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Double trouble belowground: grapevine rootstocks face drought and copper toxicity

R. Fattorini*, T. O. Caretta, F. Benyahia, M. Y. A. Zuluaga, S. Monterisi, A. Agostini, C. Andreotti, S. Cesco and Y. Pii*

Faculty of Agricultural, Environmental and Food Sciences, Free University of Bolzano, Bolzano, Italy

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EDITED BY
Anket Sharma,
Texas Tech University, United States

REVIEWED BY
Chao Song,
Ben-Gurion University of the Negev, Israel
Shabnam Heidarpour,
Urmia University, Iran

*CORRESPONDENCE
R. Fattorini
Roberto.fattorini@student.unibz.it
Y. Pii
youry.pii@unibz.it

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Background and aims: Climate change is intensifying abiotic stresses in viticulture, particularly through increased drought due to erratic rainfall. Meanwhile, copper (Cu^{2+}) toxicity, a legacy of phytosanitary treatments, may be aggravated by these environmental shifts. This study evaluated the physiological and ionomic responses of young *Vitis vinifera* cv. Pinot gris plants, grafted onto three rootstocks (M4, 1103 Paulsen, SO4), under controlled drought, Cu^{2+} toxicity, and their combined effects.

Methods: Plants were grown under greenhouse conditions and subjected to individual and combined stress treatments. Morpho-physiological traits, biomass distribution, and nutrient profiles were assessed to determine genotype-specific responses.

Results: Drought markedly reduced gas exchange and photosystem II efficiency (Fv/Fm), especially in SO4, while M4 maintained better physiological performance. Cu^{2+} toxicity alone had limited physiological impacts but significantly altered root ionomic profiles. Combined stress exacerbated water-state impairment, chlorophyll reduction, and nutrient imbalances, especially in SO4. The PCA analysis of ionomic data revealed clear separation of stress treatments among rootstocks, with M4 exhibiting the most distinct and balanced nutrient profile. In contrast, plants grafted on 1103 Paulsen and SO4 showed less coordinated nutrient responses and reduced recovery capacity.

Conclusions: Rootstock genotype strongly affected grapevine resilience under multifactorial stress. M4 emerged as the most tolerant, suggesting its suitability for future viticultural conditions marked by drought and soil contamination. These results emphasize the critical importance of belowground traits in selecting more resilient grapevine plants, integrating physiological and ionomic assessments, to enhance resilience against multifactorial stresses under climate change.

KEYWORDS

grapevine rootstocks, copper toxicity, drought stress, ionomics, abiotic stress tolerance

1 Introduction

Among all food and agricultural commodities, whether consumed fresh or processed into derived products and beverages, wine stands out as one of the most significant value-added products globally (OIV, 2022), with a cultivated area of 792 kha in the Italian peninsula as of 2023 (OIV, 2024). However, at present several threats correlated with climate change (*i.e.*, increase in temperatures, altered precipitation patterns, and enhanced frequency of extreme weather events, (Cesco et al., 2024) might seriously affect its production. In particular, the rising incidence of emerging diseases (Anderson et al., 2004; Fisher et al., 2012; Ristaino and Records, 2020) coupled with prolonged drought conditions (Masson-Delmotte et al., 2022) is causing significant pressure on grapevine cultivation. This impact can be especially pronounced during the early stages of the vineyard establishment, when grapevines are still young and particularly vulnerable (Palliotti et al., 2014; Poni et al., 2018).

From a general point of view, grapevines, unlike other crops, are known to be relatively resistant to moderate degrees of water deficit due to various physiological adaptive responses mainly based on antioxidant enzyme activities and the synthesis of secondary metabolites (Keller, 2020). These physiological responses may have negative effects on fruit yields, but, on the other hand, can positively affect berry quality, particularly when referring to the obtained wine (Medrano et al., 2003; Deluc et al., 2009; Leeuwen et al., 2009; Carbonell-Bejerano et al., 2014; Liu et al., 2015). However, under prolonged drought conditions, the ability of grapevine plants to sustain their physiological functions declines, resulting in marked effects on their physical and biological characteristics, such as slowed development, wilting, reduced water potential and turgor pressure, and impaired cell expansion (Lovisolo et al., 2010; Khan, 2011). Therefore, the grapevine ability to recover from drought conditions is a key agronomic trait, as it influences canopy regrowth, fruit set, yield stability, and overall vineyard performance across growing seasons (Gambetta et al., 2020). From the perspective of adaptive response mechanisms, the regulation of intrinsic water-use efficiency (iWUE), defined as the ratio between carbon assimilation and stomatal water loss (Briggs and Shantz, 1913; Hatfield and Dold, 2019), represents a particularly crucial strategy for grapevines under water deficit conditions. Values of iWUE increase under mild drought conditions, and subsequently decline with more severe or prolonged drought, primarily due to damage or inhibition of photosynthetic activity (Bota et al., 2016). This decline is further exacerbated by a decrease in the mesophyll conductance, which impairs CO_2 diffusion and limits photosynthetic capacity (Ouyang et al., 2017). Thus, while iWUE may initially increase under mild drought, severe or prolonged water deficits impair photosynthetic processes, ultimately reducing iWUE. Under such conditions, plants activate additional drought-response mechanisms, including osmotic adjustment, which is regulated by endogenous hormones such as abscisic acid (ABA) and methyl jasmonate (Su et al., 2020), and involves the accumulation of organic osmolytes like sugars and quaternary ammonium compounds (Jogaiah et al., 2014).

While originally developed to confer resistance to biotic stressors, rootstock selection has progressively evolved to include adaptative traits against abiotic constraints, including edaphic imbalances (such as high pH and calcareous soils), waterlogging, salinity, and drought (Rahemi et al., 2022). Noteworthy, in the context of climate change, where enhancing grapevine resilience is a growing priority, increasing attention is being directed toward the role of rootstocks in supporting grafted-plant adaptation. Among current strategies, the use of drought-resistant rootstocks has emerged as a key agronomic approach to improving vineyard performance under increasing water scarcity. Their drought tolerance is largely attributed to specific traits, including depth and branching pattern, and has been associated with the maintenance of yield, reduced irrigation requirements, and the preservation of key berry quality traits (Zhang et al., 2016). A clear example is observed in arid regions or in areas subject to irrigation restrictions, where rootstocks like 1103 Paulsen (*V. rupestris* × *V. berlandieri*) are widely used for their deep taproots and resilience (Fregoni et al., 1978; Ollat et al., 2016; Zhang et al., 2016). In contrast, the newer M4 one (41B × *V. berlandieri* Resseguier No. 4) shows promise due to its quicker recovery after re-watering, slower physiological decline under water stress, and higher hydraulic conductance (Meggio et al., 2014) when compared to the more drought-sensitive SO4 (Galbignani et al., 2016). Additionally, rootstocks can influence the regulation of various physiological mechanisms, such as ABA synthesis and signaling pathways. It is interesting to note that these physiological effects differ significantly between self-rooted *Vitis vinifera* grapevines (*e.g.*, Cabernet Sauvignon) and grapevines grafted onto selected rootstocks (Prinsi et al., 2021) in the whole plant. Grapevine rootstocks are therefore not only a structural support but also a functional component of the whole plant system, with a critical influence on vineyard efficiency. In fact, they not only supply water and mineral nutrients to the scion, but they are also crucial in determining the final tree vigor (Cookson and Ollat, 2013; Nimbolkar et al., 2016; Rossdeutsch et al., 2021).

Although significant efforts have been made to select rootstocks and scion varieties capable of withstanding individual abiotic or biotic stresses, grapevines grown under field conditions are rarely exposed to isolated stressing factors. On the contrary, in vineyard they typically face complex and simultaneous environmental challenges. A clear example is observed in temperate regions, where grapevines are often affected not only by soil-fertility related constraints including drought, but also by biotic pressures such as downy mildew (*Plasmopara viticola*) (Gessler et al., 2011), or powedry mildew (*Erysiphe necator*) (Rienth et al., 2021). To prevent these fungal infections, copper (Cu)-based fungicides are routinely applied as part of grapevine plant protection programs. However, the repeated use of Cu-based fungicides over successive growing seasons has led to the long-term accumulation of Cu^{2+} in vineyard soils, particularly in the upper layers (Kandeler et al., 1996; Merrington et al., 2002; Brunetto et al., 2016; Keiblinger et al., 2018) often exceeding the trace levels sufficient to support an equilibrate plant growth (Pietrzak and McPhail, 2004; Brunetto et al., 2016) and, in some cases, exceeding the maximum limits established for agricultural soils (Komárek et al., 2010). In this respect it should be mentioned that the Cu^{2+} excess in soils poses serious threats to environmental sustainability as well as vineyard productivity. High Cu^{2+} levels interfere with root development, callus formation, and overall plant establishment, which leads to lower success rates during propagation, making it more difficult to establish healthy young vines, complicating replanting efforts (Cesco et al., 2021). This element primarily accumulates in roots, with uptake and translocation varying by concentration (Nan and Cheng, 2001; Chaignon et al., 2002; Benimeli et al., 2010; Guan et al., 2011). When present at high availability levels, it disrupts root nutrient acquisition capacity, inhibit plant development, and affect key physiological and biochemical processes (Marastoni et al., 2019b; Feil et al., 2020, 2023). In response to this nutritional disorder, grapevines activate a range of tolerance mechanisms that include morphological alterations (*e.g.*, stunted growth, leaf chlorosis), physiological adjustments (*e.g.*, activation of antioxidant systems, accumulation of osmo-protectants, and metal chelators/ligands), and biochemical responses (*e.g.*, enhanced detoxifying enzyme activity), which intensify proportionally with soil Cu^{2+} levels (Cesco et al., 2021, 2022; Kosakivska et al., 2021). Overall, these findings highlight the complexity of grapevine responses to soil Cu^{2+} excess. They also illustrate how multiple stress factors often occur simultaneously, challenging grapevine resilience and long-term vineyard productivity. It is important to note that, during natural selection, plants have adapted to both abiotic and biotic challenges, developing a wide range of coordinated tolerance mechanisms. Whilst considerable information on cross-tolerance of plants to either multiple abiotic or multiple biotic stresses is available in literature (Cao et al., 1998; Warren et al., 1998; Wen et al., 2008; Krattinger et al., 2009; Priyanka et al., 2010; Ramegowda et al., 2012, 2014, 2017; Qin et al., 2015; Carvalho et al., 2016; Ju et al., 2021; Straffelini et al., 2024), the grapevine responses to combined abiotic and biotic stresses is quite complex to understand. Several authors have indeed highlighted that the interaction between the two types of stresses (i.e., biotic and abiotic) can have either antagonistic or synergistic effects (Atkinson and Urwin, 2012; Atkinson et al., 2014; Kissoudis et al., 2014; Ramegowda and Senthil-Kumar, 2015), thus making the prediction of grapevine plants response challenging on the bases of previously acquired data (Rizhsky et al., 2002, 2004). A notable example of such interactions is represented by vine age, an often overlooked but agronomically relevant factor. Older grapevines, thanks to their deeper root systems, are better able to avoid the upper topsoil layers (0–20 cm), where heavy metals like Cu^{2+} tend to be accumulated (Pham, 2024), and can access water from deeper horizons, improving their resilience to both metal toxicity and drought (Galet, 2000; Pourtchev, 2003; Lehnart et al., 2008). In contrast, newly planted vines, due to standard root pruning practices (~10 cm) aimed at stimulating new root formation (Fregoni, 2013), can remain confined to the most contaminated and driest soil layers during the critical establishment phase. This condition is very likely to increase the susceptibility of young vineyards to the co-occurrence of multiple stressors, further complicating replanting efforts in historically cultivated areas and, at least partially, explaining the difficulties often observed in these contexts, challenges that are expected to intensify under ongoing climate change.

