties

All tested biochars exhibited high pH, electrical conductivity and CEC. In particular, grape marc derived biochar (BM) showed interesting values (pH 11.74 ± 0.01 ; EC: 15789.6 ± 243.0 $\mu\text{S.cm}^{-1}$; CEC: 46.65 ± 1.84 cmol.kg^{-1}) (Table 2). Among the pruning-derived biochars, BP1 displayed the highest WHC potential along with high S_{BET} values (WHC: 386%; S_{BET} : $185 \text{ m}^2.\text{g}^{-1}$). Furthermore, as supporting information, preliminary unpublished copper sorption experiments also highlighted the promising Cu adsorption capacity of vineyard by-products biochar, following the Langmuir isotherm for BM and seed biochars

Table 2 Physicochemical properties of the studied soil and biochars, Cu concentrations extracted using various reagents (H_2O ; CaCl_2 0.1 M; EDTA 1 M; DTPA 1 M; total by aqua regia extraction), and Cu speciation results. Data are expressed as mean $\pm$ standard deviation ($n=4$). The different letters indicate significant differences between biochar characteristics ($p<0.05$) ($n=4$). BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

Parameters	Soil	BM	BP1	BP2	BS	BES
pH	7.81 $\pm$ 0.03	11.74 $\pm$ 0.01	a	10 $\pm$ 0.02	b	9.7 $\pm$ 0.02
EC ($\mu\text{S}\cdot\text{cm}^{-1}$)	43.3 $\pm$ 3.7	15789.6 $\pm$ 243	a	3892.3 $\pm$ 230.3	b	1502.8 $\pm$ 60.2
CEC ($\text{cmol}\cdot\text{kg}^{-1}$)	0.55 $\pm$ 0.24	46.65 $\pm$ 1.84	a	36.98 $\pm$ 0.77	b	20.7 $\pm$ 3
WHC (%)	34.6 $\pm$ 0.33	164 $\pm$ 4	d	386 $\pm$ 9	a	196 $\pm$ 5
S_{BET} ($\text{m}^2\cdot\text{g}^{-1}$)	-	55	185	253	79	214
C (%)	-	63.38 $\pm$ 1.5	c	77.51 $\pm$ 0.62	b	80.58 $\pm$ 0.9
H (%)	-	1.28 $\pm$ 0.09	bc	1.9 $\pm$ 0.11	a	1.97 $\pm$ 0.27
N (%)	-	1.41 $\pm$ 0.05	a	0.86 $\pm$ 0.03	bc	0.68 $\pm$ 0.03
O (%)	-	32.63 $\pm$ 1.47	a	18.63 $\pm$ 0.88	bc	15.53 $\pm$ 1
Bulk density ($\text{g}\cdot\text{cm}^{-3}$)	1.48 $\pm$ 0.1					
OM (%)	0.92 $\pm$ 0.07					
sand (%)	65					
silt (%)	8					
clay (%)	27					
CaCO_3 (%)	4.42 $\pm$ 0.3					
[DOC] ($\text{mg}\cdot\text{L}^{-1}$)	91.27 $\pm$ 11.47					
[TN] ($\text{mg}\cdot\text{L}^{-1}$)	9.73 $\pm$ 1.25					
Cu content ($\text{mg}\cdot\text{kg}^{-1}$)						
H_2O	1.36 $\pm$ 0.26					
CaCl_2 0.1 M	0.35 $\pm$ 0.06					
DTPA 1 M	15.07 $\pm$ 0.62					
EDTA 1 M	20.00 $\pm$ 0.72					
Total	34.69 $\pm$ 3.36					
Cu Sequential extraction						
Acid-soluble fraction (%)	10.76 $\pm$ 0.79					
Reducible fraction (%)	21.51 $\pm$ 0.92					
Oxydable fraction (%)	17.82 $\pm$ 1.84					
Residual fraction (%)	49.91 $\pm$ 4.83					
Mobility factor (%)	10.76 $\pm$ 0.83					

(BS, BES), while fitting better to the Freundlich isotherm for both pruning biochars (BP1, BP2). Additionally, the Q_{max} were 134.26, 44.03, 47.84, 56.41 and 45.77 $\text{mgCu}\cdot\text{gBiochar}^{-1}$, for BM, BS, BES, BP1 and BP2, respectively (*unpublished data*).

Vineyard soil characteristics

According to *Geoportail* (<https://www.geoportail.gouv.fr>), the soil in the studied area is classified as a brunisol, developed from unconsolidated alluvial sediments forming a sedimentary rock, as reported in *InfoTerre* (<https://infoterre.brgm.fr>). This information provides a clear pedological and geological context for the study area, allowing a better understanding and reproducibility of the observed processes. Table 2 summarizes its main physicochemical characteristics, including total and bioavailable Cu concentrations, and sequential extraction data.

The soil is slightly alkaline (pH 7.81 $\pm$ 0.03), marginally calcareous (CaCO_3 : 4.42% $\pm$ 0.3), and exhibits low cation

exchange capacity (CEC: 0.55 $\pm$ 0.24 $\text{cmol}\cdot\text{kg}^{-1}$), likely due to its sandy texture (65%), low organic matter (OM: <1%), and low clay content (Rowell 1994). X-ray diffraction (XRD) analysis (Fig. S1) confirmed a mineralogical composition dominated by quartz (88.2%) and orthoclase K-feldspar (11.8%), consistent with a siliceous, sandy matrix with low reactivity. Despite low OM, the soil exhibited a relatively high dissolved organic carbon (DOC) concentration (91.27 $\pm$ 11.47 $\text{mg}\cdot\text{L}^{-1}$), suggesting that a significant proportion of Cu^{2+} may exist as Cu–DOC complexes (Filipović et al. 2023).

Total Cu concentration (*aqua regia* extraction) averaged 35 $\text{mg}\cdot\text{kg}^{-1}$; however, bioavailable Cu levels were significantly lower (0.35 $\pm$ 0.06–20.00 $\pm$ 0.72 $\text{mg}\cdot\text{kg}^{-1}$). Although total copper content in soil is an important value, what is more relevant in pollution risk assessment is the bioavailable metal concentration (Komárek et al. 2010). Sequential extraction showed the following Cu distribution: acid-soluble Cu (water-soluble and carbonate-bound) accounted for 10.76 $\pm$ 0.79%, reducible (Fe/Mn (hydr)oxides)

21.51±0.92%, oxidizable (organic matter) 17.82±1.84%, and residual (mineral matrix) 49.91±4.83%. The calculated mobility factor (10.76±0.83%) indicates a low environmental risk of Cu release (Pérez-Cid et al. 1998; Zafeiriou et al. 2023), which is consistent with Cu being predominantly associated with soil components (Cornu et al. 2022).

Cu concentrations varied with depth (Fig. S2), being lowest at the deepest sampling region (below 40 cm, 25–29 mg·kg⁻¹), the highest between 20 and 40 cm (34–38 mg·kg⁻¹), and intermediate at the surface (35–47 mg·kg⁻¹). Indeed, Cu concentration distribution follows the organic matter profile, suggesting partial Cu complexation with OM (Table 2). Typically, Cu accumulates in surface horizons (0–20 cm) (Romi et al. 2014; Brunetto et al. 2016), but inter-row tillage may have redistributed OM and Cu to 20–40 cm (Brun et al. 1998).

Elemental analysis (Table S1) confirmed the soil's low nutrient status (7.25±2.2 mg·kg⁻¹ for PO₄³⁻;

81.48±2.48 mg·kg⁻¹ for K⁺), in line with its low electrical conductivity (43.3±3.37 μS·cm⁻¹), trace Zn and Pb, and moderate Mn and Fe levels (85.32±17.55 and 1007.03±94.03 mg·kg⁻¹, respectively), consistent with the reducible fraction from sequential extraction.

