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Heavy Metals and Nanoparticles: Impact on Protocorm-like Body Formation in Hybrid *Cymbidium*

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Abstract

Heavy metals (HMs) typically inhibit plant growth and development. Only HM accumulators are able to tolerate the presence of these toxic compounds. No studies exist on the response of any orchid cultures to HMs or to nano-particles. In this study, all HMs tested (Al, As, Cd, Cr, Cu, Hg, Ni, Zn) fully reduced neo-PLB growth at or greater than 100 μM. Some neo-PLB growth was observed at 10 and 50 μM, but the levels were significantly lower than the control treatments. Thin cell layers necrosed even at 50 μM, while levels of neo-PLB formation were extremely low at 10 μM. When 100 and 400 mg/l of nano-SiO_2 and nano-TiO_2 were added to 10 and 50 μM of all HMs, in a bid to try and mitigate the negative effects of the HMs and improve neo-PLB formation, there was no improvement to neo-PLB formation. Hybrid *Cymbidium* is thus an extremely HM-sensitive species.

Introduction

The concentrations of heavy metals (HMs) in soil range from < 1 mg/kg to as much as 100,000 mg/kg, caused by natural geological deposits or formation, or as a result of anthropogenic activity such as mining or the application of pesticides for pest control (Pilon-Smits 2005). There is a wealth of literature on the negative impacts of HMs on plant growth and an equally wide range of studies reporting on the ability of plants to take up toxic levels of HMs, i.e., phytoremediation and phytoaccumulation such as by Manousaki and Kalogerakis (2011). The *in vitro* environment has been an effective biotechnological tool for selecting for HM-tolerant lines or against HM-sensitive lines (reviewed by Doran 2009 and Rai et al. 2011). For example, Nehnevajova et al. (2007) used *in vitro* breeding and

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somaclonal variation to improve the potential of Indian mustard (*Brassica juncea* L.) to extract and accumulate toxic HMs, culturing callus on medium supplemented with 10-200 μM Cd, Zn or Pb, generating new somaclones with improved tolerance to these three HMs. However, some HMs such as Co, Cu, Fe, Mn, Mo, Ni or Zn are essential micronutrients for the physiological and metabolic functioning of plants while Pb, Cd, Cr, Ag, Sb and Hg have no known biological function as nutrients in plants (Marschner 1995); so both a deficit and excess of such HMs can negatively impact plant growth and development. There are a wide range of plant species that are resistant to arsenic (As), a metalloid (Meharg and Hartley-Whitaker 2002). The lanthanoids, or rare earth metals, have shown ability to stimulate new or neo-protocorm-like bodies (PLBs) and axillary roots in hybrid *Cymbidium* (Teixeira da Silva unpublished). There are no studies on the use of orchid *in vitro* cultures to examine the effects of HMs on growth and development nor are there any studies on the use of nanotechnology in orchid research (Hossain et al. 2013, Teixeira da Silva 2013a). Based on this gap in the literature, this study was conducted to assess the impact of HMs on neo-PLB induction and organogenesis of hybrid *Cymbidium in vitro*. Many media can support the induction and development of *Cymbidium* PLBs *in vitro* (Teixeira da Silva et al. 2005), but Teixeira Cymbidium (TC) No. 1 medium (Teixeira da Silva 2012) was used in this study. PLBs are considered to be somatic embryos in orchids (Teixeira da Silva et al. 2006a) and were thus fulfil the optimal unit of propagation for this study.

Nanomaterials, used within nanotechnology, are seen increased use in agriculture (Mousavi and Rezaei 2011). Due to their size, they can change physico-chemical properties and have a greater surface area more than their bulk materials and because of the larger surface area, their solubility and surface reactivity is also higher (Castiglione and Cermonini 2009). Nano-TiO_2 (nano-titanium dioxide) increased nutrient uptake in tomato (Haghighi et al. 2012), while Si, applied as nano-SiO_2 (nano-silicon dioxide) ameliorated the detrimental effects of salt stress (Haghighi et al. 2013). The benefits of Si in alleviating abiotic stress are well documented (Liang et al. 2007). There are other examples where nano-TiO_2 and nano-SiO_2 have shown a positive effect on plant growth, but most of these have been greenhouse studies. Furthermore, in all those studies, there has always been a threshold level between a positive effect and toxicity (e.g., Lin and Xing 2007), which is usually dependent on the plant material, e.g. cultivar, species, etc.

