



# Trolox-Mediated Alleviation of Cadmium Toxicity in Grapevines Through Enhanced Mineral Homeostasis and Metabolic Stability

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## Abstract

Heavy metal contamination poses significant challenges to sustainable viticulture, necessitating innovative approaches to protect grapevine health and productivity. However, comprehensive evaluations of antioxidant-mediated protection against cadmium toxicity in integrated physiological systems remain scarce. We assessed mineral homeostasis, organic acid metabolism, and sugar accumulation in three-year-old *Vitis vinifera* L. cv. Öküzgözü berries exposed to graduated cadmium (Cd) concentrations (0–20 ppm) with and without 50  $\mu$ M Trolox supplementation. Treatment effects were highly significant across all measured parameters ( $p < 0.001$ ). Notably, Trolox application consistently demonstrated protective efficacy, maintaining nitrogen content at 1.53% compared to 0.65% in untreated cadmium-stressed plants, while preserving essential macronutrient (phosphorus, potassium, calcium, magnesium) and micronutrient (manganese, iron, zinc) accumulation under toxic conditions. In contrast, cadmium exposure alone severely compromised mineral nutrition, organic acid biosynthesis, and carbohydrate metabolism, with total organic acids declining from 15.27 to 4.43 mg L<sup>-1</sup> and reducing sugars dropping substantially across all stress levels. Multivariate analysis revealed that Trolox treatment fundamentally reorganized the metabolic network, enhancing coordination between mineral uptake, organic acid production, and sugar metabolism. Particularly, Trolox-supplemented plants formed distinct clusters with elevated beneficial compounds, including preserved tartaric, malic, citric, and succinic acid levels, alongside maintained glucose and fructose concentrations, indicating efficient stress mitigation capacity. These findings show that targeted antioxidant application plays a crucial role in improving grapevine performance under heavy metal stress. Trolox consistently protected multiple vine systems, including nutrient balance, organic acid metabolism, and carbohydrate stability. Rather than just providing support, antioxidant supplementation proved essential for maintaining vine health and metabolic function. This suggests that carefully designed antioxidant protocols adapted to specific contamination conditions could be an effective strategy for sustaining vineyard productivity and fruit quality in heavy metal-polluted areas.

**Keywords** Antioxidant treatment · Heavy metal stress · Nutrient disruption · Oxidative protection · Viticulture sustainability

## Introduction

Abiotic stresses represent one of the most significant challenges to agricultural productivity worldwide, with drought, winter cold, late spring frosts, high temperature, lime, salinity, and heavy metal stress being recognized as critical limiting factors for numerous fruit species (Dogan et al. 2025a,b,c; Bolat et al. 2024; Bakır et al. 2022; Kose and Kaya 2022; Kaya and Kose 2022, 2020; Kaya et al. 2021a,b,c, 2020). Among these environmental stressors, heavy metal toxicity has emerged as a particularly pressing concern due to increasing industrial activities and environmental pollution. The widespread distribution of heavy

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metals in agricultural soils poses a growing threat to crop production and food safety. Cadmium (Cd) stands out as one of the most problematic heavy metals, being readily available in soils and easily absorbed by plant roots (Delavar et al. 2025). This toxic element induces severe oxidative stress in plants by promoting the accumulation of reactive oxygen species (ROS), which subsequently leads to cellular damage through lipid peroxidation, protein oxidation, and enzyme inactivation (Georgiadou et al. 2018; Pinto et al. 2003). The cascade of oxidative damage results in compromised plant growth, reduced yield potential, and deteriorated fruit quality, ultimately threatening both agricultural sustainability and human health through the food chain.

Plants have evolved sophisticated biochemical defense mechanisms to cope with environmental stresses, including the activation of antioxidant enzyme systems and the synthesis of protective compounds (Dogan et al. 2025a,b,c). However, under intense environmental pressure such as heavy metal stress, these natural defense systems often prove inadequate to maintain cellular homeostasis (Bakır et al. 2022). This limitation has prompted researchers to explore external interventions, particularly the application of antioxidant compounds, as a strategy to enhance plant stress tolerance. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) represents a promising candidate in this regard, being a potent water-soluble analog of vitamin E with well-documented antioxidant properties. The compound has gained considerable attention in biomedical research for its ability to scavenge free radicals and protect cellular structures from oxidative damage. Vitamin E and its derivatives, particularly  $\alpha$ -tocopherol, function as lipid-soluble antioxidants that neutralize free radicals and stabilize cell membranes, thereby reducing oxidative damage and enhancing cellular defense mechanisms (Said et al. 2013; Harmankaya et al. 2021; Kechiche et al. 2021; Ungurianu et al. 2021). These findings suggest that Trolox may exhibit similar protective effects in plant systems, potentially mitigating the harmful effects of environmental stresses (Daler et al. 2026). The existing literature demonstrates that exogenously applied antioxidants can alleviate heavy metal stress in plants (Delavar et al. 2025); however, nearly all of these studies have focused on compounds such as ascorbic acid, glutathione, melatonin, brassinosteroids, chitosan, and similar substances, rather than Trolox. Indeed, previous research has established that exogenous molecules including glutathione, proline, and ascorbate enhance cadmium tolerance in barley (*Hordeum vulgare*), rice, maize, grape, and patchouli (Wang et al. 2011, 2025; Cao et al. 2015; Zhang et al. 2019; Fu et al. 2025). To the best of our knowledge, none of these studies have evaluated Trolox as an agricultural antioxidant application in viticulture or under heavy metal stress conditions. The limited number of studies involving

exogenous Trolox application to plant material have predominantly concentrated on seed aging/germination viability and have not been conducted under heavy metal stress or with perennial fruit species. For instance, Adetunji et al. (2021) evaluated Trolox in aged cabbage and lettuce seeds, demonstrating its capacity to enhance germination enzymes and antioxidant activities; however, this study was not conducted under heavy metal conditions and did not include perennial fruit species. Our comprehensive literature review revealed no research investigating exogenous Trolox application to grapevine tissues under Cd toxicity.

Oxidative stress manifests in plants through complex biochemical alterations that can be monitored through various physiological and metabolic parameters (Dogan et al. 2025a,b,c). The disruption of cellular homeostasis affects fundamental metabolic processes, leading to measurable changes in mineral content, sugar metabolism, and organic acid profiles (Qi et al. 2025). Cadmium accumulation in plant tissues disrupts the delicate balance of these parameters, thereby compromising plant health and productivity (Kapoor et al. 2015). The heavy metal interferes with essential mineral uptake and transport, creating nutritional imbalances that weaken the plant's natural defense capacity. Sugar metabolism is particularly vulnerable to cadmium stress, as the metal can alter carbohydrate partitioning and accumulation patterns, subsequently affecting the plant's energy status and osmotic regulation. These metabolic disruptions not only impair normal physiological functions but also compromise the plant's ability to synthesize protective compounds. Organic acids play a crucial role in plant stress responses, serving both as metabolic intermediates and as natural antioxidants that can help mitigate oxidative damage (Khan et al. 2017). Their altered production under stress conditions reflects the plant's adaptive responses and provides valuable insights into stress tolerance mechanisms. Given the increasing demand for sustainable agricultural practices and the growing concern over heavy metal contamination in agricultural systems, investigating the protective effects of Trolox against cadmium stress in young grapevines represents a critical research priority that could contribute to the development of innovative stress management strategies.

Considering the limited research on Trolox applications in plant stress management and the urgent need for effective strategies to mitigate heavy metal toxicity in agricultural systems, this study was designed to fill the existing knowledge gap. The present investigation aims to provide comprehensive insights into the protective mechanisms of Trolox under cadmium stress conditions, with particular emphasis on metabolic and physiological responses. Therefore, the objective of this study was to: (I) determine the physiological effects of Trolox against cadmium stress in

young grapevines, (II) evaluate its impact on mineral content, (III) examine changes in sugar content and organic acid composition, and (IV) assess the potential of Trolox as a novel protective strategy against environmental stresses in plants.

## Materials and Methods

### Experimental Design and Site

This study was conducted in a controlled greenhouse located in the Central Anatolia Region of Turkey. The present investigation was designed to evaluate the protective effects of Trolox against cadmium-induced stress in young grapevines. Three-year-old grapevines of the Öküzgözü (*Vitis vinifera* L.) cultivar were selected as the

plant material due to their established vigor and adaptability to controlled experimental conditions. Individual plants were cultivated in 35-L containers filled with a standardized growing medium to maintain uniform root zone conditions across all treatments. To prevent leaching and ensure consistent Cd exposure, containers were placed on drainage collection platforms, excess solution was recovered, and irrigation volumes were carefully regulated throughout the experimental period. During the first year's dormant period, vines were systematically pruned to maintain a single trunk system, with only one shoot allowed to develop per plant. In the second year's dormant season, each vine was pruned to retain two buds per shoot, enabling the development of two primary shoots per plant. During the third year's growing season, each shoot was allowed to develop one grape cluster, resulting in two clusters per vine, ensuring uniform fruit development across all experimental units (Fig. 1).

