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Bioaccumulation, Ionome and Physiological Responses to Copper Toxicity of Iranian Grapevine Rootstocks Grafted with 'Asgari' Seedless Grape

Siamak Shirani Bidabadi and Paolo Sabbatini

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ABSTRACT

Use of proper rootstocks has the potential to improve copper (Cu) tolerance of grape. To evaluate the rootstock ability to reduce Cu transfer in the aerial portion of a grapevine, a glasshouse experiment was conducted to determine the physiological impact of Cu soil application in 'Asgari' seedless grape, either non-grafted or grafted onto different rootstocks. Vines were supplied with a nutrient solution at four levels of $CuSO_4$ concentrations (0, 10, 25 and 50 µM). Cu toxicity symptoms in leaves and reduced plant growth rate were evident in the non-grafted 'Asgari' and less pronounced in grapevines grafted onto 'Shahani' rootstock. Moreover, 'Asgari' grafted onto 'Shahani' rootstock had lower rate of Cu accumulation in the leaves when compared to the roots than those of non-grafted and 'Asgari' with 'Keshmeshi' rootstock. Although significant reduction of P in non-grafted 'Asgari' leaf tissue was found under Cu toxicity, 'Shahani' rootstock reported the highest amount of P (5.94 mg g^{-1}) in the leaves of 'Asgari' scion. However, significant fluctuations in terms of Ca and Fe were reported in leaves of both grafted and non-grafted 'Asgari'. When exposed to Cu toxicity, 'Shahani' rootstock induced significant increase in chlorophyll content, *Fv/Fm* ratio and a noticeable reduction in electrolyte leakage, H_2O_2 and malondialdehyde (MDA) compared to those having 'Keshmeshi' rootstock and non-grafted 'Asgari'. 'Shahani' rootstock improved 'Asgari' performance under Cu toxicity by slowly reducing the activity of antioxidant enzymes, preventing Cu accumulation in the aerial parts and maintaining a balanced scion nutritional status.

KEYWORDS
Cu toxicity; grapevines; translocation factor; oxidative stress; rootstocks; ionome

Introduction

Copper is a micronutrient that is essential for plant growth and development; it has an important role in photosynthesis and acts as a cofactor for a variety of enzymes such as the superoxide dismutase (SOD) (Clemens, 2001). However, this micronutrient is among the major heavy metal contaminant in the environment, as excessive accumulation of copper in plant tissues not only results in

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morphological and physiological disorders; decline in growth and photosynthetic activity, reduction in mineral nutrients uptake (Burzynski and Klobus, 2004; Fernandes and Henriques, 1991; Monni et al., 2000; Wang et al., 2004; Zheng et al., 2005), leaf chlorosis, inhibition of root growth and plant cell membrane electrolyte leakage (De Vos et al., 1991; Ouzounidou et al., 1992) but also threats human health by its accumulation in edible plants and fruits (Sun et al., 2010). The main symptoms of copper toxicity in plants are a) stunted growth, b) chlorosis and senescence of leaves and c) damages to the root cell membranes (Khellaf and Zerdaoui, 2010; Kopittke and Menzies, 2006; Michaud et al., 2008; Mourato et al., 2009; Wei et al., 2008). Long-term applications of Cu-based fungicides (e.g. copper hydroxide or copper sulfate) to control grapevine diseases such as downy mildew (*Plasmopara viticola* Berl.) have led to dangerous accumulation of copper in vineyard soils, up to toxic levels in several regions of the world (Girotto et al., 2016; Miotto et al., 2014; Santana et al., 2015; Tiecher et al., 2017). Consequently, Cu contamination in vineyard soils and the subsequent soil- grapevine- human transfer have been of global concern. In fact, Chaignon et al. (2015) reported that vineyard soils contained 50–150 mg/kg Cu, a concentration up to 300 times than the standard values. The accumulation of copper in vineyard soils and the subsequent potential exposure of humans to this heavy metal pose a dangerous health risk (Sun et al., 2010). Usually, plants adopt two strategies to achieve tolerance against copper toxicity. The first is exclusion, where plants avoid excessive uptake and transport of Cu ions, and consequently sequestration and accumulation in tissues. The second is that plants detoxify free metals by a) relegating Cu in vacuoles, b) via metal chelation by organic acids or c) using amino acids and metal-binding peptides (Clemens, 2001; Hall, 2002). A practical approach to reduce fruit production losses caused by Cu toxicity in high-yielding varieties could be to graft vines onto rootstocks capable of reducing the effect of heavy metals on vine growth and development (Rouphael et al., 2008). Although there are several research on the negative effects of Cu toxicity on grapevine growth, no published data is available on the effects of Cu toxicity on agronomical and physiological responses of grafted grapevine. Our hypothesis is that grafting could improve copper tolerance of grapevine by limiting Cu transport to the growing shoots. For this reason, grafted and non-grafted potted grapevines, irrigated with 20% Hoagland's solution (Hoagland and Arnon, 1938) were supplemented with different levels of $CuSO_4$ and compared for growth, leaf chlorophyll content, electrolyte leakage, oxidative stress, antioxidant enzyme activities, and mineral composition.

