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〈 **Report** 〉

Pre-adaptation to Cu during Plant Tissue Culture Enhances Cu Tolerance and Accumulation in Begonia (*Begonia evansiana* Andr.)

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ABSTRACT: A simple and efficient protocol was developed for culturing Cu-tolerant and Cu-accumulating plants via pre-adaptation to Cu during plant tissue culture. We induced multiple shoots from begonia (*Begonia evansiana* Andr.) leaf explants on MS medium supplemented with naphtaieneacetic acid and benzyladenine. After 3 months, small plantlets were transferred to MS medium supplemented with 100 μM $CuCl_2$ for pre-adaptation to Cu and cultured for 5 months. Then, these plantlets were individually planted in pots containing artificial soil. An additional 500 mg of Cu dissolved in 1/4 strength MS solution was applied to each pot during irrigation over the course of 2 months. We planted pre-adapted and control begonias in soil from the Il-Kwang Mine, an abandoned Cu mine in Pusan, Korea, to examine their ability to tolerate and accumulate Cu for phytoremediation. Pre-adapted begonias accumulated 1,200 μg Cu/g dry root tissue over the course of 45 days. On the other hand, non-Cu-adapted controls accumulated only 85 μg Cu/g dry root tissue. To enhance Cu extraction, chelating agents, ethylenediamine tetraacetic acid (EDTA)-dipotassiun and pyridine-2,6-dicarboxylic acid (PDA), were applied. While the chelating agents did not enhance accumulation of Cu in the roots of control begonias, EDTA application increased the level of Cu in the roots of pre-adapted begonias twofold (to 2,500 μg Cu/g dry root tissue). Because pre-adapted begonias accumulated a large amount of Cu, mainly in their roots, they could be used for phytostabilization of Cu-contaminated soils. In addition, as a flowering plant, begonias can be used to create aesthetically pleasing remediation sites.

Key words: Begonia (*Begonia evansiana* Andr.), Copper, Phytoremediation, Phytostablization, Plant tissue culture, Pre-adaptation

INTRODUCTION

Soil contamination with heavy metals such as Cu, Cr, Mn, Ni, Pb, and Zn is caused mainly by wastes from metal mining/smelting and industrial and agricultural practices (Ross 1994). Heavy metals are not readily removed or degraded by natural chemical or microbial activities, and accumulate in soil and aquatic sediments, causing environmental problems (Punshon and Dickinson 1997). Cu is an essential nutrient for both plants and animals (Baker 1990). In plants, it is a component of enzymes involved in electron flow and redox reactions (Ouzounidou et al. 1995). However, excessive amounts of Cu inhibit fatty acid and protein metabolism, respiration, and nitrogen fixation (Taiz and Zeiger 1991). At high levels, Cu causes damage to root cells, interfering with root elongation (Lin et al. 2003). Cu also bioaccumulates in the food chain, which poses a potential threat to human health. Accordingly, proper remediation measures are required for Cu-contaminated soils.

As a substitute for relatively expensive and environmentally invasive traditional remediation methods, phytoremediation is a novel approach that uses plants to remove pollutants or render them harmless (Raskin 1996, Salt et al. 1998, Suresh and Ravishankar 2004, Kramer 2005). There are two strategies involved in phytoremediation of contaminated soils. First, phytoextraction utilizes hyper-accumulator plants that can take up contaminants through their roots and concentrate them in their above-ground tissues (Wong 2003). At the end of the growth period, the plants can be harvested and the contaminated plant tissues removed. Naturally-occurring hyper-accumulator plants have been identified in contaminated soils. However, most of them have very low growth and biomass accumulation rates, which result in inefficient phytoremediation. To overcome this problem, genetic engineering has been used to deliver useful genes, such as metal-detoxifying phytochelatin and metallothionein genes (Cobbett and Goldsbrough 2002), to plants that have favorable characteristics for phytoremediation, such as high growth rates, high rates of biomass accumulation, and well-developed roots (Rugh et al. 1996, Chaney et al. 1997, Hasegawa et al. 1997). However, the transgenic approach requires the investment of substantial time and effort. Accordingly, the development of hyper-accumulator plants via simpler procedures would be useful.