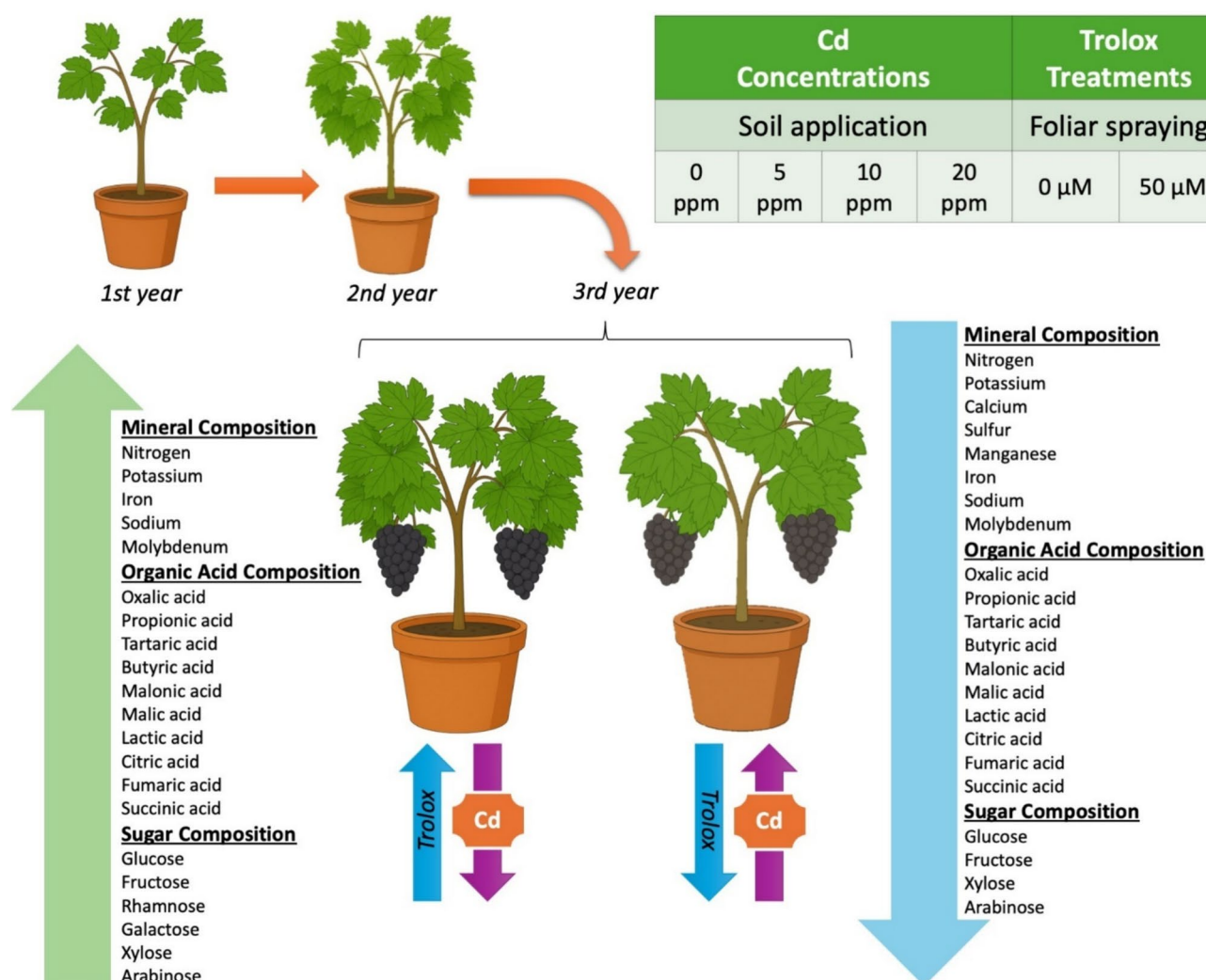


**Fig. 1** Experimental layout showing the factorial design of cadmium (Cd) concentrations (0, 5, 10, and 20 ppm) combined with two Trolox treatment levels (0 and 50 µM)

## Treatment Applications and Justification

The application of cadmium as a solution directly to the root zone, rather than using Cd-contaminated soil, was deliberately chosen to ensure equal and controllable Cd exposure across all plants while eliminating variability arising from soil heterogeneity. As is well known, although contaminated soil better mimics field conditions, soil properties such as pH, organic matter content, cation exchange capacity, and clay mineralogy significantly alter Cd bioavailability, leading to high variability and low reproducibility in controlled experiments. This situation complicates the statistical evaluation of sensitive physiological variables, including mineral nutrition, organic acid metabolism, and carbohydrate dynamics. In our study, the solution-based approach allows precise assessment of different Cd doses and Trolox effects. Additionally, this experimental design models the use of Cd-containing irrigation water, representing scenarios encountered in vineyards near industrial zones where groundwater and surface water contamination occurs. In this context, the results maintain applicability to field conditions and provide a scientific foundation for future long-term studies in Cd-contaminated soils. The Cd concentrations employed in this study (0, 5, 10, 20 ppm) are consistent with levels widely used in the literature to assess Cd tolerance in grapevine genotypes. Specifically, Cangi et al. (2022) evaluated grapevine rootstocks at 0, 5, 10, and 20 mg kg<sup>-1</sup> Cd levels. Therefore, the contamination range used here represents a valid, comparable, and scientifically established framework for examining physiological and metabolic responses of grapevines to Cd stress. It should be noted that this study focuses on stress physiology and metabolic responses rather than food safety assessment; hence, Cd levels were set according to established experimental protocols in the literature.

For the establishment of cadmium stress conditions, commercial cadmium chloride (CdCl<sub>2</sub>) obtained from Carlo Erba (CAS: 35658-65-2) was utilized as the primary stress agent. CdCl<sub>2</sub> stock solutions were prepared with distilled water immediately before application. A single root zone application was performed at the beginning of the third growing season during the active vegetative growth period (BBCH 15–17; 6–8 fully expanded leaves). Four distinct cadmium concentration levels were employed: (1) control treatment (0 ppm Cd), (2) low stress level (5 ppm Cd), (3) moderate stress level (10 ppm Cd), and (4) high stress level (20 ppm Cd). Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was obtained from Sigma-Aldrich (CAS: 53188-07-1) and served as the protective antioxidant treatment. The 50 µM Trolox concentration was selected based on literature data and preliminary optimization trials, which confirmed its biological efficacy without causing phytotoxicity. This concentration aligns

with the range used for similar exogenous antioxidant compounds (α-tocopherol) in young plants and seedlings (Ali et al. 2019). Two Trolox concentration levels were employed: a control treatment (0 µM) and an active treatment (50 µM). Trolox applications were performed using fine-mist spray equipment to ensure homogeneous coverage of leaf surfaces and shoot tissues. The application program targeted two critical developmental stages: (1) pre-veraison stage (BBCH 81, beginning of ripening), 7 days before the onset of color change, and (2) post-veraison stage (BBCH 85, berry softening), 14 days after the first application. All applications were conducted in the early morning hours (07:00–09:00) to enhance absorption and minimize evaporation. A non-ionic surfactant (Tween-20; 0.05% v/v) was added to the solution formulation to improve leaf wettability and compound penetration. The experimental design was structured as a 4 × 2 factorial arrangement, with each treatment combination replicated three times. Each replicate contained three individual plants, resulting in a total of 72 experimental units. Data from the three plants within each replicate were averaged before statistical analysis, yielding three replicate values per treatment combination for ANOVA.

## Sampling Procedures

Grape samples were collected when berries reached approximately 21.0° Brix, corresponding to harvest maturity. From each replicate, nine grape clusters were systematically selected, and five berry samples were collected from the upper, middle, and lower portions of each cluster to minimize positional effects. All mineral, sugar, and organic acid analyses reported in this study were conducted on berry tissue samples.

## Mineral Content Analysis

Treated grape berry samples were initially dried in a forced-air oven at 68 °C for 48 h, then ground into a fine, homogeneous powder. Total nitrogen (N) content was quantified using the Kjeldahl method with a Vapodest Fast Kjeldahl Distillation Unit (Gerhardt, Königswinter, Germany) following AOAC (2019) protocols. Comprehensive elemental analysis was conducted using an inductively coupled plasma optical emission spectrometer (ICP-OES; Optima 2100 DV, PerkinElmer, Shelton, CT, USA) following AOAC (2005) standardized protocols. Macroelements (P, K, Ca, Mg, S, N) were measured on a percentage (%) basis, while microelements and trace elements (Mn, Fe, Zn, B, Cu, Na, Mo, Cl, Cd) were measured in mg/kg. Calibration curves for ICP-OES analysis were prepared using certified multi-element standards (PerkinElmer Pure Plus), with R<sup>2</sup>

values exceeding 0.999 for all elements. Method detection limits ranged from 0.001 mg/kg (Mo) to 0.1 mg/kg (Cd).

### Sugar Content Analysis

Sugar composition in grape berries was determined using high-performance liquid chromatography (HPLC) equipped with a refractive index (RI) detector following established methods (Kaya et al. 2022; Kunter et al. 2024). Fresh berry samples were homogenized and juice was extracted by mechanical pressing. The extracted juice was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was filtered through 0.45 µm PTFE membrane filters. Glucose, fructose, and sucrose were quantified using a five-point calibration curve prepared with authentic standards (Sigma-Aldrich). Calibration curves exhibited linearity with  $R^2 \geq 0.998$ , and detection limits were 0.05 g L<sup>-1</sup> for all sugars. Final sugar concentrations were expressed as mg L<sup>-1</sup> fresh berry weight. Sugar separation was achieved using a Sugar Pak-1 column (300 × 6.5 mm) with HPLC-grade water as the mobile phase at a flow rate of 0.5 mL/min and column temperature maintained at 90 °C (Al-Farsi et al. 2007). The injection volume was 10 µL and the analysis time was approximately 20 min per sample.

### Organic Acid Analysis

Organic acid composition was determined using an HPLC system (Agilent 1100 Series G1362A RID) equipped with a diode array detector (G1315B DAD), adapted from Bevilacqua and Califano (1989). For extraction, 1 g of homogenized berry sample was mixed with 20 mL of 0.009 N sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), homogenized, shaken for 1 h, and centrifuged at 15,000 rpm for 15 min. The supernatant was filtered and purified using C18 Sep-Pak cartridges. Chromatographic separation was performed using an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad) at 30 °C with 0.009 N H<sub>2</sub>SO<sub>4</sub> as the mobile phase (0.4 mL/min flow rate). Detection was carried out at 214 nm. Organic acids (tartaric, malic, citric, succinic, and others) were quantified using multi-point calibration curves prepared from authentic standards (Sigma-Aldrich). Calibration curves demonstrated linearity with  $R^2 \geq 0.997$ , and detection limits ranged from 0.01 to 0.05 g L<sup>-1</sup>. Final organic acid concentrations were expressed as mg L<sup>-1</sup> fresh berry weight.

