Evaluating wild grapevine tolerance to copper toxicity

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Abbreviations: A, net photosynthetic rate; BAP, 6-benzylaminopurine; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Ci, intercellular CO_2 concentration; C*x*+*c*, carotenoids; F_0, minimal fluorescence level in the dark-adapted state; F_m, maximal fluorescence level in the dark-adapted state; F_s, steady state fluorescence yield; F_v, variable fluorescence level in the dark-adapted state; F_v/F_m, maximum quantum efficiency of PSII photochemistry; ΦPSII, quantum efficiency of PSII; Gs, stomatal conductance; NAA, naphthaleneacetic acid; RGR, relative growth rate.

Abstract

We evaluate copper tolerance and accumulation in *Vitis vinifera* ssp. *sylvestris* in populations from a copper contaminated site and an uncontaminated site, and in the grapevine rootstock "41B", investigating the effects of copper (0-23 mmol l^{-1}) on growth, photosynthetic performance and mineral nutrient content. The highest Cu treatment induced nutrient imbalances and inhibited photosynthetic function, causing a drastic reduction in growth in the three study plants. Effective concentration was higher than 23 mmol l^{-1} Cu in the wild grapevines and around 9 mmol l^{-1} in the "41B" plants. The wild grapevine accessions studied controlled root Cu concentration more efficiently than is the case with the "41B" rootstock and must be considered Cu-tolerant. Wild grapevines from the Cu-contaminated site present certain physiological characteristics that make them relatively more suitable for exploitation in the genetic improvement of vines against conditions of excess Cu, compared to wild grapevine populations from uncontaminated sites.

Keywords: Copper; tolerance; toxicity; wild grapevine.

Introduction

Copper can be highly toxic to plants when present at concentrations only slightly higher than its optimal level (Marschner, 1999). The primary effects of Cu take place in roots; however, it may also interfere with many physiological processes in the leaves when present at toxic concentrations. Excess Cu reduces plant growth and mineral nutrient uptake and may alter membrane permeability, protein synthesis, photosynthetic and respiratory processes, enzyme activities, and chromatin structure (Sandman and Böger, 1980; Van Assche and Clijsters, 1990; Fernandes and Henriques, 1991; Madejón et al., 2009). In Cu-contaminated soils, plants cope with the potential metal stress in different ways. Some species adopt an exclusion strategy to avoid excessive uptake and transport of metal ions, while accumulators can accumulate large amounts of heavy metals in plant tissues, even in aerial parts (Kabata-Pendias and Pendias, 2001).

Since the end of the 19^{th} century, the long-term application of copper-based fungicides, which have been used intensively in Europe to control vine fungal diseases, and of other Cu compounds (such as $Cu(OH)_2$ and Cu_2O), have led to considerable accumulations of Cu, reaching toxic concentrations in some vineyard soils (Komárek et al., 2010). This has a negative influence on soil flora and fauna and on human health, and may lead to phytotoxicity, yield losses and decreased wine quality (Ninkov et al., 2012). The toxicity limits, accumulation patterns and tolerance mechanisms of the grapevine in response to Cu stress remain unclear and, to date, data regarding the toxic effects of Cu are available for only a few commercial grapevine varieties (e.g. Toselli et al., 2009; Juang et al., 2012; Miotto et al., 2014).

In a recent study, our group demonstrated that plants of *Vitis vinifera* ssp. *sylvestris* from a population located in a metal-polluted site exhibit high tolerance to Cu

stress (Cambrollé et al., 2013). These findings raised new questions, the answers to which could be essential for enhancing the adaptation of vines to conditions of excess Cu. The mechanisms that determine the relatively higher tolerance exhibited by this wild subspecies compared to commercial varieties of grapevine remain unknown, since a direct comparison has never been made under the same experimental conditions. Moreover, wild grapevine populations present considerable genetic polymorphism and wide variability (McGovern et al., 1996) and it is not known whether the higher degree of Cu tolerance reported by Cambrollé et al. (2013) could be explained by inter-population differences. The present study was therefore conducted in order to clarify these issues.

The specific objectives of the study were: (1) to evaluate differences in Cu uptake, accumulation and tolerance between wild grapevine plants from two populations, grown on heavy metal contaminated and uncontaminated areas, respectively, and a commercial rootstock of grapevine, through analysis of Cu concentrations in tissues and plant growth in a range of external Cu concentrations from 0 to 23 mmol l^{-1} Cu; (2) to comparatively determine the possible mechanisms of Cu tolerance in wild grapevine by examining the extent to which Cu levels determine plant performance in terms of effects on the photosynthetic apparatus (PSII photochemistry), gas exchange characteristics, photosynthetic pigments and concentrations of N, P, S, Ca, Mg and Fe within plant tissues.

Materials and Methods

Plant material and copper treatments

Vitis vinifera (L.) ssp. *sylvestris* (Gmelin) Hegi, the wild subspecies of *Vitis vinifera* L., is the only native Eurasian subspecies and represents a valuable genetic resource for cultivated grapevines (Negrul, 1938). Two natural populations from southern Spain were selected for study; one population from a Cu-contaminated site, located on the bank of the Agrio river in Seville province ("Agrio river" population; Cambrollé et al., 2013), and the other from a non-contaminated site, located on the banks of the Anzur river in Córdoba province ("14/Rute/1" population; Ocete et al., 2007). Plants of the grapevine rootstock "41B" (*Vitis vinifera* L. cv. Chasselas x *Vitis berlandieri* Planch.) were used for comparison with the two wild grapevine populations.