Materials and Methods

All protocols (experimental design, chemicals, reagents, explant preparation and treatment analysis) applicable to PLB culture establishment and proliferation (i.e., neo-PLB formation) strictly followed Teixeira da Silva et al. (2005, 2006a, 2006b), Teixeira da Silva (2013b), and Teixeira da Silva and Dobránszki 2013). All chemicals and reagents were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, MO, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue culture grade, unless specified otherwise.

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan), originally developed from shoot-tip cultures on VW (Vacin and Went 1949) agar medium without plant growth regulators, were induced and subcultured (PLB induction and proliferation medium) every two months on Teixeira *Cymbidium* (TC) No. 1 medium (Teixeira da Silva 2012), which contains 0.1 mg/l NAA and 0.1 mg/l Kn, 2 g/l tryptone and 20 g/l sucrose, and solidified with 8 g/l Bacto agar (Difco Labs., USA). All media were adjusted to pH 5.3 prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminum foil, at 25°C, under a 16 hrs photoperiod with a light intensity of 45 μmol/m^2/s provided by 40 W plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected as two pieces of PLB (3 - 4 mm in diameter) segments (Teixeira da Silva 2013b), hereafter half-PLBs, 10/flask, were used as explants for neo-PLB induction and proliferation.

At 60 days after treatment, the growth and developmental response was represented by three parameters: (1) Percentage of half-PLBs forming neo-PLBs; (2) Number of neo-PLBs formed per half- PLB; (3) Fresh weight (mg) of half-PLB + neo-PLBs. Four concentrations (0, 10, 50, 100, 500 μM) of 8 HMs: Al as $Al_2(SO_4)_3$, As as Na_2HAsO_4, Cd as $Cd(NO_3)_2{\cdot}4H_2O$, Cr as $K_2Cr_2O_7$, Cu as $CuSO_4{\cdot}5H_2O$, Hg as $HgCl_2$, Ni as $Ni(NO_3)_2$, and Zn as $Zn(NO_3)_2{\cdot}6H_2O$. The valency of each HM has not been specified.

For example, hexavalent Cr, Cr(VI) or Cr+6 is, in this study, simply represented by the elemental code, Cr. Nano-SiO_2 and nano-TiO_2 were purchased from Sigma-Aldrich with particle sizes ranging from 10 to 20 nm. Nanoparticles were dispersed in distilled water at one concentration (1 g/l) by ultrasonication at 60 Hz for 30 min and then sterilized at 120°C and 100 KPa for 20 min. This served as the stock solution from which desired dilutions were prepared. For two concentrations of HMs (10, 50 μM), i.e., concentrations where some growth and development of neo-PLBs was registered, nano-SiO_2 and nano-TiO_2 were added to basal TC medium at two concentrations (100 and 400 mg/l).

The latter concentrations were based on anecdotal evidence of the mitigating effect of nano-SiO_2 and the growth-stimulatory effect of nano-TiO_2 on crops, horticultural plants, medicinal plants and weeds (Teixeira da Silva, unpublished data).

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment (i.e., HM concentration). All experiments were repeated in triplicate (n = 30, total sample size per treatment). The resulting organogenic outcome (neo-PLB or root response) was scored visually after 60 days, 60 days being the optimal time for sampling (Teixeira da Silva and Dobránszki 2013). Data were subjected to ANOVA with mean separation by DMRT using SAS® version 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $p \leq 0.05$.

Results and Discussion

The most notable finding of this paper is that all HMs fully reduced neo-PLB growth at or greater than 100 μM (Figs 1, 2). Some neo-PLB growth was observed at 10 and 50 μM, but the levels were significantly lower than the control treatments. Thin cell layers (TCLs), which are more sensitive explants for *in vitro* studies (Teixeira da Silva 2013b), necrosed even at 50 μM, while levels of neo-PLB formation were extremely low at 10 μM (data not shown). Based on unpublished, anecdotal evidence of the mitigating effect of nano-SiO_2 and the growth-stimulatory effect of nano-TiO_2 on crops, horticultural plants, medicinal plants and weeds (Teixeira da Silva, unpublished data), 100 and 400 mg/l of nano-SiO_2 and nano-TiO_2 were added to 10 and 50 μM of all HMs, in a bid to try and mitigate the negative effects of the HMs and improve neo-PLB formation. Regrettably, neo-PLB formation did not improve in the presence of either nanoparticle, at either concentration (data not shown). These results suggest that hybrid *Cymbidium* is an extremely HM-sensitive species, which could also lend it as a useful model plant for *in vitro* toxicity tests.

