iForest
Biogeosciences and Forestry

Short Communication
doi: 10.3832/ifor1999-009
vol. 10, pp. 93-98

A quick screening to assess the phytoextraction potential of cadmium and copper in *Quercus pubescens* plantlets

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The relevance of the environmental pollution by heavy metals warrants the necessity to develop and assess more efficient plant-based technologies. This study was conducted to evaluate a quick screening approach in order to investigate the cadmium (Cd) and copper (Cu) phytoextraction potential of *Quercus pubescens* in a micro-propagation system. Increasing concentrations of Cd (0, 5, 50, and 250 µM) and Cu (0, 5, 50, 250 and 500 µM) were separately applied to evaluate the effect of metals on their absorption and accumulation in downy oak plants. At high concentrations, Cd and Cu significantly reduced the dry biomass of shoots and roots and the plant tolerance index. Cd was toxic at increasing concentrations, inducing higher reduction of shoot dry mass than roots, whereas Cu increased dry mass at 5 µM. This study represents the first attempt to assess Cd and Cu uptake in *Q. pubescens* under *in vitro* conditions. The *in vitro* screening potential is meanly related to the following purposes: (i) proper selection of plant materials resilient to excess metals in the growth substrate; (ii) efficient removal of metals by the selected tree species; (iii) minor interference with the growth of plants accumulating metals in their tissues; (iv) rapid provision of plant materials for tree breeding programs.

Keywords: Heavy Metals, Phytoremediation, Downy Oak, Micropropagation

Introduction

The accumulation of heavy metals in soils and water poses a risk to the environmental and human health (Khan et al. 2008). Considerable progress in phytoremediation has been made with metals using hydroponically-cultured plants transplanted in metal-polluted sites, where plants eventually absorb and concentrate metals in their roots and shoots (Tognetti et al. 2013). Phytoremediation is a well-recognized, environmental-friendly strategy to control pollution by heavy metals (Ali et al. 2013). Phytoremediation includes strategies like phytoextraction and phytostabilization, which are defined by the preferential accumulation of pollutants by plants in their above- and below-ground organs, respectively (Pulford & Watson 2003, Pilon-Smits 2005).

Among heavy metals, cadmium (Cd) is one of the most threatening due to its toxicity for the environment, crops and therefore for consumers (Perfus-Barbeoch et al. 2002). Cadmium has a strong carcinogenic potential (García-Esquinas et al. 2014) and is also very toxic for plants, affecting water and nutrient uptake, as well as their photosynthetic efficiency (Pietrini et al. 2010). Copper (Cu) is a microelement essential for plant growth, though it may become potentially toxic at elevated levels, particularly as a result of agricultural practices, and industrial or municipal waste disposal on land (Ali et al. 2004).

The plant capacity to tolerate metals can rely on a variety of resistance and tolerance mechanisms (Pietrini et al. 2010, Wang et al. 2014). Plants have evolved avoidance strategies or exclusion processes, which reduce metal accumulation in cells, as well as mechanisms of surviving despite the accumulation of large amounts of metals (Rascio & Navari-Izzo 2011). Phytoextraction can be achieved through hyperaccumulator plants, which exhibit the capability to take up from soil large quantities of heavy metals, which are then translocated to the epigeous parts (Wong 2003, Yang et al. 2004). However, these plant species have low biomass accumulation and removal potential in absolute terms. Therefore, selection and/or breeding of suitable plant genotypes based on biomass production, accumulation potential, root traits, growth rate, environmental suitability and metal resilience are required for their use in the remediation of heavy metal contamination (Wang et al. 2014). In the last ten years, research was focused on highly productive crop plants, such as crops, maize, sunflower, rice (Komarek et al. 2007, Murakami & Ae 2009), as well as on short rotation forest trees, *i.e.*, willow and poplar (Fernàndez-Martínez et al. 2014, Baldantoni et al. 2014). Phytoextraction carried out with the use of other woody plants can be extremely interesting for the restoration of contaminated sites, where environmental conditions are unsuitable for growing energy plants, as well as for their longer reproductive cycles (Paoletti & Günthardt-Goerg 2006, Cocozza et al. 2012).