Based on these premises, this work was aimed at deepening our understanding of the complex interaction between abiotic stresses (*i.e.*, water stress and Cu^{2+} toxicity) that grapevine plants may simultaneously face in the current context of climate change. To this scope, three different rootstocks (*i.e.*, 1103 Paulsen, M4 and SO4) grafted with Pinot gris have been exposed either to drought stress, Cu^{2+} toxicity or the combination of the two stresses within a pot experiment in greenhouse conditions. The three rootstocks were selected based on their different levels of tolerance to drought stress (Supplementary Table S1). With regard to Cu^{2+} tolerance, several publications are available for 1103 Paulsen and SO4 (Trentin et al., 2022, 2024); however, no studies have yet been conducted for M4. Physiological and biochemical parameters have been monitored throughout the experimental period to assess the differential impact of abiotic stresses on the three rootstocks under investigation. The outcomes of this study are intended to support rootstock selection strategies aimed at improving vineyard resilience under multifactorial stress conditions, particularly in the context of replanting programs in areas affected by water scarcity and long-term soil accumulation of Cu^{2+}.

2 Materials and methods

2.1 Plant material and experimental design

The experiment was conducted in the greenhouse of the Free University of Bozen-Bolzano in Laimburg (Alto Adige, Italy 46.3827° N, 11.2877° E) during the vegetation period (from May to November 2023) using grapevine plants obtained from Vivai Cooperativi Rauscedo (Rauscedo, Italy).

The vine plants tested were a combination of the same cultivar (Pinot gris) grafted on three different rootstocks, *i.e.*, M4, 1103 Paulsen and SO4.

The grafted plants were stored in a refrigerated room at 6°C and prepared for vegetative awakening. The plants were soaked in water for 24 h, and afterwards the roots have been shortened to a length of about 10 cm and then transplanted in 6 L pots, filled with approximately 5 kg of substrate, composed of River Sand (60%), Perlite (20%), and Peat mixture (20%). The lower part of the plastic pots hosted several holes to drain excessive water from the pot, preventing any anoxia condition at the root level; moreover, the upper surface of the pots was covered with felt pads to prevent water loss by direct soil evaporation.

The pot experiment was then conducted in greenhouse with a mean air temperature of 28 ± 2°C during the day and 20° ± 2°C during the night, controlled lighting (500 µmol m^2/s) and with controlled irrigation. Protection against diseases was carried out only with Cu-free preparations.

Before the end of the growing period, each vine was pruned to retain two shoots, which were then supported using stakes.

The experiment followed a completely randomized block design (CRBD) with a minimum of nine replicates per treatment. Plants were organized in rows of 16 and randomly assigned to treatments within each block. To control spatial and positional effects, plants were rotated weekly both within a single row and between rows. This regular repositioning helped ensure uniform exposure to environmental conditions and minimized location-based bias. Plants were divided in four treatments: 1- Control (no treatments applied); 2- Cu^{2+} and drought stresses (Cu+DS); 3- drought stress (DS); 4- Cu stress (Cu). The Cu was applied as $CuSO_4$ and the first Cu^{2+} dose of 120 mg/kg was applied at ~55 days after the transplanting. Based on preliminary analyses, a maintenance Cu^{2+} dose of 60 mg/kg was applied a week after the first Cu^{2+} application. The copper dosage was selected by comparing data from previous studies with concentrations currently reported in contaminated vineyards (Toselli et al., 2009; Komárek et al., 2010; Baldi et al., 2018; Marastoni et al., 2019a, 2019b), with the objective of inducing Cu^{2+} toxicity without being lethal to grapevine. In the same moment, drought stress was induced in Cu+DS and DS plants by withholding the irrigation, until severe drought stress was reached (corresponding to approximately -1.2 MPa midday stem water potential). The other plants were manually irrigated until soil saturation. After reaching severe drought stress, the plants were watered to induce the recovery. During the different stages of the experiment — (start of the experiment (T0), induction of drought stress (T1), peak of drought stress (T2), recovery (T3)) — morpho-physiological data have been recorded, and root, leaves and soil samples were collected and stored for further chemical, biochemical and molecular analysis.

2.2 Measurements of morpho-physiological parameters

Every other day during the experimental period the midday stem water potential (Ψ_{stem} - MPa) was measured. To mitigate the effect of repeated leaf destructive samplings on the total vine leaf area, measurements were taken on each date from only one fully expanded leaf per vine. The leaves were enclosed in transparent plastic bags, covered with aluminum foil at noon, detached after approximately one hour, and immediately inserted into a pressure chamber (Pump-up Pressure Chamber, PMS Instrument Comp. USA) for the reading.

Using a portable infrared gas analyzer (LC-pro ADC, Hoddesdon Bioscientific, Ltd., Herts, United Kingdom), measurements were conducted for the photosynthetic rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}). The measurements were performed on the same day as the Ψ_{stem} assessments to ensure consistency in plants' water status. Morning readings (9:00–11:00 am) were taken under saturating light conditions (PPFD of 1800 µmol photons m^{-2} s^{-1} provided by a LED array unit) and ambient CO_2 levels (382–438 ppm), always selecting a fully expanded leaf per vine located in the intermediate section of the shoots. A and g_s values were used to calculate the intrinsic water use efficiency (iWUE= A/g_s). A total of ten measurements were collected over the course of the experiment, from the first copper application to the recovery of the plants.

Chlorophyll fluorescence parameters were measured every other day, on the same days as other physiological parameters (leaf gas exchange and midday Ψ_{stem}). Readings were obtained in the morning (9:00-11:00) utilizing a portable chlorophyll fluorometer Handy PEA (Hansatech Instruments, UK) with an excitation light at 650 nm. For each measurement, one mature and healthy leaf per vine was selected and prepared for assessment. The measurements were taken after the leaves were fully dark-adapted for at least 20 minutes, achieved by covering them with a leaf clip that occupied a total diameter of 4mm, ensuring proper illumination.

Leaf chlorophyll content was indirectly evaluated with a SPAD-502 Chlorophyll Meter (Konica Minolta, Tokyo, Japan). Measurements were performed once per week on each plant. SPAD values are reported as an average of three measurements on the third to fifth leaf of each shoot. The instrument calculated a numerical SPAD value, which correlates with the chlorophyll content in leaf tissues.

Soil humidity (m^3/m^3) from a depth of 0 to 6 cm was measured every other day during the trial using a Theta Probe ML3 (Delta-T Devices Ltd, Cambridge, England).

The total leaf area of each plant was estimated using a Leaf Area Meter (LI-3000 Leaf Area Meter coupled with the Li-3050 leaf charger, Li-Cor Inc., Nebraska, USA) by collecting all the available leaves that were present at the end of the experiment. The harvested grapevine roots (only the new growth), branches, and leaves were oven dried at 60°C for a week, and the dry weight of each sample was recorded.

2.3 ICP-MS analysis

For each treatment, all leaves and all roots from a plant were collected, dried at 65°C, and ground to a fine powder using a TissueLyser II. Three biological replicates were prepared for both leaf and root tissues. Approximately 0.2–0.3 g of each sample was weighed and acid digested with 69% ultrapure HNO_3 (Carlo Erba, Milano, Italy) in a single reaction chamber microwave digestion system (UltraWAVE, Milestone, Shelton, CT, USA). The digested samples were diluted to 2% HNO3 with ultrapure grade water (18.2 MW·cm at 25°C), and the concentrations were then determined using an inductively coupled plasma–mass spectrometer (ICP-MS, iCAP™ RQ, Thermo Scientific). Element quantification was carried out using certified multi-element standards (CPI International, https://cpiinternational.com). NIST standard reference materials 1573a (tomato leaves) and 1570a (spinach leaves) were used as external certified references, which were digested and analyzed the same way as the samples.

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2.4 Cu accumulation assessment

The methodology proposed by Lai et al. (2010) and employed by Vršič et al. (2023) was used to characterize the rate of Cu^{2+}uptake from the soil into the grapevine grafts in our experimental setup.

In accordance with the definition proposed by (Juang et al., 2012), the bioaccumulation factor (BAF) is established as the ratio of Cu^{2+}concentrations in the grapevine leaf (C_{gl}) to the Cu^{2+}concentration in the substrate (C_{su}), expressed by the equation $BAFl = C_{gl}/C_{su}$, where BAFl represents the bioaccumulation factor for the grapevine leaf, C_{gl} is the Cu^{2+} concentration in the leaf (mg kg^{-1}), and C_{su} is the Cu^{2+}concentration in the substrate (mg kg^{-1}).

The translocation factors (TFs) represent the ratios of Cu^{2+} concentrations in the roots to those in other organs of grapevine grafts. Therefore, the TFs for Cu^{2+} translocation from roots to trunk and canes were estimated according to the equation $TFl = C_{leaf}/C_{root}$ (Chopin et al., 2008; Busuioc et al., 2011), where C_{leaf} is the Cu^{2+}concentration in the leaf (mg kg^{-1}), C_{root} is the Cu concentration in the roots, and TFl signifies the translocation factor through the root to the leaf.

2.5 Data treatment, statistical analysis and data visualization

Statistical analyses were performed using R software (Team, 2025). Differences among the days of leaf gas exchanges (*A, E, gs*), chlorophyll parameters (SPAD and florescence) and Ψ_{stem} were tested by one-way ANOVA. Mean separation was performed with the Tukey HSD test ($p < 0.05$). Biometric values were also tested by one-way ANOVA and the mean separation was performed with the Tukey HSD test ($p < 0.001$). Both ANOVA and Tukey HSD tests were conducted using the stats package (v4.5.0), and *post hoc* analyses were performed using the agricolae package (de Mendiburu, 2023).

Principal component analysis (PCA) was performed to evaluate patterns in the dataset using the prcomp function in R. PCA visualization was carried out using the ggfortify package (Horikoshi and Tang, 2018) and plotted with ggplot2 (Wickham, 2016). Data wrangling and preparation were managed with dplyr (Wickham et al., 2023) and readxl (Wickham and Bryan, 2025).

