Effects of Copper Exposure in Tissue Cultured *Vitis vinifera*

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The present study determined the effects of copper treatment on some biochemical parameters in a closed system. Sauvignon grapevines were cultured in agar and exposed to copper levels ranging from 0.07 to 10 μg Cu/g medium. Chlorophylls, carotenoids, lipids, sucrose, soluble sugars, starch, cellulose, and minerals in root, leaves, and sap were determined. Copper levels over 5 μg Cu/g inhibit root and plantlet development. Copper-exposed plants have higher levels of chlorophylls and carotenoids as well as total lipids. Soluble sugars decrease without changes in starch or sucrose. Copper concentration increases dramatically in roots and leaves. Iron concentrations are lower in leaves, although they increase in roots, with respect to control plants. Sap flow and translocation of essential elements are reduced. Reduction of K translocation can be related to limited use of water by the plant and, thus, reduction in growth and physiological activity.

Keywords: *Cu; Fe; sap flow; translocation; plant growth; Vitis vinifera*

INTRODUCTION

Heavy metal pollution in the environment is continuously increasing because of increased metal use in industry and agriculture. Copper is heavily used in the Mediterranean agriculture as a fungicide, and specifically, grapevine crops are treated with copper in the form of copper sulfate or oxychloride. However, the knowledge of the effect of copper on grapevine development and grape productivity is scarce and limited (Serra et al., 1981).

Although copper is essential for the normal development of plants, large copper exposures can negatively affect plant growth and productivity. In some plants, such as rice, excessive copper exposure has been related to higher plant copper concentration and reduction of photosynthesis (Lidon et al., 1993). Similar effects have been described from exposure to other heavy metals (Moya et al., 1993). Other reported responses to copper exposure for grains include reduction in protein concentration, soluble sugars, starch, and lipids and increase in leaf Cu, Fe, and Zn levels (Lanaras et al., 1993). Reduction of chlorophyll and carotenoids in oat and other plants have also been reported (Ouzounidou et al., 1992). Copper exposure also induces changes in mineral metabolism, especially Fe and Zn (Reboredo, 1994). Also, root growth seems to be a very sensitive indicator of heavy metal exposure (Wilkins, 1978), and reduction in root growth has been reported for copper-treated plants (Ouzounidou et al., 1992).

The aim of the present study is to determine the effects of copper exposure on some physiological and biochemical parameters in a closed system (in vitro explants). These parameters include determination of essential metal concentrations in leaves, roots, and xylem sap. Other parameters include root growth, xylem flow, and biomass production. Finally, concentration of photosynthetic pigments (chlorophylls and carotenoids) and sugar, lipid, and water content have also been measured.

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MATERIALS AND METHODS

Plants. Meristems of *Vitis vinifera* (cv. Sauvignon or Cabernet sauvignon) or hybrids used as rootstock (R-110, *Vitis rupestris* × *Vitis berlandieri*) grapevines were cultured in agar containing modified Murashige and Skoog (1962) media and illuminated with 14 h daylight (2000 lux) at 25 °C in a growth chamber. Copper was added (as copper sulfate solution) at concentrations of 0.07 (control, no copper added), 2, 5, and 10 μg Cu/g medium initially to determine effects on rooting and shoot development. For the other determinations, copper was added after 4 weeks (when the plants were developed). For each copper concentration, groups of 24 plantlets were grown for 2 additional months in the presence of copper. Harvest was always done after 3 months of culture.

Samples. Roots and leaves from in vitro plantlets were processed after thorough cleaning with Milli-Q water. Roots and leaves were homogenized by grinding in a mortar after freezing in liquid nitrogen.

Determination of Relative Root Growth. For all control and copper-treated plants, relative root growth (RRG) was determined by formula 1 (Ouzounidou et al., 1992):

$$\mathrm{RRG} = \frac{\text{average length of longest root treated}}{\text{average length of longest root control}} \times 100 \quad (1)$$

Determination of Sap Flow. In vitro plantlets (five each treatment) were transferred to hydroponic medium (Cooke et al., 1993) with a continuous air flow. The shoot was eliminated under the first leaf insert. A capillary tube was connected to the root and sealed with silicone oil. Sap exudate was collected for 1−2 h and weighed. Sap flow was expressed as mg sap (g root)$^{-1}$ h^{-1}.