Plants were obtained by micropropagation of axillary buds from individuals of the three study plants described above according to López et al. (2004). The resulting plants were adapted according to Cantos et al. (1993), transferred to individual plastic pots (diameter 11 cm) filled with perlite and placed in a glasshouse with minimum-maximum temperatures of 21-25ºC, at 40-60% relative humidity and natural daylight (minimum and maximum light flux: 200 and 1000 μmol m^{-2} s^{-1}, respectively). Pots were carefully irrigated with 20% Hoagland's solution (Hoagland and Arnon, 1938), as required.

When the plantlets were around 30 cm in height, the pots were allocated to five different Cu concentration treatments: 0, 1, 2.5, 9 and 23 mmol l^{-1} Cu, applied in shallow trays within the same glasshouse (fifteen pots per tray and one tray per Cu treatment, for each study plant). Cu treatments were prepared by mixing the 20%

Hoagland's solution with $CuSO_4 \cdot 5H_2O$ at the appropriate concentration. The control, 0 mmol l^{-1} Cu treatment, in fact contained 0.0005 mmol l^{-1} of Cu, since Hoagland's solution contains a small amount of Cu as an essential trace nutrient.

At the beginning of the experiment, 3 L of the appropriate solution were placed in each of the trays to a marked depth of 1 cm. Throughout the experiment, solution levels in the trays were monitored and topped up to the marked level with 20% Hoagland's solution, (with no additional $CuSO_4 \cdot 5H_2O$) in order to limit changes in Cu concentration due to evaporation of the water in the nutrient solution. In addition, the entire solution (including $CuSO_4 \cdot 5H_2O$) was changed on a weekly basis.

Growth

From each treatment, three complete plants (roots and shoots) were harvested at the beginning, and the remaining twelve at the end of the experiment (i.e. following 30 days of treatment). These plants were dried at 80ºC for 48 h and then weighed.

Relative growth rate (RGR) of whole plants was calculated using the formula:

$$RGR = (\ln Bf - \ln Bi) \cdot D^{-1} \quad (g\ g^{-1} day^{-1})$$

where Bf = final dry mass, Bi = initial dry mass (average of the three plants from each treatment dried at the beginning of the experiment) and D = duration of experiment (days).

Plant height was measured from the base of the stem to the tip of the uppermost leaf.

Mineral analysis

At the end of the experimental period, leaf samples were carefully washed with distilled water and then dried at 80ºC for 48 h and ground. Samples of 0.5 g each were then digested by wet oxidation with concentrated HNO_3, under pressure in a microwave oven to obtain the extract. Concentrations of Cu, P, S, Ca, Mg and Fe in the extracts were determined by optical spectroscopy inductively coupled plasma (ICP-OES) (ARL-Fison 3410, USA). Total N concentration was determined by Kjeldahl digestion using an elemental analyzer (Leco CHNS-932, Spain).

Gas exchange

After 30 days of treatment, gas exchange measurements were taken from randomly selected, fully expanded leaves (for each study plant and copper treatment, n = 20, i.e. one measurement per replicate plant, plus eight extra measurements taken randomly) using an infrared gas analyzer in an open system (LI-6400, LI-COR Inc., Neb., USA). Net photosynthetic rate (A), intercellular CO_2 concentration (C_i) and stomatal conductance to CO_2 (G_s) were determined at an ambient CO_2 concentration of 400 µmol mol^{-1} at 20 - 25ºC, 50 ± 5% relative humidity and a photon flux density of 1600 µmol m^{-2} s^{-1}. Values of the parameters A, C_i and G_s were calculated using the standard formulae of Von Caemmerer and Farquhar (1981).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured in randomly selected, fully developed leaves (n = 20) using a portable modulated fluorimeter (FMS-2, Hansatech Instruments Ltd., England) following 30 days of treatment. Light- and dark-adapted fluorescence parameters were measured at dawn (stable, 50 µmol m^{-2} s^{-1} ambient light) and midday (1600 µmol m^{-2} s^{-1}) in order to investigate the effect of Cu concentration on the sensitivity of study plants to photoinhibition. Values of variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry (F_v/F_m) were calculated from F_0 and F_m. In addition, using fluorescence parameters determined in both light- and dark-adapted states, quantum efficiency of PSII ($\Phi_{PSII} = (F_m' - F_s)/ F_m'$) was calculated. This parameter measures the proportion of light absorbed by the chlorophyll associated with PSII that is used in photochemistry (Maxwell and Johnson, 2000).

Photosynthetic pigments

At the end of the experimental period, photosynthetic pigments were extracted from fully expanded leaves of plants grown under each treatment (n = 12) by using the methods described in Cambrollé et al. (2013). Pigment concentrations (µg g^{-1} fwt) were calculated following the method of Lichtenthaler (1987).

Statistical analysis

Statistical analysis was carried out using Statistica v. 6.0 (Statsoft Inc.). Pearson coefficients were calculated to assess the correlation between different variables. Data

were analyzed using one- and two-way analyses of variance (*F*-test). Data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variance with the Brown-Forsythe test. Tukey tests were applied to significant test results for identification of important contrasts. Measured differences between fluorescence at dawn and midday were compared using the Student test (*t*-test).

Results

Growth

In the case of grapevine rootstock "41B", relative growth rate (RGR) was drastically affected by external Cu concentrations of 9 mmol l^{-1} and showed no clear response to further increases in external Cu content; relative to the control, reduction in RGR in the 9 and 23 mmol l^{-1} Cu treatments was around 50%. In comparison, the RGR of the "Agrio river" and "14/Rute/1" wild grapevine populations presented no significant difference with increasing Cu concentrations up to 9 mmol l^{-1} (ANOVA, Tukey test, $p > 0.05$, in both cases), but then declined substantially on exposure to the highest Cu level, with these values being significantly lower than those of the control treatment (ANOVA, Tukey test, $p < 0.05$, in both cases; Fig. 1A). Plant height showed a similar pattern to that of RGR (Fig. 1B). Plants from the "Agrio river" and "14/Rute/1" wild populations treated with 23 mmol l^{-1} Cu exhibited chlorosis from around the third week of treatment; in the case of the "41B" plants, leaf chlorosis was detected early in plants exposed to external Cu levels from 9 mmol l^{-1}.