For visualization of mean comparisons (e.g., bar plots), customized plotting was performed using ggplot2 (Wickham, 2016) and enhanced with the ggpattern package (Mike et al., 2025) for pattern fills. Thematic styling was applied using ggthemes (Arnold, 2024), and multi-panel plot arrangements were constructed using the patchwork package (Pedersen, 2024). Additional data manipulation and formatting tasks were handled with the plyr (Wickham, 2011), stringr (Wickham, 2023) and tidyr (Wickham et al., 2024) packages.

All base functions and core graphical capabilities were supported by R's native base, graphics, grDevices, methods, utils, and datasets packages (v4.5.0; R Core Team, 2025).

3 Results

3.1 Soil humidity

Soil moisture measurements began at the onset of the drought stress (T1), as the soil was fully irrigated prior to this point. In the fully irrigated treatments (Control, Cu), soil humidity remained consistently high and constant throughout the entire growing period (Table 1), ranging from approximately 0.27 to 0.39 m^3/m^3 between the initial and final measurements (T1–T3), indicating that the soil could guarantee a sufficient availability of water for the request of the plants. In contrast, in non-irrigated treatments (DS, Cu +DS),soil humidity reaching minimum values between ~0.02 and ~0.1 m^3/m^3 at the peak of the stress (T2), indicated a severe water deficit. Following rewatering (T3), soil moisture in these treatments recovered to levels between 0.31 and 0.42 m^3/m^3. Statistical analysis confirmed significant differences in soil moisture across treatments during stress and recovery phases, although no substantial rootstock-specific differences were observed for this parameter.

3.2 Assessment of grapevine plants' physiological status

As far as Ψ_{stem} is concerned (Table 1), in the fully irrigated treatments (Control, Cu), the Ψ_{stem} ranged over the whole growing period from -0.3 to -0.6 MPa, confirming adequate water availability throughout the cycle. Under drought stress (DS, Cu+DS), Ψ_{stem} values dropped to -0.8 - -1.7 MPa, indicating severe stress (Table 1). Upon rewatering, values partially recovered to -0.4 - -0.7 MPa. At the peak of drought stress, M4 maintained much less negative Ψ_{stem} to soil moisture ratios (MPa/m^3/m^3), reaching about –12.6 under DS and –13.1 under Cu+DS, while SO4 dropped to –62.0 and –67.7 and 1103P to –105.0 and –77.2, indicating that M4 sustained higher Ψ_{stem} at the same soil moisture and therefore displayed higher water uptake efficiency.

Stomatal conductance, transpiration rate and photosynthetic rate (Table 2) exhibited consistently high intensity throughout the entire cycle under full irrigation conditions (Control, Cu), while in drought-stressed treatments (DS, Cu+DS), all three parameters progressively declined until rewatering. At the peak of drought stress, no statistically significant differences were observed between rootstocks under drought-stressed conditions (DS and Cu+DS), whereas among fully irrigated plants (Control and Cu), 1103 Paulsen exhibited the highest transpiration rate compared to M4 and SO4. Additionally, M4 plants treated with Cu^{2+} showed noticeably lower values of both transpiration and stomatal conductance compared to the control. After recovery, a general trend toward restoration was observed, though the extent varied by rootstock (Table 2). Intrinsic water use efficiency (iWUE) (Table 3) remained unaffected until the peak of drought stress in 1103 Paulsen and M4, consistent with their reported drought tolerance. Interestingly, even when subjected to combined stresses (Cu+DS), vines grafted on M4 showed values of iWUE that were not different

TABLE 1 Soil moisture and Midday stem water potential (Ψ_{stem}) measured from the start till the end of the experiment.

Cultivar	Treatment	T0	T1	T2	T3
Soil moisture (m^3/m^3)					
1103 Paulsen	Control		0.35± 0.01Aa	0.36± 0.01Ba	0.35± 0.01BCDa
	Cu		0.34± 0.01Aa	0.37± 0.01ABa	0.35± 0.00CDa
	Cu+DS		0.35± 0.01Aa	0.02± 0.00Db	0.36± 0.01BCDa
	DS		0.34± 0.01Aa	0.02± 0.01Db	0.35± 0.01BCDa
M4	Control		0.34± 0.01Aa	0.39± 0.01ABa	0.31± 0.01Da
	Cu		0.35± 0.01Aa	0.40± 0.01Aa	0.32± 0.01Da
	Cu+DS		0.32± 0.01ABa	0.10± 0.01Cb	0.31± 0.01Da
	DS		0.33± 0.01Aa	0.10± 0.01Cb	0.33± 0.02CDa
SO4	Control		0.31± 0.01ABa	0.35± 0.01Ba	0.36± 0.03ABCDa
	Cu		0.27± 0.01BCab	0.36± 0.01Ba	0.39± 0.01ABCa
	Cu+DS		0.21± 0.01Dc	0.03± 0.01Db	0.42± 0.01Aa
	DS		0.23± 0.02CDbc	0.03± 0.01Db	0.41± 0.01ABa
Ψ_{stem} *(MPa)*					
1103 Paulsen	Control	-0.55± 0.02ABa	-0.47± 0.02Aa	-0.48± 0.02Aa	-0.43± 0.01Aa
	Cu	-0.58± 0.03ABa	-0.49± 0.02Aa	-0.51± 0.01Aa	-0.48± 0.02ABa
	Cu+DS	-0.61± 0.04Aa	-0.48± 0.04Aa	-1.32± 0.05Bb	-0.49± 0.02ABa
	DS	-0.54± 0.04ABa	-0.50± 0.04Aa	-1.38± 0.10Bb	-0.48± 0.03ABa
M4	Control	-0.55± 0.01ABa	-0.50± 0.02Aa	-0.40± 0.02Aa	-0.49± 0.01ABab
	Cu	-0.54± 0.03ABa	-0.59± 0.03Aa	-0.39± 0.01Aa	-0.48± 0.02ABa
	Cu+DS	-0.52± 0.03ABa	-0.58± 0.03Aa	-1.28± 0.03Bb	-0.55± 0.01BCbc
	DS	-0.56± 0.02ABa	-0.58± 0.01Aa	-1.23± 0.02Bb	-0.57± 0.02BCDc
SO4	Control	-0.48± 0.03Ba	-0.46± 0.02Aa	-0.48± 0.02Aa	-0.52± 0.03ABCa
	Cu	-0.52± 0.01ABa	-0.51± 0.02Aa	-0.48± 0.03Aa	-0.54± 0.03BCa
	Cu+DS	-0.51± 0.02ABa	-0.51± 0.02Aa	-1.40± 0.06Bb	-0.65± 0.02Db
	DS	-0.53± 0.01ABa	-0.52± 0.02Aa	-1.39± 0.09Bb	-0.62± 0.03CDab

T0 represents the time point before the first copper application, T1 marks the onset of water stress induction, T2 corresponds to the peak of drought stress, and T3 refers to two days after the beginning of the recovery phase. Data are presented as means ± standard error. Differences between treatments, i.e., Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), within the same rootstock were assessed using Tukey's HSD test, and significant differences ($p < 0.05$) are indicated by different lowercase letters within columns. Significant differences between rootstocks are indicated by uppercase letters.

from those of vines exposed to drought or Cu^{2+}stress only. In SO4, on the other hand, iWUE showed no significant change across treatments. After rehydration, differences among treatments largely disappeared (Table 3).

The Chlorophyll Fluorescence (Fv/Fm) values (Table 4) generally ranged from 0.75 to 0.9 and remained stable in irrigated treatments (Control, Cu) in all the rootstocks considered. In contrast, drought-stressed treatments (DS and Cu+DS) caused a decline in Fv/Fm, particularly at the peak of drought stress (T2). The most severe reduction was observed in the 1103 Paulsen rootstock under DS, where Fv/Fm dropped to 0.63 at T2. A similar trend, albeit with a lower extent, was noted in SO4 and M4 under DS and Cu+DS conditions. After rewatering (T3), Fv/Fm values showed partial recovery in most treatments, although values remained slightly lower than the pre-stress phase, especially in SO4.

Leaf relative chlorophyll content, expressed as SPAD values (Table 4), varied in response to drought and Cu stress, showing rootstock-specific patterns. In 1103 Paulsen and SO4, a significant decrease in SPAD values was observed under drought (DS) and combined Cu+DS treatments, especially at the peak of drought stress (T2) and after partial recovery (T3). In contrast, M4 maintained relatively stable SPAD values across all treatments, with no significant reduction under drought stress or Cu^{2+} application. Under fully irrigated conditions (Control and Cu), all rootstocks showed minimal variation in SPAD values throughout the experimental period.

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TABLE 2 Leaf transpiration (E), Photosynthetic activity (A), and Stomatal conductance (gs).