### Statistical Analysis

All experiments were designed as a two-factorial (4 × 2) experimental design, where the first factor consisted of cadmium applications and the second factor comprised Trolox treatments. Each parameter was evaluated using three

biological replicates, with each replicate representing the average of three plants. Numerical data were systematically managed using Microsoft 365 Excel, and a two-way analysis of variance (Two-Way ANOVA) was performed using IBM SPSS version 22.0 software. Post hoc comparisons were conducted using Duncan's multiple range test at a 5% significance level ( $p \leq 0.05$ ). Data are presented as mean values ± standard deviations (SD). Hierarchical clustering heat maps were generated using the SRPLOT online platform (<https://www.bioinformatics.com.cn/en>, accessed November 11, 2024). Principal Component Analysis (PCA) was performed using GraphPad Prism version 9.3.1 (GraphPad Software, LLC, San Diego, CA, USA). Prior to PCA, all variables were standardized using z-score transformation (mean = 0, standard deviation = 1) to bring them to a common scale. GraphPad Prism automatically applies this standardization before performing PCA (Table S1). The PCA results were interpreted using biplot visualization following the methodology of Evgenidis et al. (2011). PCA loading values, eigenvalues, and variance contribution tables are provided in the supplementary materials.

## Results

### Mineral Element Composition in Berry Tissues

Cadmium stress and Trolox application significantly influenced the mineral element composition in grapevine berry tissues (Table 1). All mineral analyses presented in this study were conducted on berry samples collected at harvest maturity. Nitrogen content exhibited a consistent decline with increasing cadmium concentrations ( $p = 0.000$ ). In the absence of cadmium, Trolox application enhanced nitrogen accumulation, reaching 3.19% compared to 2.43% in the untreated control. Under 20 ppm Cd, nitrogen content dropped to 0.65%, while Trolox application partially alleviated this reduction (1.53%). A significant Cd × Trolox interaction was observed ( $p = 0.000$ ). Phosphorus content followed a similar declining trend with cadmium stress ( $p = 0.001$ ), decreasing from 0.75% in control plants to 0.14% at 20 ppm Cd. Potassium content decreased significantly under cadmium exposure ( $p = 0.000$ ), and Trolox application significantly improved potassium levels ( $p = 0.003$ ), particularly in non-stressed plants (2.64% under 0 ppm Cd + 50 µM Trolox versus 2.30% in control). Calcium and magnesium contents declined consistently with increasing cadmium levels ( $p = 0.000$  for both). Calcium dropped from 2.10% at 0 ppm Cd to 0.31% at 20 ppm Cd, while magnesium decreased from 0.34% to 0.07%. Trolox moderately enhanced both elements across treatments. Sulfur content decreased significantly under cadmium stress

**Table 1** Mineral element composition of grapevine under cadmium stress and Trolox application

| Cadmium concentrations       | Trolox treatments | Nitrogen (%)       | Phosphorus (%)      | Potassium (%)     | Calcium (%)            | Magnesium (%)         | Sulfur (%)          | Manganese (mg/kg)   | Iron (mg/kg)       |
|------------------------------|-------------------|--------------------|---------------------|-------------------|------------------------|-----------------------|---------------------|---------------------|--------------------|
| 0 ppm                        | 0 $\mu$ M         | 2.43 $\pm$ 0.13 b* | 0.75 $\pm$ 0.42     | 2.30 $\pm$ 0.16 b | 2.10 $\pm$ 0.62 a      | 0.21 $\pm$ 0.10       | 0.55 $\pm$ 0.25 a   | 33.97 $\pm$ 7.35 a  | 14.06 $\pm$ 1.35 a |
|                              | 50 $\mu$ M        | 3.19 $\pm$ 0.27 a  | 0.39 $\pm$ 0.04     | 2.64 $\pm$ 0.28 a | 1.41 $\pm$ 0.15 b      | 0.34 $\pm$ 0.05       | 0.26 $\pm$ 0.02 b   | 30.86 $\pm$ 2.19 ab | 15.77 $\pm$ 1.59 a |
| 5 ppm                        | 0 $\mu$ M         | 2.37 $\pm$ 0.21 b  | 0.29 $\pm$ 0.01     | 1.98 $\pm$ 0.10 b | 1.06 $\pm$ 0.05 bc     | 0.24 $\pm$ 0.01       | 0.18 $\pm$ 0.03 bc  | 23.78 $\pm$ 2.15 cd | 11.54 $\pm$ 1.34 b |
|                              | 50 $\mu$ M        | 2.36 $\pm$ 0.21 b  | 0.28 $\pm$ 0.03     | 2.04 $\pm$ 0.10 b | 1.07 $\pm$ 0.06 bc     | 0.21 $\pm$ 0.05       | 0.16 $\pm$ 0.01 bc  | 23.32 $\pm$ 1.61 cd | 10.62 $\pm$ 0.30 b |
| 10 ppm                       | 0 $\mu$ M         | 1.80 $\pm$ 0.06 cd | 0.21 $\pm$ 0.04     | 1.52 $\pm$ 0.20 c | 0.81 $\pm$ 0.06 c      | 0.14 $\pm$ 0.02       | 0.11 $\pm$ 0.01 bc  | 17.28 $\pm$ 2.85 de | 7.87 $\pm$ 0.67 c  |
|                              | 50 $\mu$ M        | 1.93 $\pm$ 0.09 c  | 0.22 $\pm$ 0.02     | 1.46 $\pm$ 0.24 c | 0.86 $\pm$ 0.06 c      | 0.15 $\pm$ 0.05       | 0.14 $\pm$ 0.03 bc  | 15.92 $\pm$ 2.24 ef | 8.18 $\pm$ 1.36 c  |
| 20 ppm                       | 0 $\mu$ M         | 0.65 $\pm$ 0.06 e  | 0.08 $\pm$ 0.00     | 0.54 $\pm$ 0.04 d | 0.31 $\pm$ 0.01 d      | 0.07 $\pm$ 0.01       | 0.05 $\pm$ 0.01 c   | 10.70 $\pm$ 3.26 f  | 3.34 $\pm$ 0.30 d  |
|                              | 50 $\mu$ M        | 1.53 $\pm$ 0.20 d  | 0.21 $\pm$ 0.02     | 1.21 $\pm$ 0.20 c | 0.79 $\pm$ 0.05 c      | 0.16 $\pm$ 0.04       | 0.12 $\pm$ 0.03 bc  | 26.99 $\pm$ 3.79 bc | 7.53 $\pm$ 1.28 c  |
| Average of Cd concentrations | 0 ppm             | 2.81 $\pm$ 0.46    | 0.57 $\pm$ 0.33 A** | 2.47 $\pm$ 0.28   | 1.75 $\pm$ 0.56        | 0.28 $\pm$ 0.10 A     | 0.41 $\pm$ 0.23     | 32.42 $\pm$ 5.14    | 14.92 $\pm$ 1.62   |
|                              | 5 ppm             | 2.37 $\pm$ 0.19    | 0.28 $\pm$ 0.02 B   | 2.01 $\pm$ 0.10   | 1.07 $\pm$ 0.05        | 0.23 $\pm$ 0.03 A     | 0.17 $\pm$ 0.02     | 23.55 $\pm$ 1.72    | 11.08 $\pm$ 1.00   |
|                              | 10 ppm            | 1.86 $\pm$ 0.10    | 0.22 $\pm$ 0.03 B   | 1.49 $\pm$ 0.20   | 0.84 $\pm$ 0.06        | 0.15 $\pm$ 0.03 B     | 0.13 $\pm$ 0.02     | 16.60 $\pm$ 2.41    | 8.02 $\pm$ 0.98    |
|                              | 20 ppm            | 1.09 $\pm$ 0.50    | 0.14 $\pm$ 0.07 B   | 0.87 $\pm$ 0.39   | 0.55 $\pm$ 0.26        | 0.11 $\pm$ 0.05 B     | 0.09 $\pm$ 0.04     | 18.85 $\pm$ 9.46    | 5.43 $\pm$ 2.44    |
| Average of Trolox treatments | 0 $\mu$ M         | 1.81 $\pm$ 0.75    | 0.33 $\pm$ 0.32     | 1.58 $\pm$ 0.70   | 1.07 $\pm$ 0.73        | 0.17 $\pm$ 0.08 B     | 0.22 $\pm$ 0.23     | 21.44 $\pm$ 9.72    | 9.20 $\pm$ 4.31    |
|                              | 50 $\mu$ M        | 2.25 $\pm$ 0.67    | 0.27 $\pm$ 0.08     | 1.84 $\pm$ 0.61   | 1.03 $\pm$ 0.26        | 0.22 $\pm$ 0.09 A     | 0.17 $\pm$ 0.06     | 24.27 $\pm$ 6.17    | 10.53 $\pm$ 3.55   |
| Cadmium                      | <i>p</i> -value   | 0.000              | 0.001               | 0.000             | 0.000                  | 0.000                 | 0.000               | 0.000               | 0.000              |
| Trolox                       | <i>p</i> -value   | 0.000              | 0.346               | 0.003             | 0.697                  | 0.025                 | 0.167               | 0.072               | 0.011              |
| Cadmium $\times$ Trolox      | <i>p</i> -value   | 0.000              | 0.067               | 0.015             | 0.004                  | 0.066                 | 0.016               | 0.001               | 0.008              |
| Cadmium concentrations       | Trolox treatments | Zinc (mg/kg)       | Boron (mg/kg)       | Copper (mg/kg)    | Sodium (mg/kg)         | Molybdenum (mg/kg)    | Cadmium (mg/kg)     | Chlorine (mg/kg)    |                    |
| 0 ppm                        | 0 $\mu$ M         | 2.66 $\pm$ 1.31    | 6.83 $\pm$ 7.48     | 0.08 $\pm$ 0.02 d | 652.98 $\pm$ 67.50 b   | 111.78 $\pm$ 7.16 cd  | 1.09 $\pm$ 0.07     | 0.34 $\pm$ 0.04     |                    |
|                              | 50 $\mu$ M        | 1.56 $\pm$ 0.29    | 14.57 $\pm$ 3.27    | 0.13 $\pm$ 0.01 d | 1200.49 $\pm$ 119.67 a | 356.85 $\pm$ 90.82 a  | 3.46 $\pm$ 0.88     | 0.38 $\pm$ 0.02     |                    |
| 5 ppm                        | 0 $\mu$ M         | 1.17 $\pm$ 0.08    | 9.06 $\pm$ 1.34     | 0.83 $\pm$ 0.04 b | 211.14 $\pm$ 0.84 c    | 265.59 $\pm$ 52.34 ab | 17.36 $\pm$ 3.42    | 0.39 $\pm$ 0.02     |                    |
|                              | 50 $\mu$ M        | 1.18 $\pm$ 0.31    | 7.70 $\pm$ 0.37     | 1.13 $\pm$ 0.13 a | 288.13 $\pm$ 21.55 c   | 198.74 $\pm$ 80.00 bc | 4.39 $\pm$ 1.77     | 0.34 $\pm$ 0.03     |                    |
| 10 ppm                       | 0 $\mu$ M         | 1.02 $\pm$ 0.15    | 5.19 $\pm$ 1.28     | 0.78 $\pm$ 0.07 b | 198.48 $\pm$ 34.77 c   | 110.27 $\pm$ 53.13 cd | 15.10 $\pm$ 7.28    | 0.38 $\pm$ 0.05     |                    |
|                              | 50 $\mu$ M        | 1.16 $\pm$ 0.08    | 6.29 $\pm$ 3.21     | 0.90 $\pm$ 0.12 b | 274.34 $\pm$ 13.31 c   | 142.48 $\pm$ 64.22 cd | 5.52 $\pm$ 2.49     | 0.35 $\pm$ 0.03     |                    |
| 20 ppm                       | 0 $\mu$ M         | 0.42 $\pm$ 0.09    | 2.47 $\pm$ 0.60     | 0.36 $\pm$ 0.04 c | 84.78 $\pm$ 6.76 d     | 63.70 $\pm$ 29.35 d   | 25.48 $\pm$ 11.74   | 0.43 $\pm$ 0.06     |                    |
|                              | 50 $\mu$ M        | 0.91 $\pm$ 0.06    | 6.31 $\pm$ 2.75     | 0.84 $\pm$ 0.10 b | 266.29 $\pm$ 29.30 c   | 149.60 $\pm$ 66.41 cd | 14.53 $\pm$ 6.45    | 0.43 $\pm$ 0.04     |                    |
| Average of Cd concentrations | 0 ppm             | 2.11 $\pm$ 1.04 A  | 10.70 $\pm$ 6.68 A  | 0.11 $\pm$ 0.03   | 926.74 $\pm$ 312.22    | 234.32 $\pm$ 146.07   | 2.28 $\pm$ 1.42 C   | 0.36 $\pm$ 0.04 B   |                    |
|                              | 5 ppm             | 1.18 $\pm$ 0.20 B  | 8.38 $\pm$ 1.15 AB  | 0.98 $\pm$ 0.19   | 249.64 $\pm$ 44.32     | 232.17 $\pm$ 70.69    | 10.87 $\pm$ 7.51 B  | 0.37 $\pm$ 0.04 B   |                    |
|                              | 10 ppm            | 1.09 $\pm$ 0.13 B  | 5.74 $\pm$ 2.27 B   | 0.84 $\pm$ 0.11   | 236.41 $\pm$ 47.76     | 126.38 $\pm$ 55.59    | 10.31 $\pm$ 7.16 B  | 0.37 $\pm$ 0.04 B   |                    |
|                              | 20 ppm            | 0.67 $\pm$ 0.28 B  | 4.39 $\pm$ 2.75 B   | 0.60 $\pm$ 0.27   | 175.54 $\pm$ 101.22    | 106.65 $\pm$ 65.74    | 20.00 $\pm$ 10.38 A | 0.43 $\pm$ 0.05 A   |                    |
| Average of Trolox treatments | 0 $\mu$ M         | 1.32 $\pm$ 1.03    | 5.89 $\pm$ 4.14     | 0.51 $\pm$ 0.32   | 286.84 $\pm$ 229.01    | 137.84 $\pm$ 86.72    | 14.76 $\pm$ 11.00 A | 0.39 $\pm$ 0.05     |                    |
|                              | 50 $\mu$ M        | 1.20 $\pm$ 0.30    | 8.72 $\pm$ 4.25     | 0.75 $\pm$ 0.40   | 507.31 $\pm$ 421.51    | 211.92 $\pm$ 111.20   | 6.97 $\pm$ 5.54 B   | 0.38 $\pm$ 0.05     |                    |
| Cadmium                      | <i>p</i> -value   | 0.001              | 0.021               | 0.000             | 0.000                  | 0.002                 | 0.001               | 0.017               |                    |
| Trolox                       | <i>p</i> -value   | 0.564              | 0.054               | 0.000             | 0.000                  | 0.009                 | 0.004               | 0.608               |                    |
| Cadmium $\times$ Trolox      | <i>p</i> -value   | 0.065              | 0.145               | 0.001             | 0.000                  | 0.003                 | 0.121               | 0.293               |                    |