Determination of Biochemical Parameters. Chlorophyll was determined after Arnon (1949) and carotenoids after

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Table 1. Effects of Different Concentrations of Copper on Root, Shoot, and Leaf Developed in Plantlets ($n = 24$) after 5 Weeks of in Vitro Culture (*V. vinifera* cv. Sauvignon), Expressed as Percentage of Plants That Had Developed Roots, Shoot, or Leaves

	root %					shoot %						
	1 wk	2 wk	3 wk	4 wk	5 wk	1 wk	2 wk	3 wk	4 wk	5 wk	RRG	leaf %
control	0	80	100	100	100	0	100	100	100	100	100	100
2 μg/g	0	35	85	95	95	0	85	85	95	95	61.2	51.9
5 μg/g	0	35	70	75	75	0	75	80	85	85	47.3	27.3
10 μg/g	0	0	0	0	0	0		65	85	85	0	0

Table 2. Copper Effects (5 μg/g) on Root Growth and Length of *V. vinifera* (cv. Sauvignon and Cabernet Sauvignon) and Hybrid Richter (R-110)[a]

	R-110		Cabernet sauvignon		Sauvignon	
	control	Cu	control	Cu	control	Cu
total weight(g)	1.04 ± 0.20	0.69 ± 0.27[b]	1.85 ± 0.57	0.60 ± 0.34[b]	1.12 ± 0.14	0.79 ± 0.16[b]
root weight (g)	0.33 ± 0.12	0.24 ± 0.11[b]	0.93 ± 0.40	0.26 ± 0.21[b]	0.35 ± 0.05	0.26 ± 0.04[b]
shoot weight (g)	0.23 ± 0.06	0.14 ± 0.07[b]	0.42 ± 0.11	0.17 ± 0.11[b]	0.35 ± 0.05	0.26 ± 0.06[b]
leaf weight (g)	0.44 ± 0.08	0.28 ± 0.09[b]	0.45 ± 0.09	0.22 ± 0.12[b]	0.42 ± 0.05	0.28 ± 0.06[b]
root length (cm)	10.3 ± 1.2	6.9 ± 2.9[b]	14.8 ± 5.7	8.2 ± 3.0[b]	16.0 ± 5.5	7.8 ± 3.1[b]
leaf/root ratio	1.45 ± 0.48	1.36 ± 0.62	0.59 ± 0.34	1.06 ± 0.40[b]	1.21 ± 0.02	1.09 ± 0.09[b]
shoot/root ratio	0.76 ± 0.28	0.65 ± 0.25	0.52 ± 0.23	0.77 ± 0.25[b]	1.01 ± 0.06	0.98 ± 0.10
root density (mg/cm)	32.8 ± 12.6	33.0 ± 12.7	64.7 ± 27.4	29.6 ± 15.9[b]	21.9 ± 9.1	33.3 ± 12.9
plant protein (mg/g)	5.0 ± 0.4	7.4 ± 0.9[b]	6.0 ± 1.1	12.8 ± 0.4[b]	16.9 ± 2.0	20.5 ± 0.8[b]
root protein (mg/g)	5.8 ± 0.3	7.3 ± 1.1	2.5 ± 0.1	5.6 ± 0.3[b]	9.9 ± 2.2	12.6 ± 2.3
shoot protein (mg/g)	4.2 ± 0.1	3.8 ± 0.1	3.3 ± 0.1	5.1 ± 0.3[b]	11.6 ± 3.6	13.8 ± 2.6
leaf protein (mg/g)	4.0 ± 1.8	9.3 ± 1.2[b]	15.8 ± 2.4	27.2 ± 0.5[b]	27.2 ± 3.9	33.8 ± 4.7

[a] Plants ($n = 12-15$) were exposed to copper for 2 months. Values expressed as mean ± SD. [b] $P < 0.05$.