Cultivar	Treatment	T0	T1	T2	T3
E (mmol H_2O m^{-2} s^{-1})					
1103 Paulsen	Control	2.45± 0.09Ca	2.68± 0.18ABa	4.44± 0.13Aa	3.07± 0.18Aa
	Cu	2.50± 0.15BCa	2.80± 0.13ABa	4.10± 0.15ABa	2.89± 0.22ABa
	Cu+DS	2.61± 0.17BCa	2.63± 0.13Ba	0.71± 0.14Db	2.51± 0.22ABCa
	DS	2.65± 0.12BCa	2.66± 0.11ABa	0.67± 0.23Db	2.45± 0.27ABCDa
M4	Control	2.97± 0.09BCb	2.81± 0.12ABa	3.59± 0.08Ba	3.18± 0.10Aa
	Cu	2.90± 0.16BCb	2.72± 0.12ABa	2.80± 0.15Cb	2.55± 0.14ABCb
	Cu+DS	3.93± 0.11Aa	2.95± 0.13ABa	0.95± 0.05Dc	1.81± 0.09CDc
	DS	3.04± 0.06Bb	3.04± 0.17ABa	1.13± 0.10Dc	2.08± 0.11BCDc
SO4	Control	2.83± 0.09BCa	3.29± 0.13Aa	2.52± 0.13Ca	2.64± 0.27ABCab
	Cu	2.72± 0.07BCa	2.97± 0.11ABa	2.37± 0.23Ca	2.77± 0.33ABa
	Cu+DS	2.86± 0.12BCa	3.21± 0.08ABa	0.65± 0.06Db	1.56± 0.14Dc
	DS	2.70± 0.09BCa	3.03± 0.10ABa	0.62± 0.08Db	1.75± 0.21CDbc
A (µmol CO_2 m^{-2} s^{-1})					
1103 Paulsen	Control	15.09± 1.23Aa	12.95± 1.71Aa	11.59± 0.78Aa	9.53± 1.31ABa
	Cu	13.39± 1.49Aa	10.86± 2.31Aa	8.69± 1.01ABa	8.74± 1.20ABa
	Cu+DS	14.43± 1.29Aa	14.42± 3.12Aa	1.70± 0.73Cb	8.21± 1.12ABCa
	DS	13.29± 0.82Aa	12.68± 1.38Aa	2.08± 0.91Cb	7.11± 1.21ABCa
M4	Control	13.43± 1.17Aa	12.90± 1.23Aa	11.01± 0.48ABCa	10.72± 0.45Aa
	Cu	12.41± 0.55Aa	11.09± 0.71Aa	8.42± 0.77ABCb	8.99± 0.80ABab
	Cu+DS	14.57± 0.99Aa	12.65± 0.78Aa	1.61± 0.26Cc	6.12± 0.35BCc
	DS	15.15± 0.94Aa	12.63± 0.95Aa	3.37± 0.53BCc	7.72± 0.55ABCbc
SO4	Control	11.85± 0.63Aa	12.19± 0.81Aa	10.65± 0.39ABCa	9.34± 0.91ABa
	Cu	12.21± 0.73Aa	11.30± 0.60Aa	8.13± 1.01ABCb	9.42± 0.63ABa
	Cu+DS	12.67± 0.72Aa	11.99± 0.65Aa	1.26± 0.14Cc	4.58± 0.59Cbc
	DS	12.50± 0.43Aa	11.65± 0.69Aa	1.11± 0.21Cc	6.69± 0.96ABCab
gs (mol m^{-2} s^{-1})					
1103 Paulsen	Control	0.24± 0.02Ga	0.25± 0.02Da	0.26± 0.01Aba	0.25± 0.03Aa
	Cu	0.23± 0.03Ga	0.27± 0.02Ca	0.21± 0.01BCb	0.21± 0.03ABab
	Cu+DS	0.24± 0.03Fa	0.25± 0.02Da	0.02± 0.01Dc	0.13± 0.02BCDb
	DS	0.25± 0.02EFa	0.28± 0.03Da	0.03± 0.01Dc	0.13± 0.03BCDb
M4	Control	0.23± 0.01BCab	0.24± 0.02Ca	0.31± 0.02Aa	0.21± 0.02ABa
	Cu	0.19± 0.01CDb	0.22± 0.02CDa	0.21± 0.02BCb	0.15± 0.01BCb
	Cu+DS	0.27± 0.02Aa	0.29± 0.03Ba	0.04± 0.00Dc	0.10± 0.01CDb
	DS	0.23± 0.01Bab	0.29± 0.03Ba	0.04± 0.00Dc	0.11± 0.01CDb
SO4	Control	0.20± 0.01Da	0.27± 0.01Aa	0.19± 0.02Ca	0.17± 0.02ABCa

(Continued)

TABLE 2 Continued

Cultivar	Treatment	T0	T1	T2	T3
gs (mol m^{-2} s^{-1})					
	Cu	0.22± 0.01Ea	0.29± 0.02Ba	0.19± 0.02Ca	0.20± 0.01ABA
	Cu+DS	0.21± 0.01Da	0.28± 0.01Aa	0.02± 0.00Db	0.06± 0.01DB
	DS	0.20± 0.01EFa	0.26± 0.01Ba	0.02± 0.00Db	0.08± 0.01CDb

T0 represents the time point before the first copper application, T1 marks the onset of water stress induction, T2 corresponds to the peak of drought stress, and T3 refers to two days after the beginning of the recovery phase. Data are presented as means ± standard error. Differences between treatments, i.e., Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), within the same rootstock were assessed using Tukey's HSD test, and significant differences ($p < 0.05$) are indicated by different lowercase letters within columns. Significant differences between rootstocks are indicated by uppercase letters.

3.3 Leaf area and dry biomass

The biometric data, reported in Table 5, demonstrated that water deprivation (DS and Cu+DS) had a significant negative impact on both leaf area (LA) and dry shoot biomass across all three rootstocks. In contrast, Cu^{2+}application alone did not significantly affect biomass accumulation at either the root or shoot level.

In terms of LA, 1103 Paulsen and SO4 showed the most pronounced reduction under drought conditions. For example, 1103 Paulsen decreased from 5353.3 cm^2 (Control) to 2361.6 cm^2 (DS) and 2620.2 cm^2 (Cu+DS), while SO4 declined from 5002.7 cm^2 (Control) to 1954.4 cm^2 (DS). Although M4 was also affected, it retained higher LA values under stress (e.g., 4216.5 cm^2 under DS), indicating a greater capacity to maintain leaf development under water limitation (Table 5).

Regarding dry leaf biomass, M4 retained higher values under drought compared to SO4 and 1103 Paulsen. Notably, leaf dry weight of SO4 under DS fell to 6.95 g, and 1103 Paulsen dropped to 5.44 g, M4 instead showed 15.12 g, suggesting a greater tolerance. For shoot dry weight, only SO4 under drought stress showed a significant reduction of dry biomass (Table 5). At the root level, M4 and 1103 Paulsen did not exhibit significant differences across treatments, maintaining root dry biomass between 4.0 and 5.1 g, including under drought. In contrast, SO4 showed a marked reduction in root biomass, from 3.78 g (Control) to 2.31 g (DS), reflecting lower drought resilience in below-ground organs.

3.4 Copper bioaccumulation and translocation

The ionomic data has been investigated to calculate Cu^{2+}bioaccumulation and translocation factors, to shed light on possible different behaviors depending on the stressing conditions and the rootstocks genotype. The analysis of copper content (Figure 1A), bioaccumulation factor (Figure 1B), and translocation factor (Figure 1C) revealed distinct rootstock-

TABLE 3 Intrinsic water use efficiency (iWUE).

Cultivar	Treatment	T0	T1	T2	T3
iWUE (µmol $mmol^{-1}$)					
1103 Paulsen	Control	66.90± 7.14Aa	45.32± 5.26Aa	45.56± 3.16Ab	42.11± 7.61Da
	Cu	61.10± 5.37Aa	42.55± 8.43Aa	42.04± 6.38ABb	47.39± 8.65CDa
	Cu+DS	66.32± 8.56Aa	52.52± 10.04Aa	86.74± 10.48Ca	65.26± 6.33ABCDa
	DS	59.75± 9.02Aa	42.37± 5.78Aa	130.92± 27.18Ca	57.46± 5.75BCDa
M4	Control	57.15± 2.45Aab	54.07± 4.74Aa	36.89± 2.16ABb	53.45± 4.88BCDa
	Cu	66.98± 2.86Aa	52.47± 4.26Aa	43.90± 5.69Bb	61.67± 3.85ABCDa
	Cu+DS	50.27± 2.27Ab	46.63± 4.28Aa	48.12± 7.98Ca	68.97± 8.31ABCDa
	DS	69.34± 6.29Aa	46.71± 4.17Aa	92.80± 17.11Cb	73.57± 5.32ABCa
SO4	Control	60.23± 3.77Aa	45.71± 4.15Aa	59.35± 4.23ABa	59.83± 4.48ABCDa
	Cu	58.43± 6.14Aa	41.69± 4.67Aa	45.05± 5.54Ba	50.35± 4.96BCDa
	Cu+DS	61.24± 3.63Aa	43.58± 2.08Aa	61.22± 8.80Ca	80.25± 10.80ABa
	DS	63.44± 4.75Aa	45.78± 3.69Aa	47.14± 8.04Ca	91.89± 5.92Aa

Measured from the start till the end of the experiment. T0 represents the time point before the first copper application, T1 marks the onset of drought stress induction, T2 corresponds to the peak of water stress, and T3 refers to two days after the beginning of the recovery phase. Data are presented as means ± standard error. Differences between treatments, *i.e.*, Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), within the same rootstock were assessed using Tukey's HSD test, and significant differences ($p < 0.05$) are indicated by different lowercase letters within columns. Significant differences between rootstocks are indicated by uppercase letters.

TABLE 4 Chlorophyll fluorescence (Fv/Fm) and SPAD measured from the start till the end of the experiment.

Cultivar	Treatment	T0	T1	T2	T3	T0	T1	T2	T3
		Fv/Fm				***SPAD***			
1103 Paulsen	Control	0.80± 0.01Aa	0.79± 0.01Aa	0.80± 0.01Aa	0.78± 0.01ABa	33.01± 0.45Aa	34.91± 0.74ABCa	30.10± 1.64ABCa	28.04± 1.65BCDa
	Cu	0.80± 0.00Aa	0.79± 0.02Aa	0.77± 0.01ABa	0.78± 0.00ABa	31.90± 0.81Aa	31.11± 0.75Cb	29.24± 1.52BCa	24.52± 1.57CDa
	Cu+DS	0.80± 0.00Aa	0.82± 0.00Aa	0.73± 0.07ABa	0.79± 0.01ABa	31.87± 0.54Aa	31.73± 0.71BCb	14.06± 2.90Db	13.04± 3.36EFb
	DS	0.79± 0.01Aa	0.82± 0.00Aa	0.63± 0.08Ba	0.74± 0.05ABa	32.35± 0.93Aa	31.82± 0.98BCab	17.90± 2.30Db	10.46± 1.59Fb
M4	Control	0.81± 0.00Aa	0.81± 0.00Aa	0.80± 0.00Aa	0.80± 0.00Aa	34.57± 0.72Aa	35.79± 0.75ABa	35.49± 0.94ABa	36.78± 0.97Aab
	Cu	0.81± 0.00Aa	0.81± 0.00Aa	0.81± 0.00Aa	0.80± 0.00Aa	33.44± 1.05Aa	36.19± 0.91Aa	36.15± 0.53Aa	38.23± 0.56Aa
	Cu+DS	0.80± 0.00Aa	0.80± 0.01Aa	0.80± 0.01Aa	0.79± 0.01Aa	32.54± 0.76Aa	35.71± 0.88ABa	35.30± 0.47ABCa	35.55± 0.43Aab
	DS	0.80± 0.00Aa	0.80± 0.00Aa	0.81± 0.00Aa	0.80± 0.00Aa	33.34± 0.56Aa	35.97± 0.73Aa	34.62± 0.60ABCa	35.04± 0.95ABb
SO4	Control	0.81± 0.00Aa	0.81± 0.00Aa	0.79± 0.00Aab	0.79± 0.00Aa	35.00± 0.97Aa	35.49± 1.03ABa	34.75± 0.88ABCa	31.50± 0.86ABCa
	Cu	0.81± 0.00Aa	0.81± 0.00Aa	0.80± 0.00Aa	0.78± 0.01ABa	35.34± 0.67Aa	34.37± 0.77ABCa	33.95± 0.60ABCa	33.80± 1.14ABa
	Cu+DS	0.81± 0.01Aa	0.80± 0.00Aa	0.79± 0.00Aa	0.70± 0.05Ba	35.00± 0.86Aa	35.82± 0.85ABa	27.73± 1.61Ca	21.04± 2.18Db
	DS	0.81± 0.00Aa	0.80± 0.01Aa	0.76± 0.02ABb	0.76± 0.02ABa	34.83± 0.69Aa	32.69± 1.29ABCa	18.09± 4.19Db	20.59± 2.59DEb

T0 represents the time point before the first copper application, T1 marks the onset of water stress induction, T2 corresponds to the peak of drought stress, and T3 refers to two days after the beginning of the recovery phase. Data are presented as means ± standard error. Differences between treatments, i.e., Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), within the same rootstock were assessed using Tukey's HSD test, and significant differences ($p < 0.05$) are indicated by different lowercase letters within columns. Significant differences between rootstock are indicated by uppercase letters.