Material and Methods

A glasshouse culture experiment was conducted to evaluate the response of Iranian grapevine rootstocks to Cu toxicity. One-year-old canes of 'Asgari', 'Shahani' and 'Keshmeshi' grapevines (*Vitis vinifera* L.) were collected from vineyards located at the Isfahan University of Technology, Department of Horticultural Sciences in March 2017. Canes of the cultivars 'Shahani' and 'Keshmeshi' were cut into 10 cm cuttings to use as rootstock, and single-bud cuttings of 'Asgari' (of about 5 cm) prepared to be used as scion variety of the experiment. 'Asgari' as an important and widely cultivated table grape in Iran, has a short shelf life with green and tender skin. The single-bud scions of 'Asgari' were grafted onto 'Shahani' and 'Keshmeshi' rootstock, using the wedge grafting technique. The grafting combinations were as follows: A-A non-grafted 'Asgari', A-S 'Asgari' grafted onto 'Shahani' and A-K 'Asgari' grafted onto 'Keshmeshi'. The total length of the cuttings was about 15 cm. The surface of the grafted zone of both scion and rootstocks was treated with 200 mg·L^{-1} Naphthalene Acetic Acid (NAA + 500 mg·L^{-1} Benzyl Amino Purine (BAP) according to the procedure described by Shirani Bidabadi et al. (2018). The grafted cuttings associated with non-grafted 'Asgari' grapevines were placed in glasshouse benches and filled with fine rooting sand. Sixty days after grafting, grafted cuttings with proper root systems were transferred to individual plastic pots (diameter 12 cm) filled with perlite and placed in a glasshouse. The glasshouse environmental conditions consisted of minimum and maximum temperatures of 21°C and 25°C respectively, with a 40–60% relative humidity and natural daylight (minimum and maximum light flux: 200 and 1000 μmol m^{-2}s^{-1}, respectively) according to Cambrolle et al. (2015). Before starting Cu treatments, plants were selected for homogeneity in growth and were cut to leave single main shoot for a logical comparison of vegetative development. Then, pots were irrigated with 20% Hoagland's solution (Hoagland and Arnon, 1938) of 200 mL per pot, at 3-day intervals over 30 days. The pots were then arranged in a completely randomized block design and Cu treatments were imposed by mixing the 20% Hoagland's solution with $CuSO_4$ at 0 (control), 10, 25 and 50 μM appropriate concentrations. Each treatment had seven replicates (pots). Due to the presence of only a small amount of Cu in Hoagland's solution, the control (0 μM Cu) treatment contained 0.5 μM of Cu as an essential trace nutrient. After 60 days of growth under Hoagland's solution with different Cu levels, the cuttings were harvested and measurements of morphological and physiological attributes carried on together with the analyses of Cu accumulation and nutrient uptake.

Vine Growth

For the determination of relative scion shoot growth rate (SGR) (g day^{-1} per plant), two plants from each treatment were harvested at 30-day intervals. Data collection started 60 days after grafting and the vines were transferred successfully into the pots and consequently when active growth had started. The SGR was derived by the following equation (Cambrolle et al., 2015):

$$SGR = [(\text{final weight}) - (\text{initial weight})]/30\text{days}$$

Initial weight was the fresh biomass (g) of the main scion shoot before transferring into the pots, and final weight is fresh biomass (g) of the main scion shoot 30 days after exposure to copper treatments. Moreover, the increase of main scion shoot length was calculated by the following formula:

$$I(\%) = \frac{FSL - ISL}{ISL} \times 100$$

where I is the increase of main scion shoot length; ISL is the initial scion shoot length (cm) measured before transferring into pots; FSL is the final scion shoot length (cm) measured 30 days after exposure to copper treatments.

Determination of Phosphorus (P), Sulfur (S), and Iron (Fe) Concentrations in Grafted and Non-grafted 'asgari' Grape Leaves

Leaf samples from 60-day summer shoots of grafted and non-grafted 'Asgari' grapevines dried in an oven at 80°C for 48 h, were ground to powder and then 0.2 g of tissue was digested with 5 mL HNO_3 at 110°C for 2 h, cooled, added 1 mL of H_2O_2 and boiled for 1 h. The clear digests were diluted to 50 mL with triple-deionized water. The P, S and Fe concentration was measured by flame an atomic absorption spectrometry instrument (Perkin Elmer, A- Analyst 200).

Determination of Mg and Ca Concentration in Grafted and Non-grafted 'asgari' Grape Leaves

For the determination of Mg and Ca concentrations, leaf samples were dried in an oven at 60°C for 48 h. Then, 1 g of leaves were ground using an electric grinder and scorched at 560°C. The obtained ashes were digested in 10 mL of 1 N HCl. Then, the concentration of Mg and Ca in the digested samples was determined using a flame photometer instrument (Model CL 378, Nanolytik, Germany).

Determination of Cu Concentration in Shoots and Roots of Grafted and Non-grafted 'asgari' Grapevines

Shoot and root samples from 60-day cuttings of grafted and non-grafted 'Asgari' grapevines dried in oven at 80°C for 48 h, were ground to powder and then 0.2 g was digested with 5 mL HNO_3 at 110°C for 2 h, cooled, added 1 mL of H_2O_2 and boiled for 1 h. The clear digests were diluted to 50 mL with triple deionized water. The Cu concentration was measured by a flame atomic absorption spectrometry instrument (Perkin Elmer, A- Analyst 200). To determine the Cu translocation ability (from root to shoot) of different levels of Cu applications in grafted and non-grafted grapevine, the translocation index (TI %) was calculated as proposed by Yang et al. (2016) using the following formula:

$$\text{Translocation index (TI \%)} = \frac{\text{Cu content in shoot}}{\text{Cu content in rhoot}} \times 100$$

Physiological Parameters

Relative leaf chlorophyll content was measured using a spectrophotometry (Model PD-303, Apel Co. Ltd, Japan) in fully expanded scion leaves at fourth node, based on the method described by Arnon (1949). Chlorophyll fluorescence was measured using a fluorometer (Walz, Effeltrich, Germany) and the maximum photochemical efficiency of photosystem II (*Fv/Fm*) was also measured by the same instrument after 30 min of dark adaptation. The *Fv/Fm* ratio was calculated as: *Fv/Fm* = (*Fm*- *F0*)/*Fm*. Where, *Fm* and *F0* represented the maximum and minimum yields of dark-adapted leaves, respectively. Electrolyte leakage (EL) was measured by using a conductivity meter (WTW, Cond 7110, Germany) according to Ozden et al. (2009).