Percolation experiment

Assessment of copper leaching in biochar-amended soils

To quantify the copper retention (or leaching) of this vineyard soil and the effect of the different biochars obtained from the vine byproducts, a percolation test was performed. Figure 1 shows the percentage of total soil Cu retained after water percolation. In the control without Cu or biochar (C), almost no Cu was leached, probably due to a strong soil retention (low mobility factor, Table 2). In contrast, Cu-spiked soil without biochar (SPK) exhibited significant

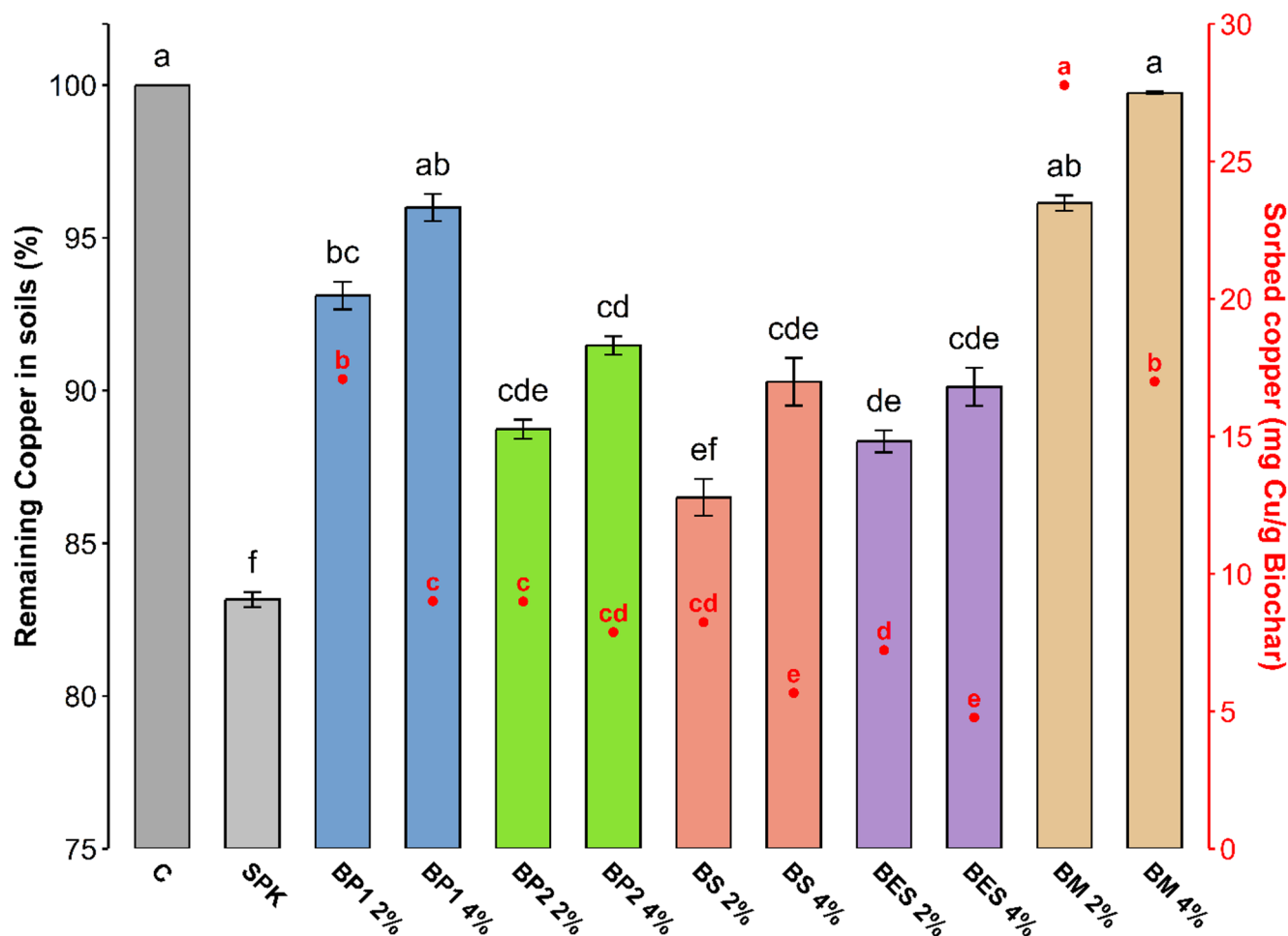


Fig. 1 Percentage of copper retained after leaching (left Y axis, black), in undoped, Cu-doped, and Cu-doped soils amended with various biochars at 2% and 4% (w/w); and Sorbed copper per gram of biochar (mg·g⁻¹) (right Y axis, red). Values are means±SD ($n=3$); different letters indicate significant differences between treatments ($p<0.05$, ω^2

= 0.92; 95% CI [0.85, 1.00] for left Y axis, $\omega^2 = 1.00$; 95% CI [1.00, 1.00] for right Y axis). C: undoped vineyard soil without biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

leaching, with $16.83 \pm 0.24\%$ of total Cu recovered in leachates. All biochar treatments (except BS at 2%) significantly increased Cu retention compared to SPK. Considering the high DOC level ($91.27 \pm 11.47 \text{ mg} \cdot \text{L}^{-1}$) (Table 2), most of the leached copper was probably in the form of Cu-DOC complex (Simms et al. 2020; Filipović et al. 2023). In soils, biochar can adsorb DOC, reducing the concentration of free DOC in solution and limiting the formation of soluble Cu-DOC complexes. This process indirectly promotes Cu immobilization while biochar could bind Cu directly, and in some cases, the adsorption of DOC can even create new functional sites that facilitate additional Cu binding, explaining the decrease of leachates percentage in biochar amended soils (He et al. 2017; Feng et al. 2021). Thus, no significant Cu was leached from soils amended with BM (2% or 4%) or BP1 (4%) with levels comparable to the unamended control (C), with BM at 4% retaining up to $99.75 \pm 0.04\%$ of added Cu. These findings confirm biochar's capacity to retain Cu in soils and prevent leaching as reported in Zhang et al. (2013) and Simms et al. (2020). This retention is likely due to surface sorption properties (Liang et al. 2021; Meng et al. 2025), since results showed that BM and BP1 had the highest Cu sorption capacities in solution (134.26 , $56.41 \text{ mg} \cdot \text{g}^{-1}$) and thus retained the most Cu in soil. Effective Cu sorption onto biochar surfaces can be verified using complementary approaches such as FTIR spectroscopy, zeta potential measurements, SEM–EDS analyses before and after sorption, pH-controlled sorption experiments, or sequential extractions indicating Cu incorporation into the residual soil fraction.

Additionally, the soil alkalization derived from biochar application participated in Cu mobility decrease (Simms et al. 2020; Ibrahim et al. 2022). BM (pH 11.74 ± 0.01) and BP1 (pH 10.00 ± 0.02) significantly raised soil pH (Table S2 – pH and EC of the 4th leachates), counteracting Cu-induced acidification (pH 5.24 ± 0.05) that may result from replacement of $\text{H}^+/\text{Al}^{3+}$ by Cu in the soil matrix (Simms et al. 2020). This pH rise was particularly marked in BM (2% and 4%) and BP1 (4%), and above the control (pH 7.23 ± 0.08) for BM 4% (pH 7.71 ± 0.10). As expected, during the successive leaching events, the amount of exported copper decreased as reported also in (Simms et al. 2020). Cu addition (SPK) increased EC tenfold compared to the control. Biochar further enhanced EC, particularly BM (2% and 4%, up to 19-fold) and pruning's biochars (4%) due to their ash content. High leachate pH (BM and BP1 4%) are correlated with stronger Cu soil retention as previously determined (Cornu et al. 2017; Deng et al. 2025), while EC may reflect ash content (e.g., for BM 27.9%). Elevated EC suggests potential interactions between Cu and co-occurring cations (Ca, Mg, K, or P), present in the biochar (Meng et al. 2022), supporting additional Cu immobilization by

precipitation and possible nutrient addition, contributing to fertilization (Ibn Ferjani et al. 2019; Andelini et al. 2023).