TABLE 5 Leaf area, leaves dry weight, shoot dry weight, root dry weight measured at the end of the experiment.

Cultivar	Treatment	Total leaf area per vine (cm^2)	Dry Leaves (g)	Dry Shoots (g)	Dry Roots (g)
1103 Paulsen	Control	5353.32 ± 423.24ABa	39.63 ± 4.52Aa	35.66 ± 6.42ABCa	4.48 ± 0.61ABa
	Cu	4772.25 ± 535.92ABCa	19.72 ± 2.75Bb	39.03 ± 6.17Aa	4.88 ± 0.71ABa
	Cu+DS	2620.19 ± 327.65DEb	6.28 ± 1.35EFc	36.24 ± 3.71ABa	4.05 ± 0.59ABa
	DS	2361.61 ± 408.17DEb	5.44 ± 1.75Fc	30.93 ± 3.05ABCa	4.25 ± 0.54ABa
M4	Control	5868.78 ± 430.05Aa	17.76 ± 1.63BCa	28.58 ± 2.93ABCa	4.93 ± 0.66Aa
	Cu	5105.07 ± 527.32ABCab	17.24 ± 2.31BCDa	27.99 ± 3.74ABCa	4.12 ± 0.50ABa
	Cu+DS	3671.64 ± 209.52BCDEb	14.46 ± 1.40BCDEFa	19.68 ± 2.20BCa	3.99 ± 0.71ABa
	DS	3832.86 ± 340.47BCDEb	15.12 ± 1.52BCDEa	21.19 ± 2.74BCa	4.19 ± 0.55ABa
SO4	Control	4113.87 ± 357.23ABCDa	14.19 ± 1.30BCDEFa	29.24 ± 3.33ABCa	3.78 ± 0.36ABa
	Cu	3515.66 ± 376.30CDEab	9.43 ± 1.56CDEFb	22.54 ± 2.91ABCab	2.75 ± 0.43ABab
	Cu+DS	2762.92 ± 192.18DEbc	8.13 ± 0.53DEFb	21.89 ± 2.10ABCab	2.31 ± 0.22Bb
	DS	2246.76 ± 177.52Ec	6.95 ± 0.54EFb	18.58 ± 2.27Cb	2.55 ± 0.36ABab

Differences between treatments, i.e., Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), in the same rootstock were determined using Tukey HSD test and significant differences ($p < 0.05$) are indicated by different lowercase letters when comparing means in columns; difference between all the rootstocks are indicated by uppercase letters.

specific responses to copper supplementation and drought stress. Across all treatments, Cu^{2+}accumulation in root tissues was significantly higher in Cu and Cu+DS conditions compared to the control and DS treatments ($p < 0.05$), whilst it remained largely unaffected in leaves, except for the specific case of drought and heavy metal stressed 1103 Paulsen (Figure 1A). Among rootstocks, SO4 accumulated the highest Cu^{2+} levels in roots, particularly under Cu+DS, with root concentrations exceeding ~1400 mg/kg, reflecting its higher sensitivity to combined stress. In contrast, M4 exhibited the lowest root Cu^{2+}accumulation under both Cu and Cu+DS treatments (e.g., ~290 mg/kg), suggesting a superior exclusion or sequestration capacity at the root level (Figure 1A).

These trends were mirrored by the Bioaccumulation factor (BAF), where M4 consistently showed lower BAF values, indicating limited translocation from soil to roots. For instance, M4 under Cu treatment had a BAF in the root of approximately ~2.45, significantly lower than the ~7.70 observed in SO4 under the same condition, and values of ~ 11 under Cu+DS (Figure 1B). The translocation factor (TF) from roots to leaves also differed between rootstocks (Figure 1C). Whilst all Cu-treated plants displayed lower TFs compared to controls, M4 maintained relatively higher TF values under Cu+DS and Cu, with values of ~0.14, possibly due to reduced root accumulation. Conversely, SO4 and 1103 Paulsen showed strong Cu^{2+}retention in roots with limited translocation to aerial parts.

3.5 Ionomics

To gain a better understanding about the effects of treatments on plant nutrients balance, ionomic data (Supplementary Table S2) obtained from the leaves and roots were subjected to multivariate statistical analyses, specifically principal component analysis (PCA). The PCA model obtained for root samples explained approximately 66% of the total variance in the dataset and revealed a clear separation of samples along PC1 (Figure 2A), determined by the different rootstock's genotype. Notably, the M4 treatment exhibited distinct separation, primarily driven by phosphorus (P), manganese (Mn), and potassium (K). Similarly, samples obtained from 1103 Paulsen rootstock clustered together, independently from the treatments, while those of SO4 were separated primarily along PC2 (Figure 2A). When the ionome of shoot tissue is considered, the PCA model generated explained 62% of the total variance and showed a distinct behavior depending on the rootstock considered (Figure 2B). In particular, SO4 samples created a single cluster that was partially overlapping with 1103 Paulsen samples. Nevertheless, 1103 Paulsen samples showed a separation in two distinct groups within the cluster (Figure 2B). On the other hand, M4 samples showed a clear separation with respect to SO4 and 1103 Paulsen along the PC1, albeit a significant segregation of M4 samples was also observed along PC2 (Figure 2B).

To further investigate the treatment effects with a focus on the different rootstocks, additional PCA were performed by subsetting the dataset according to rootstock type (Figure 3). The model generated with SO4 roots dataset explained approximately 73% of the total variance (Figure 3A). Along PC1, drought-stressed samples (i.e., DS and Cu+DS) resulted separated from the others, suggesting a higher hierarchy of the stress respect to Cu. Nevertheless, the overall samples distribution showed the separation of independent clusters (Figure 3A), possibly suggesting that each single treatment could represent a peculiar condition, as also confirmed by the alteration in the correlations between nutrients (Supplementary Figure S1). Interestingly, a similar behavior was also observed at leaf level (Figure 3B and Supplementary Figure S2).

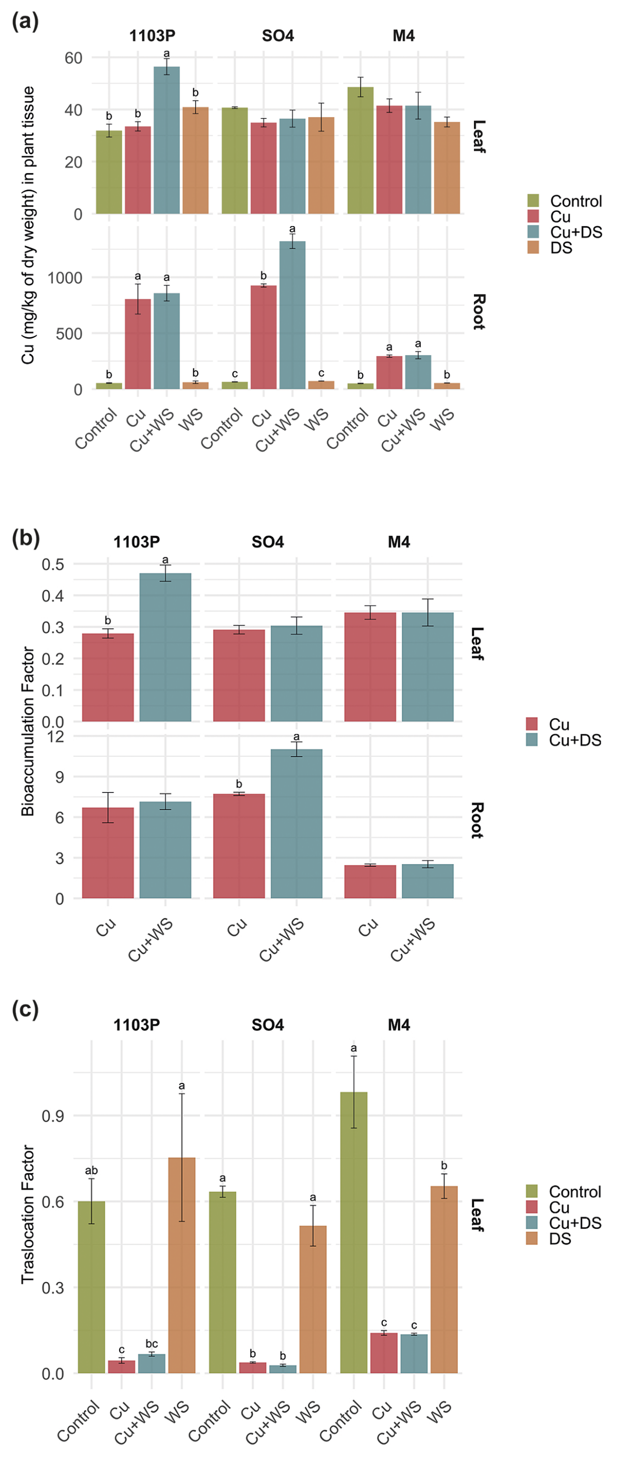

FIGURE 1
Copper concentration (mg kg^{-1} dry weight) **(a)**, Bioaccumulation Factor **(b)** and Translocation Factor of Copper from roots to leaves **(c)** in grapevine plants subjected to different treatments, *i.e.*, Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS). SO4, 1103 Paulsen and M4 are the grapevine rootstock used. Data are reported as means ± SE, n = 3. Differences between treatments in the same rootstock were determined using Tukey HSD test and significant differences (p < 0.05) are indicated by different lowercase letters comparing means in columns.

The PCA model obtained for the roots of 1103 Paulsen explained approximately 67% of the dataset variance and showed a main sample separation along the PC1 (Figure 3C). In particular, roots subjected to drought stress (DS) showed a significantly different ionome as compared to the other samples. Interestingly, when correlations among elements were examined, they showed only synergisms (Supplementary Figure S1). On the other hand, Control, Cu and Cu+DS samples separated along the PC2, with Cu and Cu+DS samples being richer in Cu^{2+}as expected (Figure 3C). Nevertheless, the reciprocal dynamics established by mineral nutrients within Cu and Cu+DS samples are different, highlighting different physiological outcomes of single and combined stresses (Supplementary Figure S1). This distribution could suggest that in 1103 Paulsen rootstock, at root levels, the Cu^{2+}stress might have prevalence on drought stress, at least when the uptake of mineral nutrients is concerned. Contrarily, at leaf level, a clear separation of samples driven by the DS (i.e., along PC1) was observed (Figure 3D and Supplementary Figure S2).