\* Different lower-case letters indicate differences according to the interaction of 'Cd  $\times$  Trolox' ( $p < 0.05$ )\*\* Different capital letters indicate differences according to 'Cd' and 'Trolox' factors ( $p < 0.05$ ). Data are presented as mean  $\pm$  standard deviation (SD)

( $p=0.000$ ), falling from 0.55% to 0.05%, with a significant Cd  $\times$  Trolox interaction ( $p=0.016$ ). Among micronutrients, manganese content was highest under control conditions (33.97 mg/kg) and dropped to 10.70 mg/kg under 20 ppm Cd ( $p=0.000$ ). Trolox significantly improved Mn levels, with a highly significant interaction effect ( $p=0.001$ ). Iron content followed a similar trend ( $p=0.000$ ), with the highest value recorded under 0 ppm Cd+Trolox (15.77 mg/kg) and the lowest (3.34 mg/kg) at 20 ppm Cd without Trolox. Trolox significantly increased iron accumulation ( $p=0.011$ ), and the Cd  $\times$  Trolox interaction was also significant ( $p=0.008$ ).

Zinc content declined significantly under cadmium stress ( $p=0.001$ ), from 2.66 mg/kg to 0.42 mg/kg at 20 ppm Cd. Copper content increased with cadmium stress ( $p=0.000$ ), reaching 1.13 mg/kg under 5 ppm Cd+Trolox, with Trolox significantly enhancing copper accumulation ( $p=0.000$ ) and significant interaction effects ( $p=0.001$ ). Sodium content dropped significantly under cadmium stress ( $p=0.000$ ), with Trolox significantly improving sodium retention ( $p=0.000$ ) and a strong interaction effect ( $p=0.000$ ). Cadmium accumulation in berry tissues increased sharply with external Cd supply ( $p=0.001$ ), reaching 25.48 mg/kg at 20 ppm Cd without Trolox. Notably, Trolox application significantly reduced Cd accumulation at all levels ( $p=0.004$ ), although the interaction effect was not significant ( $p=0.121$ ). The mechanism underlying Trolox-mediated reduction in berry Cd content may involve multiple physiological processes. First, Trolox's antioxidant activity may enhance the structural integrity and selective permeability of root cell membranes, potentially limiting initial Cd uptake. Second, Trolox may stimulate the synthesis of phytochelatins and other metal-binding peptides that sequester Cd in root tissues, thereby reducing its translocation to aerial parts and berries. Third, enhanced antioxidant capacity may support more efficient function of xylem and phloem transporters involved in mineral partitioning, favoring retention of Cd in vegetative tissues rather than reproductive organs. While our study focused on berry composition as the primary endpoint for fruit quality assessment, future research incorporating foliar Cd analysis would provide valuable insights into whole-plant Cd distribution patterns and translocation dynamics under Trolox treatment. Overall, cadmium stress significantly reduced the concentration of essential macro- and micronutrients in grapevine berries, while Trolox application mitigated many of these reductions, particularly for nitrogen, potassium, calcium, magnesium, iron, and sodium.

### Organic Acid Composition in Berry Tissues

Cadmium stress and Trolox application significantly altered the organic acid composition of grapevine berry tissues (Table 2). Total organic acids (OA) were highest in the

0 ppm Cd+Trolox treatment (20.14 mg L<sup>-1</sup>) and declined with increasing Cd levels, reaching 2.46 mg L<sup>-1</sup> under 20 ppm Cd without Trolox ( $p=0.000$ ). Trolox significantly increased OA levels overall ( $p=0.001$ ), with a significant Cd  $\times$  Trolox interaction ( $p=0.000$ ). Pyruvic acid (PA), tartaric acid (TA), and benzoic acid (BA) contents followed similar trends, with the highest levels observed under no Cd stress and significant reductions under severe Cd exposure ( $p=0.000$ ). Trolox significantly enhanced their accumulation ( $p\leq 0.005$ ). Malic acid (MA), malonic acid (ML), lactic acid (LA), and caffeic acid (CA) levels decreased significantly with increasing Cd concentrations ( $p=0.000$ ). Trolox consistently improved these acids, particularly under moderate Cd levels, with statistically significant interaction terms ( $p<0.005$ ). Mesaconic acid (ME) declined with stress, with the highest average (58.91 mg L<sup>-1</sup>) at 0 ppm Cd and the lowest (18.12 mg L<sup>-1</sup>) at 20 ppm Cd. Trolox significantly increased ME overall ( $p=0.005$ ), though interaction was not significant ( $p=0.074$ ). Fumaric acid (FA) and succinic acid (SA) contents were highest at low cadmium levels and dropped under stress. FA showed a strong interaction effect ( $p=0.000$ ), while SA was significantly influenced by both Cd and Trolox ( $p=0.000$  for both), with a notable interaction as well.

