Journal of Horticultural Research 2014, vol. 22(1): 67-75
DOI: 10.2478/johr-2014-0008

# HOW DO RARE EARTH ELEMENTS (LANTHANOIDS) AFFECT ROOT DEVELOPMENT AND PROTOCORM-LIKE BODY FORMATION IN HYBRID *CYMBIDIUM*?

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Received: January 9, 2014; Accepted: March 20, 2014

ABSTRACT

Only few studies in the plant tissue culture literature have examined the impact of lanthanoids, or rare earth elements, on *in vitro* plant organogenesis. In this study, using a model plant, hybrid *Cymbidium* Twilight Moon 'Day Light', the impact of six lanthanoids (lanthanum (III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$), cerium (III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$), neodymium (III) nitrate hexahydrate ($Nd(NO_3)_3 \cdot 6H_2O$), praseodymium (III) nitrate hexahydrate ($Pr(NO_3)_3 \cdot 6H_2O$), samarium (III) nitrate hexahydrate ($Sm(NO_3)_3 \cdot 6H_2O$), gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$) on new protocorm-like body (*neo*-PLB) formation on Teixeira *Cymbidium* (TC) medium was examined. 0 (control), 1, 2, 4 and 8 $mg \cdot dm^{-3}$ of each lanthanoid was tested. All lanthanoids could produce more *neo*-PLBs and *neo*-PLB fresh weight than TC medium lacking plant growth regulators (PGRs), suggesting some PGR-like ability of lanthanoids, although PLB-related traits (percentage of half-PLBs forming *neo*-PLBs; number of *neo*-PLBs formed per half-PLB; fresh weight of half-PLB + *neo*-PLBs) was always significantly lower than TC medium containing PGRs. Except for Gd, all other lanthanoids had no negative impact on the number of new leaves from *neo*-PLB-derived shoots, but all lanthanoids showed a significantly lower plant height, shoot fresh weight and shoot dry weight and, in most cases, SPAD (chlorophyll content) value. In addition, using the same concentration of the six lanthanoids, the ability to fortify root formation of *neo*-PLB-derived plantlets was also assessed. Except for Sm, all other lanthanoids significantly increased the number of roots, root fresh and dry weight.

**Key words:** lanthanoid, orchid, PLB, rare earth element, root formation, Teixeira *Cymbidium* (TC) medium

## INTRODUCTION

*In vitro* regeneration protocols for the most common orchid genera such as *Paphiopedilum*, *Dendrobium*, *Phalaenopsis*, *Vanda*, *Oncidium*, *Epidendrum* and some other orchids have been well developed (Hossain et al. 2013). In almost all cases, the use of plant growth regulators (PGRs) has been used to induce protocorm-like bodies (PLBs). These PLBs, in general, when maintained on the same medium, within 3-6 months, depending on the orchid genus, spuriously form shoots from the apical terminal of the PLB and roots from the basal portion, developing plantlets within 6-12 months, also depending on the genus or even cultivar. PGRs are the most commonly used growth-regulating substance used in orchid biotechnology, although substances such as phloroglucinol or coconut water have growth-promoting and growth-inhibiting properties, also depending on the concentration applied to medium (Teixeira da Silva 2013a; Teixeira da Silva et al. 2013). In this study, hybrid *Cymbidium* (Orchidaceae) has been used because its developmental response *in vitro* using its clonal propagule, the PLB, equivalent to a somatic embryo, has been well studied (Teixeira da Silva

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& Tanaka 2006; Teixeira da Silva 2013b; Teixeira da Silva & Dobránszki 2013). PLBs, when encapsulated, can form synthetic seeds (synseed) (Teixeira da Silva 2012a), which are useful units for cryopreservation (Sharma et al. 2013).