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The second approach, phytostabilization, uses vegetation to store and immobilize contaminants in roots or rhizospheres (Wong 2003, Weis and Weis 2004). In this method, contaminants are adsorbed onto or accumulated in roots, preventing erosion by wind and water and introduction of contaminants to the food chain in the surrounding environment. Phytostabilization can be especially useful for heavily contaminated soils that are difficult to clean using phytoextraction.

In this study, we present a simple and efficient protocol to develop Cu-tolerant and Cu-accumulating plants via Cu pre-adaptation during plant tissue culture. A flowering plant, the begonia (*Begonia evansiana* Andr.), was chosen for 1) its production of attractive flowers, which would result in aesthetically-pleasing remediation sites, 2) its numerous lateral roots, and 3) its easy cultivation and propagation. We developed Cu pre-adapted begonias, and then conducted experiments in which these begonias were used for phytoremediation of Cu-contaminated soil from the Il-Kwang Mine. The Il-Kwang Mine in Pusan, southeast Korea, was the largest Cu mine in Korea until the 1980s. However, after its closure, metalliferous wastes were discharged into nearby rivers and soil, polluting the surrounding areas.

MATERIALS AND METHODS

Cu Pre-adaptation during Plant Tissue Culture

Leaf tissues of begonia (*Begonia evansiana* Andr.) were sterilized in 35% (v/v) ethanol for 30 sec and 0.5% (v/v) sodium hypochlorite solution for 60 sec, and then rinsed five times in sterile deionized water. We then cut the leaf tissues into 7 mm squares and placed them on MS medium (Murashige and Skoog 1962) supplemented with 5.4 μM naphtaieneacetic acid (NAA), 22 μM benzyladenine (BA), and 8 g/L agar. Multiple shoots developed on the cut edges of the explants, reaching 2 cm in length and starting to develop roots at 3 months after culture initiation. At this point, we transferred small plantlets to MS medium supplemented with 100 μM $CuCl_2$ and cultured them for 5 months. We adjusted the pH of all culture media to pH 5.8 prior to autoclaving, and filter-sterilized plant growth regulators and $CuCl_2$ before adding them to the autoclaved media. Cultures were transferred to fresh media once every 4 weeks and incubated under a 16/8 h (day/night) photoperiod at 100 mol/m^2/s of light supplied by cool-white fluorescent lamps at temperatures ranging from 24 to 26℃ (night/day). During this stage of Cu pre-adaptation, we discarded plants that showed necrotic tissues or decreased growth rates compared to controls (tissue-cultured begonias not treated for Cu pre-adaptation).

Enhanced Cu Tolerance of Pre-adapted Begonia

We cut sterilized leaf tissues of control and pre-adapted begonias into 7 mm squares and placed them on MS medium supplemented with 5.4 μM NAA, 22 μM BA, and 50 μM $CuCl_2$. We added Cu to the shoot induction medium from culture initiation to examine if tissues of pre-adapted begonias can tolerate and initiate plant regeneration in the presence of Cu. The number of explants that survived and induced multiple shoots on the Cu-containing medium was counted at 2 and 4 weeks.

Cu Pre-adaptation in Artificial Soil

Cu pre-adapted begonias reaching 10 cm in shoot length at 8 months after culture initiation were individually planted in pots (15 cm diameter, 20 cm length) containing 1 kg of artificial soil [1:1:1 (v/v/v) = vermiculite: perlite: peat moss]. For further Cu pre-adaptation, Cu was applied to the plants during irrigation for 2 months: on the first day of irrigation, 5 mg of Cu, in the form of $CuCl_2$, was dissolved in 200 mL of 1/4 strength MS solution (pH 4) and applied to each pot. Plants were irrigated at 4-day intervals, and we increased the amount of Cu in the irrigation medium by 5 mg per pot at each subsequent irrigation until the level reached 50 mg, after which 50 mg of Cu was applied at each irrigation for the remainder of the 2 month period. A total of approximately 500 mg of Cu was applied to each pot during the pre-adaptation period.