Biotechnologies are currently available for better understanding the process of heavy metal uptake by trees and exploring

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Received: Jan 29, 2016 - Accepted: Aug 02, 2016

Citation: Di Santo P, Cocozza C, Tognetti R, Palumbo G, Di Iorio E, Paura B (2016). A quick screening to assess the phytoextraction potential of cadmium and copper in *Quercus pubescens* plantlets. iForest 10: 93-98. – doi: 10.3832/ifor1999-009 [online 2016-10-13]

Communicated by: Tamir Klein

their potential and exploitability in the remediation improvement (Capuana 2011). *In vitro* cultures provide several advantages when the plant cell tolerance to toxic elements is to be examined in experimental studies (Golan-Goldhirsh et al. 2004, Doran 2009). In this sense, *in vitro* screening is a preliminary tool for testing woody plant materials, reducing the growth period and the treatment duration, as well as the space needed for the experiments (Confalonieri et al. 2003, Di Lonardo et al. 2011). We anticipate that *in vitro* cultures represent a valuable research tool for phytoremediation of contaminated substrates and provide the required axenic conditions for screening the potential of a plant species to tolerate and accumulate heavy metals (Di Lonardo et al. 2011, Iori et al. 2012). Particularly, Di Lonardo et al. (2011) set up a test for the quick selection and assessment of poplar clones aimed to characterize the most suitable material for phytoremediation. This approach helps the quick evaluation of the most promising candidates (plant species and their clones) for further trials on polluted soils. Poplar has been used as model species in this screening test (Di Lonardo et al. 2011). However, the efficacy of this approach to test other woody plant species, not traditionally used for phytoremediation purposes, is unknown.

Quercus species are long-living forest trees whose micropropagation *in vitro* is sometimes troublesome. Nonetheless, preliminary results on the tolerance of downy oak (*Quercus pubescens* Willd.) seedlings to metal-polluted substrates has proven this species as an interesting candidate for the phytostabilization of contaminated sites, particularly in marginal areas (Cocozza et al. 2012). In this context, the aims of the present study were to: (i) assess growth perfomances of micropropagated plantlets of downy oak (*Q. pubescens* Willd.) cultivated on Cd and Cu artificially-contaminated substrate; (ii) define their uptake potential and metal bioaccumulation in roots and shoots at increasing concentrations of metals in the substrate.

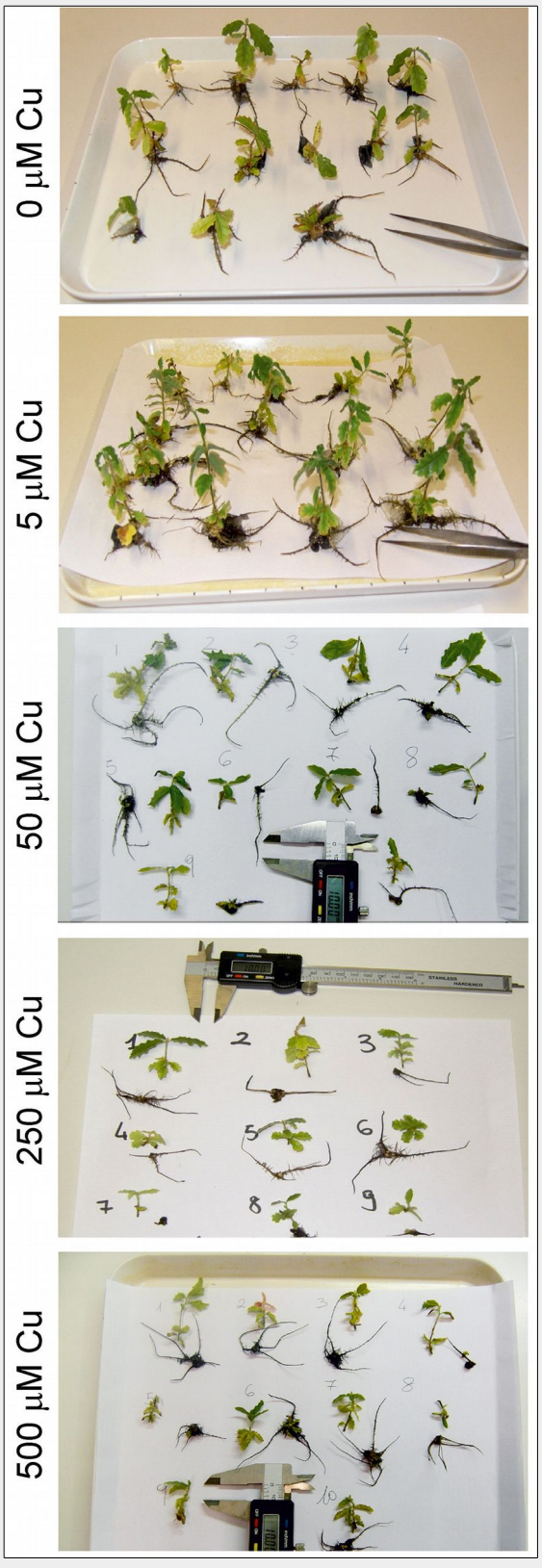

Fig. 1 - Plantlets grown in micro-propagation (five glass jars per treatment containing five rooted shoots each) after 4 weeks of Cu treatment.