Rare earth elements (REEs) or lanthanoids are a group of 15 metallic chemical elements with atomic numbers 57-76 (Wikipedia 2013). Only few studies in the literature exist on the effect of lanthanoids on *in vitro* plant growth, one of them, interestingly enough, on another orchid, *Dendrobium densiflorum* (Luo et al. 2008). In that study, lanthanoids were shown to improve rooting. Improved rooting through the application of lanthanoids has already been shown for several crops. Lanthanum (La) and/or cerium (Ce), significantly elongated roots in *Zea mays* (corn) and *Vigna radiata* L.R. Wilczek (mungbean) or flowering in *Arabidopsis thaliana* (Diatloff et al. 1995a, b; He & Loh 2000; Liu & Hasenstein 2005). However, these studies examined the effects of lanthanoids *in planta* and in an *ex vitro* environment and thus have limited application for *in vitro* studies.

On the basis of the Luo et al. (2008) study, and departing from the premise that lanthanoids would have some similar effect on other orchids, a wider range of lanthanoids, six in total, was tested to assess the impact on hybrid *Cymbidium* organogenesis. Many media can support the induction and development of *Cymbidium* PLBs *in vitro* (Teixeira da Silva et al. 2005), Teixeira *Cymbidium* (TC) No. 1 medium (Teixeira da Silva 2012b) was used in this study.

## MATERIALS AND METHODS

All protocols (experimental design, chemicals, reagents, explant preparation and treatment analysis) strictly follow Teixeira da Silva (2013c), almost *verbatim* in parts.

### Chemicals and reagents

All chemicals and reagents were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

### Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) originally developed from shoot-tip culture on Vacin & Went (1949) (VW) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium) every two months on TC medium (Teixeira da Silva 2012b), which contains a unique composition of macro- and micronutrients, and was supplemented with 0.1 $mg \cdot dm^{-3}$ α-naphthaleneacetic acid (NAA) and 0.1 $mg \cdot dm^{-3}$ kinetin (Kin), 2 $g \cdot dm^{-3}$ tryptone and 20 $g \cdot dm^{-3}$ sucrose and solidified with 8 $g \cdot dm^{-3}$ Bacto agar (Difco Labs., USA), following Teixeira da Silva et al. (2005) and Teixeira da Silva & Tanaka (2006). All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were kept on 40 ml medium in 100 ml Erlenmeyer flasks, double-capped with aluminium foil, at 25 °C, under a 16 h photoperiod with a light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ 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, 10/flask, were used as explants for PLB induction and proliferation. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva et al. 2005), biotic (Teixeira da Silva et al. 2006b) and abiotic factors (Teixeira da Silva et al. 2006a) for PLB induction, formation and proliferation.

### Response of *Cymbidium* to lanthanoids

The effect of six lanthanoids (lanthanum (III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$), cerium (III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$), neodymium (III) nitrate hexahydrate ($Nd(NO_3)_3 \cdot 6H_2O$), praseodymium (III) nitrate hexahydrate ($Pr(NO_3)_3 \cdot 6H_2O$), samarium (III) nitrate hexahydrate ($Sm(NO_3)_3 \cdot 6H_2O$), gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$) on *neo*-PLB induction from half-PLBs was assessed by adding 0 (control), 1, 2, 4 and 8 $mg \cdot dm^{-3}$ of each lanthanoid to solid or liquid TC medium (without PGRs) using the experimental design of Teixeira da Silva (2012b). The logic behind removing PGRs was to assess whether lanthanoids could effectively induce PLBs or roots in the absence of PGRs and thus test their PGR-like ability.

All lanthanoids were dissolved in distilled water and needed concentrations were made up from 100x stock solutions fresh for each repetition. Lanthanoids were only added to solid or liquid TC medium after cooling at room temperature and filtering through 22 µm Millipore filters.

Using the protocol of Teixeira da Silva et al. (2007) for the acclimatisation of *Cymbidium* to *ex vitro* conditions, shoots 4 cm long containing three ensheathed leaves were placed in PGR-free TC medium solidified with 2 $g \cdot dm^{-3}$ Gellan gum, but containing 0 (control), 1, 2, 4 and 8 $mg \cdot dm^{-3}$ of each lanthanoid listed above. Plantlet growth was quantified by the number of new leaves and roots, plant height, fresh and dry weight of shoots and roots. Chlorophyll content in the third fully developed leaf (counting downward from the top, except for IPM in Table 2) of the plantlets was measured as the SPAD value by a chlorophyll metre (SPAD-502, Minolta, Japan).