### Sugar Metabolism in Berry Tissues

Cadmium stress significantly affected sugar metabolism in grapevine berry tissues, while Trolox application partially mitigated these effects (Table 3). Sucrose content decreased significantly with increasing cadmium concentration, ranging from 0.50 mg L<sup>-1</sup> at 0 ppm Cd to 0.26 mg L<sup>-1</sup> at 20 ppm ( $p=0.000$ ). Trolox significantly enhanced sucrose accumulation (0.46 mg L<sup>-1</sup>) compared to untreated plants (0.32 mg L<sup>-1</sup>), though the interaction effect was not significant ( $p=0.127$ ). Glucose and fructose contents declined steadily under cadmium stress, from  $7.35\pm 1.67$  and  $7.95\pm 1.68$  mg L<sup>-1</sup> at 0 ppm to 3.63 and 3.91 mg L<sup>-1</sup> at 20 ppm, respectively ( $p=0.000$  for both). Trolox application significantly increased both sugars, with significant Cd  $\times$  Trolox interactions ( $p=0.000$  and  $p=0.001$ , respectively).

Rhamnose and galactose also decreased with cadmium exposure. Trolox enhanced their accumulation under all Cd levels, with both main effects and interactions being statistically significant ( $p<0.01$ ). Xylose and arabinose were significantly reduced by cadmium stress, with mean values dropping from 2.50 and 1.62 mg L<sup>-1</sup> at 0 ppm to 1.23 and 0.80 mg L<sup>-1</sup> at 20 ppm Cd, respectively. Trolox treatment increased their contents significantly, with strong interaction effects ( $p\leq 0.002$ ).

**Table 2** Organic acid composition (mg L<sup>-1</sup>) of grapevine under cadmium stress and Trolox application

| Cadmium Concentrations       | Trolox Treatments | OA              | PA            | TA            | BA               | MA             | ML            | LA              | CA              | ME             | FA            | SA              |
|------------------------------|-------------------|-----------------|---------------|---------------|------------------|----------------|---------------|-----------------|-----------------|----------------|---------------|-----------------|
| 0 ppm                        | 0 µM              | 10.40±1.27 b-d* | 21.89±2.30 cd | 40.07±3.72 bc | 128.39±14.64 b-d | 101.24±10.07 c | 41.17±7.17 bc | 155.18±19.86 cd | 122.51±18.11 cd | 59.97±12.92    | 41.92±8.10 bc | 171.62±10.28 bc |
|                              | 50 µM             | 20.14±3.58 a    | 39.95±3.98 a  | 76.97±12.18 a | 225.93±46.87 a   | 179.90±19.91 a | 60.31±4.77 a  | 292.55±33.55 a  | 275.10±24.98 a  | 57.84±6.70     | 68.32±7.74 a  | 376.53±60.98 a  |
|                              | 50 µM             | 13.40±1.53 b    | 29.83±6.12 b  | 47.02±6.08 b  | 154.29±22.39 b   | 155.76±17.66 b | 54.06±8.76 a  | 210.39±37.36 b  | 184.39±18.37 b  | 41.18±6.52     | 68.44±15.85 a | 192.28±23.19 b  |
| 5 ppm                        | 0 µM              | 12.53±1.52 bc   | 26.36±2.76 bc | 48.26±4.48 b  | 142.41±16.24 bc  | 112.29±11.17 c | 49.57±8.63 ab | 186.87±23.91 bc | 147.53±21.81 c  | 66.52±14.33    | 46.50±8.98 bc | 190.36±11.41 b  |
|                              | 0 µM              | 10.11±1.15 cd   | 22.51±4.62 cd | 35.48±4.59 c  | 113.27±16.43 cd  | 114.35±12.97 c | 40.79±6.61 bc | 158.74±28.19 cd | 139.12±13.86 c  | 30.23±4.78     | 50.24±11.63 b | 141.15±17.02 c  |
|                              | 50 µM             | 8.52±1.04 de    | 17.93±1.88 de | 32.82±3.05 cd | 99.54±11.35 de   | 78.49±7.80 d   | 33.71±5.87 cd | 127.08±16.26 de | 100.33±14.83 d  | 46.49±10.02    | 32.50±6.28 c  | 133.05±7.97 c   |
| 20 ppm                       | 0 µM              | 2.46±0.30 f     | 4.92±0.52 f   | 10.14±0.94 e  | 31.30±3.57 f     | 22.50±2.23 e   | 8.73±1.52 e   | 36.35±4.65 f    | 34.91±5.16 e    | 14.69±3.17     | 9.33±1.80 d   | 36.90±2.21 e    |
|                              | 50 µM             | 6.40±0.73 e     | 14.31±2.93 e  | 24.05±3.11 d  | 73.31±10.64 e    | 73.95±8.39 d   | 23.18±3.75 d  | 90.91±16.14 e   | 106.28±10.59 d  | 21.55±3.41     | 29.70±6.88 c  | 80.56±9.72 d    |
|                              | 0 ppm             | 15.27±5.85      | 30.92±10.31   | 58.52±21.76   | 177.16±61.79     | 140.57±45.33   | 50.74±11.82   | 223.87±79.18    | 198.81±85.82    | 58.91±9.28 A** | 55.12±16.10   | 274.08±118.85   |
| Average of Cd Concentrations | 5 ppm             | 12.97±1.45      | 28.10±4.65    | 47.64±4.82    | 148.35±18.66     | 134.03±27.23   | 51.82±8.16    | 198.63±30.87    | 165.96±27.07    | 53.85±17.08 A  | 57.47±16.65   | 191.32±16.38    |
|                              | 10 ppm            | 9.32±1.31       | 20.22±4.03    | 34.15±3.78    | 106.40±14.70     | 96.42±21.85    | 37.25±6.80    | 142.91±26.92    | 119.73±24.83    | 38.36±11.34 B  | 41.37±12.82   | 137.10±12.69    |
|                              | 20 ppm            | 4.43±2.22       | 9.61±5.48     | 17.10±7.89    | 52.31±24.08      | 48.22±28.71    | 15.95±8.32    | 63.63±31.72     | 70.60±39.79     | 18.12±4.77 C   | 19.52±12.03   | 58.73±24.73     |
| Average of Trolox Treatments | 0 µM              | 9.09±4.34       | 19.79±10.14   | 33.18±14.99   | 106.82±49.90     | 98.46±51.45    | 36.19±18.36   | 140.17±70.10    | 120.23±58.03    | 36.52±18.46 B  | 42.48±24.14   | 135.49±63.76    |
|                              | 50 µM             | 11.90±5.75      | 24.64±10.61   | 45.53±21.81   | 135.30±64.36     | 111.16±45.57   | 41.69±15.78   | 174.35±82.25    | 157.31±75.27    | 48.10±19.42 A  | 44.25±17.21   | 195.13±119.75   |
|                              | p-value           | 0.000           | 0.000         | 0.000         | 0.000            | 0.000          | 0.000         | 0.000           | 0.000           | 0.000          | 0.000         | 0.000           |
| Cadmium                      | Trolox            | 0.001           | 0.004         | 0.000         | 0.005            | 0.024          | 0.049         | 0.004           | 0.000           | 0.005          | 0.645         | 0.000           |
|                              | Cadmium×Trolox    | 0.000           | 0.000         | 0.000         | 0.001            | 0.000          | 0.004         | 0.000           | 0.000           | 0.074          | 0.000         | 0.000           |

OA: Oxalic acid, PA: Propionic acid, TA: Tartaric acid, BA: Butyric acid, MA: Malonic acid, ML: Malic acid, LA: Lactic acid, CA: Citric acid, ME: Maleic acid, FA: Fumaric acid, SA: Succinic acid

\* Different lower-case letters indicate differences according to the interaction of 'Cd×Trolox' ( $p<0.05$ )\*\* Different capital letters indicate differences according to 'Cd' and 'Trolox' factors ( $p<0.05$ ). Data are presented as mean±standard deviation (SD)

## Multivariate Analysis of Treatment Effects

The correlation analysis revealed significant relationships among mineral nutrients, sugars, and organic acids in grape berries under cadmium stress and Trolox application (Fig. 2). Strong positive correlations were observed between glucose and fructose ( $r=0.99$ ), tartaric and malic acids ( $r=0.99$ ), and citric and malic acids ( $r=0.97$ ). Essential nutrients such as nitrogen and phosphorus ( $r=0.95$ ), potassium and phosphorus ( $r=0.91$ ), and calcium and phosphorus ( $r=0.92$ ) showed strong associations. In contrast, cadmium displayed marked negative correlations with zinc ( $r=-0.83$ ), manganese ( $r=-0.78$ ), iron ( $r=-0.84$ ), glucose ( $r=-0.91$ ), sucrose ( $r=-0.92$ ), and fructose ( $r=-0.92$ ). Principal component analysis (PCA) revealed clear separation between treatments based on their nutrient and metabolite profiles (Fig. 3). PC1 explained 74.26% of the total variation, while PC2 accounted for 14.55%. Samples exposed to 20 ppm Cd alone (20Cd0TR) clustered far right along PC1, strongly correlated with increased Cd, Cl, and Cu. Control samples (0Cd0TR) and Trolox-treated samples (5Cd50TR) were positioned far left, associated with higher levels of P, S, Zn, Ca, Mn, fructose, glucose, and organic acids. Trolox treatment mitigated the Cd-induced shift, as seen in samples like 10Cd50TR and 5Cd50TR, which moved closer to the control group. The heatmap illustrates the relative abundance of mineral elements, sugars, and organic acids across treatments (Fig. 4). Cadmium-only treatment (20Cd0TR) showed strong Cd and Cu accumulation, while essential elements like P, S, Zn, Ca, and Mn were significantly depleted. Sugars and organic acids declined sharply under Cd stress, while Trolox co-treatment restored their levels toward control values. The pattern confirms the protective role of Trolox against Cd-induced biochemical disturbances.