Davies (1976) based on the optical density at 480, 645, and 663 nm. The concentrations were determined as follows:

$$\text{chlorophyll A (mg/L)} = 12.7\text{abs}_{663} - 2.60\text{abs}_{645} \quad (2)$$

$$\text{chlorophyll B (mg/L)} = 22.9\text{abs}_{645} - 4.68\text{abs}_{663} \quad (3)$$

$$\text{total chlorophyll} = \text{chlorophyll A} + \text{chlorophyll B} \quad (4)$$

$$\text{carotenoids (mg/L)} = 20\text{abs}_{480} + 2.28\text{abs}_{663} - 12.76\text{abs}_{645} \quad (5)$$

After ethanol extraction, soluble sugars were determined according to Buysse and Merckx (1993), a modification of the Dubois method. After fructose digestion, sucrose was determined with the Boeringer Mannheim kit. Starch and cellulose were determined as soluble sugars after differential acid digestion as in Moya et al. (1993). Proteins were estimated by the Bradford method (1976) using bovine serum albumin as a standard. Total lipids were determined after Zöllner and Kirsch (1962), and the concentration was determined as absorbance at 530 nm with oleic and estearic acids as standards. Ca, Mg, Cu, Zn, Fe, and Mn concentrations were determined by AAS (Hitachi Z-8200) after wet nitric/hydrochloric acid (3:1) digestion. Potassium was determined by AES (Rubio et al., 1994).

Statistical analyses were done by the Student *t* test at a significance level set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Shoot and root growth from meristems is strongly affected by copper treatment with a 100% inhibition at 10 μg/g (Table 1). Reduction of leaf production and RRG is also observed. Plant growth has been traditionally estimated by parameters such as dry weight (DW), fresh weight (FW), and RRG, which have been used as indicators of metal exposure in plants (Punz and Sieghardt, 1993). The ratio DW/FW was increased in cases of heavy metal toxicity (Moya et al., 1993). However, we do not observe a change in that ratio (the ratios being 83% in leaves and 85% in roots) as in other cases of copper toxicity (Lidon et al., 1993). Although the ratio remains constant, there is a decrease in leaf production and RRG, thus indictaing that root growth is a sensitive parameter for metal exposure (Wilkins, 1978).

As copper exposure to 10 μg/g limited growth and development strongly and 2 μg/g had little or no effect, further copper treatments were carried out at concentrations of 5 μg/g after the plant was fully developed (4 weeks).

Differences due to grape variety are also evident (Table 2). Thus, Cabernet sauvignon cultivars, which are the most vigorous plants in the test tube, are the most affected by copper exposure with a dramatic decrease in total biomass, which is mostly due to a reduction in root growth and, to a lesser extent, shoot growth. In contrast, cultivars from Richter 110 rootstock or Sauvignon with similar growth are similarly affected by copper exposure. Reduction of leaf production and shoot growth is similar in the three varieties.

As seen above, root growth is very sensitive to metal exposure, and growth reduction has been attributed to inhibition of cell division (Eleftheriou and Karataglis, 1989), cell elongation, or both together (Arduini et al., 1994). However, a greater inhibition of cell elongation than cell division would produce higher root density, as reported by Arduini et al. (1994). Both processes are observed for these data, depending on the variety. Thus, in Sauvignon and R-110 cultivars, no significant differences in root density are observed, showing that both cell division and elongation are affected similarly. Nonetheless, in C. sauvignon, the root biomass decreases more than the root length, resulting in lower root density, which can be related to reduced cell division. Instead, in young meristems, evidence is provided (see Table 1) that cell elongation is completely affected by copper at concentrations lower than 10 μg/g as seen by the differences between root development and the root length, which was much more affected. At copper concentration of 10 μg/g, cell division ceases, as seen by the lack of root development.

However, addition of copper after root development also affects plant growth (Sauvignon), with chlorosis and