Finally, and simultaneously to the second experiment, using the experimental design of Teixeira da Silva (2013c), 5 mm long roots with intact root tips were excised with a feather blade from 6 month-old shoots. These shoots were derived from control *neo*-PLBs derived from standard TC medium. Roots were grown on PGR-free TC medium solidified with 2 $g \cdot dm^{-3}$ Gellan gum (see figures in Teixeira da Silva 2013c). Using excised root tips (5 mm long), the effect of lanthanoids on root growth (as isolated organs as opposed to organs attached to the rest of the plant) was assessed. Growth of roots on solid and liquid medium was assessed after 60 days. Liquid medium was agar- and PGR-free liquid TC medium containing the same concentration of lanthanoids as the solid medium trials. Ten roots with intact root tips were cultured in 25 ml of this medium in 250 ml Erlenmeyer flasks and placed on a shaker at constant 84 rpm under the same light and temperature conditions as solid TC medium. 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 & Dobránszki 2013). Explants and roots were photographed using stereo light microscopy and/or a digital camera.

**Statistical analyses**

Experiments were organised according to a randomised complete block design (RCBD) with 10 replicates per treatment (i.e., lanthanoid concentration). All experiments were repeated in triplicate (n = 30, total sample size per treatment i.e., over three blocks). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (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 (original) finding of this paper is that all six lanthanoids at all concentrations reduced *neo*-PLB formation and all three *neo*-PLB-related parameters (Table 1; Fig. 1B-H). Except for Gd, all other lanthanoids reduced shoot-related parameters (Table 2) and stimulated root growth, development and formation, as exemplified by significantly higher number of roots, root fresh and dry weight (Table 3). On a fresh and dry weight basis, most treatments with lanthanoids, and both controls showed a root : shoot ratio of <1.0, indicating that the formation of shoots was favoured (Table 3). In the control, shoots were strongly favoured. In several instances, on a dry weight basis, root formation was strongly favoured or enhanced, namely with 4 $mg \cdot dm^{-3}$ La, 1 or 2 $mg \cdot dm^{-3}$ Ce, Nd or Pr (Table 3). This was strengthened by an increase in root tip biomass (Fig. 1A) when grown in the presence of all lanthanoids relative to both controls (data not shown). This study indicates that lanthanoids could be considered as a new class of PGR, and hence stimulate pure and applied research, although their high cost would likely make their use prohibitive to many researchers.

Table 1. The growth and developmental response of hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLBs to different lanthanoids at different concentrations after 60 days in culture

| Medium lanthanoids in mg·dm$^{-3}$ | Percentage of half-PLBs forming *neo*-PLBs (%)* | Number of *neo*-PLBs formed per half-PLB | Fresh weight (mg) of half-PLB + *neo*-PLBs[3] |
|---|---|---|---|
| TC + PGRs + no lanthanoids[1] | 100 a | 8.3 a | 526 a |
| TC – PGRs + no lanthanoids[2] | 26 de | 1.6 de | 146 f |
| TC – PGRs + 1 La | 51 bc | 4.7 c | 381 bc |
| TC – PGRs + 2 La | 64 b | 5.1 bc | 367 c |
| TC – PGRs + 4 La | 23 cd | 1.2 de | 106 g |
| TC – PGRs + 8 La | 6 ef | 0.3 e | 38 h |
| TC – PGRs + 1 Ce | 55 bc | 4.9 c | 396 bc |
| TC – PGRs + 2 Ce | 41 c | 4.6 c | 361 c |
| TC – PGRs + 4 Ce | 16 e | 0.6 e | 44 h |
| TC – PGRs + 8 Ce | 0 f | 0 e | 0 i |
| TC – PGRs + 1 Nd | 66 b | 6.1 b | 408 bc |
| TC – PGRs + 2 Nd | 48 c | 4.1 c | 327 d |
| TC – PGRs + 4 Nd | 12 e | 0.6 e | 51 h |
| TC – PGRs + 8 Nd | 0 f | 0 e | 0 i |
| TC – PGRs + 1 Pr | 59 bc | 5.8 bc | 406 bc |
| TC – PGRs + 2 Pr | 32 d | 2.3 d | 174 ef |
| TC – PGRs + 4 Pr | 7 ef | 0.1 e | 28 hi |
| TC – PGRs + 8 Pr | 0 f | 0 e | 0 i |
| TC – PGRs + 1 Sm | 32 d | 2.8 d | 203 e |
| TC – PGRs + 2 Sm | 14 e | 0.4 e | 56 h |
| TC – PGRs + 4 Sm | 0 f | 0 e | 0 i |
| TC – PGRs + 8 Sm | 0 f | 0 e | 0 i |
| TC – PGRs + 1 Gd | 69 b | 6.6 b | 436 b |
| TC – PGRs + 2 Gd | 48 c | 4.6 c | 373 c |
| TC – PGRs + 4 Gd | 16 e | 0.8 e | 62 h |
| TC – PGRs + 8 Gd | 0 f | 0 e | 0 i |