Characterization of the Soil of Il-Kwang Mine

We examined the soil around the Il-Kwang Mine as described below. We measured soil pH in a 1:2 (w/v) = soil:deionized water slurry. Organic matter content was determined by measuring weight loss upon ignition at 600℃ (Steele and Pichtel 1998). Particle size was measured as described by Day (1965). Heavy metal contents were analyzed at the Korean Basic Science Institute (Daegu branch). Chemical fractions of Cu in the soil were determined as described by Steele and Pichtel (1998). The soil was incubated with 0.5 M KNO_3 (25 mL per 2 g of soil) for 16 h to determine the exchangeable fraction, with distilled water (25 mL per 2 g of soil) for 2 h to determine the soluble fraction, with 0.05 M NaOH (25 mL per 2 g of soil) for 16 h to determine the organic fraction, with 0.05 M Na_2EDTA (25 mL per 2 g of soil) for 6 h to determine the carbonate fraction, and with 4 M HNO_3 (13 mL per 2 g of soil) at 80℃ for 16 h, followed by the addition of 4 M HNO_3 (12 mL) after heating to determine the residual fraction (Steele and Pichtel 1998). After incubation, soil slurries were centrifuged at 3,000 rpm (1,620 g) for 20 min and supernatants were filtered using Millipore membranes (pore size: 0.45 μm; Bedford, MA, USA). The concentration of Cu in the supernatant was analyzed using a flame atomic absorption spectrometer (FAAS; Varian, Palo Alto, CA, USA). The results were presented in Table 1.

Table 1. Characterization of the soil of the Il-Kwang Mine

Parameters		Values
pH		5.8
Organic matter		8.7%
Particle size	Sand	6.7%
	Silt	20%
	Clay	73.3%
Heavy metals	As	19±4 μg/g
	Pb	21±0.9 μg/g
	Cd	3±0.2 μg/g
	Cu	308±82 μg/g
Cu chemical fraction	Exchangeable	16.6 μg/g (4.8%)
	Soluble	4.5 μg/g (1.3%)
	Organic	106.9 μg/g (30.6%)
	Carbonate	162.3 μg/g (46.5%)
	Residual	58.4 μg/g (16.7%)

Concentration of Cu in Pre-adapted Begonias

During Cu pre-adaptation, begonias were exposed to Cu, so they accumulated elevated levels of Cu in their tissues. To measure the baseline concentration of Cu in pre-adapted begonia, dried tissues (leaf, stem, and root; 0.4 g) were homogenized in a 3 mL nitric acid (HNO_3, 65%) and 1 mL perchloric acid ($HClO_4$, 70%) mixture using a microwave digester (Milestone Microwave Laboratory System MLS 1200, Monroe, CT, USA) according to the manufacturer's instructions. Tissue lysate was diluted with de-ionized water in the ratio of 1:100 and the baseline concentration of Cu was analyzed using FAAS.

Cu Phytoextraction

Soil from the Il-Kwang Mine was mixed with peat in the ratio of 3:1 (v/v). Control (tissue-cultured begonias without Cu pre-adaptation) and pre-adapted begonias were planted individually in pots containing 1 kg of the mixed soil. Plants were regularly irrigated with 1/4 strength MS solution. After 1 month, each plant was given one of three treatments. The first two treatments involved application of one of two soil amendments, ethylenediamine tetraacetic acid (EDTA)-dipotassiun or pyridine-2,6-dicarboxylic acid (PDA), (7 mM in 100 mL 1/4 strength MS solution per pot). As a control, we applied 100 mL 1/4 strength MS solution to plants in the third treatment group. Soil amendments were applied 3 times at 3-day intervals. Six days after the last application, plants were harvested, separated into their constituent tissues, and dried at 75 ℃ overnight. The concentration of Cu in each tissue was measured as described above. Baseline levels of Cu in pre-adapted plants were subtracted from levels measured after the Cu phytoextraction experiment to measure the amount of Cu the pre-adapted begonias extracted from the soil in the phytoextraction experiment. The experiments were repeated 3 times with 9 plants per condition.