The PCA model generated with the M4 roots dataset explained approximately 77% of the total variance and showed the separation of samples in three independent clusters (Figure 3E). The first group encompassed Control and DS samples, whereas Cu and Cu+DS samples were separated and independent. Such observation further confirms that M4 rootstock is tolerant to drought stress and highlights that single Cu or combined Cu+DS stress can have significantly different effects in the accumulation of minerals, as also confirmed by the correlations analyses (Supplementary Figure S1). Interestingly, a coherent behavior has been also observed at leaf level (Figure 3F and Supplementary Figure S2).

4 Discussion

The present study investigated the physiological, biochemical, and ionomic responses of grapevines (Pinot gris variety) grafted onto three rootstocks (*i.e.*, M4, SO4, and 1103 Paulsen), following exposure to drought, Cu^{2+}toxicity, or their combination under controlled greenhouse conditions. The results are then discussed in terms of genotype-specific stress-response strategies, with particular attention to how stress interactions affect plant water relations, photosynthetic recovery, and nutrient homeostasis, providing insights into rootstock selection for viticultural systems increasingly exposed to multifactorial stress.

Results reported in Table 1 and referred to the midday Ψ_{stem} measurements in leaves of Pinot gris vines grafted onto the three different rootstocks show that, as expected, fully irrigated vines maintained stable and adequate water status throughout the experiment, whereas non-irrigated plants experienced a clear and pronounced drought stress. Following rewatering, only a partial recovery of Ψ_{stem} values was observed, suggesting that the effects of severe water limitation were only partially reversible within the short time frame of this study. Interestingly, the decline in Ψ_{stem} values under drought stress did not consistently mirror changes in soil moisture content, particularly during the early phases of

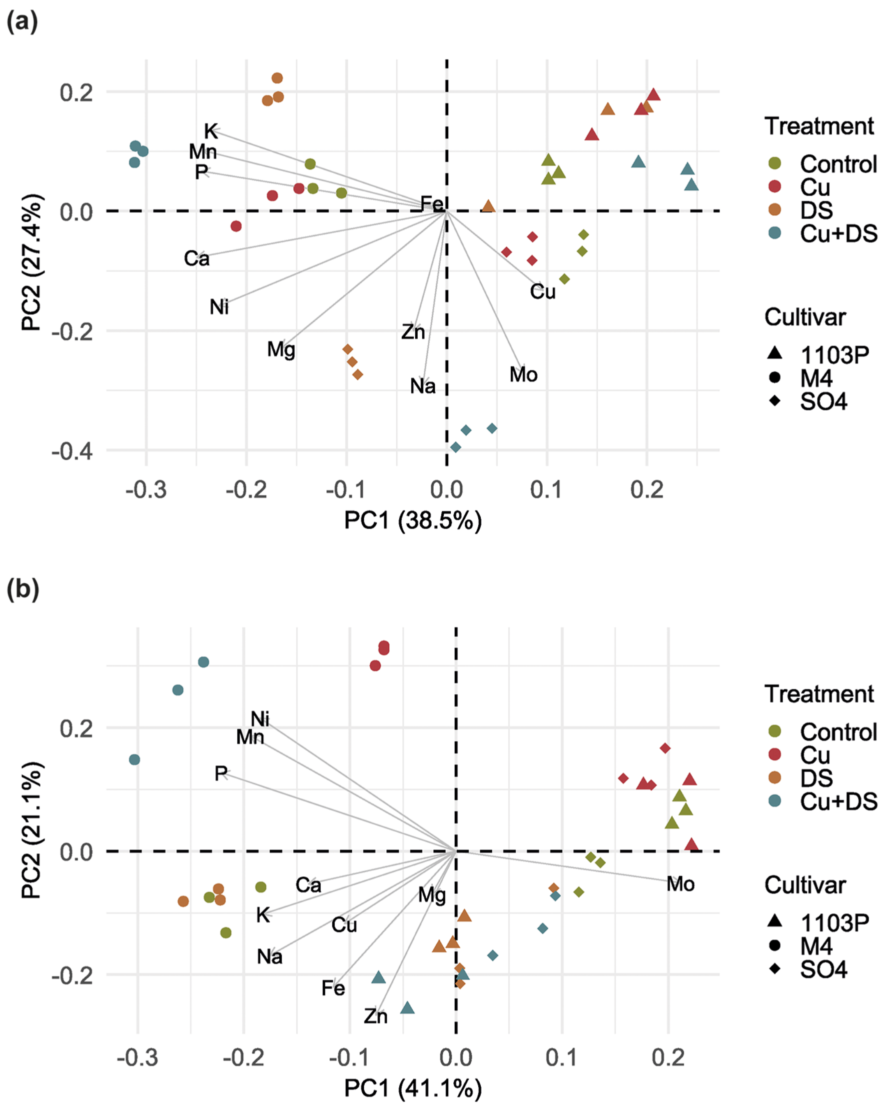

FIGURE 2
Principal component analysis of ionomic data of root **(a)** and leaf **(b)** samples of SO4, 1103 Paulsen and M4 grapevine rootstock. Each point on the plot represents an individual tissue sample, with shapes representing different grapevine cultivars and color representing different treatments, *i.e.*, Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS).

drought (data not shown). This decoupling indicates a degree of physiological buffering or delayed stress perception, supporting previous findings that grapevines may exhibit a lag between decreasing soil water availability and measurable physiological responses at the leaf or whole-plant level (Chaves et al., 2010). Such temporal uncoupling may reflect the ability of rootstocks to temporarily compensate the deficit through hydraulic redistribution, osmotic adjustment, or internal water reserves. Notably, in treatments subjected to both drought and Cu stress, the reduction in Ψ_{stem} was, on average, 2% more pronounced, and its recovery appeared slightly limited compared to drought alone. This suggests that Cu^{2+}toxicity may exacerbate hydraulic dysfunction, possibly by damaging root tissues, inhibiting water uptake, or interfering with aquaporin activity (Fatemi et al., 2020). Copper-induced root stress may therefore amplify drought effects by limiting the plant's capacity to maintain water transport and recover after rehydration, particularly in more sensitive genotypes such as SO4. Therefore, these findings highlight how crucial strong hydraulic resilience in rootstocks is to withstand the compounded effects of drought and Cu^{2+} toxicity, which can severely impair water transport and recovery capacity.

Results in Table 2 showed that drought stress mainly impacted leaf-level biochemical and physiological processes across all rootstocks used, leading to significant decrease in transpiration, photosynthetic rate and stomatal conductance. These declines were most evident at the peak of drought stress and are consistent with classical drought response mechanisms aimed at limiting water loss (Benyahia et al., 2023; Shtai et al., 2024). Following rehydration, recovery of gas exchange was only partial after three days in plants grafted onto M4 and SO4 rootstocks, suggesting persistent

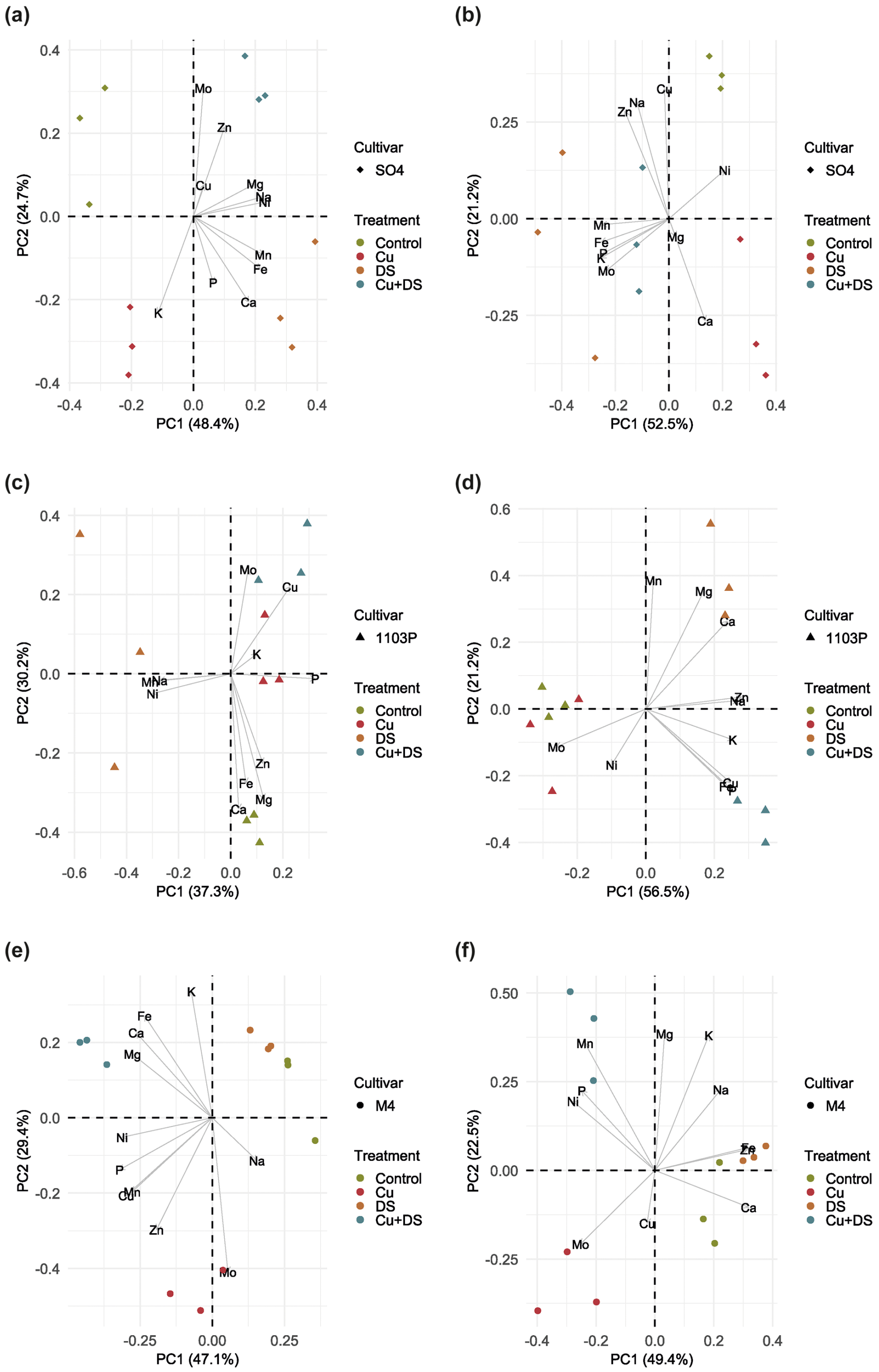

FIGURE 3
Principal component analysis of ionomic data of root and leaf samples of SO4 **(a, b)**, 1103 Paulsen **(c, d)** and M4 **(e, f)** grapevine rootstock. Each point on the plot represents an individual tissue sample, with colors indicating different treatment groups, *i.e.*, Control (untreated), Cu toxicity (Cu), combined Cu toxicity and drought stress (Cu+DS) and drought stress (DS), and shapes representing different rootstocks. The proximity of points on the plot suggests similarities or differences in elemental composition among the samples.

inhibition or delayed reactivation of both stomatal and mesophyll conductance (Flexas et al., 2006). In contrast, those grafted onto1103 Paulsen exhibited a faster recovery, which may be ascribed to its diminished leaf area post-stress, potentially lowering transpiration demand, and a more conservative water-use strategy. This observation aligns with previous findings (Pou et al., 2008), which indicates that the full stomatal recovery in water-stressed vines may require up to two weeks, particularly under midday Ψ_{stem} values comparable to those imposed in this work (−1.4 to −1.5 MPa). Additionally, in the presence of combined Cu^{2+}

toxicity and drought stress, recovery was even more limited compared to drought stress alone, particularly in plant grafted onto SO4, with drops of approximately 11% in E, 31% in A, and 23% in gs, suggesting that Cu^{2+} toxicity may exacerbate impairment of stomatal function or delay hydraulic reactivation during the recovery phase (Tashakorizadeh et al., 2023). More in general, the incomplete recovery of gas exchange parameters after rehydration may reflect persistent impairments in mesophyll conductance (Flexas et al., 2008), delayed reactivation of aquaporins (Pou et al., 2008), or continued ABA-mediated stomatal regulation (Zhang et al., 2006). The physiological bottlenecks here highlighted can limit the re-establishment of full photosynthetic capacity even when water availability is restored. These observations emphasize the need to consider rootstock-specific recovery dynamics, as the persistence of stress effects, especially under combined drought and Cu^{2+} toxicity, may affect short-term physiological functionality and, consequently, vineyard management decisions.