PLB = protocorm-like body; SON = sonication; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012b). Lanthanoid abbreviations: La = lanthanum (III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$); Ce = cerium (III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$); Nd = neodymium (III) nitrate hexahydrate ($Nd(NO_3)_3 \cdot 6H_2O$); Pr = praseodymium (III) nitrate hexahydrate ($Pr(NO_3)_3 \cdot 6H_2O$); Sm = samarium (III) nitrate hexahydrate ($Sm(NO_3)_3 \cdot 6H_2O$); Gd = gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$)

*All percentage data was arc-sine transformed prior to analysis

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$). $n = 90$ ($10 \times 3 \times 3$). Data was untransformed.

[1]Control with no lanthanoids, but including TC PGRs: 0.1 mg·dm$^{-3}$ NAA + 0.1 mg·dm$^{-3}$ Kin.

[2]Control with no lanthanoids and excluding PGRs.

[3]In fact, the average fresh weight of initial half-PLB explants is 54 mg ($n = 10$).

Table 2. Shoot (derived from *neo*-PLBs) growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to different lanthanoids at different concentrations 60 days in culture after transfer to PGR-free TC medium solidified with 2 g·dm$^{-3}$ Gellan gum

| Medium lanthanoids in mg·dm$^{-3}$ | Number of new leaves[3] | Plant height (mm)[4] | Shoot fresh weight (mg)[5] | Shoot dry weight (mg)[6] | SPAD value[7] |
|---|---|---|---|---|---|
| TC + PGRs + no lanthanoids[1] | 3.5 a | 6.8 a | 1294 a | 206 a | 44.3 a |
| TC – PGRs + no lanthanoids[2] | 1.1 c | 2.4 cd | 463 h | 56 d | 36.2 b |
| TC – PGRs + 1 La | 3.6 a | 4.6 b | 1083 b | 162 b | 40.8 ab |
| TC – PGRs + 2 La | 3.2 a | 4.2 bc | 962 c | 88 c | 38.6 ab |
| TC – PGRs + 4 La | 2.2 b | 3.1 c | 704 e | 63 cd | 34.8 b |
| TC – PGRs + 8 La | IPM | IPM | IPM | IPM | 31.6 c |
| TC – PGRs + 1 Ce | 3.8 a | 4.4 b | 951 c | 91 c | 39.6 ab |
| TC – PGRs + 2 Ce | 3.3 a | 3.6 bc | 693 e | 71 cd | 34.6 bc |
| TC – PGRs + 4 Ce | IPM | IPM | IPM | IPM | 32.1 bc |
| TC – PGRs + 8 Ce | IPM | IPM | IPM | IPM | 30.3 c |
| TC – PGRs + 1 Nd | 3.4 a | 3.9 bc | 801 d | 73 cd | 38.4 ab |
| TC – PGRs + 2 Nd | 2.9 a | 3.1 c | 644 ef | 56 d | 34.0 bc |
| TC – PGRs + 4 Nd | IPM | IPM | IPM | IPM | 30.4 c |
| TC – PGRs + 8 Nd | IPM | IPM | IPM | IPM | 30.1 c |
| TC – PGRs + 1 Pr | 3.1 a | 4.1 bc | 816 d | 78 cd | 40.4 ab |
| TC – PGRs + 2 Pr | 3.3 a | 2.9 c | 607 f | 51 d | 37.3 b |
| TC – PGRs + 4 Pr | IPM | IPM | IPM | IPM | 33.6 bc |
| TC – PGRs + 8 Pr | IPM | IPM | IPM | IPM | 31.1 c |
| TC – PGRs + 1 Sm | 1.8 b | 2.6 cd | 603 f | 60 cd | 36.1 b |
| TC – PGRs + 2 Sm | 1.2 c | 2.1 d | 529 g | 48 d | 33.8 bc |
| TC – PGRs + 4 Sm | IPM | IPM | IPM | IPM | 31.4 c |
| TC – PGRs + 8 Sm | IPM | IPM | IPM | IPM | 29.6 c |
| TC – PGRs + 1 Gd | 3.2 a | 4.9 b | 1118 ab | 179 ab | 42.3 a |
| TC – PGRs + 2 Gd | 2.8 a | 4.1 bc | 956 c | 83 cd | 39.1 ab |
| TC – PGRs + 4 Gd | IPM | IPM | IPM | IPM | 38.7 ab |
| TC – PGRs + 8 Gd | IPM | IPM | IPM | IPM | 34.3 bc |

