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ORIGINAL ARTICLE

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on Shoot Induction and Seedling Growth of *in vitro* Cultured *Anoectochilus roxburghii*

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Abstract *Anoectochilus roxburghii*, a highly valuable medicinal plant species, is threatened in its native habitat. To ensure the sustainability of this useful resource, we studied the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on plant tissue culture-propagated *A. roxburghii*. Apical buds, mid-stem segments, and basal rhizome segments were taken from aseptic seedlings of *A. roxburghii* and cultured *in vitro* on half-strength Murashige-Skoog medium containing different concentrations (up to 5.0 mg/L) of $La(NO_3)_3$ or $Ce(NO_3)_3$. After 100 d of culturing, average heights of plantlets derived from apical buds were 6.0 cm with 5.0 mg/L $La(NO_3)_3$ and 6.2 cm with 2.0 mg/L $Ce(NO_3)_3$, which respectively increased by 28.0% and 32.0% as compared with that in the non-treated control group. The optimum concentration for shoot induction from mid-stem segments was 1.0 mg/L $Ce(NO_3)_3$ which had a better proliferation times of 1.5-fold and an average length of 3.0 cm compared with 1.0-fold and 2.2 cm in the control group. Optimum growth from basal rhizome segments was achieved on media supplemented with 3.0 mg/L $Ce(NO_3)_3$, which provided a better proliferation times of 5.1-fold and an average shoot length of 4.5 cm compared with corresponding control values of 2.0-fold and 3.5 cm. Our results showed that $La(NO_3)_3$ and $Ce(NO_3)_3$ can accelerate *A. roxburghii* regeneration, which was probably due to the effect on chlorophyll contents, enzymes activity (superoxide dismutase, catalase and peroxidase) and malonldialedhyde contents caused by the addition of $La(NO_3)_3$ or $Ce(NO_3)_3$.

Keywords: *Anoectochilus roxburghii,* Cesium nitrate, *in vitro* culture, Lanthanum nitrate, Plant regeneration

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Introduction

Anoectochilus roxburghii (Orchidaceae) is a rare perennial, medicinal herb distributed in tropical areas of China, India, Bhutan, Nepal, Japan, Bangladesh, Vietnam, Laos, and Thailand (Chen et al. 2009). *Anoectochilus roxburghii* is both valued in traditional medicine and prized as an ornamental plant. The species has been reported to have anti-inflammatory, hepatoprotective, antioxidant, anti-tumor, and immunostimulating activities (Lin et al. 1993, 2000; Wang et al. 2002; Tseng et al. 2006), and has been used to treat cancer, hypertension, diabetes mellitus, and nephritis in Taiwan and Chinese mainland (Du et al. 2008). In addition, *A. roxburghii* is a desirable indoor houseplant because of its attractive, golden-yellow- veined foliage. In its native range, *A. roxburghii* occupies an extremely narrow habitat and is very sensitive to the surrounding environment. Natural populations of *A. roxburghii* are becoming increasingly rare because of continued habitat destruction, and natural propagation of *A. roxburghii* is very difficult. To effectively solve this problem, artificial cultivation techniques based on plant tissue culture have been used to establish a rapid propagation system for *A. roxburghii* (Luo et al. 2013; Yang et al. 2013). Our research group has obtained the *in vitro* propagation system for *A. roxburghii.* The main problem encountered is that *A. roxburghii* seedlings obtained from *in vitro* germination of wild seeds are very spindly, exhibit poor growth, grow very slowly, and require a prolonged cultivation period before transplanting although some plant hormones and organic additives have been applied.

However, in China, scientists have applied inorganic compounds of rare earth elements (REEs) [such as REE $(NO_3)_3$] to the soil to act as a microelement fertilizer and studied their effects on crop yield since the 1970s (Ni 1995). And the rare earth element (REEs) of La, Ce, and Nd have been reported to have interesting biological effects on plants, algae, and microorganisms, such as increases in growth, secondary metabolite biosynthesis, and crop yields (Wu et al.