RESULTS AND DISCUSSION

Enhanced Cu Tolerance of Cu Pre-adapted Begonia

Begonias were exposed and pre-adapted to Cu during plant tissue culture to examine whether Cu pre-adaptation can increase Cu tolerance or accumulation in this plant, and experiments were conducted to determine whether Cu pre-adapted begonias can be used for phytoremediation of Cu-contaminated soils. Our results suggest that metal-tolerant and metal-accumulating plants can be engineered via a relatively simple pre-adaptation procedure during plant tissue culture.

To examine if Cu pre-adaptation enhances tolerance to Cu in begonias, explants of Cu pre-adapted begonias were placed on MS medium supplemented with 5.4 μM NAA and 22 μM BA, and 50 μM $CuCl_2$. Cu (50 μM $CuCl_2$) was added to the shoot induction medium from culture initiation to examine whether explants of Cu pre-adapted begonias can tolerate and induce multiple shoots in the presence of Cu. Explants from pre-adapted begonias showed a higher survival rate (62%) after two weeks on shoot induction medium containing Cu than controls (35%; Fig. 1A). After 4 weeks, 43% of pre-adapted explants were still alive, whereas no control explants survived (Fig. 1B). Our results show that begonias can acquire tolerance to Cu through a process of pre-adaptation during plant tissue culture.

After 8 months of *in vitro* culture, Cu pre-adapted begonias were transferred to artificial soil. For further Cu pre-adaptation, approximately 500 mg of additional Cu was applied per plant over the course of 2 months. At the end of the culture period, the Cu pre-adapted begonia shoots were 10 cm in length and had well-developed roots.

Characterization of Soil from the Il-Kwang Mine

We measured a number of parameters (pH, organic matter content, soil particle size, heavy metal contents, and Cu chemical fraction) to characterize soil from the Il-Kwang Mine (Table 1). Soil from the Il-Kwang Mine had a high level of organic matter (8.7%) and clay (73.3%), compared to the national average of 2% and 19% (paddy soil), respectively. Because Cu shows a high affinity to organic matter and clay (Baker 1990), it is difficult to remove Cu from the soil. As expected, soil from the Il-Kwang Mine was heavily contaminated by Cu (308±82 μg/g). More than 90% of the

Cu in the soil was found in the organic, carbonate, and residual fractions of the soil, suggesting that the soil conditions are unfavorable for the extraction of Cu.

Accumulation of Cu in Non Pre-adapted Begonia

Control and Cu pre-adapted begonias were planted in pots of soil from the Il-Kwang Mine. One of two soil amendments, EDTA and PDA, which showed the highest rates of Cu desorption from the soil among 21 soil amendments tested in a preliminary screening experiment, or an amendment-free treatment was applied to each pot. Six days after the last soil amendment application, the plants were harvested and the amount of Cu in each tissue was measured. In plants not treated with soil amendments, non pre-adapted begonias accumulated Cu at a higher level in root tissues (85 μg/g dry tissue) than leaf or stem tissues (less than 15 μg/g dry tissue; Fig. 2A). EDTA enhanced accumulation of Cu in leaf and stem tissues 3- and 4-fold, respectively, but decreased Cu accumulation in root tissues to 60 μg/g dry tissue. The other chelating agent, PDA, did not affect the levels of Cu accumulated in any type of tissue.