IPM = insufficient plant material (i.e., not enough shoots from *neo*-PLBs); PLB = protocorm-like body; SON = sonication; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012b). Lanthanoid abbreviations: La = lanthanum (III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$); Ce = cerium (III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$); Nd = neodymium (III) nitrate hexahydrate ($Nd(NO_3)_3 \cdot 6H_2O$); Pr = praseodymium (III) nitrate hexahydrate ($Pr(NO_3)_3 \cdot 6H_2O$); Sm = samarium (III) nitrate hexahydrate ($Sm(NO_3)_3 \cdot 6H_2O$); Gd = gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$).
*All percentage data was arc-sine transformed prior to analysis.
Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$). $n = 90$ ($10 \times 3 \times 3$). Data was untransformed.
[1]Control with no sonication, but including TC PGRs: 0.1 mg·dm$^{-3}$ NAA + 0.1 mg·dm$^{-3}$ Kin.
[2]Control with no sonication and excluding PGRs.
[3]At the beginning of each treatment, rootless shoots contained three full-grown shoots.
[4]Measured from the level of medium to the tallest leaf tip.
[5]Shoots were dabbed on dry tissue paper to remove *in vitro* flask moisture before weighing.
[6]Shoots were cut at the base from roots, wrapped in two layers of newspaper, labelled, and dry in a hot-air convection oven at 65 °C for 1 week.
[7]SPAD value is a measurement of the chlorophyll content; For IPM treatments, n = 10.

Table 3. Root (derived from shoots derived from *neo*-PLBs) growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to different lanthanoids at different concentrations 60 days in culture after transfer to PGR-free TC medium solidified with 2 g·dm$^{-3}$ Gellan gum