2001; Chen et al. 2003; Feng et al. 2006). Positive effects of La on crop production of various species include faster development, greener foliage, larger roots, and improved fruit color (Hu et al. 2004). In addition, La has been found to alleviate decreased osmotic potential in leaves of maize seedlings (Feng et al. 1999). Furthermore, La promotes growth and increases chlorophyll content and photosynthetic rates in spinach (Hong et al. 2002a; 2002b; Song et al. 2003). Other beneficial effects of the REEs La and Ce include enhanced drought tolerance, improved nutrient uptake (Brown et al. 1990), and successful rooting of regenerated shoots and acclimation of regenerated plantlets (Guo et al. 2012).

Chlorophyll, whose amounts can affect the plant photosynthesis, is the best physiological indicator to measure the strength of plant photosynthesis (Hong et al. 2002b). Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) have been viewed as primary enzymatic defense systems (Guo et al. 2012; Peng et al. 2013). Moreover, the concentration of malondialdehyde (MDA) could reflect the extent of body peroxide damage (Ippolito et al. 2007; Wang et al. 2009). Thus, the changing chlorophyll contents, enzymes activity (SOD, CAT and POD) and MDA contents in plant seedlings may be significant in explaining the possible regulatory mechanism of $La(NO_3)_3$ and $Ce(NO_3)_3$. So far, the usefulness of $La(NO_3)_3$ and $Ce(NO_3)_3$ during culture of *A. roxburghii* has not been examined, thus, we investigated the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on *A. roxburghii* shoot induction and growth of regenerated plantlets, and in order to reveal the possible mechanism of $La(NO_3)_3$ and $Ce(NO_3)_3$.

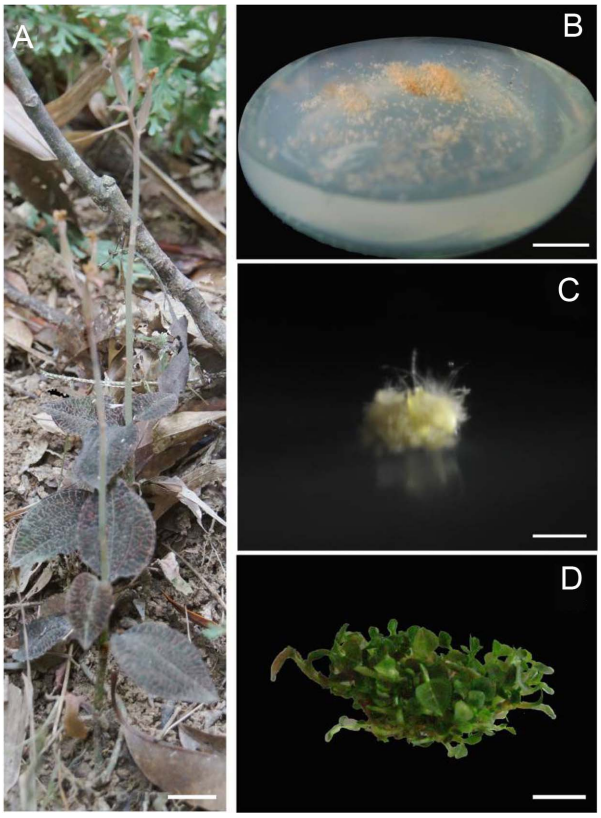

Fig. 1. Generation of aseptic seedlings of *Anoectochilus roxburghii*. (A) Capsules of wild-grown *A. roxburghii* from Hainan, China (bar = 1 cm). (B) Seeds from sterilized capsules inoculated onto half-strength Murashige–Skoog medium (bar = 1 cm). (C) Germinated protocorm from seeds after 90 d culture (bar = 5 mm). (D) Leaves emerging from protocorms transforming into seedlings (bar = 1 cm).