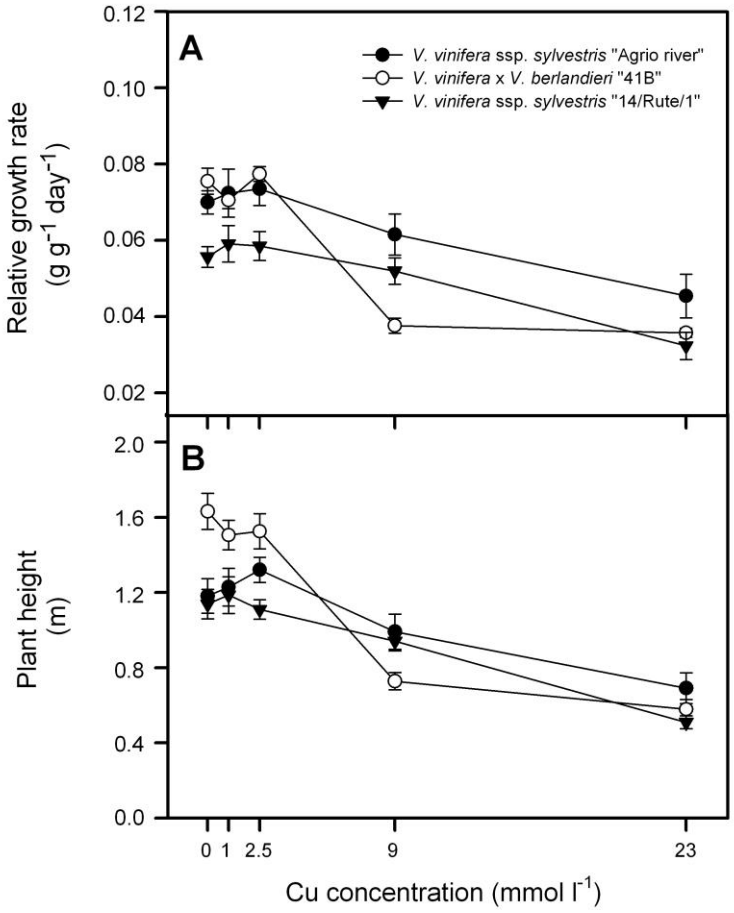

A
B
V. vinifera ssp. sylvestris "Agrio river"
V. vinifera x V. berlandieri "41B"
V. vinifera ssp. sylvestris "14/Rute/1"
Relative growth rate (g g-1 day-1)
Plant height (m)
0.12
0.10
0.08
0.06
0.04
0.02
2.0
1.6
1.2
0.8
0.4
0.0
0 1 2.5 9 23
Cu concentration (mmol l-1)

Figure 1

Chemical analysis of plant samples

Leaf copper concentration increased significantly with external Cu level ($r = 0.98$, $p < 0.0001$; $r = 0.99$, $p < 0.0001$; $r = 0.98$, $p < 0.0001$, for "Agrio river", "41B" and "14/Rute/1" plants, respectively; Table 1). Under exposure to 9 and 23 mmol l^{-1} Cu, leaf Cu concentration was significantly higher in plants of the "41B" rootstock than in the "Agrio river" and "14/Rute/1" wild grapevine plants (ANOVA, Tukey test, $p < 0.01$, in both cases). There was a significantly increasing trend in root Cu concentration with increasing external Cu level ($r = 0.99$, $p < 0.0001$; $r = 0.98$, $p < 0.0001$; $r = 0.94$, $p < 0.0001$, for "Agrio river", "41B" and "14/Rute/1" plants, respectively; Table 1). At 9 and 23 mmol l^{-1} Cu, wild grapevine plants from the "Agrio river" and "14/Rute/1" populations presented significantly lower values of root Cu than plants of "41B" rootstock (ANOVA, Tukey test, $p < 0.0005$, in both cases; Table 1). Furthermore, at 1, 2.5 and 9 mmol l^{-1} Cu, the "Agrio river" plants presented significantly lower values of root Cu concentration than those of "14/Rute/1" plants (ANOVA, Tukey test, $p < 0.0005$, in all cases).

Table 1. Total copper concentrations in the leaves and roots of plants of *V. vinifera* x *V. berlandieri* "41B", *V. vinifera* ssp. *sylvestris* from "Agrio river" population and *V. vinifera* ssp. *sylvestris* from "14/Rute/1" population, in response to treatment with a range of external Cu concentrations for 30 days. Values represent mean ± SE, n = 3.

		Copper treatment				
		0 mmol l^{-1}	1 mmol l^{-1}	2.5 mmol l^{-1}	9 mmol l^{-1}	23 mmol l^{-1}
Leaf Cu concentration (mg g^{-1})	"41B"	0.006 ± 0.0007	0.013 ± 0.0000	0.016 ± 0.0002	0.075 ± 0.0029	0.141 ± 0.0065
	"Agrio river"	0.010 ± 0.0010	0.014 ± 0.0012	0.017 ± 0.0007	0.032 ± .00012	0.083 ± 0.0060
	"14/Rute/1"	0.016 ± 0.0012	0.012 ± 0.0022	0.021 ± 0.0005	0.036 ± 0.0014	0.101 ± 0.0064
Root Cu concentration (mg g^{-1})	"41B"	0.023 ± 0.0037	0.310 ± 0.0220	0.461 ± 0.0321	4.554 ± 0.4997	22.515 ± 0.8028
	"Agrio river"	0.021 ± 0.0027	0.087 ± 0.0061	0.253 ± 0.0009	0.459 ± 0.0047	1.333 ± 0.0140
	"14/Rute/1"	0.012 ± 0.0008	0.298 ± 0.0052	0.392 ± 0.0247	0.929 ± 0.0714	1.372 ± 0.0578