The *intrinsic* water use efficiency (iWUE)was introduced to compare photosynthetic properties regardless of the evaporative demand (Osmond et al., 1980). The increase of iWUE values at the peak of drought stress is coherent with the drought-related traits of 1103 Paulsen and M4 rootstocks (Bianchi et al., 2020), which appear to reduce stomatal conductance as a conservative strategy to limit water loss, while maintaining relatively stable rates of photosynthesis. This adaptive response suggests a preferential regulation of gas exchange in these genotypes, enabling sustained C assimilation under conditions of reduced water availability (Bertolino et al., 2019; Barl et al., 2025). In contrast, the lack of significant changes of iWUE values in plants grafted onto SO4 under similar stress conditions may indicate a less effective stomatal regulation or a decline in photosynthetic capacity, reinforcing its classification as a drought-sensitive genotype (Bertolino et al., 2019; Barl et al., 2025). Consistently, the normalization of iWUE values after rehydration might indicate partial recovery of stomatal function and photosynthetic activity, although with varying speeds among rootstocks (Table 3). From an agronomic perspective, these results underscore the importance of selecting rootstocks that support efficient water use regulation under drought, as demonstrated by grafted plants onto M4 and 1103 Paulsen.

The Fv/Fm ratio, a widely used indicator of the maximum quantum efficiency of PSII, decreased under increasing drought stress, as previously described in other studies focused on grapevine (Murchie and Lawson, 2013; Mashilo et al., 2018; Giorgi et al., 2019; Díaz-Barradas et al., 2020; Benyahia et al., 2023; Shtai et al., 2024). Under our experimental conditions, statistically significant differences in Fv/Fm were evident only for plants grafted onto 1103 Paulsen and SO4 at the peak of drought stress and for the latter also after the recovery (Table 4). These findings are consistent with existing literature, where Fv/Fm ratios typically range from 0.6 to 0.8 under drought and heat stress (Palliotti et al., 2015; Ju et al., 2018; Bernardo et al., 2022). Indeed, our results indicate that photosystem II efficiency is impaired under severe drought conditions, especially in plants grafted onto the more sensitive rootstock SO4 and align with previously reported thresholds for photoinhibition under abiotic stress in grapevines (Tzortzakis et al., 2020; Benyahia et al., 2023). The relatively stable Fv/Fm values in M4-grafted plants, even under the stresses, further support its already mentioned drought resilience. Overall, chlorophyll fluorescence proved to be a sensitive early indicator of drought stress across genotypes. On the other hand, stable SPAD values in fully irrigated plants could suggest that Cu^{2+} application alone did not significantly impair chlorophyll biosynthesis or N status, at least at the concentration used for this work. However, under drought (DS) and combined stress (Cu+DS), SPAD values dropped significantly in both plants grafted onto 1103 Paulsen and SO4, particularly at the peak of water deficit (T2) and following the recovery phase (T3). The observed decline likely reflects stress-induced chlorophyll degradation or impaired N metabolism, consistent with previous reports of drought-associated decrease in leaf greenness (Monteoliva et al., 2021; Hu et al., 2023; Zhao et al., 2023). In contrast, vines grafted onto M4 rootstock maintained stable SPAD values across all treatments, with no significant variation even under severe stress, suggesting a greater capacity to preserve chlorophyll integrity and N assimilation under adverse conditions. From an agronomic perspective, these results clearly confirm the M4 potential as a resilient rootstock for sustaining vine performance in increasingly harsh growing conditions, like under drought and combined (Cu^{2+} toxicity and drought) stress.

Biometric data (Table 5) indicate that plants grafted onto M4 and 1103 Paulsen maintained the root biomass accumulation under drought and combined drought-Cu^{2+} stresses, suggesting robust below-ground resilience. This is consistent with previous studies highlighting the importance of deep, well-developed root systems in drought-tolerant rootstocks such as 1103 Paulsen (Zhang et al., 2016). However, 1103 Paulsen exhibited a marked decrease in leaf area and shoot biomass under drought stress, suggesting a resource allocation strategy that prioritizes root maintenance over canopy development. Such a trade-off is characteristic of isohydric behavior, where water-conserving responses, like stomatal closure and growth inhibition, limit shoot expansion to preserve hydraulic integrity (Schultz, 2003; Lovisolo et al., 2010). This strategy may help ensure survival under prolonged drought but could impair C assimilation and delay post-stress recovery (Pou et al., 2008). Therefore, evaluating rootstock performance under stress should consider not only the ability to sustain root function but also the capacity to support rapid shoot regrowth once favorable conditions return, that is particularly important for young vines establishing their canopy in challenging environments (Cuneo et al., 2021). Copper stress alone, on the other hand, reduced shoot biomass in vines grafted onto SO4 and 1103 Paulsen but had minimal effects on the M4-grafted ones. This suggests that SO4 rootstock may lack effective Cu^{2+} detoxification or metal exclusion mechanisms, leading to impaired shoot function (Marastoni et al., 2019c; Cesco et al., 2021). Interestingly, the ability of M4 to preserve both root and shoot biomass under stress, including combined Cu^{2+} toxicity and drought, might underscores its integrated stress tolerance. Previous research has associated the rootstock M4 with high hydraulic conductance, enhanced rewatering recovery, and better physiological stability under water deficit (Meggio et al., 2014; Bianchi et al., 2020). Altogether these elements highlight the agronomic relevance of selecting rootstocks capable of sustaining both root and shoot growth under abiotic stress, with the M4 one demonstrating integrated stress resilience and representing a promising candidate for vineyard replanting in drought- and Cu-affected soils, while 1103 Paulsen may limit canopy recovery due to its conservative growth strategy.

Although drought drove the largest shifts, Cu^{2+} imposed a root-centered constraint that became clear under co-stress with an extra drop in Ψ_{stem} and slower post-rewatering recovery of *E, A* and g_s, strongest in SO4. This pattern fits oxidative damage and aquaporin inhibition lowering root hydraulic conductivity; at our dose, Cu^{2+} alone didn't reduce SPAD. Ionomically, Cu^{2+} shifted nutrient balance: PCA highlighted K, P, Mn. M4 maintained higher root K–P–Mn with lower Cu accumulation but higher translocation (chelation/compartmentation), sustaining Fv/Fm, biomass, and a faster rebound; 1103P was intermediate; SO4 showed dispersed ionomics and weak K/P/Mn control, matching its largest photochemical and gas-exchange losses.

Ionomic analyses and PCA (Figure 2) revealed rootstock-specific responses to Cu^{2+} toxicity and water scarcity. In vines grafted onto 1103 Paulsen, Cu^{2+} stress had a more pronounced impact on root ionome, while drought stress dominated leaf responses. M4-grafted plants displayed clear separation between Cu, Cu+DS, and DS treatments in both roots and leaves, indicating distinct effects of combined stresses and a strong tolerance to water scarcity. Plants grafted onto SO4 rootstock showed specific and distinct responses to each stressor, with minimal interaction between Cu^{2+} and drought stress, although drought stress was more impactful at the leaf level. The PCA loadings indicated that PC1 in the root datasets was primarily driven by elements such as potassium (K), phosphorus (P), and manganese (Mn), which are crucial for osmoregulation, membrane integrity, and antioxidant activity under stress (Hasanuzzaman et al., 2018; Alejandro et al., 2020; Sardans and Peñuelas, 2021; Khan et al., 2023). In vines grafted onto M4, the clustering of Cu and Cu+DS samples away from Control and DS along PC1 suggests that Cu^{2+} availability strongly modulated the uptake of these key elements, likely due through adaptive regulation of root transporters (Marastoni et al., 2019b). Differently, the close grouping of 1103 Paulsen root samples under Cu and Cu+DS along PC2 may reflect Cu^{2+} accumulation rather than active redistribution. For rootstock SO4, the less distinct clustering and more dispersed samples might suggest a weaker nutrient homeostasis and a limited capacity to prioritize specific elemental adjustments under stress (Marastoni et al., 2019a). Therefore, rootstock choice proves crucial in shaping nutrient uptake and maintaining homeostasis under combined drought and Cu^{2+} stress, with M4 showing the most favorable response.

The physiological effects of Cu^{2+} stress were less pronounced than those of drought, as also demonstrated by the iWUE, although interesting trends were observed in mineral translocation patterns (Figure 1C). In vines exposed to Cu^{2+} stress, translocation of Cu^{2+} appeared limited, possibly due to protective mechanisms that limit its systemic diffusion or impair translocation pathways. Notably, M4 exhibited a higher Cu^{2+} translocation factor compared to other rootstocks, which could be attributed to its lower Cu^{2+} accumulation factor. This may reduce the toxic response in M4, enabling greater tolerance to Cu^{2+} exposure. Several physiological mechanisms might be responsible of these observations. Indeed, one key strategy could be represented by sequestration of complexed Cu^{2+} into vacuoles through tonoplast-localized transporters, thus reducing its cytoplasmic toxicity (Ejaz et al., 2023). Moreover, the exuded organic acids (*e.g.*, citrate or malate) or phenolic compounds can chelate Cu^{2+} in the rhizosphere, decreasing its bioavailability (Marastoni et al., 2019c, 2019a). Beside these mechanisms, also a modulation of high-affinity Cu^{2+} transporters as well as other divalent cations' transporters could contribute to a reduced Cu^{2+} uptake at root level (Marastoni et al., 2019b). These mechanisms, alone or in combination, could explain the lower Cu^{2+} accumulation factor observed in M4 (Kopittke and Menzies, 2006; Cesco et al., 2020). Additionally, the capacity of M4 rootstock to translocate Cu^{2+} without excessive accumulation in leaves might suggests an efficient chelation by ligands such as phytochelatins or glutathione, reducing free Cu^{2+} ions in the cytoplasm (Seregin and Kozhevnikova, 2023). In addition, Cu^{2+} toxicity has been reported to reduce the shoot content of macronutrients such as calcium (Ca), magnesium (Mg), K, and P, likely due to interference with ion uptake and translocation (Kopittke and Menzies, 2006). Similarly, drought stress can also impair nutrient dynamics by limiting mass flow and diffusion of ions in the soil, reducing root uptake efficiency, and altering translocation within the plant (Hsiao and Xu, 2000; Farooq et al., 2009). The combination of both stressors, as observed in our study, may thus create a complex physiological context where antagonistic or synergistic effects impact the ionomic balance in a unique manner, also depending on plants' genotype (Mishra et al., 2024) highlighting the agronomic relevance of selecting rootstocks like M4 that ensure efficient Cu^{2+} detoxification and nutrient homeostasis under multifactorial stress conditions.