Malondialdehyde and H_2O_2 Concentrations

The concentration of malondialdehyde (MDA) which is a product of lipid peroxidation was assessed spectrophotometrically by the thiobarbituric acid (TBA) method according to Wang et al. (2009) and was calculated on a fresh weight basis, using the following formula:

$$\left(\mu\text{mol MDA g}^{-1}\text{FW}\right) = 6.45(\text{OD}_{532} - -\text{OD}_{600}) - -0.56(\text{OD}_{450})$$

Hydrogen peroxide (H_2O_2) was assessed spectrophotometrically after a reaction with potassium iodide (KI), according to the method developed by Velikova and Loreto (2005). The content of H_2O_2 was determined using a standard curve.

Enzyme Extraction and Assay

Fresh foliar tissue (0.2 g) from scion shoots (uppermost leaves) was separated from the 60-day shoots, weighed, washed with distilled water and then homogenized with a mortar and pestle with 5 mL chilled sodium phosphate buffer (50 mM, pH 7.8). The homogenates were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was stored at 4°C and used for Catalase (CAT), Ascorbate Peroxidase (APX) and Peroxidase (POX) assays. POX activity was determined spectrophotometrically, by measuring the oxidation of o-dianisidine (3, 3-dimethoxybenzidine) at 460 nm as described by Ranieri et al. (2000) and expressed as units (µmol of dianisidine oxidized per minute) per mg of protein. APX activity was measured by ascorbate oxidation at 290 nm in the presence of 100 mL of 1 mM H_2O_2 as described by the methods of Nakano and Asada (1981). The unit for APX activity was µmol of ascorbate oxidized per min per mg of protein. CAT activity was measured by the method of Blume and McClure (1980). The unit for CAT activity was µmol of hydrogen peroxide oxidized per min per mg of protein.

Statistical Analysis

The recorded data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) for comparison of means using SAS (ver. 8.2, SAS Institute, Cary, NC, USA) and MSTAT-C software (MSTAT Development Team, Michigan State University, East Lansing, MI).

Results

Visual Toxicity Symptoms and Vine Growth

Cu toxicity symptoms in the non-grafted 'Asgari' grapevines were more pronounced than those of the grafted onto 'Shahani' rootstocks (Figure 1). After 60 d of exposure, non-grafted 'Asgari' grapevines grown in increasing levels of Cu toxicity (up to 50 µM) exhibited a pattern of tip necrosis in the older leaves, which was associated with an intense defoliation at the highest concentration (50 µM) (Figure 1). The results obtained from Table 1, demonstrated the significant main effects (at 5% and 1% probability levels) of rootstock and copper levels on scion growth rate, increase of main scion shoot length, chlorophyll content, *Fv/Fm* ratio, electrolyte leakage, MDA accumulation, H_2O_2 generation, CAT, APX and POX activities of grapevines. However, among the above-mentioned attributes, the interactive effects of rootstock × copper level were regarded as significant only in terms of scion growth rate and H_2O_2 generation (at 5% probability level) and *Fv/Fm*, CAT, APX, POX activities (at 1% probability level). Higher doses of Cu in the nutrient solution showed a reduction in main shoot growth rate of grafted and non-grafted 'Asgari' grapevines (Figure 2a). However, there was a lower decline in scion growth when 'Shahani' rootstock (A-S) used when compared with the non-grafted 'Asgari' (A-A) and 'Asgari' grafted onto 'Keshmeshi' (A-K) rootstocks. At the two higher Cu concentrations (25 and 50 µM $CuSO_4$), A-K had a decrease in scion growth rate by 61.9% and 87.5%, respectively when compared to A-S. When different $CuSO_4$ levels were imposed, 'Shahani' rootstock resulted in an increased shoot length of

Figure 1. Effects of 20% Hoagland's solution contain 0 (a), 10 (b), 25 (c) and 50 (d) µM Cu on non-grafted cuttings of 'Asgari' seedless grapevine (A-A) (top row) and on 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) (bottom row).

'Asgari' scions (A-S) than those of grafted onto 'Keshmeshi' and non – grafted one (A-K). However, no significant difference was observed in the shoot length among the different grafting combinations (Figure 2b).

Ionome Parameters

Results from an analysis of variance (Table 2) revealed that rootstock, copper levels, and rootstock × copper levels at 1% and 5% probability levels had a significant impact on leaf and root Cu concentrations, translocation factor, the amount of phosphorus, magnesium, sulfur, calcium and Fe in the leaf tissue. However, among the above-mentioned attributes, the interactive effects of rootstock × copper level were regarded as significant in terms of the amount of Mg in the leaf tissue (at 5% probability level) and leaf and root

Table 1. Values of mean squares in the two-way analysis of variance of scion growth rate (SGR) (g day^{-1} per plant), increase of main scion shoot length (IL) (cm), chlorophyll content (Chl.) (µg mL^{-1} fresh leaf weight) *Fv/Fm* ratio, electrolyte leakage (EL%), lipid peroxidation indicated by malondialdehyde (MDA) accumulation (µmol g^{-1} FW), H_2O_2 content (µmole g^{-1} FW), catalase (CAT) activity (u mg^{-1} protein), ascorbate peroxidase (APX) activity (u mg^{-1} protein) and peroxidase (POX) activity (u mg^{-1} protein) in 'Asgari' grapevine.