Accumulation of Cu in Pre-adapted Begonias

Due to the exposure to Cu during pre-adaptation, Cu pre-adapted begonias accumulated higher levels of Cu in their tissues than non pre-adapted controls, even before they were planted in soil from the Il-Kwang Mine. Therefore, to accurately assess the amount of Cu

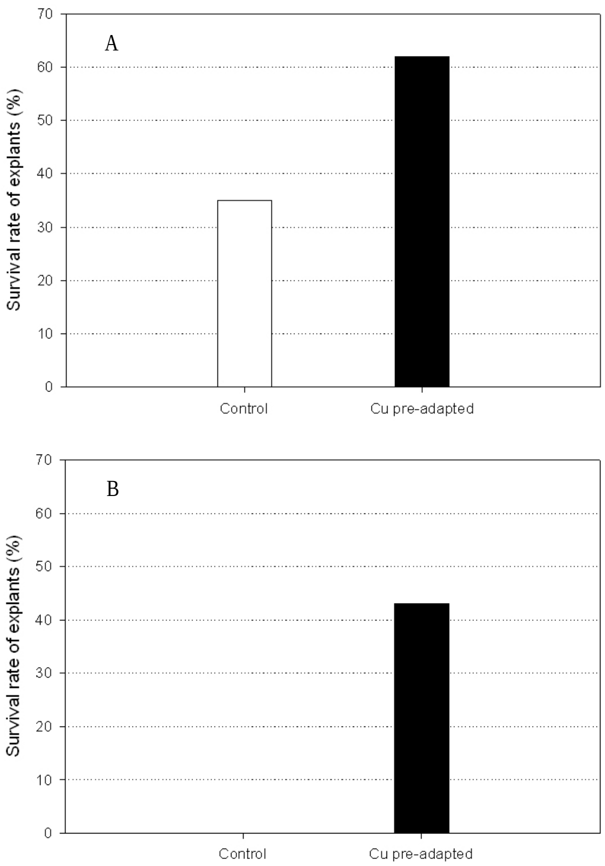

Fig. 1. Survival and regeneration rates of control and Cu pre-adapted begonia on shoot induction medium containing Cu (MS medium supplemented with 5.4 μM NAA, 22 μM BA, and 50 μM $CuCl_2$). A total of 30 pieces of leaf explants (7 mm squares) were placed on the medium and the number of explants surviving and regenerating in the presence of Cu was counted at (A) 2 weeks and (B) 4 weeks.

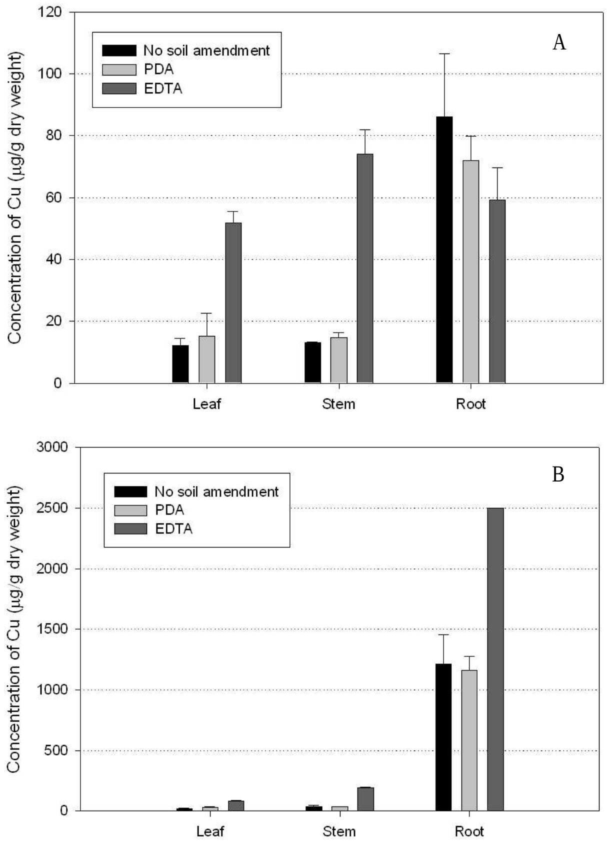

Fig. 2. Cu accumulation in A) control begonias (tissue cultured without Cu pre-adaptation) and B) Cu pre-adapted begonias. Begonias were planted individually in pots containing soil from the Il-Kwang Mine and cultured for 1 month. Then, one of two soil amendments, EDTA and PDA (7 mM in 100 mL 1/4 strength MS solution), or an amendment-free solution was applied to the soil 3 times at 3-day intervals. Six days after the last application, plants were harvested and dried, and the amount of Cu in their tissues was measured. For the Cu pre-adapted begonias, the baseline amount of Cu in each tissue prior to planting was subtracted from the Cu level after the Cu extraction experiment to accurately determine the amount of Cu extracted from the soil.