| Medium lanthanoids in mg·dm$^{-3}$ | Number of roots[3] | Root fresh weight (mg)[4] | Root dry weight (mg)[5] | Root : shoot ratio FW/DW basis[6] |
|---|---|---|---|---|
| TC + PGRs + no lanthanoids[1] | 3.4 c | 206 de | 58 bc | 0.159 : 0.282 |
| TC – PGRs + no lanthanoids[2] | 1.1 d | 85 f | 27 cd | 0.184 : 0.482 |
| TC – PGRs + 1 La | 6.3 a | 518 a | 144 a | 0.478 : 0.889 |
| TC – PGRs + 2 La | 5.4 ab | 486 b | 106 b | 0.505 : 0.807 |
| TC – PGRs + 4 La | 3.6 c | 241 d | 71 bc | 0.342 : 1.127 |
| TC – PGRs + 8 La | IPM | IPM | IPM | IPM |
| TC – PGRs + 1 Ce | 5.8 ab | 501 ab | 121 ab | 0.527 : 1.330 |
| TC – PGRs + 2 Ce | 4.1 bc | 394 c | 94 b | 0.569 : 1.324 |
| TC – PGRs + 4 Ce | IPM | IPM | IPM | IPM |
| TC – PGRs + 8 Ce | IPM | IPM | IPM | IPM |
| TC – PGRs + 1 Nd | 6.1 a | 512 a | 143 a | 0.639 : 1.959 |
| TC – PGRs + 2 Nd | 3.1 c | 191 de | 63 bc | 0.297 : 1.125 |
| TC – PGRs + 4 Nd | IPM | IPM | IPM | IPM |
| TC – PGRs + 8 Nd | IPM | IPM | IPM | IPM |
| TC – PGRs + 1 Pr | 5.6 ab | 472 b | 101 b | 0.578 : 1.295 |
| TC – PGRs + 2 Pr | 4.4 bc | 408 c | 98 b | 0.672 : 1.922 |
| TC – PGRs + 4 Pr | IPM | IPM | IPM | IPM |
| TC – PGRs + 8 Pr | IPM | IPM | IPM | IPM |
| TC – PGRs + 1 Sm | 2.6 cd | 124 e | 44 c | 0.206 : 0.733 |
| TC – PGRs + 2 Sm | 0.9 d | 57 g | 16 d | 0.108 : 0.333 |
| TC – PGRs + 4 Sm | IPM | IPM | IPM | IPM |
| TC – PGRs + 8 Sm | IPM | IPM | IPM | IPM |
| TC – PGRs + 1 Gd | 4.9 b | 441 bc | 102 b | 0.394 : 0.570 |
| TC – PGRs + 2 Gd | 3.7 c | 237 d | 66 bc | 0.248 : 0.795 |
| TC – PGRs + 4 Gd | IPM | IPM | IPM | IPM |
| TC – PGRs + 8 Gd | IPM | IPM | IPM | IPM |

IPM = insufficient plant material (i.e., not enough roots since shoots from *neo*-PLBs did not form, or developed poorly); SON = sonication. Lanthanoid abbreviations: La = lanthanum (III) nitrate hexahydrate ($La(NO_3)_3 \cdot 6H_2O$); Ce = cerium (III) nitrate hexahydrate ($Ce(NO_3)_3 \cdot 6H_2O$); Nd = neodymium (III) nitrate hexahydrate ($Nd(NO_3)_3 \cdot 6H_2O$); Pr = praseodymium (III) nitrate hexahydrate ($Pr(NO_3)_3 \cdot 6H_2O$); Sm = samarium (III) nitrate hexahydrate ($Sm(NO_3)_3 \cdot 6H_2O$); Gd = gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$)

*All percentage data was arc-sine transformed prior to analysis.

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$). $n = 90$ ($10 \times 3 \times 3$). Data was untransformed.

PG = phloroglucinol; PLB = protocorm-like body; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012b).

[1]Control with no sonication, but including TC PGRs: 0.1 mg·dm$^{-3}$ NAA + 0.1 mg·dm$^{-3}$ Kin.

[2]Control with no sonication and excluding PGRs.

[3]Unlike shoots, no roots existed on shoots when first plated, thus number of roots = new roots.

[4]Roots were dabbed on dry tissue paper to remove *in vitro* flask moisture before weighing; roots were cut off at the point adjoining them to the shoot.

[5]Roots were cut at the base from shoots, wrapped in two layers of newspaper, labelled, and dry in a hot-air convection oven at 65 °C for 1 week.

[6]FW = fresh weight; DW = dry weight; a ratio < 1.0 = favoured shoot formation; a ratio > 1.0 = favoured root formation.