Additionally, in Fig. 1, is shown Cu sorption per gram of biochar in soil. While the amount of adsorbed Cu *per* gram of biochar decreased, especially at higher application rates (e.g., BM: $28.27 \pm 0.33 \text{ mg}$ at 2%, $17.29 \pm 0.02 \text{ mg}$ at 4%), the total Cu retained was the highest at 4%, doubling the 2% biochar retention on average. This may reflect a pH-induced shift in Cu speciation, promoting copper carbonates and hydroxides precipitation over sorption at high biochar doses (Wang et al. 2024). Additionally, the biochar maximum sorption capacities observed in Cu solution were not reached in soil, probably due to copper competition with other soil components, e.g., organic matter, clay, and Fe/Mn oxides (Tomeczyk et al. 2019; Cesco et al. 2021).

As BM was the most effective at retaining Cu in soil, Cu retention was further characterized across a gradient of BM doses (Fig. S3). In control samples lacking spiked Cu or biochar (C), nearly all Cu was retained in soil, while spiked Cu in the absence of biochar led to 18% of Cu leaching. When biochar was added, Cu retention significantly improved, even at the lower tested concentration (0.5% BM). The retention increased with biochar concentration up to 4%, which showed no significant difference from the control (no added copper and no biochar), confirming the biochar-dose-dependent efficiency described in Fig. 1 (BM 2% and 4%). These findings confirm that BM helps to retain doped Cu in the soil, reducing leaching, especially under high Cu contamination. Figure S3 also shows adsorbed Cu *per* gram of biochar, which decreases with increasing dose. However, total Cu retention increased, with the 4% treatment adsorbing five times more Cu than the 0.5% treatment—similar to trends observed in Fig. 1 for BM.

This dose-dependent retention is also linked to pH increase following biochar application (BM pH: 11.74 ± 0.01). Cu is less mobile from neutral to alkaline pHs, highlighting pH's key role in Cu mobility (Cornu et al. 2017; Zhang et al. 2021) and promoting its precipitation with carbonates and hydroxides (Wang et al. 2024). Therefore, the maximum sorption capacity observed in batch sorption tests was not reached in soil due to both pH effects and competition with other soil components. Table S3 (pH and EC of the 4th leachates) supports these findings. Copper addition (SPK) significantly acidified the soil (pH 5.24 ± 0.05), whereas BM addition progressively increased pH from 1% onwards, reaching 7.74 ± 0.13 at 4%. Similarly, EC rose ten times following Cu addition (1568.43 ± 118.21 compared to the control) and further increased with the BM addition (up to 2455.18 ± 646.57 at 4%). These high pH and EC values correlate with enhanced Cu retention, suggesting improved sorption by both biochar and soil component, thus reducing Cu mobility and bioavailability. As previously observed,

higher biochar doses led to greater Cu adsorption in soil (Tomczyk et al. 2019).

In summary, biochar application effectively reduces the availability of artificially supplemented Cu in the studied soil by stabilizing it into less mobile forms; thus, reducing the environmental risk and potential phytotoxicity. Using sequential extraction, many studies revealed biochar ability to reduce labile Cu (exchangeable – acid soluble fraction) while enhancing Cu bound to more stable fractions (organic matter, reducible and residual fraction) (Gonzaga et al. 2020; Simms et al. 2020; Gholami and Rahimi 2021; Salam et al. 2019; Meier et al. 2017a). The observed soil pH rise, toward neutral or slightly alkaline values leading to deprotonation, resulting in a soil colloidal particle's negatively charged increment [(hydr)oxide minerals and organic matter], that binds copper, and promotes the precipitation of copper ions in $\text{Cu}(\text{OH})_2$. The copper increase in the residual fraction could be associated with copper retention on biochar surfaces through complexation reactions (Salam et al. 2019; Gonzaga et al. 2020). Overall, reduction in labile Cu decreased the pool of bioavailable – leachable Cu (Gonzaga et al. 2020; Simms et al. 2020).

Phytotoxicity assessment

Effect of copper stress and biochar amendment on Soil Pore Water (SPW) composition

In the results shown above we demonstrated the biochar's Cu retention ability in the soil matrix (Fig. 1) reducing Cu leaching (Pietrzak and McPhail 2004; Banas et al. 2010). Here we studied the Cu bioavailability and their phytotoxicity in soil by testing the growth of *Phaseolus vulgaris* plant under the conditions explained above; soil pore water was analyzed for its Cu content, availability as well as pH and electrical conductivity. Regarding the plant, their growth and metal content were determined.

Biochar's effect on Cu concentrations in soil pore water (SPW) is shown in Fig. 2, which reflects the bioavailable fraction (Moreno-Jiménez et al. 2011). Although in arable soils, Cu concentration in soil solution typically ranges from 0.001 to 1 $\text{mg}\cdot\text{L}^{-1}$ (Cesco et al. 2021; Cornu 2023), in control (C), Cu concentration in the SPW (SPW-Cu) was below detection limits. However, the Cu-doped unamended soil (SPK) showed a concentration of $8.86 \pm 0.36 \text{ mg}\cdot\text{L}^{-1}$, far exceeding regular values. This relatively low SPW-Cu in the Cu-control reflects Cu's strong affinity for solid phases, limiting its presence in solution even in contaminated areas ($< 10 \text{ mg}\cdot\text{L}^{-1}$) (Cornu et al. 2017).

All biochar treatments, regardless of their concentration, significantly reduced Cu levels in SPW compared with the unamended control (SPK). Nonetheless, due to the

extremely high Cu levels, none of the biochars was able to reduce Cu concentration in SPW below detection limits. Among the tested biochars, BM at 2% and 4%, and BP1 and BP2 at 4% showed the greatest reductions, up to tenfold compared with SPK. A dose-dependent effect was observed only for BP2. Notably, SPW-Cu concentrations for BM 4% and BP1 4% (0.81 ± 0.07 and $0.76 \pm 0.09 \text{ mg}\cdot\text{L}^{-1}$, respectively) fell within typical arable soil ranges, consistent with soil Cu retention trends shown in Fig. 1. These findings may indicate that biochar promotes Cu retention in soil solid phases through both direct (surface adsorption) and indirect (pH-induced precipitation) mechanisms, even under elevated Cu loads. These results led to a strong exchangeable Cu fraction reduction and corresponding low bioavailable Cu in SPW. These effects have been widely demonstrated in Cu and other metal trace elements under multiple conditions also demonstrating biochar effect in plant growth promotion under metal stress (Lebrun et al. 2017; Meier et al. 2017a; Forján et al. 2018; Norini et al. 2019).

Complementary to SPW Cu concentrations, pH and electrical conductivity (EC) values were measured (Table S4). Cu spiked soil without biochar showed a 9% pH decrease (6.17 ± 0.22), likely due to Cu loading. In contrast, most biochar treatments increased soil pH, neutralizing Cu acidification with no significant differences from the control soil (C). Solely, BM displayed a significantly higher pH (8.45 ± 0.18 for BM 4%), reflecting its higher alkalinity. The alkalizing effect enhances Cu complexation with soil constituents, thereby further reducing its mobility (Simms et al. 2020). Regarding EC, Cu addition raised EC levels further increased by biochar addition (especially at 4% for all biochar and from 2% for BM), indicating the release of soluble mineral salts from the biochar into SPW (Salam et al. 2019).