The literature is full of studies related to the (mostly) negative impacts of HMs on plant growth, *in vitro* or in hydroponic or greenhouse studies, so only a handful of representative studies will be discussed below.

Seed germination, as well as shoot and root growth of alfalfa were negatively impacted by 20 mg/l of Cd and Cr, and by 40 mg/l of Cu and Ni (Aydinalp and Marinova 2009). Ar at 20 - 500 μM, Cd at 10 - 500 μM and Hg at 5 - 50 μM negatively impacted root and shoot biomass of artichoke and savory grown hydroponically (Karimi *et al.* 2013). HMS negatively impacted wheat growth as

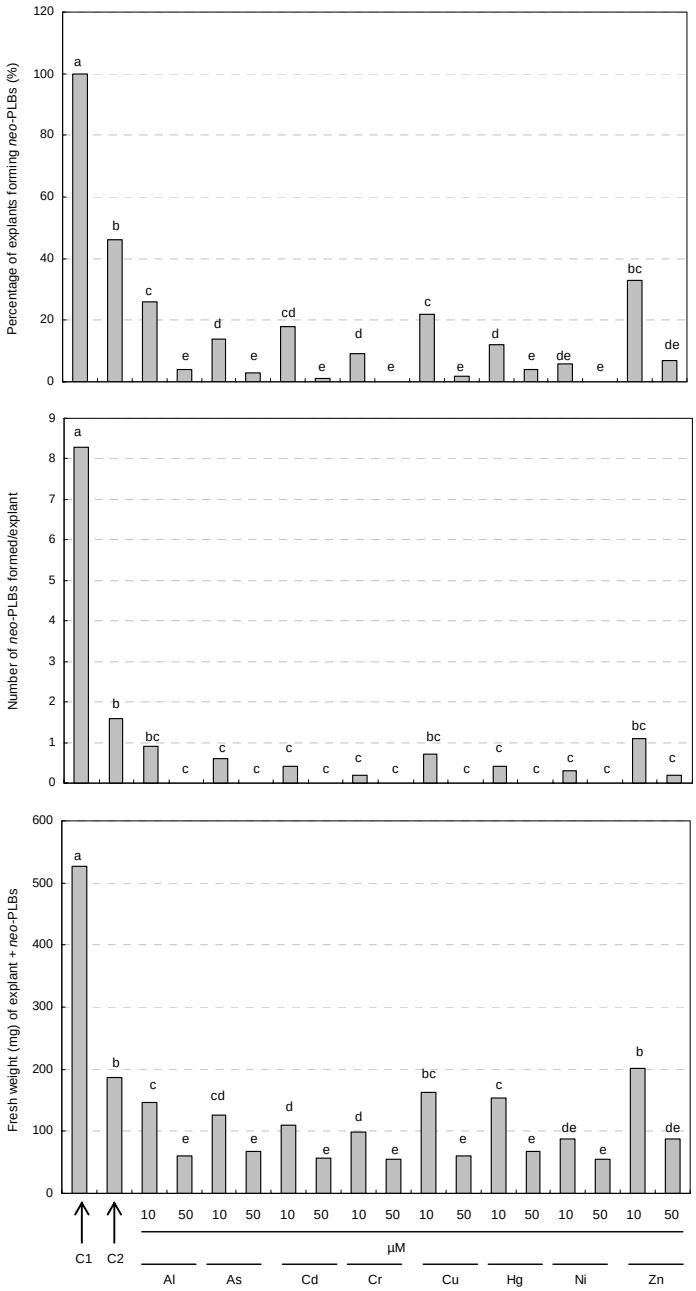

Fig. 1. The response of hybrid *Cymbidium* Twilight Moon 'Day Light' to different concentrations of 8 heavy metals (Al, As, Cd, Cr, Cu, Hg, Ni, Zn). Since no *neo*-PLB formation occurred at or above 100 μM, graphs only represent 10 and 50 μM. Mean values with by the same letter are not significantly different based on DMRT ($p = 0.05$). n = 90 (10 × 3 × 3). C = Control; C1 = TC + PGRs (0.1 mg/l NAA + 0.1 mg/l Kn); C2 = TC - PGRs; PGR, plant growth regulator; PLB = Protocorm-like body; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012).