291 Leaf N concentration of both "Agrio river" and "14/Rute/1" wild grapevine
292 populations showed little variation with external Cu concentration up to 9 mmol l^{-1}, but
293 decreased under exposure to 23 mmol l^{-1} Cu, reaching values that were significantly
294 lower than those of the control treatment (ANOVA, Tukey test, $p < 0.05$, in both cases).
295 In the case of "41B" rootstock, N concentration significantly declined at 9 mmol l^{-1} Cu
296 and did not respond to further increases in external Cu content (Fig. 2A).
297 Leaf P concentration in "Agrio river" wild grapevine plants was virtually
298 unaffected up to the 9 mmol l^{-1} Cu treatment but then decreased significantly at 23
299 mmol l^{-1} Cu, whereas in the case of the "14/Rute/1" and "41B" plants, leaf P
300 significantly decreased with external Cu level ($r = -0.70$, $p < 0.005$; $r = 0.83$, $p < 0.0005$,
301 for "14/Rute/1" and "41B" plants, respectively; Fig. 2B). Leaf S concentration was
302 significantly lower in "41B" plants than in "Agrio river" and "14/Rute/1" wild grapevine
303 plants (two-way ANOVA, $p < 0.001$). In the "Agrio river" and "14/Rute/1" plants, there
304 was no significant effect of external Cu on leaf S concentration up to the 9 mmol l^{-1}
305 treatment, but a significant reduction was detected under exposure to 23 mmol l^{-1} Cu
306 (ANOVA, Tukey test, $p < 0.05$, in both cases); in contrast, leaf S concentration was
307 drastically reduced in the "41B" plants at 9 and 23 mmol l^{-1} Cu (Fig. 2C).
308 In both "Agrio river" and "14/Rute/1" wild grapevine plants, leaf Fe showed no
309 clear relationship with external Cu concentration (ANOVA, Tukey test, $p > 0.05$, in
310 both cases), whereas in the "41B" grapevine rootstock, there was a drastic reduction in
311 leaf Fe concentration at 9 and 23 mmol l^{-1} Cu (Fig. 2D). On the other hand, while leaf
312 Ca and Mg declined with increasing external Cu concentration in the "41B" grapevine
313 rootstock ($r = -0.89$, $p < 0.0001$; $r = -0.73$, $p < 0.005$, for Ca and Mg, respectively), Ca
314 and Mg concentrations in "Agrio river" and "14/Rute/1" plants were only significantly

decreased on exposure to 23 mmol l^{-1} Cu (ANOVA, Tukey test, $p < 0.05$, in all cases; Fig. 2E and F).

Under exposure to 9 mmol l^{-1} Cu, concentrations of leaf N, P, S, Ca and Mg were significantly lower in plants of the "41B" rootstock than in the "Agrio river" and "14/Rute/1" wild grapevine plants (ANOVA, Tukey test, $p < 0.005$, in all cases).