Table 3. Effects of Copper (5 μg/g) upon Leaf and Root Parameters in *V. vinifera* cv. Sauvignon after 2 Months of Exposure[a]

	leaf		root	
	control	Cu	control	Cu
total chlorophylls	2.5 ± 0.4	3.3 ± 0.1[b]		
chlorophyll A	1.9 ± 0.3	2.3 ± 0.2		
chlorophyll B	0.79 ± 0.12	1.06 ± 0.11		
carotenoids	2.5 ± 0.4	3.3 ± 0.1		
lipids	12.6 ± 1.5	17.7 ± 0.2[b]	10.3 ± 1.2	7.2 ± 1.5
sucrose	3.88 ± 0.89	3.84 ± 0.40	1.36 ± 0.40	1.07 ± 0.53
soluble sugars	21.3 ± 2.1	17.9 ± 1.6[b]	13.4 ± 1.1	7.6 ± 1.4[b]
starch	11.8 ± 2.8	9.6 ± 0.8	9.3 ± 1.5	10.9 ± 2.5
cellulose	11.3 ± 0.7	9.6 ± 1.6	8.2 ± 1.8	8.7 ± 1.3

[a] Data expressed in mg/g wet weight as mean ± SD of three determinations. [b] $P < 0.05$.

Table 4. Effects of Copper (5 μg/g) upon Leaf and Root Metal Concentrations after 2 Months of Exposure[a]

Leaf

	R-110		Cabernet sauvignon		Sauvignon	
	control	Cu	control	Cu	control	Cu
Cu	1.1 ± 0.2	15.6 ± 10.3[b]	1.3 ± 0.2	16.9 ± 1.2[b]	0.9 ± 0.1	19.4 ± 7.5[b]
Zn	10.0 ± 1.1	7.5 ± 4.1	12.2 ± 1.1	12.0 ± 1.3	7.9 ± 0.7	6.5 ± 0.6
Fe	46.3 ± 17.3	51.0 ± 35.7	89.3 ± 15.0	25.0 ± 3.2[b]	57.5 ± 1.6	33.9 ± 6.3[b]
Mn	77.0 ± 25.5	131.9 ± 52.3	172.7 ± 37.7	127.3 ± 18.4	46.8 ± 4.2	52.8 ± 17.2

Root

	R-110		Cabernet sauvignon		Sauvignon	
	control	Cu	control	Cu	control	Cu
Cu	17.8 ± 14.1	89.8 ± 16.2[b]	0.37 ± 0.19	77.1 ± 46.0[b]	0.87 ± 0.14	102.2 ± 39.6[b]
Zn	52.8 ± 47.2	72.3 ± 20.1	39.0 ± 11.6	53.3 ± 3.8	24.4 ± 9.1	20.5 ± 4.5
Fe	177.0 ± 87.3	318.4 ± 138.8	73.0 ± 24.7	200.9 ± 76.9[b]	233 ± 78	584 ± 175[b]
Mn	57.3 ± 32.9	56.4 ± 15.8	37.0 ± 6.4	52.8 ± 10.4	15.4 ± 4.5	20.1 ± 5.3

[a] Data expressed in μg/g wet weight as mean ± SD of three determinations. [b] $P < 0.05$.

death at 10 μg/g. In vitro grapevines are more sensitive to copper toxicty than other plants, with complete growth inhibition at 10 μg/g, levels at which other plants survived although the periods of exposure were shorter (15 days; Ouzounidou et al., 1992).

Total chlorophylls and carotenoids increased by 32%, total lipids by 40% in copper-exposed plants as compared to untreated plants, while soluble sugar levels (in leaves and roots) were lower than those of control plants (Table 3).

As the photosynthate was not increased, the increase in pigments can be related to a reduction of CO_2 fixation in response to metal exposure (Moya et al., 1993), through inhibition of RUBISCO activity (Stiborová et al., 1986) along with other metabolically related enzymes (De Filippis and Ziegler, 1993). Thus, the increase in photosynthetic pigments may balance the reduced photosynthesis, as has been observed in other plants (Ouzounidou et al., 1993). However, it has been suggested that the lack of sugar utilization for growth due to excessive heavy metals is stronger than the lack of CO_2 fixation, ultimately increasing storage glucans (Moya et al., 1993). Also, a decrease in water content could explain the observed increase in starch but was not observed. Nonetheless, it is also known that in vitro cultures take up sucrose directly from the medium, which can be similarly affected by copper, as has been reported in sugar beet by cadmium (Greger and Bertell, 1992).