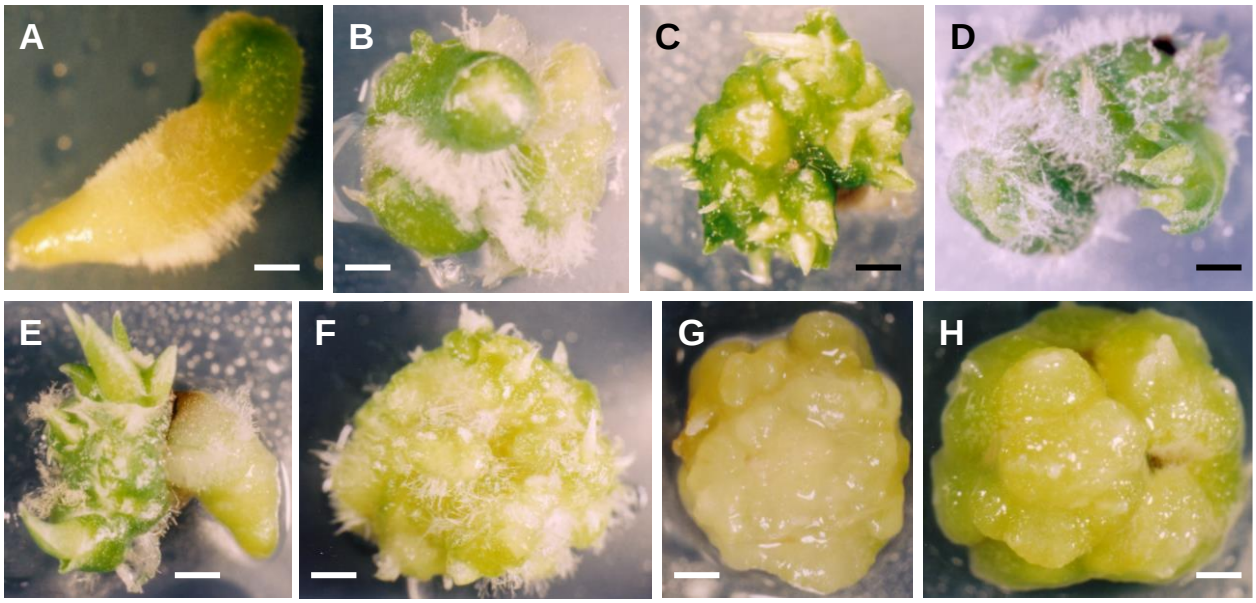


Fig. 1. Growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' *neo*-PLBs and roots in response to lanthanoids. (A) Swelling of excised root in the presence of 1 mg·dm$^{-3}$ $La(NO_3)_3 \cdot 6H_2O$. (B) Control *neo*-PLB (lanthanoid-free + PGRs) growing on solid basal TC (Teixeira da Silva 2012b) medium. *Neo*-PLB formation in the presence of (C) 2 mg·dm$^{-3}$ $La(NO_3)_3 \cdot 6H_2O$, (D) 1 mg·dm$^{-3}$ $Ce(NO_3)_3 \cdot 6H_2O$, (E) 1 mg·dm$^{-3}$ $Nd(NO_3)_3 \cdot 6H_2O$, (F) 1 mg·dm$^{-3}$ $Pr(NO_3)_3 \cdot 6H_2O$, (G) 1 mg·dm$^{-3}$ $Sm(NO_3)_3 \cdot 6H_2O$, (H) 1 mg·dm$^{-3}$ $Gd(NO_3)_3 \cdot 6H_2O$. Bars = 1 mm