Ion chromatography (Table S5) confirmed Cl^- input into SPW from CuCl_2 spiking. While some negative effects may partly result from Cl^- rather than Cu alone, the consistent biochar-induced improvements, even when Cl^- levels were higher than in SPK (e.g., BP2 4%; BM 4%), indicate that Cu immobilization and pH regulation were the main drivers of Cu concentration decrease in SPW. In contrast, biochar appeared to release nutrients into the soil solution, mostly anions such as NO_3^- , SO_4^{2-} , and PO_4^{3-} . On the other hand, a 134-fold K^+ increase was detected in the BM4% treatment compared to the control, highlighting the fertilizing potential of biochar amendments. Interestingly, Cu treatments led to a decrease in Ca^{2+} concentrations in SPW, probably through precipitation mechanisms involving calcium (Nguyen et al. 2023).

To assess Cu-induced phytotoxicity, a toxicity bioassay was performed using SPW samples after exposure to *P. vulgaris*. Here SPW phytotoxicity is estimated with the normalized *Lepidium sativum* seed vigor index (SVI); SPW

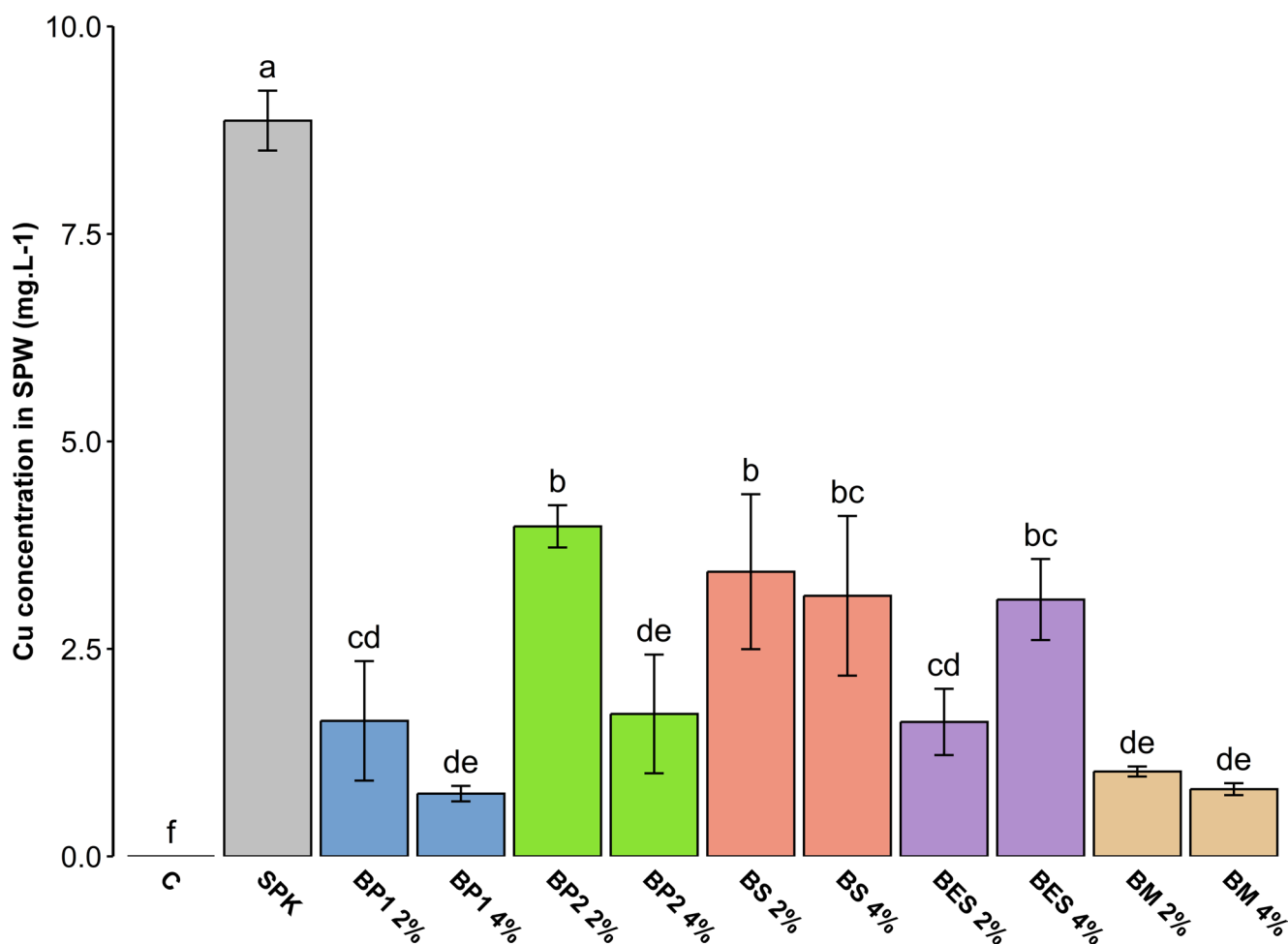


Fig. 2 Copper concentration (mg.L^{-1}) in soil pore water (SPW) for undoped, Cu-doped, and Cu-doped soils amended with different biochars at 2% and 4% (w/w). Values are means $\pm$ SD ($n=3$); letters indicate significant differences between treatments ($p < 0.05$, $\omega^2 = 0.86$;

95% CI [0.73, 1.00]). C: undoped vineyard soil without biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

from the Cu doped soil (SPK) showed clear phytotoxicity in comparison to control (C) (Fig. S7), likely due to both high Cu levels (Fig. 2) and slight pH diminution (Table S4). Biochar treatments with BP1 2%, BP2 2%, BS 4% and BES 4% reduced Cu toxicity compared to SPK. Nonetheless, BS and BES at 2% showed no positive effects. Conversely, amendments with pruning (BP1, BP2 at 4%) and marc (BM at 2 and 4%) biochars mitigated Cu toxicity, even improving the growth of *Lepidium sativum* above the soil control (C) values. This improvement is probably due to the combined effects of pH alkalization and higher nutrient level in SPWs after biochar application (Salam et al. 2019), along with the reduced oxidative seedlings stress derived from lower Cu content (Fig. 2) (Wang et al. 2019).

Growth response of *Phaseolus vulgaris* to copper stress and biochar amendment

Table 3 summarizes the effects of the different biochar types, applied at two rates, on *Phaseolus vulgaris* growth in copper-contaminated soil. Copper addition (SPK) strongly inhibited aerial growth, reducing biomass up to ninefold compared to the undoped control (C). No biochar effect was observed for BS and BES at both doses, or for BP1 and BP2 at 2%. In contrast, BM (at both 2% and 4%) and BP1 and BP2 at 4%, significantly enhanced aerial plant growth (BM 4%: 17.67 ± 2.52 cm) compared to the copper treatment, although none reached control growth (C: 27.67 ± 2.31 cm).