Materials and methods

Plant material and in vitro growth condition

Plant material was obtained from cut buds of two years old downy oak seedlings. The buds were washed with tap water and their surface sterilized by Cl 2%. *In vitro* proliferating microshoots of *Q. pubescens* were subcultured on woody plant medium (WPM – Lloyd & McCown 1980), added with 2% sucrose, 0.5 mg l^{-1} BA at pH 5.6, in baby food-glass jars sterilized by autoclaving at 121 °C and 108 kPa for 20 min. The aseptic cultures were incubated in a growth chamber at 23 ± 1 °C with a 16-h photoperiod (40 µE m^{-2} s^{-1}) and routinely subcultured every 4 weeks. To promote rooting, microshoots were placed in contact with 20 mg l^{-1} IBA half strength WPM medium for 24 h in growth chamber. Finally, microshoots were transferred to phytohormone-free WPM medium for four weeks, added with 1 g l^{-1} charcoal to remove hormone carry-over effect and enhance shoot and root elongation. At this point of the experiment, the lack of sucrose in the medium induced well-developed rooted plants to autotrophic phase; gas exchange was improved with autotrophic micro-propagation (Fig. 1).

Metal treatment

Well-developed rooted plantlets were transferred to phytohormone-free WPM medium containing different metal concentrations: 0, 5, 50, 250 µM Cd and 0, 5, 50, 250 and 500 µM Cu, supplied as $CdSO_4$ and

$CuSO_4$, respectively. Twenty plants per concentration (four glass jars per treatment, containing five rooted shoots each) were used in the experiment. Other 20 plants were dried at 70 °C for 24 h and then weighed to estimate a mean value of the dry biomass at the beginning of the experiment.

Determination of metal content

After 15 days of treatment, plantlets were gently removed from the medium and roots were carefully washed. Roots were desorbed with 10 mM $CaCl_2$ solution for 10 min to remove the adhering metals from the cell walls and determine the intracellular fraction of heavy metals. $CaCl_2$ was used as extractant to avoid plasma membrane alteration and metal displacement. Plantlets were separated into shoots and roots, dried at 70 °C for 24 h and then weighed. The dry biomass production (dry biomass at the end of the experiment minus that previously estimated at the beginning of the experiment) was used as a measurement of the metal toxic effects (Baker & Walker 1989). The tolerance index (Ti) was calculated as the ratio between the dry mass of plants (shoots and roots) grown in the solution with metal and that of plants grown in the control solution (Zacchini et al. 2009).

Roots and shoots of plantlets exposed to $CdSO_4$ and $CuSO_4$ were ground in a stainless steel plant mill and mineralized in 4 ml concentrated HNO_3 and 1 ml concentrated $HClO_4$ at 225 °C using an automated heating block (Digester DK 42/26®, Velp Scientifica, Milano, Italy). After digestion, Cu and Cd were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista-MPX). The procedure was validated with reference materials of poplar leaves (GBW 07604 – limit of detection 1 µg l^{-1}). Metal contents were calculated as the product between mean dry biomass of plantlets (n = 5 seedlings) and mean metal concentration in each plant.

Statistical analysis

Treatments were performed in quintuplicate and repeated at least in four independent experiments. Normal distribution of the data was tested using the Kolmogorov-Smirnov test; since no significant departures from normality were found, parametric comparison methods were used (Razali 2011). Data were averaged on a plant basis and the individual means were used for the analysis. The effects of metal treatment on the development traits and metal uptake were tested using the repeated-measures analysis of variance (ANOVA). To assess the differences between treatments for the measured parameters, a *post hoc* comparison of means was performed using the least significant difference (LSD) test or the Tukey's test with α = 0.05. Statistical analysis was performed using the software packages STATISTICA® (StafSoft Inc., Tulsa, OK, USA) and SPSS® ver. 16.0 (SPSS Inc., Chicago, IL, USA) for Windows™.

