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Growth and photosynthetic responses to copper in wild grapevine

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HIGHLIGHTS

- We evaluate physiological responses to Cu in *Vitis vinifera* ssp. *sylvestris*.
- *V. vinifera* ssp. *sylvestris* can survive external Cu levels of up to 23 mmol L^{-1}.
- Growth of *V. vinifera* ssp. *sylvestris* is unaffected by up to 35 mg kg^{-1} of foliar Cu.
- This subspecies is more tolerant to Cu than cultivated varieties of grapevine.

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ABSTRACT

The present study evaluates the tolerance and accumulation potential of *Vitis vinifera* ssp. *sylvestris* under moderate and high external Cu levels. A greenhouse experiment was conducted in order to investigate the effects of a range of external Cu concentrations (0–23 mmol L^{-1}) on growth and photosynthetic performance by measuring gas exchange, chlorophyll fluorescence parameters and photosynthetic pigments. We also measured the total copper, nitrogen, phosphorus, sulphur, calcium, magnesium, iron, potassium and sodium concentrations in the plant tissues. All the experimental plants survived even with external Cu concentrations as high as 23 mmol L^{-1} (1500 mg Cu L^{-1}), although the excess of metal resulted in a biomass reduction of 35%. The effects of Cu on growth were linked to a reduction in net photosynthesis, which may be related to the effect of the high concentration of the metal on photosynthetic electron transport. *V. vinifera* ssp. *sylvestris* survived with leaf Cu concentrations as high as 80 mg kg^{-1} DW and growth parameters were unaffected by leaf tissue concentrations of 35 mg Cu kg^{-1} DW. The results of our study indicate that plants of *V. vinifera* ssp. *sylvestris* from the studied population are more tolerant to Cu than the commercial varieties of grapevine that have been studied in the literature, and could constitute a basis for the genetic improvement of Cu tolerance in grapevine.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most widely cultivated and economically important fruit crops in the world (De Andrés et al., 2012; Mattia et al., 2008). One major challenge for viniculture in this century will be the maintenance of a sustainable production of high quality grapes in a changing environment (Martínez-Zapater et al., 2010). In this regard, future viniculture must consider, among other relevant issues such as genetic erosion, the changing physical and chemical environment of the vineyard. Increased organic and inorganic contamination of soils negatively affects the sustainability of agroecosystems, and has become a major problem in recent decades.

Copper-based fungicides have been used intensively in Europe since the end of the 19th century to control vine fungal diseases, such as downy mildew caused by *Plasmopara viticola*. Furthermore, other Cu compounds have been introduced (such as $Cu(OH)_2$ and Cu_2O) and their long term application and subsequent wash-off from the treated plants have led to considerable Cu accumulation up to toxic concentrations in 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).

Despite the current importance of Cu contamination in vineyards, to our knowledge, data regarding the toxic effects of Cu

Abbreviations: A, net photosynthetic rate; BAP, 6-benzylaminopurine; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Ci, 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; ΦPSII, quantum efficiency of PSII; G_s, stomatal conductance; NAA, naphthaleneacetic acid; RGR, relative growth rate.

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are available for only a few commercial varieties of grapevine to date, and the phytotoxicity limits and tolerance mechanisms of grapevine in response to Cu stress remain unclear. A thorough characterization of the physiological responses of different species of the genus *Vitis* to the negative conditions of the vineyard is essential to provide ways to improve current classic cultivars through the incorporation of traits that are well adapted to the prevailing environmental and growing conditions.

V. vinifera (L.) ssp. *sylvestris* (Gmelin) Hegi, the wild subspecies of *V. vinifera* L., is the only Eurasian native subspecies and constitutes a valuable genetic resource for cultivated grapevines (Negrul, 1938). Wild grapevine populations maintain considerable genetic polymorphism and manifest wide variability (McGovern et al., 1996). The disappearance of these populations from their natural habitat would be an irreversible loss for the environment and for breeding programs (Grassi et al., 2006). The aim of the present study was to evaluate the tolerance and accumulation potential of wild grapevine under exposure to moderate and high Cu levels. The specific objectives were: (1) to determine the Cu phytotoxicity thresholds of the study species through analysis of the growth of plants in a range of external Cu concentrations, from 0 to 23 mmol L^{-1} Cu; (2) to ascertain the extent to which Cu levels determine plant performance, in terms of influence on the photosynthetic apparatus (PSII photochemistry), gas exchange characteristics and photosynthetic pigments; and (3) to examine the possible relationship between the effects of Cu on growth and concentrations of N, P, S, Ca, Mg, Fe, Na and K within plant tissues.

2. Materials and methods

2.1. Plant material and copper treatments

Plant material was collected from a non-described wild grapevine population located on the bank of the Agrio river, in Seville province (SW Spain) (37° 30′ 45.7″N – 6° 13′ 24.6″ W). The site is located within the Ossa-Morena mountain range, and the river, which flows through the Aznalcóllar mining zone, carries high levels of heavy metals. In 1998, the river was suddenly flooded with 4–5 million cubic meters of mine wastes, coming from a holding dam that failed at a mine near Aznalcóllar. The dam contained very dangerous levels of several heavy metals (e.g. cadmium, copper and zinc).

Axillary buds were taken from individuals of *V. vinifera* ssp. *sylvestris* of the population described above and washed with water and household detergent before gently rinsing with distilled water. The buds were then sterilized by immersion in absolute ethanol (75 s) and immediately in a solution of sodium hypochlorite 12% (10% of active chloride) with some drops of Tween-20, for 15 m and finally rinsed three times with sterilized water (5 min each time). They were then placed individually into sterile test tubes (21 × 150 mm) with 8 mL of the nutritive medium reported by Troncoso et al. (1990), modified to include 0.32 μM BAP and 0.13 μM NAA as growth regulators. The tubes were covered with polypropylene caps, sealed with parafilm and placed in a culture chamber at 24 °C, 30 $\mu Em^{-2} s^{-1}$ of light intensity and a photoperiod of 16 h of light. Buds from the obtained plantlets were subcultured for 45 d in the same medium to obtain a very homogeneous group of plants. 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 $\mu mol\ m^{-2} s^{-1}$, 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, one tray per Cu treatment). Cu treatments were prepared by mixing the 20% Hoagland's solution with $CuSO_4 \cdot 5H_2O$ of 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. The range of Cu concentrations was chosen based on previous experiments to detect the phytotoxicity thresholds of the study species.

At the beginning of the experiment, 3 L of the appropriate solution was 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 change 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:

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

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).

Leaf area was determined from the projected area by scanning and digitalising the leaves (Epson V30, Seiko Epson Corp., Nagano, Japan), and using appropriate software (MideBMP v. 4.2.; Ordiales-Plaza, 2000) for processing and analysis. 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 and root samples were carefully washed with distilled water and then dried at 80 °C for 48 h and ground, in accordance with the protocols of Redondo-Gómez et al. (2007). 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, Fe, K and Na 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

Gas exchange measurements were taken from randomly selected, fully expanded leaves (n = 20, one measurement per plant plus eight extra measurements taken randomly), following 10 and 30 d of treatment, 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 $\mu mol\ mol^{-1}$ at 20–25 °C, 50 ± 5% relative humidity and a photon flux density of 1600 $\mu mol\ m^{-2} s^{-1}$. Values of the parameters A, C_i and G_s were calculated using the standard formulae of Von Caemmerer and Farquhar (1981).

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