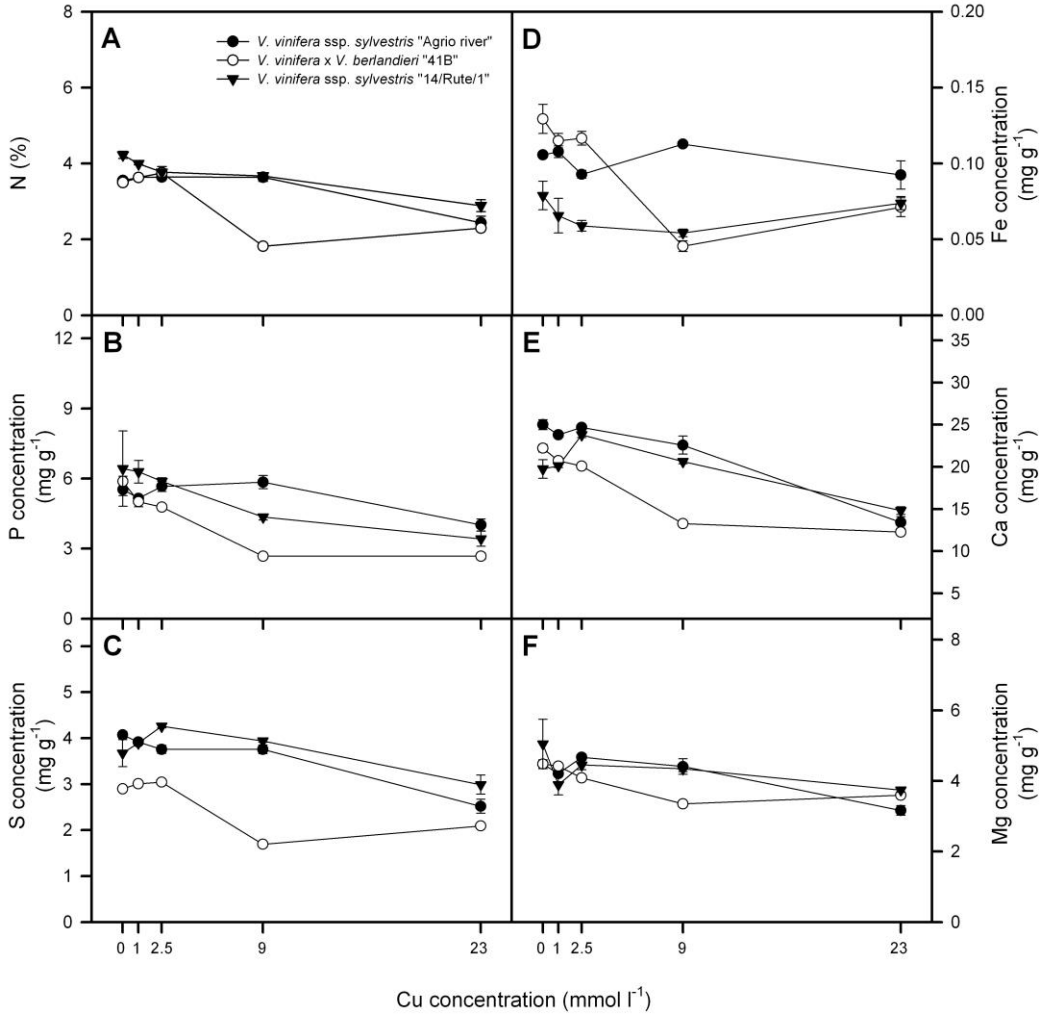


Figure 2

Gas exchange

Net photosynthesis rate (A) decreased significantly with increasing external Cu level ($r = -0.90$, $p < 0.001$; $r = -0.87$, $p < 0.0001$; $r = -0.82$, $p < 0.0001$, for "Agrio river", "41B" and "14/Rute/1" plants, respectively). Under exposure to 9 and 23 mmol l^{-1} Cu in the nutrient solution, values of A in wild grapevine plants from the "Agrio river" and "14/Rute/1" populations were significantly higher than those obtained in the "41B" plants (ANOVA, Tukey test, $p < 0.0005$, in both cases). Relative to the control, the reduction of A in the 9 mmol l^{-1} Cu treatment was around 42% in "Agrio river" and "14/Rute/1" wild grapevine plants, and around 88% in "41B" plants (Fig. 3A).

In all three cases, stomatal conductance (Gs) was strongly correlated with A ($r = 0.89$, $p < 0.001$; $r = 0.96$, $p < 0.001$; $r = 0.70$, $p < 0.0001$, for "Agrio river", "41B" and "14/Rute/1" plants, respectively; Fig. 3B). Intercellular CO_2 concentration (Ci) in the "Agrio river" and "14/Rute/1" plants showed no significant variations up to the 9 mmol l^{-1} Cu treatment, but an increase was observed at the highest external Cu level. In contrast, external Cu concentrations greater than 2.5 mmol l^{-1} caused a marked increase in the Ci of the "41B" plants (Fig. 3C).

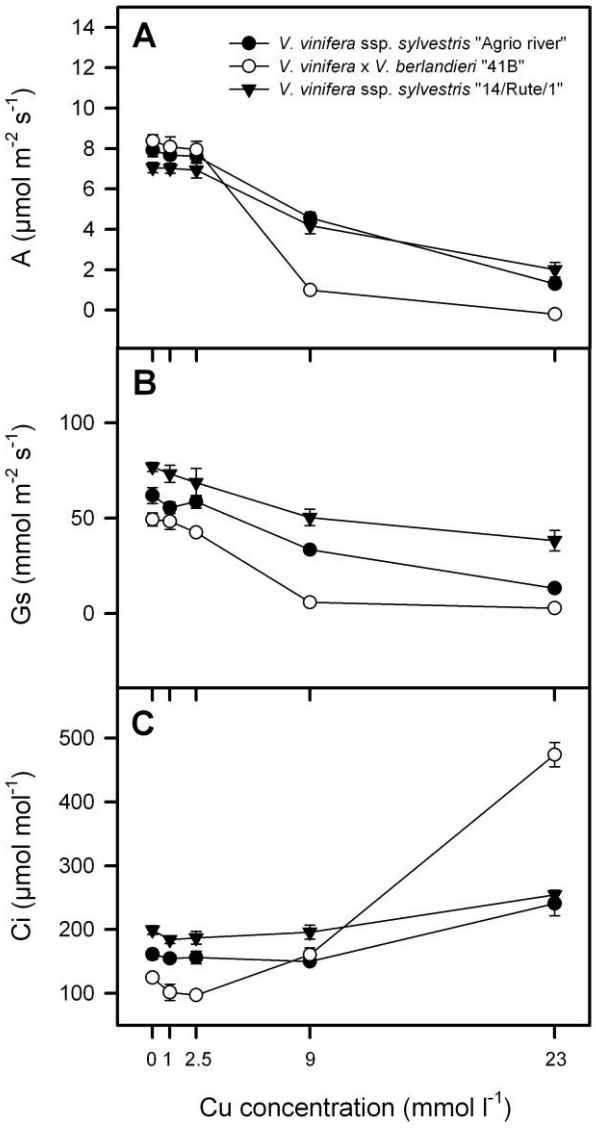

A
B
C
V. vinifera ssp. sylvestris "Agrio river"
V. vinifera x V. berlandieri "41B"
V. vinifera ssp. sylvestris "14/Rute/1"
A (µmol m-2 s-1)
Gs (mmol m-2 s-1)
Ci (µmol mol-1)
0 1 2.5 9 23
Cu concentration (mmol l-1)