Lipid synthesis has been reported to be affected by copper exposure (Lidon et al., 1993). In copper-sensitive plants, a reduction of unsaturated fatty acid synthesis has been observed, whereas in copper-tolerant plants, there was no change or even an increase in the rate of synthesis (Ric de Vos et al., 1993). It was pointed out that copper exposure could induce peroxidation of unsaturated fatty acids, although it was not clear if this was a cause or a result. The observed increase of lipids in *Vitis* as a result of copper exposure suggests it may be a mechanism of copper tolerance.

Regardless of the differences in metal concentrations among the three varieties, their changes after copper exposure are very similar (Table 4). As expected, copper levels increase in both leaves and roots, but to higher concentrations in roots, suggesting an immobilizing mechanism in this organ. Iron concentration significantly decreases in leaves and increases in roots (except in R-110). The reduction of iron in leaves of copper-exposed plants along with higher levels in roots indicates that the immobilizing mechanism for copper in roots can involve other metals, specifically Fe (Reboredo, 1994). It is known that roots can act as storage organs for trace elements (Punz and Sieghardt, 1993).

Sap flow is reduced by more than 60% after copper exposure (76.4 mg/(g h) in the control vs 28.6 mg/(g h) in copper-exposed plants). This reduction is not reversed by reducing agents such as mercaptoethanol (results not shown). As far as the translocation of essential elements is concerned, the concentrations are not significantly different from control plants (Table 5). However, due to the reduction of sap flow in the copper-treated plants, the overall translocation of the four trace elements analyzed is reduced significantly.

The reduction of Fe in leaves can be easily correlated with the reduction in sap flow. This reduction affects copper as well, resulting in increased root concentration, although the high level in roots ultimately produces the increase in leaf Cu concentration as observed.

Table 5. Effects of Copper (5 μg/g) upon Cu, Zn, Fe, and Mn in Sap Flow, Expressed as Concentration (μg/L) and Overall Translocation Rate (μg/(g h)) in *V. vinifera* cv. Sauvignon[a]

	Cu	Zn	Fe	Mn
		Control		
μg/L	340 ± 58	1167 ± 289	237 ± 79	960 ± 27
μg/(g h)	25.3 ± 4.4	76.4 ± 5.0	15.8 ± 6.9	72.3 ± 5.9
		Cu Exposed		
μg/L	290 ± 13	1030 ± 107	170 ± 47	1215 ± 195
μg/(g h)	10.6 ± 0.5[b]	36.6 ± 0.1[b]	6.2 ± 1.7[b]	44.4 ± 7.1[b]

[a] Expressed as mean ± SD of five determinations. [b] $P < 0.05$.

Table 6. Effects of Copper (5 μg/g) upon Ca, K, and Mg in Sap Flow, Expressed as Concentration (mg/L) and Overall Translocation (mg/(g h)) in *V. vinifera* cv. Sauvignon[a]

	Ca	K	Mg
		Control	
mg/L	58.8 ± 17.0	266.0 ± 57.0	39.0 ± 28.2
mg/(g h)	4.4 ± 1.3	20.3 ± 4.3	3.0 ± 2.1
		Cu Exposed	
mg/L	127.8 ± 13.7[b]	55.2 ± 28.9[b]	26.8 ± 11.2
mg/(g h)	3.8 ± 0.4	1.6 ± 0.9[b]	0.8 ± 0.3

[a] Expressed as mean ± SD of five determinations. [b] $P < 0.05$.

Determination of the major mineral nutrients (Mg, Ca, and K) shows significant differences for Ca and K, but not for Mg (Table 6). Although Ca concentration increases and K concentration decreases, due to the reduction in sap flow, the overall translocation is reduced. However, K translocation is reduced to less than 10% of the control levels. The lower concentration of K in sap can be explained by the effects of copper on the plasma membrane (Ric de Vos et al., 1989), which affects membrane ATPase activity (Mas et al., 1994) and thus the overall movement across it, which easily affects K regulation. This dramatic decrease of K translocation may have a role in overall water flow. Thus, regardless of the primary effect of copper in water transport systems, the reduced flow may also be affected by this reduction in K concentration. The reduction of both water flow and K translocation can reduce plant growth in response to copper exposure.

These results show that grapes can adapt to copper exposure by a physiological response that involves restriction of water transport, which may be maintained by reduced K translocation. The reduction of water flow correlates with the observed restriction in metabolic activity.

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