Results and discussion

The micro-propagation system highlighted that high concentrations of Cd and Cu were toxic for downy oak. The study clearly illustrated the detrimental effects of excess metal on plant growth, as shown by the smaller dry biomass accumulation in shoots and roots of Cd-treated plants compared with non-contaminated plantlets (Fig. 2). As expected, Cd-treated plantlets showed a decreased shoot biomass at increasing concentration of the contaminant, while the biomass of roots was negatively affected in a similar way by all Cd treatments (Fig. 2). Regarding the Cu treatments, the dry biomass of shoots and roots of plantlets was significantly higher at 5 µM compared with other treatments and control conditions, suggesting a positive effect of low Cu concentration on plant growth (Kunjam et al. 2015). However, copper became highly toxic at higher concentrations (Fig. 2). For both metals, the accumulations in seedlings, shoot and root tissues increased with increasing metal contamination (Fig. 3). The accumulation of Cd and Cu was significantly higher at the highest contamination (50 and 250 µM Cd treatment; 250 and 500 µM Cu treatment - Fig. 3). Thus, the reduced growth of micro-propagated downy oak plantlets was likely due to the high metal accumulation in the roots and to a moderate metal translocation to the shoots.

The tolerance index in shoots of Cd-treated plants was higher at low (5 µM) than at high (50 and 250 µM) metal concentration, while no significant differences in tolerance index of roots were observed between Cd treatments (Fig. 4). The tolerance index of shoots in Cu-treated plants was higher at 5 µM and 250 µM compared to other concentrations, while the value in roots was higher at low than at high metal concentration (Fig. 4). However, the low tolerance index at increasing contamination in both metal treatments corresponded to a reduction in plant growth. The high concentration of non-essential (Cd) and essential (Cu) metals could have negatively

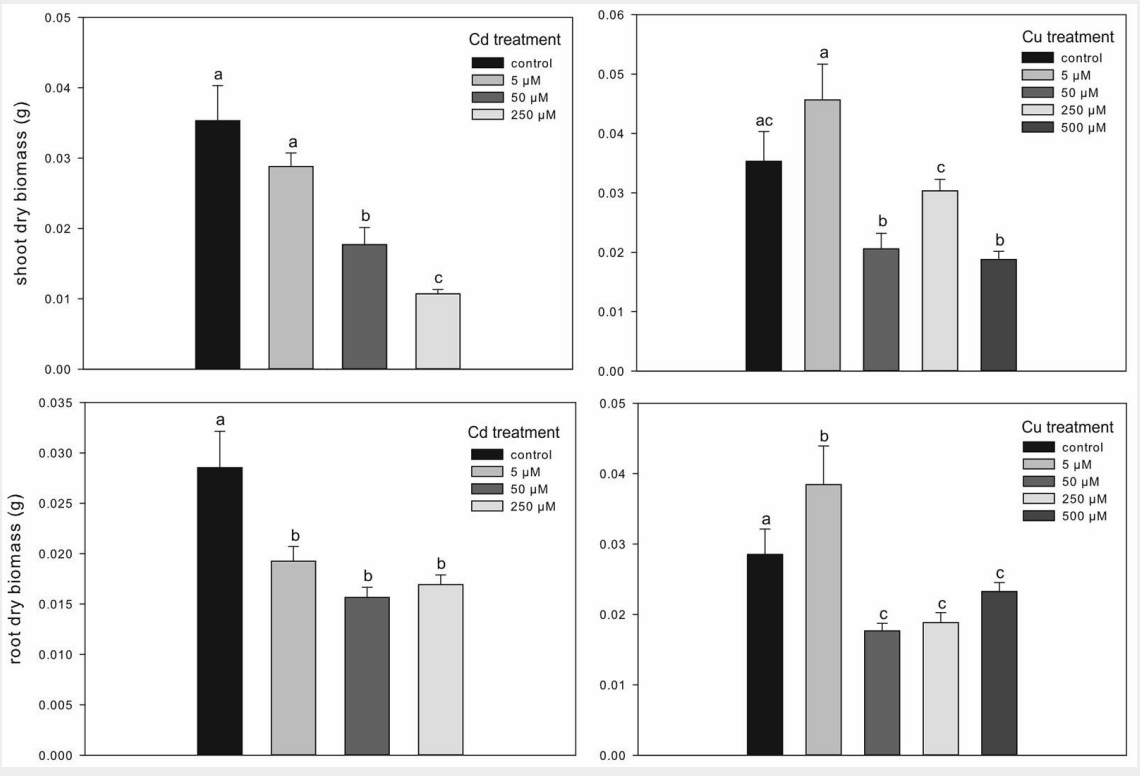

Fig. 2 - Treatment effects on shoot and root dry biomass in Cd- and Cu-treated plants. Mean values (± standard errors) followed by the same letter are not significantly different after LSD test ($p \leq 0.05$).