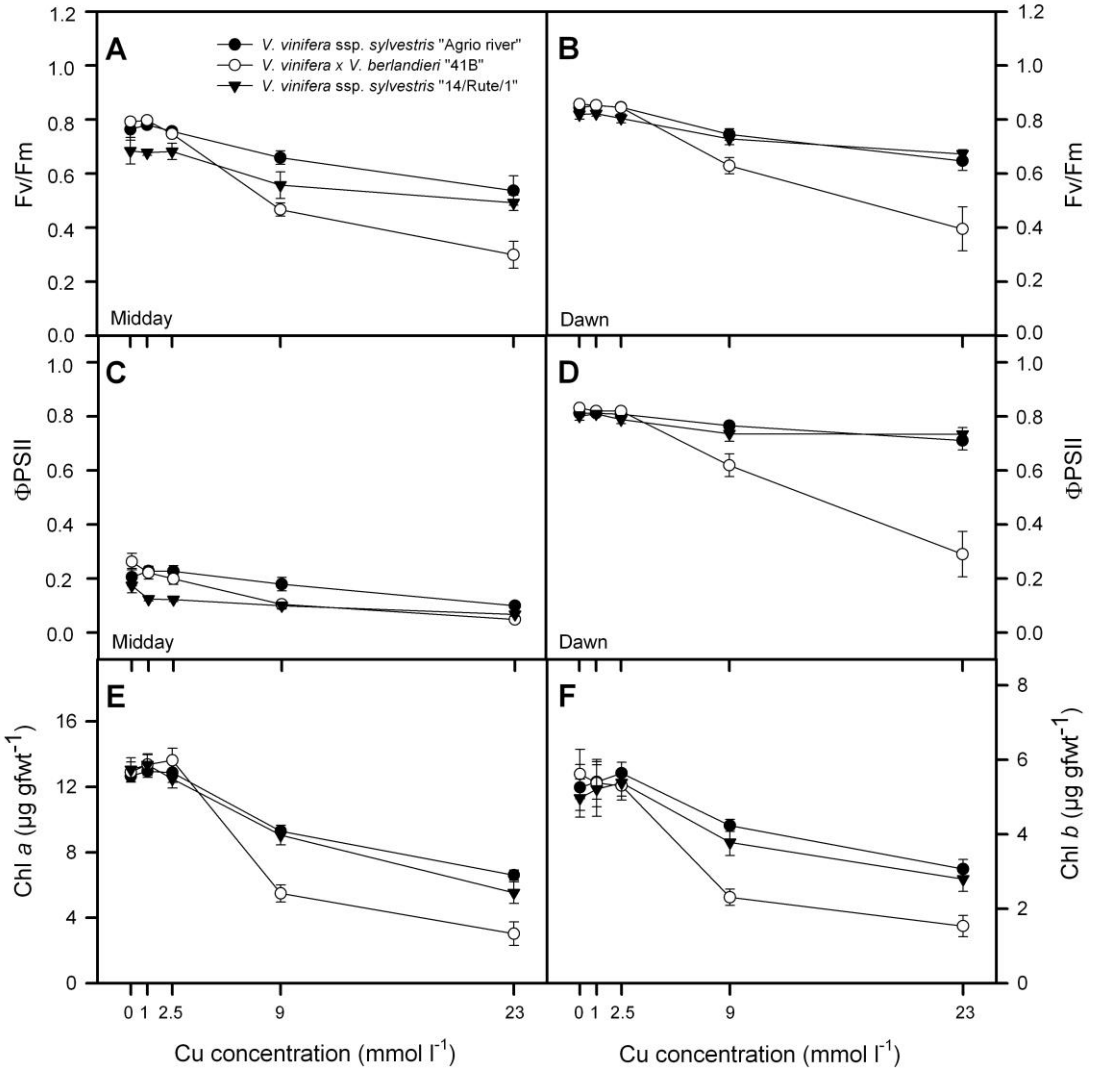
Figure 3

Chlorophyll fluorescence and photosynthetic pigments

Values of maximum quantum efficiency of PSII (F_v/F_m) were lower at midday than at dawn (*t*-test, $p < 0.01$, in all cases). In the "Agrio river" and "14/Rute/1" wild grapevine plants, F_v/F_m, both at dawn and midday, decreased at external Cu concentrations from 9 mmol l^{-1}, but differences relative to the control were only significant at the highest Cu level (ANOVA, Tukey test, $p < 0.05$, in all cases). In comparison, there was a significant decrease in F_v/F_m at Cu concentrations above 2.5 mmol l^{-1} in the "41B" plants (Fig. 4A and B). There were no significant differences between the dawn F_v/F_m values of the "Agrio river" and "14/Rute/1" plants (two-way ANOVA, $p > 0.05$). Furthermore, in the 9 and 23 mmol l^{-1} Cu treatments, the dawn F_v/F_m of the "41B" plants was significantly lower than in both the "Agrio river" and "14/Rute/1" plants (ANOVA, Tukey test, $p < 0.005$, in both cases), with values varying around 0.60 and 0.40, for the 9 and 23 mmol l^{-1} Cu treatments, respectively (Fig. 4B).

Quantum efficiency of PSII (ΦPSII) was significantly lower at midday than at dawn (*t*-test, $p < 0.001$, in all cases). At both dawn and midday, ΦPSII showed a similar pattern to that of F_v/F_m in all three cases, with minimum values at 23 mmol l^{-1} Cu, and a more pronounced decline in ΦPSII values of "41B" plants at 9 mmol l^{-1} Cu (Fig. 4C and D).

In all three cases, pigment concentrations significantly decreased on exposure to external Cu concentrations from 9 mmol l^{-1} (ANOVA, Tukey test, $p < 0.05$, in all cases) but this decrease was considerably more pronounced in the "41B" plants (Fig. 4E and F).