Source of Variation	d.f.	SGR	IL	Chl.	*Fv/Fm*	EL	MDA	H_2O_2	CAT	APX	POX
Rootstock	2	0.0004**	15.19*	2.53**	0.03**	0.09**	0.02**	3.18**	0.65**	0.48**	0.03**
Coper levels	3	0.002**	475.98**	36.98**	0.1**	1.99**	0.31**	29.12**	14.99**	8.61**	0.28**
Rootstock × Copper levels	6	0.00001*	1.69^{ns}	0.08^{ns}	0.001**	0.007^{ns}	0.002^{ns}	0.28*	0.25**	0.11**	0.01**
Error	24	0.000007	4.79	0.17	0.0003	0.005	0.001	0.08	0.05	0.03	0.002
CV (%)		12.29	13.22	5.04	9.04	5.06	5.10	4.52	4.16	4.46	8.47

* and **: Significant at the 5% and 1% probability levels, respectively., ^{ns}: Non-Significant.

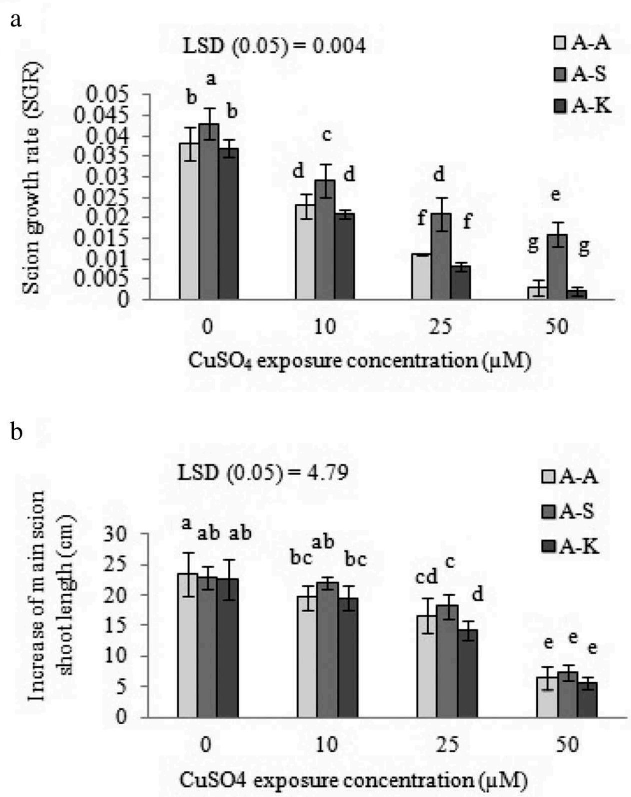

Figure 2. Scion growth rate (A) and increase of main scion shoot length (B), of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM $CuSO_4$ for a 60-day period. Different letters indicate significant differences according to LSD test (*P* = .05). Values are the means of three replicate samples.

Cu concentrations, translocation factor and the amount of phosphorus, sulfur, calcium and Fe in the leaf tissue (at 1% probability level). Under control growing conditions (0 µM Cu), some traces of Cu were also found in leaves and roots of both non grafted and grafted 'Asgari' grapevines; however, there were no significant differences among rootstocks and control grapevines in relation to the initial Cu concentration in leaf or root tissue (Table 3). Nevertheless, leaf and root Cu concentrations increased when nutrient solution was supplemented with $CuSO_4$ levels (Table 3). Interestingly, 'Asgari' grapevine grafted onto 'Shahani' rootstock (A-S) had a lower concentration of Cu in their leaves and a higher Cu accumulation in their roots than those of non-grafted 'Asgari' (A-A) and 'Asgari' grafted onto 'Keshmeshi' (A-K) rootstocks. Therefore, total Cu deposited in leaves to total Cu in roots (translocation factor) of Asgari' grapevine grafted onto 'Shahani' rootstock under Cu toxicity was lower compared to those of non-grafted and 'Asgari' grafted onto 'Keshmeshi' rootstock (Table 3). Under 0 mM Cu,

Table 2. Values of mean squares in the two-way analysis of variance of leaf Cu concentration (mg/g), Cu concentration in root tissue (mg/g), Translocation Factor (TF), the amount of phosphorus in the leaf tissue (mg/g) P, the amount of magnesium in the leaf tissue (mg/g) Mg, the amount of sulfur in the leaf tissue (mg/g) S, the amount of calcium in the leaf tissue (mg/g) Ca, the amount of Fe in the leaf tissue (mg/g) Fe in 'Asgari' grapevine.

Source of Variation	d. f.	Cu concentration			Nutrient concentration				
		Leaf	Root	TF	*P*	Mg	S	Ca	Fe
Rootstock	2	0.002**	6.12**	10.88**	4.65**	0.02 ns	1.66**	14.76**	0.0008**
Coper levels	3	0.09**	893.05**	625.74**	26.83**	2.74**	0.61**	59.63**	0.01**
Rootstock × Copper levels	6	0.003**	1.25**	16.15**	0.78**	0.05*	0.15**	0.7**	0.0007**
Error	24	0.00002	0.14	2.04	0.05	0.02	0.03	0.16	0.00003
CV (%)		6.23	4.34	7.85	5.67	3.92	4.58	1.89	6.60

* and **: Significant at the 5% and 1% probability levels, respectively., ns: Non-Significant.