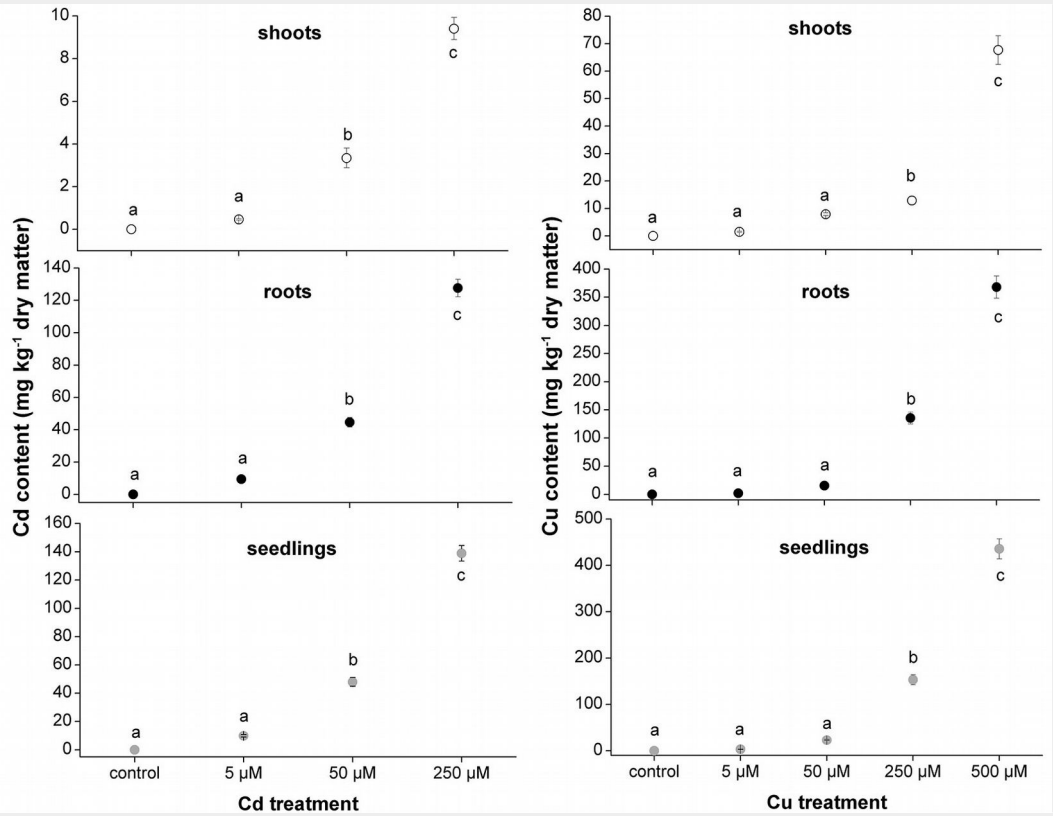

Fig. 3 - Cadmium and Cu contents (milligram per kilogram of dry matter) in shoots, roots and whole seedlings of Cd- and Cu-treated plants. Mean values (± standard errors) followed by the same letter are not significantly different after LSD test ($p \leq 0.05$).