follows: Cd > Cu > Ni > Zn > Pb > Cr (Athar and Masood 2002) while poplar *in vitro* cultures were most affected by Cu, followed by Pb, then Al (Bojarczuk 2004). *Nopalea cochenillifera* was tolerant up to 100 μM $K_2Cr_2O_7$, and was thus considered to be a Cr-hyper-accumulator (Adki et al. 2013). Cr is a widespread industrial pollutant (Shanker et al. 2005). Low levels of Cd (100 - 200 μM) increased the biomass of potato *in vitro* Gonçalves et al. (2009). 100 μM $CuSO_4$ and 300 μM of $ZnSO_4$ improved *Withania somnifera* shoot formation (Fatima et al. 2011). *In vitro* screening is an effective way of screening for HM-tolerant clones (Di Lonardo et al. 2011). The physiological basis behind HM detoxification by plants is explained in detail by Hossain et al. (2012), and specifically for Cd by Gill et al. (2011).

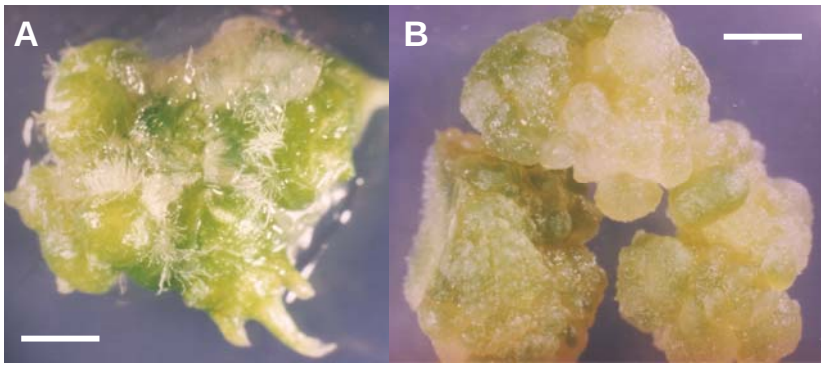

Fig. 2. Growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' neo-PLBs in solid basal TC (Teixeira da Silva 2012) medium under control conditions (no HMs) (A). Typical response under 10 and 50 μM of any HM, even in the presence of 100 and 400 mg/l of nano-SiO_2 and nano-TiO_2 (B). Bar = 1 mm.

Not all reports on the use of nanoparticles are positive. For example, Yang and Watts (2005) reported that 2000 mg/l of nano-Al_2O_3 significantly inhibited root elongation in maize, cucumber, soybean, cabbage and carrot. Lin and Xing (2007) showed that nano-Al_2O_3 particles was phytotoxic to maize root elongation, while Salama (2012) showed that 80 and 100 mg/l of Ag nanoparticles significantly inhibited shoot and root elongation of common bean (*Phaseolus vulgaris* L.) and corn (*Zea mays* L.). TiO_2 NPs improved wheat (*Triticum aestivum* L.) root and shoot fresh weights in the presence of 10 and 100 mg/l but higher concentrations (1000-2000 mg/l) decreased them (Mahmoodzadeh et al. 2013). Pd-nanoparticles were toxic to kiwi-fruit pollen (Speranza et al. 2010).

A search of the literature did not reveal any study on the use of HMs for *in vitro* orchid regeneration or toxicity assays.

The logic, however, is that orchids would most likely never be exposed to HMs under normal *in vitro* or greenhouse growth conditions. Nonetheless, it would be important to examine this potential to phytoremediate or tolerate HMs, even if only as a fundamental study. At an extreme stretch of the imagination, and as an example, one could envision the use of HM-tolerant orchids, developed through *in vitro* screening, for the use of phytoremediation using ornamental plants. Key questions that need still to be answered are: (a) What is the biochemical reason for the toxicity observed? (b) What is the mechanism by which an orchid plant takes up and responds to a HM, and is it similar to other higher plants? (c) To what level and in what organelles and parts of the plant are HMs accumulated, or used?

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