Table 3. Cu concentration in leaf and root tissues and translocation index of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-SG2) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM $CuSO_4$ for a 60-day period.

Graft combinations	$CuSO_4$ exposure concentration (µM)	Cu concentration (mg g^{-1})		
		Leaf	Root	Translocation Index (TI, %)
A-A	0	0.005 h	0.035 g	14.43 b
	10	0.032 g	2.54 ef	1.24 c
	25	0.093 d	8.91 d	1.04 c
	50	0.152 a	21.68 b	0.70 c
A-S	0	0.006 h	0.028 g	22.68 a
	10	0.030 g	3.17 e	0.95 c
	25	0.078 e	10.95 c	0.71 c
	50	0.104 c	23.92 a	0.44 c
A-K	0	0.006 h	0.036 g	15.79 b
	10	0.041 f	2.52 f	1.63 c
	25	0.117 b	9.07 d	1.29 c
	50	0.152 a	21.44	0.71 c
$LSD_{(0.05)}$		0.008	0.63	2.41

Different letters between the Cu concentrations of the respective rootstock-grafted plants indicate significant differences according to LSD test.

rootstock effect did not present considerable variations in leaf Mg (Figure 3b) and S (Figure 4a) concentrations. In relation to leaf P (Figure 3a) and Ca (Figure 4b) content, 'Asgari' with 'Shahani' (A-S) rootstock showed the highest values (5.02 and 24.09, respectively). Unlike other elements, Fe was found to be the highest in non-grafted 'Asgari' grape leaves compared to those of grafted 'Asgari' grapevine onto 'Shahani' and 'Keshmeshi' (Figure 5). Increasing Cu toxicity caused reductions in P content in leaves of 'Asgari' grapevines when compared to the control (0 µM) (Figure 3a). Contrarily, 'Shahani' rootstock caused the highest amount of P content in the leaves of 'Asgari' scion (Figure 3a). Significant fluctuations in the amount of Ca (Figure 4b) and somewhat about Fe (Figure 5) in leaves of non – grafted 'Asgari' and 'Asgari' grafted onto 'Shahani' and 'Keshmeshi' also were

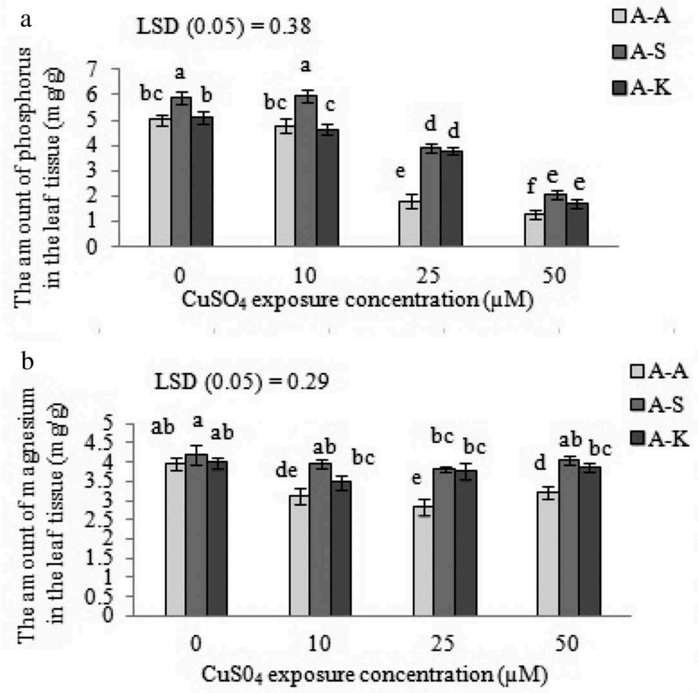

Figure 3. Concentration of P (A) and Mg (B) in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM $CuSO_4$ for a 60-day period. Different letters indicate significant differences according to LSD test (P = .05). Values are the means of three replicate samples.

recorded at different concentrations of $CuSO_4$ although fluctuations of the amount of Mg (Figure 3b) and S (Figure 4a) in leaves of both grafted and non-grafted 'Asgari' were not significant.

Leaf Relative Chlorophyll Content (LRCC), Chlorophyll Fluorescence (Fv/Fm), H_2O_2 Content, Lipid Peroxidation, and Electrolyte Leakage

Exposing 'Asgari' grapevines to rising levels of copper toxicity, the amount of chlorophyll (Figure 6a) and *Fv/Fm* (Figure 6b) decreased significantly. However, 'Asgari' grapes having 'Shahani' root system maintained higher values of chlorophyll content (Figure 6a) and *Fv/Fm* ratio (Figure 6b) compared to those having 'Keshmeshi' root system and to the non-grafted 'Asgari' grapevines. In the control treatment (0 µM $CuSO_4$), the rootstocks caused no significant difference in terms of MDA production (Figure 7a), H_2O_2 generation (Figure 7b), and Electrolyte Leakage (EL) (Figure 8). However, under increasing levels of Cu toxicity, 'Asgari' grapes grafted onto 'Shahani' (A-S) rootstock had significantly lower amounts of H_2O_2, MDA and EL in