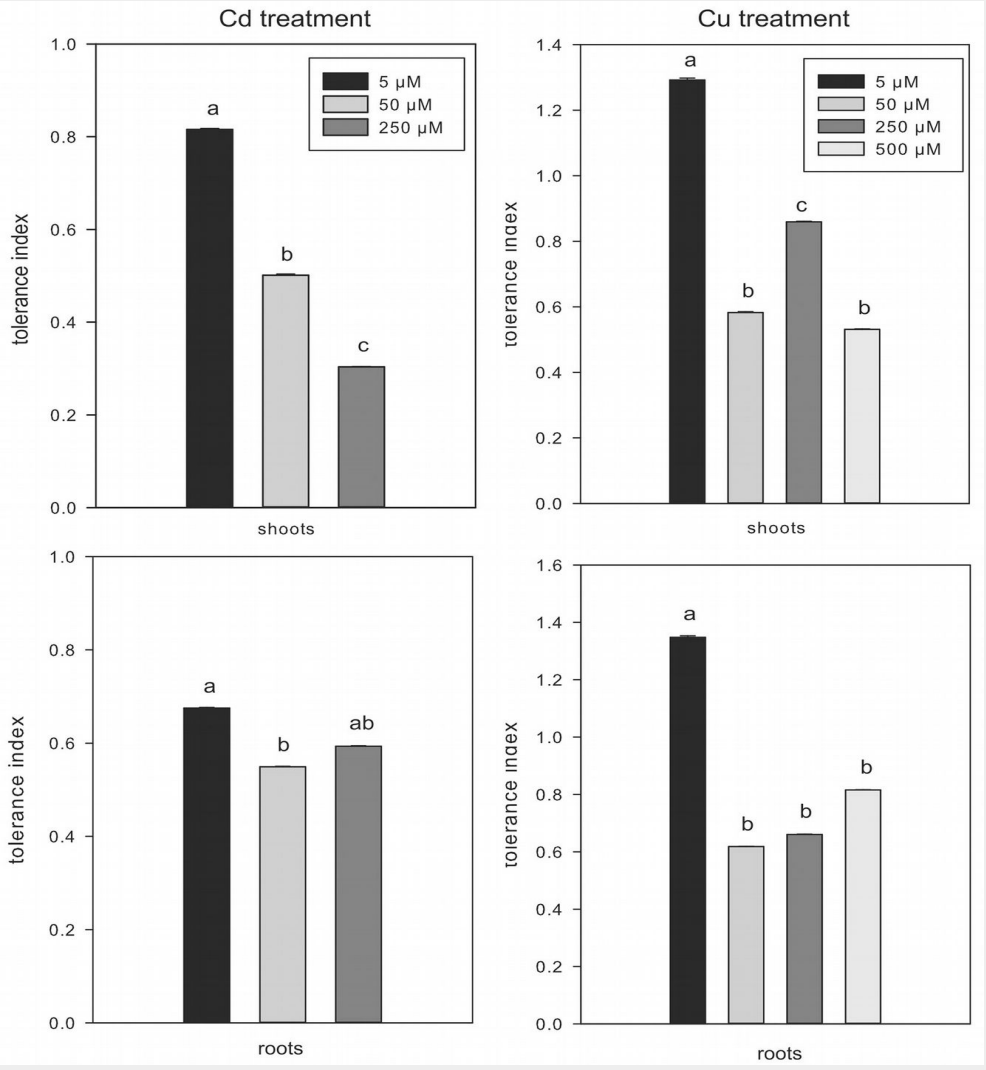

Fig. 4 - Tolerance index in shoots and roots of Cd- and Cu-treated plants. Mean values (± standard errors) followed by the same letter are not significantly different after LSD test ($p \leq 0.05$).

affected the photosynthetic machinery in these woody plants (Di Lonardo et al. 2011). Nevertheless, the potential for metal accumulation at the root level might have been affected by the release into the rizosphere of chelating agents with high affinity for metals, thus favoring metal sequestration (Schat et al. 2002, Shah & Nongkynrih 2007).

The *in vitro* culture approach adopted in this study has proven to be effective to test whether downy oak could be further assessed for resilience to metal contamination over longer experimental periods and/or under *in situ* conditions. Indeed, the metal concentrations used in our micropropagation conditions were very high, in particular when considering that downy oak is not widely recognized as an efficient forest species for decontamination purposes. Nonetheless, its potential Cd accumulation is high, as detected in pot experiments (Cocozza et al. 2012), and this might advocate its use in ecological restoration of polluted sites in marginal areas. Our results provide a preliminary evidence of the remediation potential of downy oak *in vitro*, although they should not be extrapolated to predict the effects of heavy metals on the growth of mature oak trees. The *in vitro* approach offered a stable growth medium, where plants freely absorbed metals from the substrate and translocate them to shoots, with minor interactions with surrounding conditions during the phytoextraction process. Common-garden investigations and provenance trials are planned in this species to test whether geographic variations of adaptive traits should be considered in the selection of material to be used in the restoration of contaminated sites in marginal areas of Italy. The *in vitro* screening carried out in this study allowed for: (i) testing the tolerance of plant materials to high-concentration metals in a short period of time; (ii) providing a suitable environment for the study of plant processes in response to metal treatments. This approach can be further improved towards appropriate breeding strategies and field trials.

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