## Discussion

### Mineral Element Composition and Mechanisms of Trolox Protection

The most striking result to emerge from our data is that cadmium stress caused severe disruptions in the mineral element composition of grapevine berries, with all measured macro- and micronutrients showing significant declines under increasing cadmium concentrations. The dramatic reduction in nitrogen content from 2.43% in control plants to 0.65% under 20 ppm Cd stress represents a particularly concerning outcome, as nitrogen is fundamental for protein synthesis, chlorophyll formation, and overall plant metabolism (Rizwan et al. 2016). This widespread nutritional disruption can be attributed to the fact that cadmium competes

with essential nutrients for the same membrane transporters. Specifically,  $\text{Cd}^{2+}$  can be transported through ZIP (ZRT-IRT-like protein) family transporters that normally facilitate  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  uptake, through NRAMP (Natural Resistance-Associated Macrophage Protein) transporters involved in  $\text{Mn}^{2+}$  and  $\text{Fe}^{2+}$  transport, and through calcium channels that mediate  $\text{Ca}^{2+}$  influx (Clemens et al. 2013; Sasaki et al. 2012). This competitive inhibition at the transporter level explains why cadmium toxicity results in disturbance in the uptake and translocation of mineral nutrients, ultimately inhibiting plant growth and development. The reduction in micronutrient concentrations (manganese, iron, zinc, and boron) under cadmium stress further demonstrates the comprehensive nature of cadmium-induced nutritional disorders (Haider et al. 2021). Interestingly, copper content showed an opposite trend, increasing with cadmium stress, which may reflect the plant's attempt to enhance antioxidant enzyme activities, as copper is a cofactor for several antioxidant enzymes including copper/zinc superoxide dismutase (Liu et al. 2008). However, excessive copper accumulation can itself become problematic, contributing to oxidative stress through the generation of reactive oxygen species via Fenton-like reactions (Benavides et al. 2005).

Remarkably, Trolox application significantly mitigated many of the cadmium-induced reductions in mineral nutrients, particularly for nitrogen, potassium, calcium, magnesium, iron, and sodium. The ameliorative effects of Trolox operate through multiple interconnected mechanisms. First, as a water-soluble analog of vitamin E, Trolox functions as a potent lipid-soluble antioxidant that prevents membrane oxidative damage by scavenging lipid peroxyl radicals and inhibiting polyunsaturated fatty acid peroxidation (Satarug et al. 2003). By maintaining membrane integrity, Trolox preserves the proper functioning of ion channels, transporters, and ATPase pumps responsible for nutrient uptake and translocation (Tran and Popova 2013). Second, Trolox's antioxidant activity reduces reactive oxygen species (ROS) accumulation that would otherwise damage membrane lipids and proteins, thereby maintaining the selectivity and efficiency of nutrient transport systems. Under cadmium stress, plants experience increased oxidative damage due to disruption of electron transport chains in chloroplasts and mitochondria, leading to superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $\bullet\text{OH}$ ) generation (Rizwan et al. 2016). By neutralizing these ROS, Trolox helps preserve cellular redox homeostasis and protects critical transport proteins from oxidative inactivation. The observed reduction in berry cadmium accumulation following Trolox application (from 25.48 mg/kg to lower levels) suggests additional regulatory mechanisms beyond simple ROS scavenging. Trolox may influence the expression or activity of metal transporters involved in long-distance Cd

**Table 3** Sugar composition ( $\text{mg L}^{-1}$ ) of grapevine under cadmium stress and Trolox application

| Cadmium concentrations       | Trolox treatments | Sucrose       | Glucose      | Fructose     | Rhamnose     | Galactose    | Xylose        | Arabinose   |
|------------------------------|-------------------|---------------|--------------|--------------|--------------|--------------|---------------|-------------|
| 0 ppm                        | 0 $\mu\text{M}$   | 0.40±0.09     | 5.89±0.54 c* | 6.52±0.87 cd | 0.82±1.22 cd | 0.61±0.75 cd | 2.04±0.17 bc  | 1.28±0.13 c |
|                              | 50 $\mu\text{M}$  | 0.60±0.09     | 8.81±0.53 a  | 9.38±0.46 a  | 3.00±0.32 a  | 2.02±0.11 a  | 2.96±0.31 a   | 1.96±0.11 a |
| 5 ppm                        | 0 $\mu\text{M}$   | 0.42±0.10     | 5.94±0.55 c  | 6.58±0.87 cd | 2.09±0.17 b  | 1.34±0.14 b  | 2.06±0.17 bc  | 1.30±0.14 c |
|                              | 50 $\mu\text{M}$  | 0.46±0.07     | 6.71±0.41 b  | 7.14±0.36 b  | 2.29±0.24 ab | 1.54±0.09 ab | 2.25±0.24 b   | 1.49±0.09 b |
| 10 ppm                       | 0 $\mu\text{M}$   | 0.32±0.07     | 4.49±0.42 d  | 4.98±0.66 d  | 1.58±0.13 bc | 1.01±0.11 bc | 1.56±0.13 d   | 0.98±0.10 d |
|                              | 50 $\mu\text{M}$  | 0.40±0.06     | 5.63±0.34 c  | 6.00±0.30 cd | 1.91±0.20 b  | 1.29±0.07 b  | 1.89±0.20 b-d | 1.25±0.07 c |
| 20 ppm                       | 0 $\mu\text{M}$   | 0.14±0.04     | 1.94±0.18 e  | 2.14±0.28 e  | 0.68±0.06 d  | 0.44±0.05 d  | 0.67±0.06 e   | 0.42±0.04 e |
|                              | 50 $\mu\text{M}$  | 0.38±0.06     | 5.32±0.32 c  | 5.67±0.28 cd | 1.81±0.19 b  | 1.22±0.07 b  | 1.79±0.19 cd  | 1.18±0.07 c |
| Average of Cd concentrations | 0 ppm             | 0.50±0.13 A** | 7.35±1.67    | 7.95±1.68    | 1.91±1.43    | 1.31±0.91    | 2.50±0.55     | 1.62±0.38   |
|                              | 5 ppm             | 0.44±0.08 AB  | 6.33±0.60    | 6.86±0.67    | 2.19±0.22    | 1.44±0.15    | 2.16±0.21     | 1.39±0.15   |
|                              | 10 ppm            | 0.36±0.07 B   | 5.06±0.71    | 5.49±0.72    | 1.75±0.24    | 1.15±0.17    | 1.73±0.24     | 1.12±0.17   |
|                              | 20 ppm            | 0.26±0.14 C   | 3.63±1.87    | 3.91±1.95    | 1.25±0.63    | 0.83±0.43    | 1.23±0.63     | 0.80±0.42   |
| Average of Trolox treatments | 0 $\mu\text{M}$   | 0.32±0.13 B   | 4.57±1.74    | 5.06±1.98    | 1.29±0.80    | 0.85±0.49    | 1.58±0.60     | 1.00±0.38   |
|                              | 50 $\mu\text{M}$  | 0.46±0.11 A   | 6.62±1.47    | 7.05±1.55    | 2.25±0.53    | 1.52±0.33    | 2.22±0.52     | 1.47±0.33   |
| Cadmium                      | <i>p</i> -value   | 0.000         | 0.000        | 0.000        | 0.022        | 0.010        | 0.000         | 0.000       |
| Trolox                       | <i>p</i> -value   | 0.000         | 0.000        | 0.000        | 0.000        | 0.000        | 0.000         | 0.000       |
| Cadmium × Trolox             | <i>p</i> -value   | 0.127         | 0.000        | 0.001        | 0.008        | 0.007        | 0.002         | 0.000       |

\* Different lower-case letters indicate differences according to the interaction of 'Cd × Trolox' ( $p < 0.05$ )\*\* Different capital letters indicate differences according to 'Cd' and 'Trolox' factors ( $p < 0.05$ ). Data are presented as mean + standard deviation (SD)