Stems and roots dry weights were lower under copper stress, with no significant differences with any of the biochars tested. However, a positive trend in biomass was observed at 4% applications of BS, BP1, BP2, and BM. Supporting this, Fig. S5 illustrates root deformation in

Table 3 Aerial growth (cm) and dry weight (DW)(g) of leaves, stems, and roots of *Phaseolus vulgaris* grown for 20 days on untreated, Cu-doped, and Cu-doped soils amended with biochars at 2% and 4% (w/w). Data are means $\pm$ SD ($n=3$); letters indicate significant differences between modalities for each measurement ($p<0.05$, $\omega^2=0.92$; 95% CI [0.86, 1.00] for Aerial length, $\omega^2=0.9$; 95% CI [0.79, 1.00] for DW, $\omega^2=0.91$; 95% CI [0.82, 1.00] for DW leaves, $\omega^2=0.83$; 95% CI [0.64, 1.00] for DW stems, $\omega^2=0.82$; 95% CI [0.63, 1.00] for DW roots). C: undoped vineyard soil without biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

Parameters	Aerial length (cm)		DW <i>P. vulgaris</i> (g)		DW leaves (g)		DW stems (g)		DW roots (g)	
Control	27.67 $\pm$ 2.31	a	1.17 $\pm$ 0.17	a	0.48 $\pm$ 0.09	a	0.44 $\pm$ 0.07	a	0.25 $\pm$ 0.06	a
SPK	3.1 $\pm$ 0.1	e	0.09 $\pm$ 0.02	d	0.02 $\pm$ 0	d	0.06 $\pm$ 0.01	b	0.02 $\pm$ 0.01	b
BP1 2%	7.67 $\pm$ 2.08	cde	0.15 $\pm$ 0.02	d	0.05 $\pm$ 0.03	d	0.08 $\pm$ 0.01	b	0.03 $\pm$ 0.01	b
BP1 4%	17.33 $\pm$ 1.15	b	0.57 $\pm$ 0.06	b	0.31 $\pm$ 0.02	ab	0.19 $\pm$ 0.02	b	0.07 $\pm$ 0.02	b
BP2 2%	4.67 $\pm$ 0.76	e	0.13 $\pm$ 0.02	d	0.03 $\pm$ 0.01	d	0.07 $\pm$ 0.02	b	0.03 $\pm$ 0.01	b
BP2 4%	12.33 $\pm$ 1.53	bcd	0.54 $\pm$ 0.13	bc	0.27 $\pm$ 0.05	ab	0.21 $\pm$ 0.07	b	0.06 $\pm$ 0.01	b
BS 2%	5 $\pm$ 1.73	e	0.16 $\pm$ 0.06	d	0.04 $\pm$ 0.03	d	0.08 $\pm$ 0.02	b	0.04 $\pm$ 0.01	b
BS 4%	8.5 $\pm$ 2.29	cde	0.2 $\pm$ 0.12	cd	0.03 $\pm$ 0.02	d	0.12 $\pm$ 0.08	b	0.05 $\pm$ 0.04	b
BES 2%	3.5 $\pm$ 0.5	e	0.09 $\pm$ 0.04	d	0.02 $\pm$ 0.01	d	0.05 $\pm$ 0.02	b	0.02 $\pm$ 0.01	b
BES 4%	6.67 $\pm$ 3.51	de	0.24 $\pm$ 0.06	bcd	0.07 $\pm$ 0.03	cd	0.13 $\pm$ 0.02	b	0.04 $\pm$ 0.01	b
BM 2%	12.67 $\pm$ 2.52	bc	0.37 $\pm$ 0.05	bcd	0.17 $\pm$ 0.03	bc	0.15 $\pm$ 0.04	b	0.05 $\pm$ 0.01	b
BM 4%	17.67 $\pm$ 2.52	b	0.56 $\pm$ 0.31	b	0.29 $\pm$ 0.18	ab	0.2 $\pm$ 0.1	b	0.07 $\pm$ 0.03	b

copper-exposed BP1 plants, including thickening, stunted growth and poor branching; typical symptoms of Cu toxicity (Cesco et al. 2021; Wang et al. 2024). At 2%, root development appears similar to SPK, whereas 4% biochar improved root growth; indicating dose-dependent mitigation of toxicity for this treatment. Leaf dry weights, for BP1, BP2, and BM at 4% were comparable to the control (C), suggesting partial alleviation of copper stress. In contrast, BS, BES, and pruning's biochars at 2% produced a leaf biomass similar to SPK. Thus, 4% applications of marc and pruning biochar showed the most notable benefits for leaf growth, which largely explained total dry weight variations. Overall, copper spiking reduced total dry weight by up to 13-fold compared to the control (C). While most biochar treatments at 2% did not differ significantly from SPK, BP1 and BP2 at 4% and BM treatments significantly improved total biomass, consistent with aerial biomass patterns.

Visual observations (Fig. S4) aligned with these results: clearly, copper addition drastically reduced plant size, while biochar amendment led to slight growth increases at 4% for BS, BES, and BP2, and stronger effects for BM at both doses, and BP1, BP2 at 4%. The dose effect was also visually evident, especially for BM and BP1, with increased growth along with biochar dose; supporting biochar's role in reducing Cu toxicity.

To summarize, biochar mitigates Cu toxicity in plants through direct or indirect effects, resulting in improved growth compared to SPK. This is attributed to bioavailable Cu reduction, which lowers toxicity and restores growth and metabolic functions. Similar results have been previously reported by Deng et al. (2025) with pear twig biochar at 1% and 3% increasing *Yunnanopilia longistaminea* height by 8.5% and 20% respectively in soil containing 300 mg Cukg⁻¹; and, by Simms et al. (2020), with 1% pine needle

biochar raising sweet potato biomass sevenfold and height tenfold for soil with 400 mg Cu kg⁻¹. Sheng et al. (2024) reported a 55% height increase in *Brassica napus* in soil containing 1000 mg Cu kg⁻¹ amended with 10% of rice straw biochar. Additionally, biochar improved metabolic parameters such as gas exchange (Buss et al. 2012; Abideen et al. 2023) and chlorophyll content (Rehman et al. 2019; Fontanive et al. 2023; Deng et al. 2025). Excess Cu induces oxidative damage via reactive oxygen species (ROS) generation that disrupt cellular integrity (Wang et al. 2024; Xu et al. 2024). Antioxidant enzymes like peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) detoxify ROS and serve as stress markers. Their activities decreased after biochar application in Cu-contaminated soils, indicating reduced oxidative stress (Rehman et al. 2019; Sheng et al. 2024; Deng et al. 2025).

Uptake and Translocation of Metals from Soil to Aerial Parts in Plant.

The mobility index (MI) was calculated to assess Cu bioavailability and translocation in soils cultivated with *P. vulgaris*. MI1, MI2, and MI3 correspond to Cu uptake from soil to roots, transfer from roots to stems, and stems to leaves, respectively (Table 4).

In the absence of Cu contamination, MI significantly increased along the soil–leaf axis (MI1 < MI2 < MI3), suggesting limited root uptake but important translocation to leaves, likely due to Cu's essential role in photosynthesis (Wang et al. 2024). Under Cu constraint (SPK), MI1 and MI3 exceeded MI2, suggesting restricted root-to-stem Cu movement and preferential Cu retention in roots; which aligned with previous findings (Shabbir et al. 2020; Cesco et al. 2021). Figure S6C shows a root Cu concentration ten times higher than in aerial parts. The elevated MI3 compared to MI2 suggests that once Cu reached stems, it was

Table 4 Copper mobility index (MI) in leaves, stems, and roots of *P. vulgaris* grown for 20 days on untreated, Cu-doped, and Cu-doped soils amended with various biochars at 2% and 4% (w/w). MI₁, MI₂, and MI₃ represent translocation from soil to roots, roots to stems, and stems to leaves, respectively. Values are means $\pm$ SD ($n=3$). Lowercase letters indicate significant differences among treatments for the same MI (1, 2 or 3) ($p<0.05$, $\omega^2=0.78$; 95% CI [0.56, 1.00] for MI₁, $\omega^2=0.26$; 95% CI [0.00, 1.00] for MI₂, $\omega^2=0.22$; 95% CI [0.00, 1.00] for MI₃), while uppercase letters denote differences among MI for the same treatments ($p<0.05$). C: undoped vineyard soil without biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