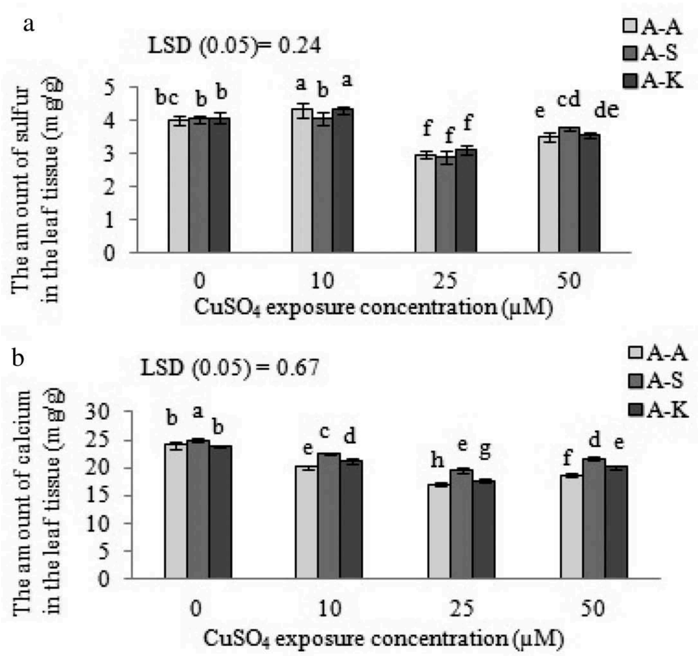

Figure 4. Concentration of S (A) and Ca (B) in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM $CuSO_4$ for a 60-day period. Different letters indicate significant differences according to LSD test (*P* = .05). Values are the means of three replicate samples.

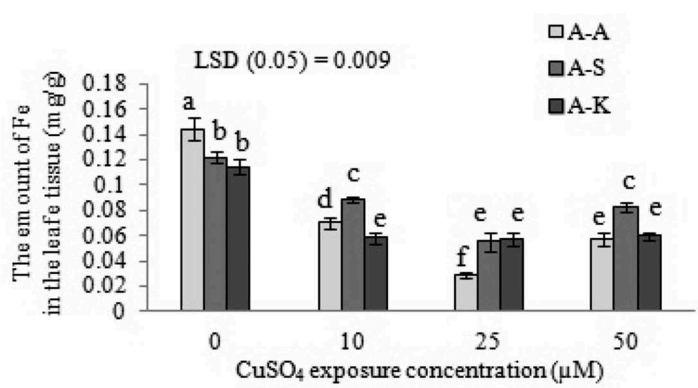

Figure 5. Concentration of Fe in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM CuSO4 for a 60-day period. Different letters indicate significant differences according to LSD test (*P* = .05). Values are the means of three replicate samples.

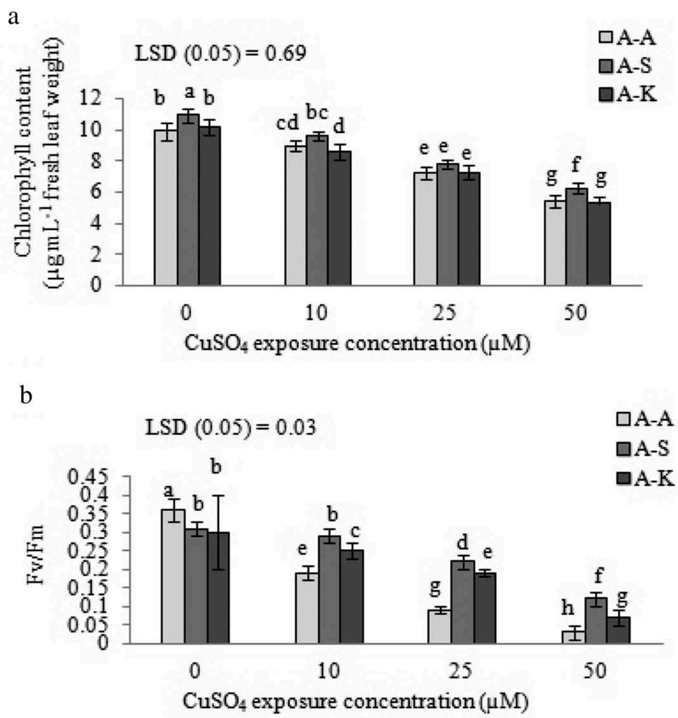

Figure 6. Concentration of chlorophyll content in the leaf tissue (A) and *Fv/Fm* (B) of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM CuSO4 for a 60-day period. Different letters indicate significant differences according to LSD test (P = .05). Values are the means of three replicate samples.

their leaves when compared to the non-grafted 'Asgari' (A-A) and those having 'Keshmeshi' rootstock (A-K) (Figures 7(a, b and 8)).

Antioxidant Activities

As shown in Figure 9, the two rootstocks used in this experiment caused no significant changes in the activities of CAT, APX and POX in the control treatment (0 µM CuSO4). Contrarily, under Cu toxicity, significant fluctuations were observed in all the Cu levels of the rootstocks (Figure 9). However, 'Shahani' rootstock slightly decreased the activity of all the enzymes measured during the experiment in leaves of 'Asgari' grapes at different Cu toxicities. In general, we recorded that 'Asgari' with 'Shahani' rootstock (A-S) caused a lower enzymatic activities in the leaves of the scion than those having 'Keshmeshi' rootstock (A-K).

