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Evaluating wild grapevine tolerance to copper toxicity

J. Cambrollé [b,*], J.L. García [a], M.E. Figueroa [b], M. Cantos [a]

[a] Instituto de Recursos Naturales y Agrobiología de Sevilla (C.S.I.C.), P.O. Box 1052, 41080 Sevilla, Spain
[b] Facultad de Biología, Universidad de Sevilla, P.O. Box 1095, 41080 Sevilla, Spain

HIGHLIGHTS

- We investigate Cu tolerance and accumulation in *Vitis vinifera* ssp. *sylvestris*.
- Effective concentration was higher in wild grapevine than in 41B rootstock.
- Wild grapevine can be considered a Cu-tolerant subspecies of *Vitis vinifera*.
- Plants from the contaminated site are more efficient in controlling root Cu content.

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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 mM) 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 mM Cu in the wild grapevines and around 9 mM 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.

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1. 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 (Sandmann 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 19th 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

Abbreviations: A, net photosynthetic rate; BAP, 6-benzylaminopurine; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; C_i, intercellular CO_2 concentration; $Cx+c$, 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; Φ_{PSII}, quantum efficiency of PSII; G_s, stomatal conductance; NAA, naphthaleneacetic acid; RGR, relative growth rate.

* Corresponding author. Address: Dpto. Biología Vegetal y Ecología, Facultad de Biología, Universidad de Sevilla, Av. Reina Mercedes 6, 41012 Seville, Spain. Tel.: +34 95 4557165.
E-mail address: cambrolle@us.es (J. Cambrollé).

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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 mM 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.

2. Materials and methods

2.1. 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 × *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 21–25 °C, 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 mM 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 mM Cu treatment, in fact contained 0.0005 mM 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.

2.2. 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 d of treatment). These plants were dried at 80 °C for 48 h and then weighed.

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

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

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.

2.3. Mineral analysis

At the end of the experimental period, leaf samples were carefully washed with distilled water and then dried at 80 °C 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).

2.4. Gas exchange

After 30 d 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 20–25 °C, 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).

2.5. 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 d 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

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