Mobility Index (MI)	MI ₁		MI ₂		MI ₃	
Control	0.08 $\pm$ 0.03	dB	0.52 $\pm$ 0.33	aAB	0.81 $\pm$ 0.23	abA
SPK	6.23 $\pm$ 4.82	aA	0.15 $\pm$ 0.14	abA	1.14 $\pm$ 0.87	abA
BP1 2%	1.30 $\pm$ 0.42	abcA	0.13 $\pm$ 0.11	abA	1.98 $\pm$ 1.84	abA
BP1 4%	0.62 $\pm$ 0.22	cA	0.15 $\pm$ 0.08	abB	0.21 $\pm$ 0.09	bB
BP2 2%	1.32 $\pm$ 0.44	abcAB	0.11 $\pm$ 0.05	abB	2.90 $\pm$ 1.24	aA
BP2 4%	0.68 $\pm$ 0.23	bcA	0.16 $\pm$ 0.17	abA	1.08 $\pm$ 0.66	abA
BS 2%	1.81 $\pm$ 0.38	abcA	0.22 $\pm$ 0.08	abB	0.69 $\pm$ 0.45	abB
BS 4%	1.72 $\pm$ 1.14	abcA	0.11 $\pm$ 0.04	abA	1.65 $\pm$ 0.43	abA
BES 2%	2.95 $\pm$ 1.92	abA	0.11 $\pm$ 0.07	abA	1.77 $\pm$ 1.28	abA
BES 4%	1.20 $\pm$ 0.56	bcA	0.10 $\pm$ 0.09	abA	1.14 $\pm$ 1.19	abA
BM 2%	0.80 $\pm$ 0.29	bcA	0.07 $\pm$ 0.04	bB	1.20 $\pm$ 0.25	abA
BM 4%	0.77 $\pm$ 0.15	bcA	0.07 $\pm$ 0.03	bB	0.59 $\pm$ 0.21	abA

effectively transferred to leaves, possibly for vacuolar sequestration (Wang et al. 2024).

Biochar addition strongly reduced MI₁ under Cu stress—by up to 9-fold for BP1 4%, BP2 4%, BM 2%, and BM 4%—in line with the decrease in root Cu concentrations (Fig. S6C). Reductions in MI₂ and MI₃ were less pronounced, reflecting smaller differences in Cu distributions across plant organs (Fig. S6A/B). This behavior is likely due to reduced soil Cu bioavailability (Fig. 2) following biochar-induced pH increase and metal sorption (Rehman et al. 2019; Sheng et al. 2024; Deng et al. 2025). Root Cu immobilization acts as a defense mechanism, limiting translocation to more sensitive aerial parts (Deng et al. 2025).

MI₁ was highest in SPK and lowest in the uncontaminated control (C), with biochar lowering MI₁ below SPK but remained higher than in C. Among biochar treatments, BS and BES showed the highest MI₁, whereas BM (2% and 4%) and BP1/2 4% had the lowest. A dose-dependent trend was observed (except for BM), with 4% biochar generally more effective than 2%. These results confirm that biochar can limit Cu uptake from soil (Fontanive et al. 2023), with BM showing a beneficial effect starting at 2% application.

Interestingly, for MI₂ the highest value was for the control (C), 2–5 times higher than other treatments, despite being not significant, suggesting unique translocation behavior in non-contaminated soils. MI₂ values in SPK and biochar treatments were similar, indicating limited influence of biochar on root-to-stem Cu translocation. Conversely, MI₃ tended to be reduced by biochar addition (vs. SPK), but only at 4% application for BM and pruning's biochar. At 2%, MI₃ increased in all modalities, suggesting enhanced Cu translocation to leaves, possibly due to more elevated tissue Cu levels than in 4% biochar application, triggering vacuolar storage mechanisms. Indeed, plants often detoxify excess

Cu by sequestering it into vacuoles of leaf cells, where it is chelated by organic acids, amino acids, or metallothioneins. This reduces free Cu²⁺ in the cytoplasm, thereby limiting damage to sensitive metabolic processes (Wang et al. 2024). Results for MI₂ and MI₃ should be interpreted with caution due to $\omega^2 \leq 0.26$.

The choice of *P. vulgaris* as an indicator plant likely influenced trace element mobility in the soil. This species is known for its ability to absorb and translocate high levels of metals including Cu from roots to leaves. Under metal stress, this species produces reactive oxygen species (ROS), but also activates antioxidant defense mechanisms that neutralize these toxic compounds. This tolerance to metal-induced stress may explain the high mobility indices observed, particularly the efficient translocation from stems to leaves (Silva-Gigante et al. 2023; Chafik et al. 2025b). Moreover, biochar application led to trace element toxicity mitigation in *P. vulgaris* consistent with previous report (Ibrahim et al. 2017; Dadasoglu et al. 2022; Hammami et al. 2022; Chafik et al. 2025a).

Overall, except for control (C), measured Cu concentrations (Fig. S6) in plant tissues exceeded known phytotoxicity thresholds (100–400 mg kg⁻¹ DW in roots, 20–30 mg kg⁻¹ DW in leaves; (Lamichhane et al. 2018; Cesco et al. 2021), even in the presence of biochar. Thus, although BM and BP1 (4%) reduced Cu uptake, they did not eliminate toxicity, consistent with growth differences and previous findings (Fontanive et al. 2023). Increasing biochar doses could enhance remediation but is impractical economically (Yang et al. 2021) and ecotoxicologically (Li et al. 2015; Gascó et al. 2016).

Soil enzymatic activities and microbial response

Figure 3 presents soil enzymatic hydrolytic activities estimated by fluorescein diacetate hydrolysis measured in unamended, Cu-doped and Cu-doped amended soils with different biochar at 2% and 4% (w/w) which was used as an indicator of soil health (Dick 1994).

Copper addition (SPK) drastically reduced (4.5-fold) hydrolytic activity compared to control (C) which might be due to the reduction of microbial biomass (Xu et al. 2016) and changes in bacteria community structure (Dell'Amico et al. 2008). Overall, impact of copper on FDA activity was mitigated by the presence of biochar. Notably, BP1 2% and BM4% enhanced activity up to four times over Cu-control (SPK). Nonetheless, only BP1 at 2% restored microbial function to undoped control soil values. BES at 4% showed the lowest value, with a non-significant difference from the Cu-control.

These results support prior work on biochar's role in the restoration of soil microbial activity in Cu-contaminated soils (Mackie et al. 2015; Meier et al. 2017b; Moore et al. 2018; Meier et al. 2021). The effect of biochar on bacterial populations is likely due to a reduction in Cu bioavailability (as shown in Fig. 2), and to its beneficial effect on bacterial communities, supplying nutrients and shelter for microorganisms (Moore et al. 2018). Other enzymatic activities (β -glucosidase, alkaline phosphatase, urease) showed high variability and limited diagnostic value, and were therefore not used (*data not shown*), whereas FDA hydrolysis, integrating overall microbial hydrolytic activity, provides a sensitive indicator of microbial metabolic potential under Cu stress and biochar amendment.

Additionally, a BiologTM EcoPlate experiment was conducted using Cu doped soil either unamended or amended with different biochar (BM, BES, BS, BP1) at 2% and 4% doses, and compared to a non-contaminated control soil (T).