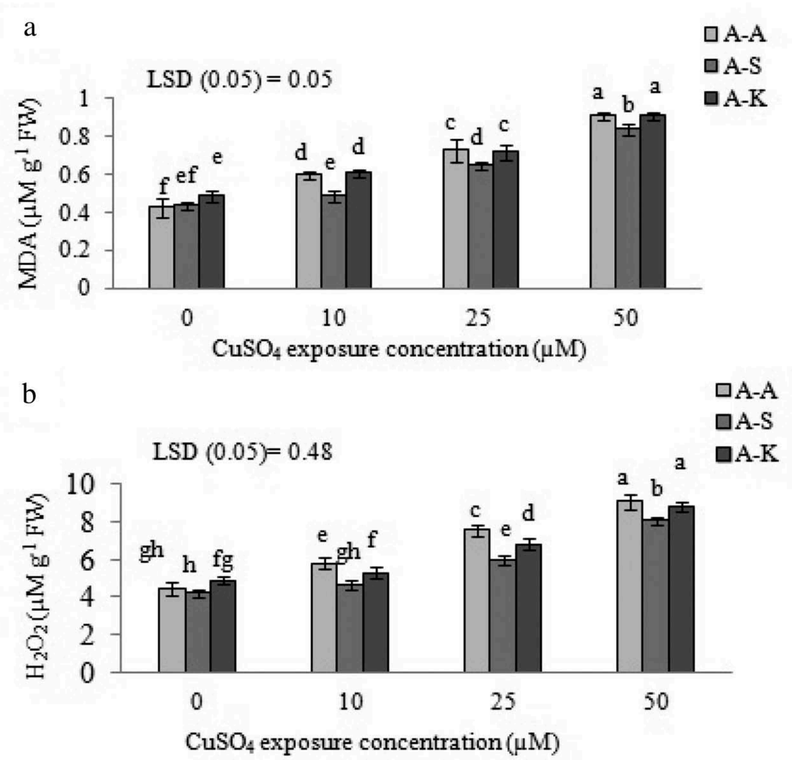

Figure 7. MDA accumulation (A) and H_2O_2 generation (B) in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM CuSO4 for a 60-day period. Different letters indicate significant differences according to LSD test (*P* = .05). Values are the means of three replicate samples.

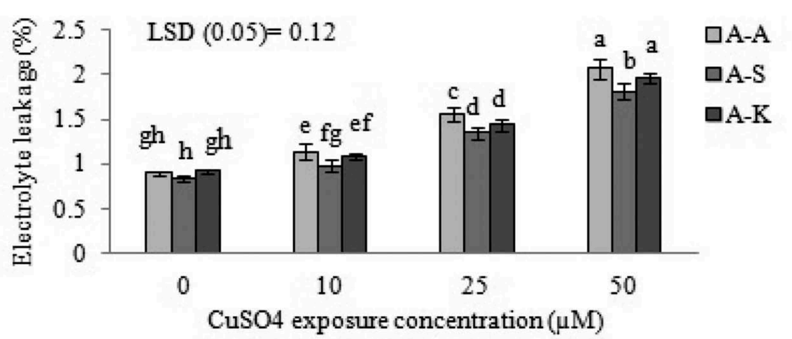

Figure 8. Electrolyte leakage in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM CuSO4 for a 60-day period. Different letters indicate significant differences according to LSD test (*P* = .05). Values are the means of three replicate samples.

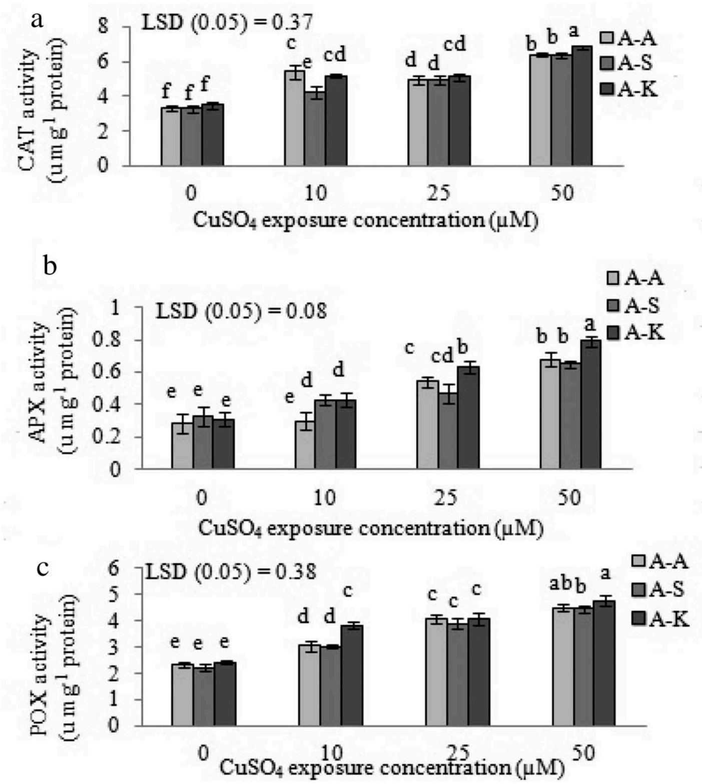

Figure 9. CAT, APX and POX activities (A, B, and C, respectively) in the leaf tissue of non-grafted 'Asgari' seedless grapevine (A-A), 'Asgari' scion grafted onto 'Shahani' rootstock (A-S) and 'Asgari' scion grafted onto 'Keshmeshi' rootstock (A-K) under 0, 10, 25 and 50 µM $CuSO_4$ for a 60-day period. Different letters indicate significant differences according to LSD test (P = .05). Values are the means of three replicate samples.