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384 Figure 4

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Discussion

Vitis vinifera ssp. *sylvestris* from both studied populations were shown to be more tolerant to Cu stress than the "41B" grapevine rootstock. The effective concentration of wild grapevine plants (EC50, substrate copper concentration resulting in 50% biomass reduction; Paschke et al., 2000) was higher than 23 mmol l^{-1} Cu (approx. 1500 mg Cu kg^{-1}). In contrast, in the "41B" grapevine rootstock, Cu toxicity symptoms such as growth inhibition were absent at concentrations of up to 2.5 mmol l^{-1} and the EC50 value was around 9 mmol l^{-1}. Toselli et al. (2009) reported that the Cu toxicity threshold in *Vitis vinifera* cv Sangiovese, growing in sand-enriched soil, can be established at 200 mg kg^{-1} external Cu.

A common strategy of metal tolerance is to avoid excessive uptake and transport of metal ions (Kabata-Pendias and Pendias, 2001). It is well known that root exudation of metal-chelating substances can reduce the uptake, and thus the effective toxicity, of metals (Pal and Rai, 2010). The bioaccumulation factor (BF), which is defined as the ratio of the metal concentration in the plant tissue to that in the soil (Sun et al., 2007), is highly species-specific. In our study, the root BF values at 9 and 23 mmol l^{-1} Cu in the "41B" grapevine rootstock were 7.5 and 14.7, implying 7- and 16-fold higher values than in the wild grapevine plants, respectively. These results indicate that, under exposure to elevated external Cu levels, the roots of wild grapevine plants exhibit a stronger capability for actively avoiding Cu uptake from the nutrient solution, and/or excluding Cu from roots, than is the case in the "41B" grapevine rootstock. Moreover, it is interesting to note that, at external Cu levels from 1 to 9 mmol l^{-1} Cu, the root BF values of "Agrio river" plants were around half that of the "14/Rute/1" plants, indicating that the mechanisms of reduced uptake and/or Cu exclusion are more efficient in the

418 wild grapevine plants of the "Agrio river" population. Efficient exclusion of Cu from
419 whole roots has been found in Cu-tolerant ecotypes of different other species (e.g.
420 Llugany et al., 2003; Ke et al., 2007).

421 Previous studies regarding Cu distribution in grapevine indicate that the roots of
422 this species effectively restrict Cu transport into the shoots (e.g. Juang et al., 2012;
423 Cambrollé et al., 2013). Our study shows that the "41B" plants could only limit Cu
424 transport into the shoots effectively up to an external concentration of 2.5 mmol l^{-1} Cu,
425 since leaf metal levels increased considerably when external Cu exceeded this
426 concentration, in all probability as a consequence of the extremely high Cu content in
427 root tissues of plants exposed to 9 and 23 mmol Cu l^{-1}. The previously described lower
428 root Cu concentrations detected in the wild grapevine plants at the highest Cu
429 treatments also implied considerably lower leaf Cu contents than those detected in
430 "41B" rootstock plants.

431 In our experiment, the reduced growth recorded in plants exposed to the highest
432 external Cu concentrations can be attributed to the reduction in photosynthetic carbon
433 assimilation. The photosynthetic process was affected by external Cu concentrations
434 above 2.5 mmol l^{-1} in all "41B" rootstock plants and wild grapevines from both
435 populations. The highest external Cu levels induced considerable effects on net
436 photosynthesis rate (A) and stomatal conductance (Gs), with no direct relationship
437 between both parameters since there was no reduction in intercellular CO_2 concentration
438 (Ci). These results suggest that the reduction of A could be ascribed to the different
439 effects of Cu on the integrity or function of the photochemical apparatus, as well as to
440 its impact on chlorophyll concentrations in the leaves. Moreover, our fluorescence
441 analysis showed that the reduction in photosynthetic activity at the highest Cu treatment
442 could be partially due to the effects of the metal on the photosynthetic apparatus. In all

443 plants, the maximum quantum efficiency of PSII (F_v/F_m) and the quantum efficiency of
444 PSII (ΦPSII) were considerably affected by external Cu concentrations of 23 mmol l^{-1},
445 suggesting that an excess of Cu enhances the photoinhibition induced by light stress.
446 Moreover, midday values of F_v/F_m at the highest external Cu concentration did not
447 recover at dawn and, in fact, remained lower than the control parameters for unstressed
448 plants (Björkman and Demmig, 1987), indicating the occurrence of chronic
449 photoinhibition or photodamage. The reported decline in F_v/F_m in plants exposed to 23
450 mmol l^{-1} was possibly due to the recorded decrease in the concentration of chlorophyll.
451 It should be highlighted that the deleterious effects of Cu on photosynthetic
452 function were more severe in the "41B" grapevine rootstock than in the wild grapevine
453 plants, and this difference between study plants was especially marked at 9 mmol l^{-1} Cu.
454 Moreover, at this external Cu level, the reduction in net photosynthesis rate and pigment
455 concentration of the "Agrio river" and "14/Rute/1" wild grapevines was around half that
456 of the "41B" plants, and dawn F_v/F_m values in these plants remained around the optimal
457 values for unstressed plants. It should be noted that, in contrast to the "41B" plants, the
458 negative effects on the photosynthetic function of both populations of wild grapevine
459 plants at 9 mmol l^{-1} external Cu did not lead to a reduction of plant growth. Integration
460 of these results indicates that the wild grapevines exposed to this external Cu level
461 experienced little overall effects on photosynthetic function over most of the
462 experimental period.
463 Heavy metals may interfere with essential nutrient uptake and transport and
464 thereby disturb the mineral nutrition composition of plants (Kabata-Pendias and
465 Pendias, 2001; Ke et al., 2007). In our experiment, the "41B" grapevine rootstock
466 suffered considerable reductions in the concentration of all the analyzed nutrients at
467 external Cu levels from 9 mmol l^{-1}, whereas plants from the "Agrio river" and

"14/Rute/1" wild grapevine populations demonstrated a more efficient control of their nutrient status under Cu stress. Previous studies indicate that some mineral elements are less affected or even unaffected by Cu and other heavy metals in tolerant populations and/or species (Ali et al., 2002; Ke et al., 2007).