Among the vines grafted onto the three rootstocks considered, the M4-grafted ones demonstrated the most stable ionomic profile under stress conditions. Notably, M4 roots accumulated higher levels of K, P, and Mn under both Cu and Cu+DS treatments. These elements are critical in abiotic stress resilience: K regulates stomatal function and xylem hydraulic conductance (Brodersen et al., 2010), P supports ATP-driven transport processes and stress-related signaling (Khan et al., 2023), while Mn is a cofactor in antioxidant enzymes and lignin biosynthesis (Alejandro et al., 2020; Cesco et al., 2020). The higher root accumulation of these elements suggests that M4-grafted plants may actively prioritize root nutrient uptake and retention mechanisms to mitigate stress impact. In contrast, plants grafted onto SO4 exhibited pronounced ionomic imbalance under stress, particularly in roots, where the lack of coherent elemental clustering and greater dispersion in PCA plots suggest weaker ionomic regulation and a limited capacity to reprogram nutrient uptake under dual stress. Interestingly, vines grafted onto 1103 Paulsen presented an intermediate profile, thereby showing divergent responses across tissues. Most importantly, the analysis of the ionomic signature in root and leaves possibly highlighted a different hierarchy of drought and Cu^{2+} stress, underscoring the central role of rootstock genotype in determining nutrient plasticity and homeostatic regulation under combined abiotic stress.

Taken together, the physiological, biochemical, and ionomic responses observed in this study reveal distinct stress-response strategies among the vines grafted onto the three tested rootstocks. Those grafted onto M4 consistently exhibited the most balanced and integrated tolerance to both drought and Cu^{2+} toxicity, maintaining photosynthetic efficiency, biomass accumulation, and nutrient homeostasis across all treatments. This rootstock demonstrated strong physiological plasticity, likely supported by active regulation of mineral uptake and Cu^{2+} detoxification mechanisms (Figure 4). Although vines grafted onto 1103 Paulsen showed robust root maintenance and some drought resilience, the recovery of shoot functionality and mineral balance under stress was more limited, possibly reflecting a more conservative drought strategy prioritizing below-ground function (Figure 4). In contrast, the SO4-grafted vines were the most sensitive, with significant declines in both shoot and root biomass, reduced Fv/Fm ratios, and nutrient imbalances, particularly under combined Cu^{2+} toxicity and drought stress (Figure 4). Moreover, the rootstock-specific responses observed in this study have clear implications for vineyard management in areas increasingly affected by both drought stress and heavy metal accumulation.

Specifically, M4 coupled stable Ψ_{stem} and gas exchange after rewatering with sustained SPAD and Fv/Fm under WSDS and Cu+DS and preserved both root and shoot biomass. Its ionomic signature, e.g.higher root K, P and Mn under Cu and Cu+DS with a lower Cu^{2+} accumulation but higher translocation factor, points to efficient compartmentation and nutrient prioritization that support osmotic balance, ATP supply and antioxidant capacity, explaining faster photosynthetic recovery. 1103 Paulsen behaved as drought-hardy but more Cu-sensitive, thereby showing elevated iWUE and maintained roots, yet reduced leaf area/shoot biomass and weaker K/P retention under Cu conditions. SO4 showed the largest drops in Fv/Fm, SPAD, photosynthesis and stomatal conductance and the poorest recovery under Cu+DS; its dispersed PCA pattern and lack of coherent K/P/Mn adjustments indicate limited ionomic homeostasis and detoxification capacity, amplifying Cu^{2+}-related hydraulic and photochemical impairment. These trait-linked differences might be used to inform site-specific rootstock choice: M4 for Cu^{2+} contaminated, drought-prone vineyards where rapid recovery is needed; 1103P for severe-drought, lower-Cu sites with canopy-supporting management; and SO4 avoided where water deficits and legacy Cu co-occur.

The sensitivity manifested by SO4 mirrors the behavior of its parental *V. riparia* which confers limited drought tolerance and a more conservative, isohydric stomatal regulation that depresses gas exchange as soil water potential declines, whereas *V. rupestris* confers drought hardiness, complemented by *V. berlandieri* adaptation to dry, calcareous soils (Serra et al., 2014; Keller, 2020; Lucini et al., 2020; Jiao et al., 2023).

The superior performance of M4 under combined stress conditions suggests that it could be a suitable candidate for replanting programs in Cu^{2+} contaminated soils and/or limited water resource environments, especially in Mediterranean basin where these stressors often co-occur. Additionally, understanding Cu^{2+} uptake and translocation patterns under field conditions is critical for developing strategies to reduce metal accumulation in

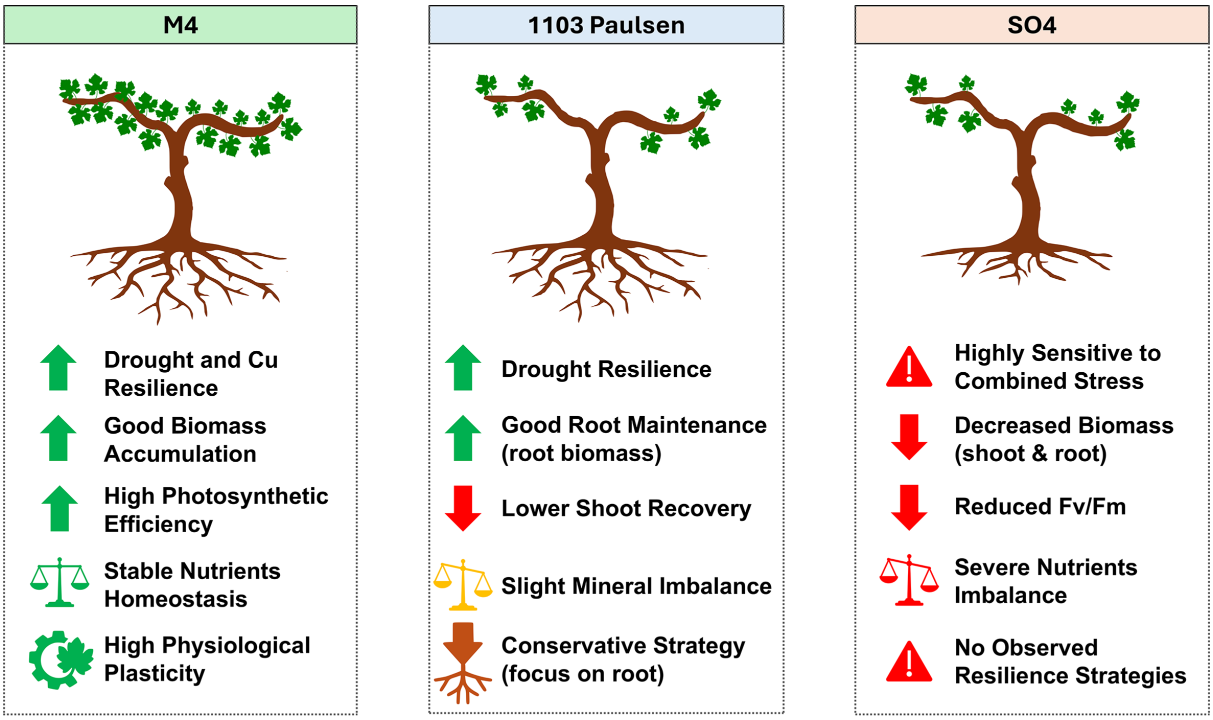

FIGURE 4
Schematic representation of the physiological response activated by the different rootstock towards the combined Cu and drought stress.

edible plant parts and limit environmental toxicity. These findings emphasize the importance of selecting rootstocks not only for their resistance to individual stressors but also for their integrated performance under multiple abiotic challenges, an approach that is becoming increasingly relevant under future climate change scenarios. However, it should be noted that this study was conducted under short-term pot conditions in a greenhouse environment, which may not fully reproduce vineyard field dynamics. Future research under field conditions will be essential to confirm the stress-response patterns reported here.

5 Conclusion

Overall, this study highlights contrasting stress responses among grapevine rootstocks under drought and Cu^{2+} toxicity, identifying M4 as the most resilient genotype. Its ability to maintain physiological functions, photosynthetic efficiency, and ionomic stability under both single and combined stress conditions underscores its strong potential for use in viticultural systems increasingly affected by abiotic pressures and multifactorial environmental stress. By contrast, SO4 displayed the most pronounced sensitivity. From an agronomic perspective, these findings reinforce the importance of selecting rootstocks capable of supporting water uptake, nutrient homeostasis, and shoot growth recovery, particularly in young vineyards or replanting context where altered soil fertility and limited water availability are critical issues. These findings underline the importance of rootstock selection as a strategic tool to enhance vineyard resilience by optimizing the soil-plant functional system under multiple scenarios. The observed genotype-specific responses, especially at root-soil interface, demonstrate how root traits and transporter regulation can influence whole-plant resilience under complex environment. Future research should focus on elucidating the molecular mechanisms underpinning M4's stress tolerance and validating these traits under long-term field conditions. The deployment of tolerant rootstocks such as M4 may represent a sustainable strategy for managing grapevine performance and soil resource use in marginal or contaminated areas, particularly within Mediterranean viticulture systems.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

RF: Visualization, Writing – review & editing, Methodology, Formal analysis, Writing – original draft, Data curation. TC: Investigation, Writing – review & editing, Methodology. FB: Investigation, Formal analysis, Writing – review & editing, Data curation, Methodology. MZ: Writing – review & editing, Investigation, Methodology. SM: Writing – review & editing, Methodology, Investigation. AA: Methodology, Writing – review & editing, Investigation. CA: Writing – review & editing, Conceptualization, Supervision, Funding acquisition. SC: Writing – review & editing, Project administration, Funding acquisition, Supervision, Conceptualization. YP: Conceptualization, Funding acquisition, Writing – original draft, Project administration, Writing – review & editing, Supervision.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2025.1682753/full#supplementary-material

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