Discussion

Our results indicated that increasing levels of Cu toxicity, up to 50 µM in the nutrient solution applied to non-grafted 'Asgari' grapes caused high Cu accumulation and nutritional disorder in the leaves and roots. The decrease in chlorophyll we observed (Figure 7) could be due to peroxidation of chloroplast membranes mediated by Cu as reported earlier for spinach and cucumber (Baszynski et al., 1988; Rouphael et al., 2008). Our results also showed that Cu toxicity enhanced the oxidative injury through the disruption of photosynthesis, lipid peroxidation, and cell membrane stability as indexed by the enhanced electrolyte leakage; all the damages resulted in reduced growth rate of the grapevines treated with a Cu nutrient solution. Tiecher et al. (2017) reported that Cu toxicity caused a decrease in plant growth and in photochemical efficiency, which further support our results. 'Asgari' grafted on 'Shahani' (A-S) had a lower percentage of ion leakage after the treatments with Cu, indicating that grafting has potentially facilitated the protection of the membrane functionality and integrity. Moreover, Cu toxicity resulted in a significant loss in chlorophyll content, especially with non-grafted 'Asgari' grapes (A-A). This result is in accordance with the data reported by Rouphael et al. (2008), using cucumber plants in their experiments. Moreover, Girotto et al. (2013) also reported similar results to our findings, where Cu toxicity may cause oxidative stress due to imbalance between antioxidant responses and increased production of reactive oxygen species (ROS). However, when grafted onto 'Shahani' rootstock (A-S), 'Asgari' vines were more tolerant to Cu toxicity than those grafted onto 'Keshmeshi' (A-K) rootstocks and non-grafted ones. In fact, 'Asgari' on 'Shahani' had a reduced decrease in growth rate, photosynthesis efficiency and less oxidative damage as demonstrated by decreased amount of MDA and H_2O_2 accumulation. Higher tolerance to Cu toxicity in 'Asgari' grapevines when grafted on 'Shahani' root system (A-S) could be due to the fact that this rootstock had lower translocation factor, suggested by the lower Cu accumulation in the leaves of 'Asgari' grapes. The improved 'Asgari' scion growth rate under Cu toxicity could be also attributed to a lower accumulation of Cu in scion leaves. According to previous reports, reduced growth and reduced biomass production are general responses of grapevines to Cu toxicity (Toselli et al., 2009). Our results demonstrated that excessive Cu in the nutrient solution affect nutrient uptake such as P, Ca and Fe of 'Asgari' grapes, which was supported by the findings also of Marschner (1995) and Toselli et al. (2009). All the authors asserted that the negative effects of excessive Cu are related to the interference of the toxicity with the root absorption of essential elements. Nawaz et al. (2016) and Shirani Bidabadi et al. (2018) reported that using proper rootstock could significantly reduce heavy metal translocation from the root to shoots and it could also impact the transport of absorbed nutrients. Moreover, several recent finding showed that rootstocks could modify the ionome of plant crops resulting in different mineral status than non-grafted plants (Nawaz et al., 2016; Shirani Bidabadi et al., 2018). All of these recent reports further support our research results in demonstrating the rootstock control in absorption and translocation of mineral elements, such as the Cu–Fe antagonism, common in plant crops grown under conditions of Cu toxicity (Foy et al., 1978; Wallace and Cha, 1989). With increasing Cu concentration up to 25 μm, leaf iron content decreased significantly, but with increasing Cu concentration up to 50 μm, the amount of iron in the leaf was reversed and showed an increasing trend. Reduced chlorophyll and leaf chlorosis symptoms of 'Asgari' grapes subjected to excessive Cu could be attributed to a reduced leaf Fe concentration. However, 'Asgari' with 'Shahani' root system (A-S) showed a more enhanced amount of leaf Fe concentration than that was in non-grafted and 'Asgari' with 'Keshmeshi' rootstocks at all Cu levels tested in this experiment. Cell membrane stability has been extensively applied as a metric to determine tolerance against stress as in some cases higher membrane stability could be closely correlated with stress tolerance (Blum and Ebercon, 1981; Premchandra et al., 1992). The use of the 'Shahani' rootstock provided a counterbalance in the absorption of copper under Cu deficit and toxicity by adjusting Cu transport to the growing shoots of 'Asgari' seedless grape.

Conclusion

Continuous applications of Cu-based fungicides to control the threatening of grapevine diseases, have led to the accumulation of copper in vineyard soils, to a dangerous levels for vineyards around the world. The present study reveals noticeable differences in the ionome and physiological responses between grafted and non-grafted 'Asgari' seedless grape in response to excessive Cu. The toxicity symptoms on leaves in the non-grafted grapes and those grafted onto 'Keshmeshi' rootstocks were more pronounced than those having 'Shahani' root systems. Grafted 'Asgari' grapes onto 'Shahani' rootstocks exposed to excessive external Cu levels were capable of maintaining higher growth rates, improved photosynthesis efficiency, better resistance to oxidative stress, lower electrolyte leakage and a better nutritional status in the scion shoot tissues in comparison with non-grafted ones and 'Asgari' with 'Keshmeshi' root systems. Undoubtedly, studies at the molecular levels seem to be necessary to further clarify the physiological mechanisms of copper tolerance in specific rootstocks such as 'Shahani' or rootstock-scion combinations. Further research will try to evaluate the responses of the grafted grapevines to excessive Cu supply when associated with a higher ability of their root system to exclude Cu from the scion shoots, as we reported in this experiment.

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