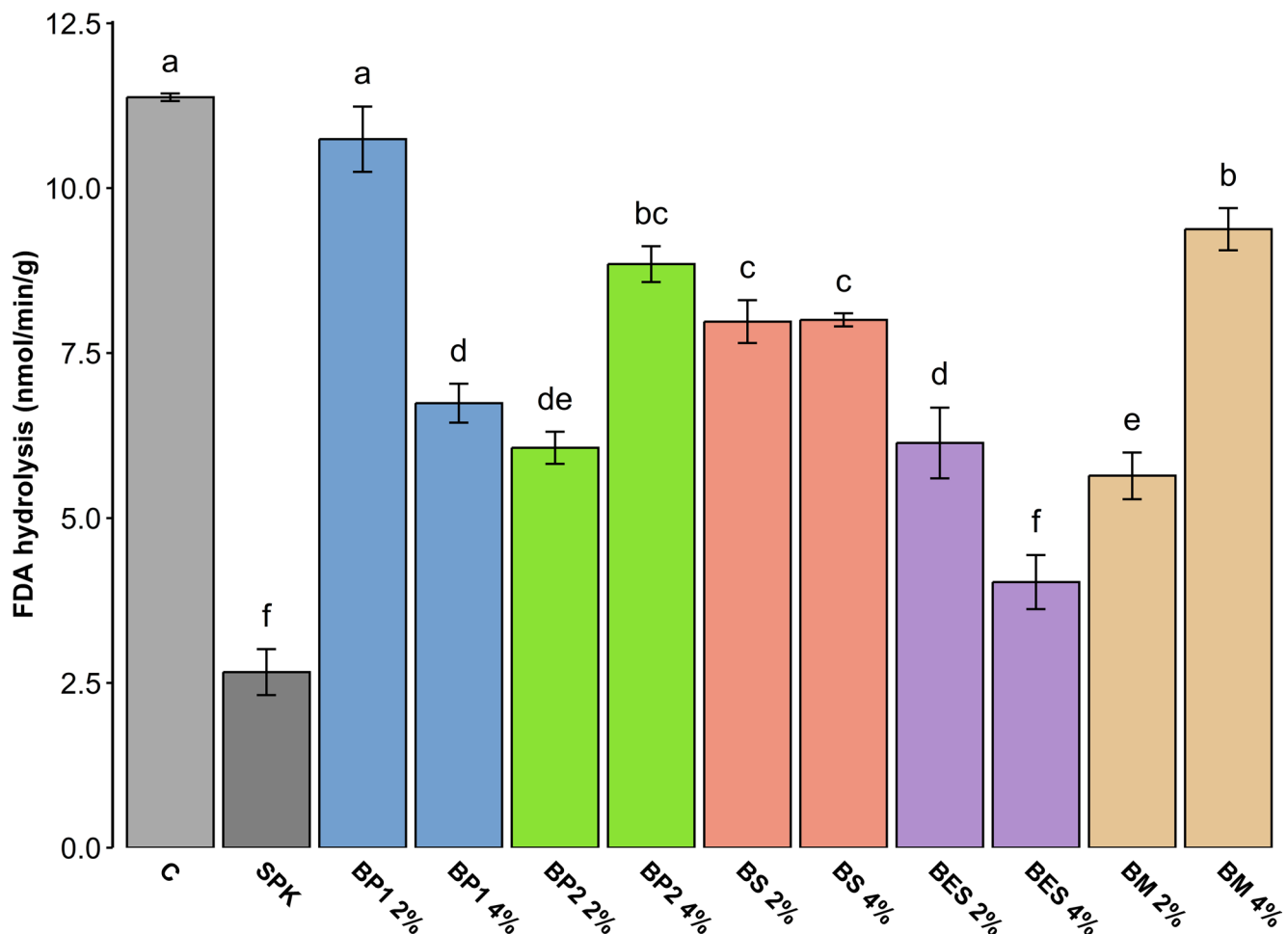


Fig. 3 Soil enzymatic hydrolytic activity (fluorescein diacetate hydrolysis, $\text{nmol FDA} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$) measured in untreated, Cu-doped, and Cu-doped soils amended with different biochars at 2% and 4% (w/w). Results are means $\pm$ SD ($n=3$); letters indicate significant differences between modalities for each measurement ($p < 0.05$, $\omega^2 = 0.98$; 95%

CI [0.96, 1.00]). C: undoped vineyard soil without biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

Table S6 summarizes three key microbial indicators: (1) AWCD (Average Well Color Development) reflecting overall microbial metabolic activity; (2) H' (Shannon Diversity Index) indicating functional diversity of the microbial community and (3) E (Evenness) showing the uniformity of substrates utilization across the microbial community.

Copper contamination led to a marked increase in microbial activity and diversity. AWCD rose from 0.014 (T) to 0.031 (SPK), while both H' and E also increased. These changes suggest a shift in microbial community structure, possibly driven by stress adaptation or enrichment of copper-tolerant species (*Low20. DellAmico08*). The impact of biochar amendments varied by type and dose: (1) BM at 2% significantly enhanced all metrics ($H' = 2.819$; $E = 0.821$), indicating a strong positive effect by biochar as already reported (Gałazka et al. 2019; Wang et al. 2023; Xiang et al. 2023). However, the 4% dose resulted in reduced microbial activity and diversity, pointing to potential inhibitory effects at higher concentrations (Zhu et al. 2017). (2) BES, both 2% and 4% doses improved AWCD, H' , and E compared to SPK. A slight increase in H' and E from 2% to 4% suggests a dose-dependent benefit to microbial diversity and evenness. (3) BS at 2%, yielded the highest H' (2.830) and E (0.824), indicating the most functionally diverse and balanced microbial community. The 4% dose led to the highest AWCD (0.032), but a sharp drop in E (0.084), suggesting dominance by a few highly active species and reduced community balance. (4) BP1 with 2% dose provided good enhancement ($H' = 2.481$; $E = 0.722$), but microbial activity and diversity dropped markedly at 4% ($H' = 1.143$; $E = 0.333$), suggesting possible toxicity or overstimulation at higher concentrations.

In conclusion, copper pollution (SPK) stimulated microbial activity and diversity, likely due to Cu stress-induced community shifts. Biochar amendments significantly influenced microbial function, with effects depending strongly on biochar type and application rate. The beneficial effects of biochars likely result from nutrient release, microbial habitat provision (Moore et al. 2018), and reduction in Cu levels. In contrast, the reductions in H' and E observed at 4% application might be attributed to pH stress and excessive nutrient release in SPW (e.g., pH = 8.45; EC = 2546.2 ± 90.9 for BM4%; Table S4). Finally, BS at 2% showed the most favorable balance of microbial activity and functional diversity. Moreover, high-dose applications (4%), particularly of BM and BP1, may negatively affect microbial evenness and diversity.

Statistical Exploration of Parameter Interactions

Pearson Correlation Matrix

A correlation matrix (Fig. S8) summarizing the relationships among plant and soil parameters. Positive correlations indicate that both variables increase together, while negative correlations denote inverse relationship.

The variable “Cu retained in soil (%)” (see Fig. 1) was strongly positively correlated with SPW pH (Table S4) and negatively correlated with Cu concentration in SPW (Fig. 2), indicating the central role of pH in Cu mobility (Cornu et al. 2017). These findings suggest that Cu retained in the soil – either directly or indirectly potentially through biochar action – results in lower Cu concentrations in SPW.

Growth parameters of *P. vulgaris* (aerial length, organ dry weight; Table 3) were positively intercorrelated, as expected. They also correlated positively with Cu retained in soil (Fig. 1) and negatively with Cu concentration in SPW (Fig. 2) and Cu concentration in plant organs (Fig. S6), which were themselves positively correlated. This highlights that Cu toxicity is driven primarily by bioavailable Cu, rather than total Cu content (Komárek et al. 2010; Cornu et al. 2022). The plant growth was also positively correlated with microbial activity (FDA; Fig. 3), which in turn was negatively correlated with Cu in SPW and plant Cu uptake, supporting the view that Cu toxicity affects both microbial and plants function (Xu et al. 2016; Mir et al. 2021). Finally, the toxicity index (SVI, Fig. S7) was positively correlated with all plant growth parameters (and slightly with microbial activity) but negatively with Cu concentration in SPW and plant Cu uptake. This reinforces SVI's utility as an indicator of trace element toxicity (Bożym et al. 2021).