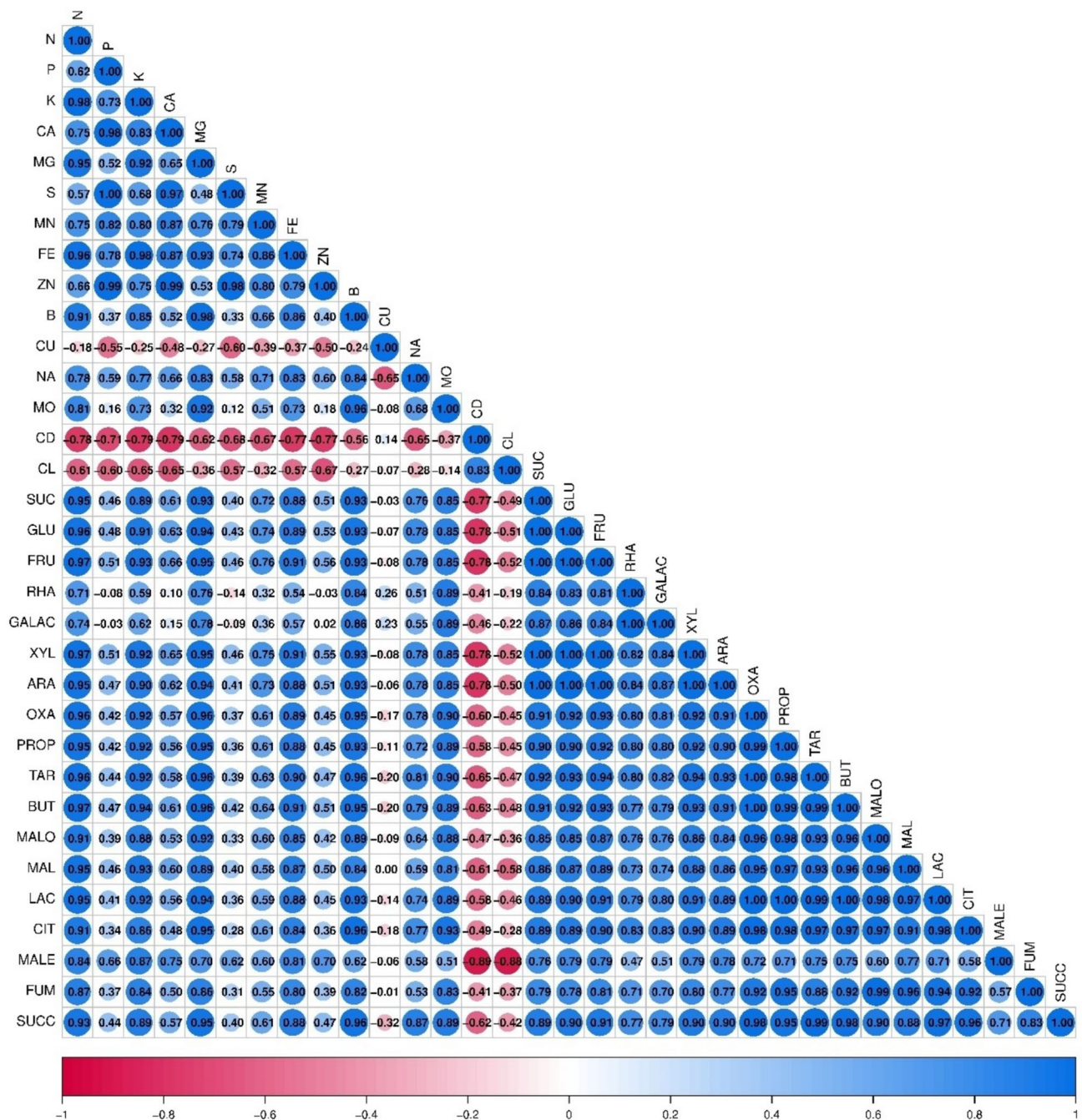


**Fig. 2** Correlation matrix of mineral nutrients, sugars, and organic acids in grapevine berries under Cadmium Stress and Trolox Application. N – Nitrogen, P – Phosphorus, K – Potassium, Ca – Calcium, Mg – Magnesium, S – Sulfur, Mn – Manganese, Fe – Iron, Zn – Zinc, B – Boron, Cu – Copper, Na – Sodium, Mo – Molybdenum, Cd – Cadmium, Cl – Chlorine, SUC – Sucrose, GLU – Glucose, FRU – Fructose,

RHA – Rhamnose, GALAC – Galactose, XYL – Xylose, ARA – Arabinose, OXA – Oxalic acid, PROP – Propionic acid, TAR – Tartaric acid, BUT – Butyric acid, MALO – Malonic acid, MAL – Malic acid, LAC – Lactic acid, CIT – Citric acid, MALE – Maleic acid, FUM – Fumaric acid, SUCC – Succinic acid

translocation. For instance, members of the HMA (Heavy Metal ATPase) family, such as HMA2 and HMA4, are responsible for loading metals into the xylem for root-to-shoot transport (Clemens et al. 2013). By reducing oxidative stress and maintaining cellular energy status, Trolox

may help preserve the normal regulation of these transporters, potentially reducing their affinity for  $\text{Cd}^{2+}$  or promoting Cd sequestration in root vacuoles through enhanced expression of vacuolar transporters. Furthermore, Trolox may indirectly stimulate the synthesis of phytochelatins

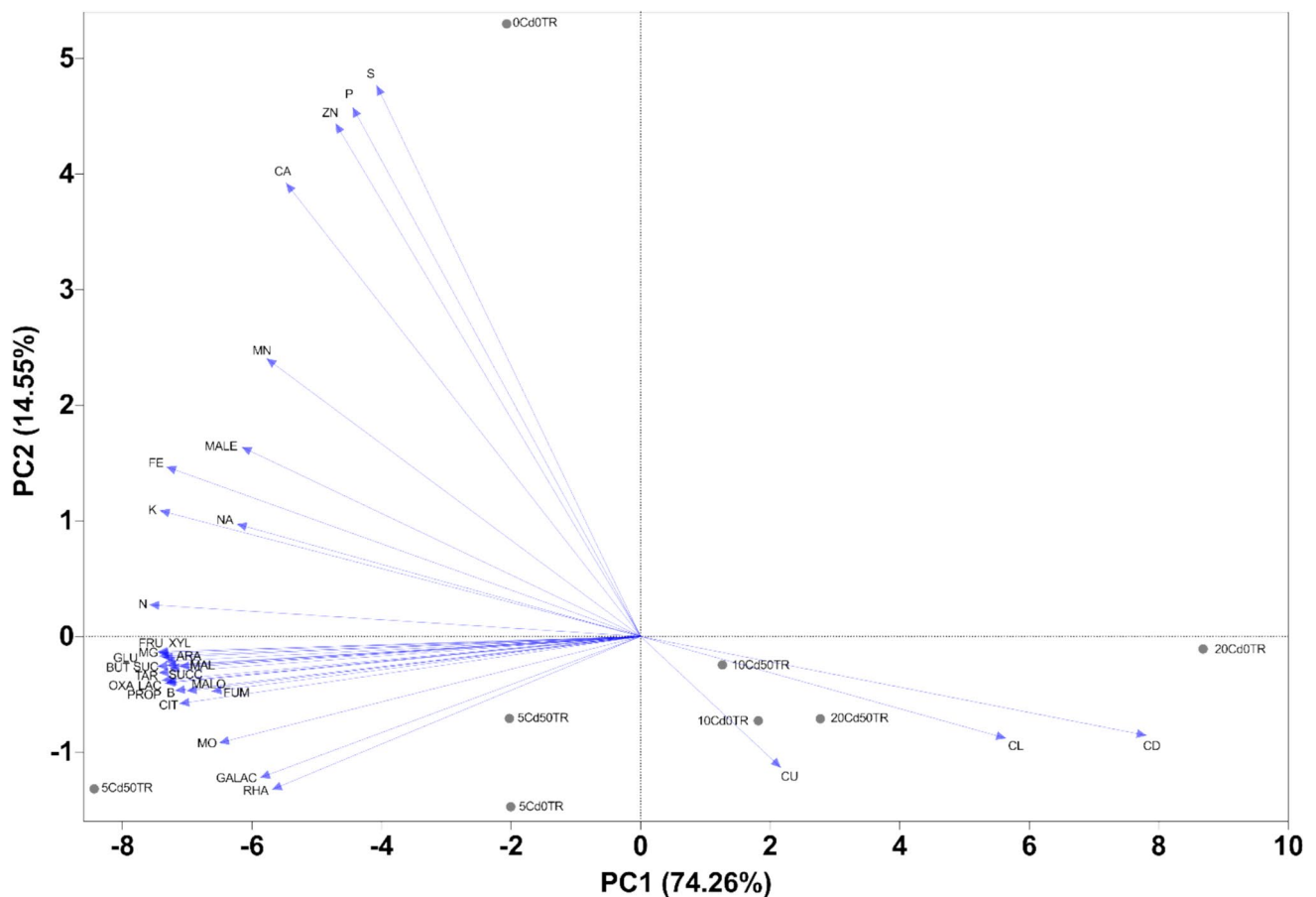


**Fig. 3** Principal component analysis of mineral nutrients, sugars, and organic acids in grapevine berries under cadmium stress and Trolox application

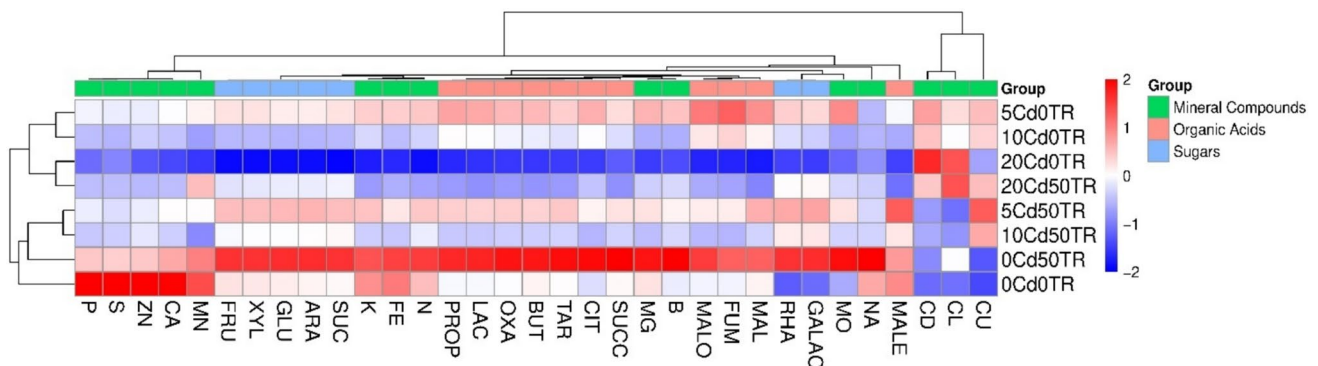


**Fig. 4** Heatmap analysis of mineral nutrients, sugars, and organic acids in grapevine berries under cadmium and Trolox treatments. P – Phosphorus, S – Sulfur, Zn – Zinc, CA – Calcium, CN – (Probably a typo: should be Ca or Cn not defined), MN – Manganese, FE – Iron, XYL – Xylose, FRU – Fructose, GLU – Glucose, ARA – Arabinose, N – Nitrogen, K – Potassium, SUC – Sucrose, FE – Iron, N – Nitrogen, P – Phosphorus, OXA – Oxalic acid, PROP – Propionic acid, LAC

– Lactic acid, CIT – Citric acid, TAR – Tartaric acid, BUT – Butyric acid, SUCC – Succinic acid, MG – Magnesium, B – Boron, MG – Magnesium, SUCC – Succinic acid, MALO – Malonic acid, MAL – Malic acid, FUM – Fumaric acid, RHA – Rhamnose, MAL – Malic acid, GALAC – Galactose, NA – Sodium, MO – Molybdenum, NA – Sodium, MALE – Maleic acid, CD – Cadmium, CL – Chlorine, CU – Copper

(PCs) and metallothioneins (MTs), metal-binding peptides that chelate Cd in root tissues and limit its translocation to aerial parts and reproductive organs (Haider et al. 2021). The preservation of membrane selectivity by Trolox likely

maintains proper ion transport kinetics, as damaged membranes become more permeable to toxic metals while losing their ability to selectively transport essential nutrients. The interaction effects observed between cadmium and Trolox

for several nutrients (nitrogen, manganese, iron, copper, sodium, and molybdenum) indicate that Trolox's protective effects become more pronounced under stress conditions, suggesting a stress-dependent response mechanism. This stress-dependent response indicates that Trolox's benefits represent a targeted response to oxidative damage rather than simply additive effects (Liu et al. 2008).