Our results show that the effects of external Cu on leaf N, S, Ca and Mg concentrations were similar in plants from both wild grapevine populations, with significant reductions observed only at the highest external Cu level (23 mmol l^{-1}). Although Cu-Fe antagonism is common in plants grown under conditions of Cu toxicity (Foy et al., 1978; Wallace and Cha, 1989), the leaf Fe concentration in both wild grapevine plants was virtually unaltered, even at the highest external Cu concentration. A synergistic effect of Cu and Fe has been described by Kitagishi and Yamane (1981) in rice seedlings and by Lanaras et al. (1993) in wheat plants. It is interesting to note that plants from the "Agrio river" population seem to be more efficient at controlling P uptake under Cu stress than the "14/Rute/1" plants, which presented a considerable reduction in leaf P content at external Cu concentrations from 9 mmol l^{-1}. Ke et al. (2007) reported a positive correlation between Cu tolerance and P accumulation in *Rumex japonicus*, suggesting that P could play an important role in controlling Cu accumulation and transport. Moreover, other studies have shown that P plays an important role in governing Cu accumulation in *Phaseolus vulgaris* (Wallace and Cha, 1989) and *Brassica pekinensis* (Xiong et al., 2002).

Copper concentrations of between 20 and 100 mg Cu kg^{-1} DW in mature leaf tissue are considered excessive or toxic (Kabata-Pendias and Pendias, 2001). Summarizing our results, we can state that despite the capacity of root tissues of the three study plants to retain large quantities of Cu, the highest external Cu level tested (23 mmol l^{-1}) caused excessive Cu concentrations in aerial tissues, which induced

493 nutrient imbalances and significantly inhibited photosynthetic function, thus causing an
494 overall reduction in carbon gain and consequently reduced plant growth. In contrast, the
495 physiological response of the wild grapevines and "41B" rootstock was different under
496 exposure to 9 mmol Cu l^{-1}: At this external Cu level, roots of both the "Agrio river" and
497 "14/Rute/1" wild grapevine plants were shown to be more efficient in controlling Cu
498 uptake and/or excluding the metal, than was the case with the "41B" plants. This
499 explains why leaf metal levels were considerably higher in "41B" plants, leading to
500 more severe physiological effects that resulted in considerable reduction of growth.
501
502 **Conclusions**
503 In comparison to the "41B" grapevine rootstock, the studied accessions of *Vitis*
504 *vinifera* ssp. *sylvestris* are considerably more efficient in controlling root Cu
505 concentration on exposure to extremely high external Cu contents. Heavy metal
506 contamination of soils or sediments can constitute a powerful selective force in plant
507 evolution (Antonovics et al., 1971; Ye et al., 2003); specific mechanisms may have
508 developed in resistant populations through natural selection in response to heavy metal
509 contamination of the soils (Liu et al., 2004). By comparing the physiological response
510 of wild grapevine plants from both studied populations, we detected that the plants from
511 the metal contaminated site ("Agrio river" population) seem to present a stronger
512 physiological capacity for controlling root Cu content and their mineral composition is
513 certainly less affected by Cu stress than in the "14/Rute/1" plants. We conclude that
514 wild grapevine can be considered a Cu-tolerant subspecies of *Vitis vinifera*, and plants
515 from the "Agrio river" population could represent a valuable genetic resource to exploit
516 in the improvement of Cu tolerance in grapevine compared to other wild grapevine
517 populations.

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Fig 1. Relative growth rate (A) and plant height (B) in plants of *V. vinifera* x *V. berlandieri* "41B" (○), *V. vinifera* ssp. *sylvestris* from the "Agrio river" population (●) and *V. vinifera* ssp. *sylvestris* from the "14/Rute/1" population (∇), in response to treatment with a range of external Cu concentrations for 30 days. Values represent the mean ± SE, n = 12.

Fig 2. Total nitrogen (A), phosphorus (B), sulphur (C), iron (D), calcium (E) and magnesium (F) concentrations in the leaves of plants of *V. vinifera* x *V. berlandieri* "41B" (○), *V. vinifera* ssp. *sylvestris* from the "Agrio river" population (●) and *V. vinifera* ssp. *sylvestris* from the "14/Rute/1" population (∇), in response to treatment with a range of external Cu concentrations for 30 days. Values represent the mean ± SE, n = 3.

Fig 3. Net photosynthetic rate, A (A), stomatal conductance, G_s (B), and intercellular CO_2 concentration, C_i (C) in randomly selected, fully developed leaves of plants of *V. vinifera* x *V. berlandieri* rootstock "41B" (○), *V. vinifera* ssp. *sylvestris* from the "Agrio river" population (●) and *V. vinifera* ssp. *sylvestris* from the "14/Rute/1" population (∇), in response to treatment with a range of external Cu concentrations for 30 days. Values represent the mean ± SE, n = 20.

Fig 4. Maximum quantum efficiency of PSII photochemistry, F_v/F_m, and quantum efficiency of PSII, ΦPSII, at midday (A, C) and at dawn (B, D), and Chlorophyll *a* (chl *a*) (E) and Chlorophyll *b* (chl *b*) (F) in randomly selected, fully developed leaves of *V. vinifera* x *V. berlandieri* rootstock "41B" (○), *V. vinifera* ssp. *sylvestris* from the "Agrio

685 river" population (●) and *V. vinifera* ssp. *sylvestris* from the "14/Rute/1" population
686 (∇), in response to treatment with a range of external Cu concentrations for 30 days.
687 Values represent the mean ± SE, n = 20, for F_v/F_m and ΦPSII, and n = 12, for chl *a* and
688 chl *b*.
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