Together, these correlations suggest that recovery of plant growth, enzymatic activity, and reduction in toxicity index are mainly driven by SPW Cu concentrations, which are themselves influenced by SPW pH and soil Cu retention. Nonetheless, although statistically significant, the correlation coefficients were not maximal (i.e., < 1 or > -1), indicating that other factors also contribute. Variability in plant performance may also reflect additional biochar effects, including other trace element sorption, microbial stimulation, nutrient input, improved water retention and bulk density reduction (Joseph et al. 2021); as well as the influence of root exudates (Wang et al. 2024; He et al. 2024) and microbial extracellular polymeric substances (Prabhakaran et al. 2016; Lin et al. 2020), which may affect copper toxicity.

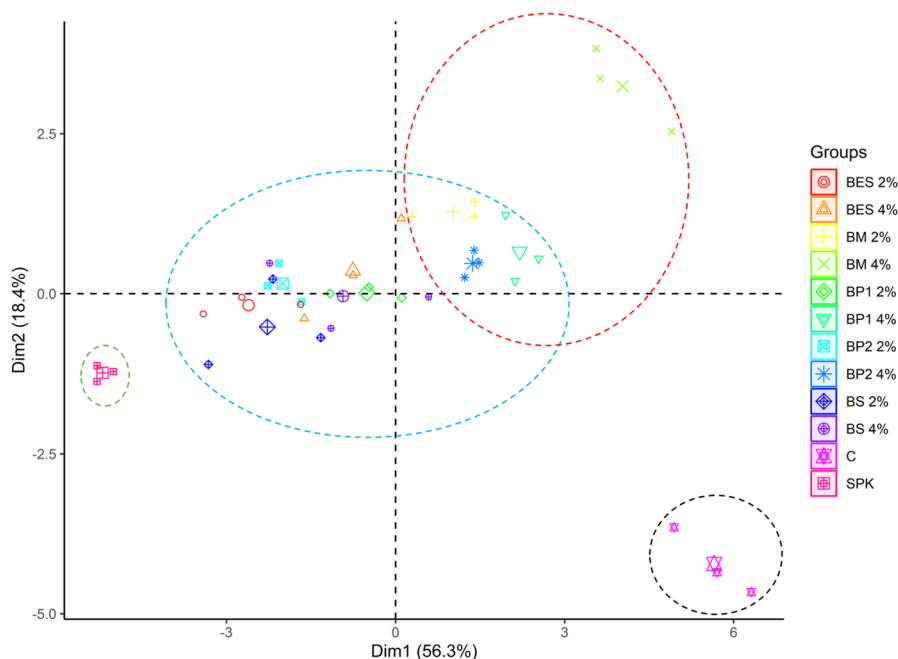


Fig. 4 Principal Component Analysis (PCA) showing projection of treatments on the first two dimensions (Dim1 and Dim2). Ellipses indicate clustering of individuals. C: undoped vineyard soil without

biochar; SPK: doped vineyard soil without biochar; BP1: Pruning Biochar; BP2: Pruning Biochar with two pyrolysis; BS: Seed Biochar; BES: Biochar Extracted seed; BM: Marc Biochar

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed to explore the structure of relationships between quantitative variables. The results indicated that the first two principal components (PC1 and PC2) together accounted for approximately 75% of the total variance, with PC1 explaining 56.3% and PC2 18.4%. This suggested that these two components were the most significant for understanding the data's variability and that dimensionality reduction was possible by focusing on these two axes. In terms of variable contribution, elements such as Cu retained (%), aerial length, and dry mass (DW) of *P. vulgaris* organs had a high positive coefficients and Cu concentration in SPW had a high negative coefficient in PC1, indicating that they were strongly associated with this component. Furthermore, variables such as pH and electrical conductivity (EC) of SPW showed marked positive contributions in PC2, suggesting they were crucial for differentiating samples according to this component. The subsequent components, PC3 to PC14, explained a marginal share of the variance (below 10%), with almost negligible contribution from the variables, reflecting that most of the information could be captured by the first two components.

The projection of modalities on the PC1-PC2 plane (Fig. 4) revealed distinct clusters. The position of the control (C) at the bottom right (*black cluster*) indicated that it was strongly influenced by PC1 (positive values) and weakly influenced by PC2 (negative values). The control was thus characterized by high Cu retention in soil, low Cu content in SPW and significant *P. vulgaris* growth, but more acidic soil conditions and low conductivity. Cu control (SPK) formed a cluster (*green cluster*) negatively correlated with PC1, indicating low Cu retention, high Cu content in SPW and low plant growth. Its position in the middle of the PC2 axis suggested that its soil conditions (pH and conductivity) were not decisive in explaining differences from this modality. The majority of biochar was concentrated in a central cloud (*blue cluster*) around the origin of the axes, indicating intermediate effects on Cu retention, plant growth, and soil pore water pH and EC parameters. This suggested that most biochars did not lead to marked differentiation according to PC1 or PC2. The presence of BM 4% at the top right indicated that this sample was clearly distinguished by high values for PC1 and PC2. Its position reflected very high Cu soil retention, low Cu content in SPW and strong plant growth (positive PC1), as well as high pH and electrical conductivity (positive PC2), suggesting that this biochar significantly

influenced soil properties. The three other biochars closest to BM4% (within the central cloud) were BM2%, BP1 4% and BP2 4%, as expected and formed the last cluster (*red cluster*).

These results demonstrate that biochar (BM, BP1), particularly at higher doses, significantly improved Cu retention, reduced bioavailable Cu (lowering Cu plant uptake), and promoted plant growth and enzymatic activity, likely through soil alkalization and enhanced Cu sorption capacity. Although the study demonstrates effective short-term Cu immobilization and reduced phytotoxicity, the relatively short incubation and plant growth periods do not allow conclusions on long-term stability. Biochar ageing processes, such as surface oxidation and changes in functional group availability, may alter Cu binding mechanisms over time (Liu et al. 2022). Concurrently, Cu speciation may evolve due to mineral transformations and organic matter dynamics, affecting its mobility and bioavailability (Tomczyk et al. 2019). Moreover, the persistence of pH-driven immobilization under field conditions may be reduced by soil buffering capacity, leaching, and biological activity (Apostolović et al. 2024). Therefore, long-term field studies are required to assess the durability of biochar-induced Cu stabilization under realistic agronomic conditions.

Conclusion and prospects

This study evaluated the potential of biochars derived from viticultural by-products to mitigate copper toxicity in contaminated soils.

The results show that:

- (1) The addition of biochar, particularly grape marc biochar (BM) at 2% and 4% and vine shoot biochar (BP1) at 4%, reduced the bioavailable copper load in soil pore water while increasing the copper fraction bound to the solid matrix.
- (2) This reduction in copper toxicity was linked to both the copper sorption capacity of biochar, and its indirect effect on soil pH, thereby influencing copper speciation and mobility.
- (3) As a result, soil microbial activities improved and *P. vulgaris* growth was restored, likely due to decreased copper uptake and limited translocation to aerial parts facilitated by the biochar.
- (4) However, due to the high copper loading in this study, biochar addition only attenuated copper toxicity rather than completely eliminating it.

These results confirm previous observations demonstrating the effectiveness of biochar in reducing copper toxicity on plants, mainly by decreasing the bioavailable fraction of copper. Importantly, this work highlights the potential of using bio-sourced materials from viticultural by-products, as a sustainable remediation strategy for copper-contaminated soils, including vineyards, orchards, olive groves and tea or coffee plantations, supporting circular economy and greenhouse gas emission mitigation. However, the study was conducted under controlled conditions, using a model plant species (*P. vulgaris*) and artificially copper addition, which may not fully reflect the complexity of natural soils and long-term field exposure. Thus, further research is needed under real copper contaminated conditions, or after a long aging process to validate these findings, including measurements of plant metabolic parameters (chlorophyll content, gas exchange) and also oxidative stress markers. Finally, future work should also evaluate the effects of grape marc biochar—identified as the most effective material—on *Vitis vinifera*, and characterize changes in the microbiological parameters of copper-contaminated soils.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

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