The lanthanoids remain a relatively unexplored area of research in the biological sciences, particularly in the plant sciences, and most notably with only one other study related to *in vitro* culture. Even so, La has been used to monitor the movement of calcium in plant tissues (Liu & Hasenstein 2005). Hu et al. (2004) showed the use of lanthanoids in agriculture. Spurred by a promising initial study on lanthanoids that showed that three of these rare earth metals could stimulate rooting in *Dendrobium densiflorum* (Luo et al. 2008), this study was born. In their study, Luo et al. found that after shoot induction in the presence of 6-benzyladenine, that the application of 2 mg·dm$^{-3}$ $Nd(NO_3)_3$ could fortify root growth of plantlets, significantly more than commonly used auxins such as NAA, IAA and IBA. Plantlets could then be successfully acclimatised; 2.5 µM IAA, when combined with 100 µM $La^{3+}$, resulted in 96% rooting efficiency in *Saussurea involucrata* Kar. et Kir *in vitro* shoots (Guo et al. 2012). Peroxidase (POX) and superoxide dismutase activity increased in tissue of $La^{3+}$-containing medium. Hong et al. (2005) noted that nitrate reductase (NR), glutamine synthetase and glutamate dehydrogenase activity in the roots of peach [(*Prunus persica* (L.) Stokes)] *in vitro* plantlets increased significantly in response to 0.3 µmol·dm$^{-3}$ $CeCl_3$. Song et al. (2003) also noted that 1.0-3.0 µmol·dm$^{-3}$ $La(NO_3)_3$ in loquat (*Eriobotrya japonica* Lindi) *in vitro* rooting medium increased the rooting rate, root fresh weight, promoted root length and increase POX and NR activities.

Some rudimentary evidence for the mechanism of action exists. Lanthanoids increase the content of endogenous IAA by stimulating the synthesis of IAA precursor tryptophan and/or inhibiting the enzyme activity for IAA decomposition (Hu et al. 2004). Some relation with the stabilisation of the cytoskeleton of root cells also exists (Liu & Hasenstein 2005) while mitochondrial metabolic activity is enhanced in the presence of La in rice (*Oryza sativa* L.) (Dai et al. 2008). Guo et al. (2012) supported this theory with the following mechanistic proposal 'La enhanced IAA-induced rooting by acting as a mild abiotic stress to stimulate POX and SOD activities in plant cells. Then, IAA reacted with oxygen and POX to form the ternary complex enzyme-IAA-$O_2$ that dissociated into IAA radicals and $O_2^-$. Subsequently, IAA-induced $O_2^-$ readily

converted to hydroxyl radical ($HO^{\cdot}$) via SOD-catalysed dismutation. Finally, cell wall loosening and cell elongation occurred as a consequence of HO-dependent scission of wall components, leading to root growth'. The involvement of stress-inducible enzymes systems had already been shown in the roots of peach and loquat *in vitro* cultures grown in the presence of lanthanoids (Song et al. 2003; Hong et al. 2005) or in salt-stressed *Vigna radiata* (Shan & Zhao 2014). Ruíz-Herrera et al. (2012) suggested that lanthanoids precipitate phosphate, creating P deficiency conditions in the growth medium, inhibiting primary root growth and increasing root hair and lateral root development. $CeCl_3$ promoted nitrogen metabolism through the transformation of $NO_3^-$ to $NH_4^+$ in peach *in vitro* roots (Hong et al. 2005). Küpper et al. (2006) claim that photosynthesis and thus shoot/leaf growth could be affected by lanthanoids which can substitute the central $Mg^{2+}$ ion of chlorophyll. Interestingly, in what appears to be support of this heavy-metal substitution of $Mg^{2+}$ in chloroplasts, growth of *Dryopteris erythrosora*, a lanthanoid-accumulating fern species, was enhanced in response to La (Ozaki et al. 2000).

However, apart from five studies, including two on orchids, few other studies have examined the effects of lanthanoids on plant growth and development *in vitro*. Consequently, this area of research is at a nascent phase of development and many more trials would be required on more plant species, both edible, horticultural and agronomic, to assess the broad range of effects *in vitro* and under greenhouse and field trials. Key questions that still need to be answered: (A) what is the toxicity of lanthanoids, as assessed by toxicity assays? (B) What is the mechanism by which a plant takes up lanthanoids? (C) To what level and in what organelles and parts of the plant are lanthanoids accumulated, or used? The expense of these metallic chemical elements (between 60 and 200 US$/25 g) may make future research prohibitive or restricted, although only milligram amounts are in fact required.

**Acknowledgement**

The author thanks Prof. Michio Tanaka for research support and Dr. Judit Dobránszki for literature.

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