### Organic Acid Composition and Metabolic Pathway Protection

This investigation reveals a dramatic dose-dependent relationship between cadmium exposure and organic acid depletion in grapevine berries, with Trolox application providing remarkable protective benefits. The observed decline in total organic acids from 15.27 mg L<sup>-1</sup> under control conditions to 4.43 mg L<sup>-1</sup> under severe cadmium stress is consistent with previous findings demonstrating that heavy metal stress disrupts organic acid metabolism in fruit tissues (Khan et al. 2022; Nazima et al. 2015). The mechanisms underlying organic acid depletion under Cd stress involve multiple metabolic disruptions. First, cadmium interferes with photosynthetic electron transport, reducing ATP and NADPH production, which in turn limits the availability of energy and reducing power required for carboxylate synthesis (Nazar et al. 2012). Second, Cd-induced ROS accumulation damages enzymes involved in the tricarboxylic acid (TCA) cycle, including citrate synthase, aconitase, and malate dehydrogenase, thereby reducing the flux through this central metabolic pathway (Geigenberger and Fernie 2014). Third, oxidative damage to mitochondrial membranes impairs substrate transport and disrupts the electrochemical gradients necessary for ATP synthesis, further compromising energy-dependent biosynthetic processes. Significantly, Trolox application enhanced organic acid accumulation across all stress levels, with the highest total organic acid content (20.14 mg L<sup>-1</sup>) observed in the Trolox-treated control group. The dramatic reduction in individual acids such as tartaric acid, malic acid, and citric acid under cadmium stress demonstrates the vulnerability of primary grape acids to metal toxicity, which has significant implications for wine quality (Oliveira et al. 2015). Trolox's protective effect on organic acid metabolism likely operates through preservation of TCA cycle enzyme activity by preventing oxidative modifications of critical cysteine and methionine residues. Additionally, by maintaining mitochondrial membrane integrity and respiratory chain function, Trolox ensures adequate ATP production for biosynthetic reactions. The preservation of citric acid levels is particularly important, as citric acid serves as both a key TCA cycle intermediate and a natural metal chelator that

can complex with Cd<sup>2+</sup>, potentially reducing its reactivity within cells (Roberts et al. 2004). The protective effect of Trolox was most pronounced for succinic acid, which decreased from 376.53 mg L<sup>-1</sup> in the Trolox-treated control to only 2.21 mg L<sup>-1</sup> under severe cadmium stress without antioxidant protection. This dramatic response suggests that succinate dehydrogenase, the enzyme catalyzing succinic acid oxidation in the TCA cycle, is particularly vulnerable to Cd-induced oxidative inactivation. The significant Cd × Trolox interaction effects ( $p < 0.001$ ) for most organic acids indicate that antioxidant protection becomes increasingly important as stress intensity increases.

### Sugar Metabolism and Carbohydrate Partitioning Under Stress

Our investigation demonstrates that cadmium stress severely disrupts sugar metabolism in grapevine berries, with glucose and fructose being particularly vulnerable to metal toxicity. The observed decline in glucose content from 7.35 mg L<sup>-1</sup> under control conditions to 3.63 mg L<sup>-1</sup> at 20 ppm cadmium stress confirms previous findings that heavy metals interfere with carbohydrate metabolism in fruit tissues (Lorenc-Plucinska et al. 2005; Savvas et al. 2010). The mechanisms underlying sugar metabolism disruption involve impaired photosynthetic activity, reduced phloem loading efficiency, and altered sink strength in developing berries. Cadmium interferes with the Calvin cycle by inhibiting ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and other photosynthetic enzymes through ROS-mediated oxidative modifications (Nazar et al. 2012). Additionally, Cd disrupts sugar transporters (SUTs and SWEETs) responsible for sucrose loading into phloem and sugar unloading in berry cells, thereby reducing photosynthate translocation to developing fruits (Emamverdian et al. 2015). Remarkably, Trolox application enhanced both glucose and fructose levels across all stress conditions, with the most pronounced effects observed under moderate cadmium exposure. The significant Cd × Trolox interaction effects ( $p \leq 0.001$ ) indicate that antioxidant protection becomes increasingly critical as metal stress intensifies. Trolox's protective effect on sugar accumulation likely involves maintaining the activity of invertases and other carbohydrate-metabolizing enzymes, preserving phloem transport efficiency, and protecting sugar transporter proteins from oxidative damage. By maintaining cellular energy status and redox balance, Trolox ensures continued function of energy-dependent sugar transport processes. The differential responses of various sugar types to cadmium stress reveal that while hexose sugars (glucose and

fructose) showed dramatic reductions, pentose sugars (xylose and arabinose) demonstrated relatively greater resilience, though still benefiting significantly from antioxidant treatment. This differential sensitivity may reflect differences in biosynthetic pathways, with pentose sugars derived from the oxidative pentose phosphate pathway being somewhat more insulated from Cd effects on primary photosynthesis. The preservation of rhamnose and galactose levels through Trolox treatment underscores the broad-spectrum protective effects of antioxidants on carbohydrate metabolism (Shivashankara et al. 2016).

### Study Limitations and Future Research Directions

While this study provides valuable insights into Trolox's protective effects against cadmium stress in grapevines, several limitations should be acknowledged. First, only a single Trolox concentration (50  $\mu\text{M}$ ) was tested, which limits our understanding of potential dose–response relationships. Future research should examine multiple Trolox concentrations to identify optimal application rates for different stress intensities. Second, we did not measure enzymatic antioxidant activities (superoxide dismutase, catalase, ascorbate peroxidase) or direct oxidative stress indicators (hydrogen peroxide, malondialdehyde, lipid peroxidation products). Including these parameters in future studies would provide direct evidence for the proposed ROS-scavenging mechanisms and clarify the interplay between exogenous antioxidant application and endogenous antioxidant systems. Third, our analysis focused exclusively on berry tissues and did not assess cadmium distribution in roots, stems, or leaves. Comprehensive tissue analysis in future research would elucidate whole-plant Cd translocation dynamics and reveal whether Trolox primarily reduces Cd uptake at the root level or limits its subsequent translocation to fruits. Fourth, molecular analyses examining the expression of metal transporter genes (ZIP, NRAMP, HMA families), phytochelatin synthase genes, and antioxidant enzyme genes would provide mechanistic insights into how Trolox modulates cadmium tolerance at the transcriptional level. Finally, as noted in the Materials and Methods section, our experimental design employed solution-based Cd application rather than contaminated soil to ensure controlled, reproducible conditions. While this approach allowed precise dose–response assessment and minimized experimental variability, it differs from field contamination scenarios. Future long-term field trials using naturally Cd-contaminated soils would be valuable for validating the practical applicability of Trolox treatments under realistic agricultural conditions and for assessing the sustainability of this intervention

strategy across multiple growing seasons. Despite these limitations, our findings establish that Trolox application represents a promising strategy for mitigating cadmium-induced physiological and metabolic disruptions in grapevines, with potential applications in contaminated vineyard sites.

### Conclusion

Based on our results, this work has provided comprehensive evidence that cadmium stress severely disrupts mineral element composition, organic acid metabolism, and sugar accumulation in grapevine berries, while Trolox application offers significant protective benefits against these detrimental effects. The evidence from this study suggests that cadmium exposure creates a cascade of metabolic disruptions, with essential nutrients like nitrogen, phosphorus, and potassium experiencing dramatic declines under severe stress conditions. We have obtained satisfactory results demonstrating that organic acid content decreased substantially, with total organic acids showing marked reductions under maximum cadmium exposure. Taken together, these results suggest that sugar metabolism is equally vulnerable, with glucose and fructose contents experiencing considerable declines under severe cadmium stress. Our research has highlighted the importance of antioxidant intervention, as Trolox application consistently mitigated cadmium-induced damage across all measured parameters. We have found a novel solution for maintaining berry quality under metal contamination through strategic antioxidant supplementation. The strength of our contribution lies in demonstrating that a single antioxidant compound can provide broad-spectrum protection against heavy metal toxicity in fruit crops. These findings add substantially to our understanding of plant stress physiology and fruit quality preservation strategies. Our data suggest that Trolox could be exploited as a practical intervention in viticulture for regions affected by soil metal contamination, potentially helping decision makers develop sustainable grape production systems under adverse environmental conditions.

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## Declarations

**Conflict of Interest** The authors declare no competing financial or non-financial interests that could have influenced the work reported in this manuscript.

**Ethical Approval and Participant Consent** This research did not involve human subjects or animals requiring ethical approval.

**Publication Consent** No individual participant data requiring consent for publication were included in this study.

**Publication Authorization** All authors provided explicit consent for manuscript publication and agree to be accountable for all aspects of the work.

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