extracted from soil from the Il-Kwang Mine, the baseline amount of Cu in each tissue of Cu pre-adapted begonias before the phyto-extraction experiment (leaf and stem: 35 ug/g dry weight; root: 350 ug/g dry weight) was subtracted from Cu levels measured after the experiment. These corrected values are shown in Fig. 2B. As in the control begonias, the level of Cu in pre-adapted begonias was much higher in root tissues than leaves and stems. In plants grown on contaminated soil without additional soil amendments, roots accumulated approximately 1,200 μg Cu/g dry weight, while leaf and stem tissues accumulated less than 40 μg Cu/g dry weight. Our results suggest that Cu pre-adapted begonias contain accumulated Cu to their roots, preventing it from migrating to above-ground tissues.

Cu accumulation in pre-adapted begonias was significantly enhanced by treatment with EDTA. EDTA-treated roots showed a more than twofold increase in Cu levels relative to non-treated roots, accumulating approximately 2,500 μg/g of dry tissue. EDTA also enhanced the accumulation of Cu in leaf and stem tissues 3- to 4-fold, respectively. The other chelating agent, PDA, did not affect Cu accumulation in any type of tissue in Cu pre-adapted begonias.

EDTA is an effective chelating agent for Pb, Zn, Cu, and Cd (Huang and Cunningham 1996, Brun et al. 1998, Ebbs and Kochian 1998, Ebbs et al. 1998, Steele and Pichtel 1998, Vassil et al. 1998), and has been frequently used for soil remediation, including phytoremediation. The plasma membranes of root cells are considered to be a primary barrier that controls uptake of chemicals from soil. Vassil et al. (1998) speculated that synthetic chelating agents, such as EDTA, destabilize plasma membranes of root cells, allowing a high level of chemical inflow.

Effect of Cu Pre-adaptation on Cu Accumulation

Our results clearly show that Cu pre-adaptation enhanced Cu accumulation in begonias, especially in root tissues (Fig. 2A and B). In plants not treated with soil amendments, Cu pre-adapted begonias accumulated 1,200 μg Cu per 1 g of dry root tissue, while non- pre-adapted begonias accumulated only 85 μg Cu per 1 g of dry root tissue. When EDTA was applied, the pre-adapted begonias accumulated 2,500 μg Cu per 1 g of dry root tissue, but the non-pre- adapted plants accumulated only 60 μg Cu per 1 g of dry root tissue.

Enhanced accumulation of Cu in pre-adapted begonias (especially in the root tissues) suggests that they developed intracellular Cu detoxification and/or sequestration mechanisms during pre-adaptation. In plants, metal-binding metallothionein and phytochelatin are known to be involved in tolerance to heavy metals, such as Cu (Cobbett and Goldsgrough 2002). Further studies are required to examine the molecular mechanisms of enhanced accumulation of Cu, such as metal-binding protein synthesis, in pre-adapted begonias.

In summary, we developed a simple and effective protocol to enhance Cu tolerance and accumulation in begonias via Cu pre-adaptation and selection during plant tissue culture. Pre-adapted begonias accumulated Cu mainly in their roots and did not allow Cu to migrate to above-ground tissues at high levels, so they do not appear to be Cu hyper-accumulators. However, their ability to accumulate Cu in roots suggests that Cu pre-adapted begonias may be useful for phytostabilization of Cu-contaminated soils, preventing Cu from spreading to the surrounding environment and entering the food chain. Given the practical difficulties involved in developing heavy metal hyper-accumulators for phytoextraction, phytostabilization using metal-tolerant plants appears to be a realistic alternative for stabilizing and beautifying contaminated soils.

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