Results

Seed Germination and Seedling Development

After culturing for 30 d, mature seeds (Fig. 1B) from capsules of *A. roxburghii* (Fig. 1A) were swollen and yellow, with embryos enlarged and testae beginning to rupture. Germinated protocorms were observed after an additional 30 d (Fig. 1C). Leaves subsequently emerged from the protocorms and elongation occurred, with seedlings (Fig. 1D) evident after another 2 months. At this time, the seedlings were transferred to other media for subculturing.

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on Growth of Plantlets Regenerated from Apical Buds

We evaluated the effect of various $La(NO_3)_3$ and $Ce(NO_3)_3$ concentrations on the growth of apical bud-regenerated plantlets. As shown in Table 1 and Fig. 2A-D, $La(NO_3)_3$ and $Ce(NO_3)_3$ at concentrations ranging from 1.0 to 5.0 mg/L showed positive effects on plantlet growth. As indicated by the average heights of *A. roxburghii* plantlets after culturing for 30, 60, and 100 d (Table 1), the effects of $La(NO_3)_3$ and $Ce(NO_3)_3$ on plant growth were only slightly apparent after 30 d but were very obvious after 60-d culture. This delayed response may be due to adaptation of plant tissues to the REE-containing medium during later growth stages, leading to faster growth (Wang et al. 2007). After 100 d, non-treated control plantlets had average heights of 4.7 cm, with narrow stems and small, light-green leaves (Fig. 2B). Application of 5.0 mg/L $La(NO_3)_3$ yielded average plantlet heights of 6.0 cm at 100 d (Table 1). The optimum concentration of $Ce(NO_3)_3$ was 2.0 mg/L (Table 1); at this concentration, plantlet heights after 100 d averaged 6.2 cm. Plantlets from both treatment groups displayed vigorous growth and possessed large, dark-green leaves, sturdy stems, and numerous thick roots (Fig. 2C, D). These data provide further evidence that $La(NO_3)_3$ and $Ce(NO_3)_3$ can stimulate plant growth. As can be seen in Table 1 and Fig. 2A-D, plantlets cultured on media containing $Ce(NO_3)_3$ at concentrations of 1.0-5.0 mg/L grew better than those cultured on media containing the same concentrations of $La(NO_3)_3$.

Table 1. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on growth of plantlets regenerated from *Anoectochilus roxburghii* apical buds

Number	Concentration(mg/L)		Average height of plantlets(cm)[a]			Plantlet quality
	$La(NO_3)_3$	$Ce(NO_3)_3$	30d	60d	100d	
0	0	0	3.3±0.03a	3.8±0.03a	4.7±0.07a	+
1	1.0		3.5±0.23ab	4.2±0.12b	5.3±0.13b	+
2	2.0		3.5±0.10ab	4.3±0.12b	5.3±0.18bc	+
3	3.0		3.6±0.21abc	4.4±0.09bc	5.5±0.15bcd	+
4	4.0		3.7±0.12bcd	4.5±0.12bc	5.5±0.15bcd	+
5	5.0		4.0±0.06cd	5.0±0.09de	6.0±0.09ef	+++
6		1.0	3.9±0.13bcd	4.7±0.12cd	5.8±0.12def	++
7		2.0	4.1±0.03d	5.1±0.12e	6.2±0.09e	+++
8		3.0	3.8±0.07bcd	4.7±0.10cd	5.7±0.12dce	++
9		4.0	3.7±0.20abc	4.5±0.20bc	5.5±0.15bcd	+
10		5.0	3.5±0.06ab	4.4±0.03bc	5.4±0.03bcd	+

Mean values within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).
[a]Values are means ± SE of three independent experiments. + Plantlets had narrow stems with small, light-green leaves and only a few roots; ++ plantlets were taller, with slightly thicker stems; small, green leaves; and many roots; +++ plantlets were much taller, with thick, sturdy stems; large, dark green leaves; and many thick roots

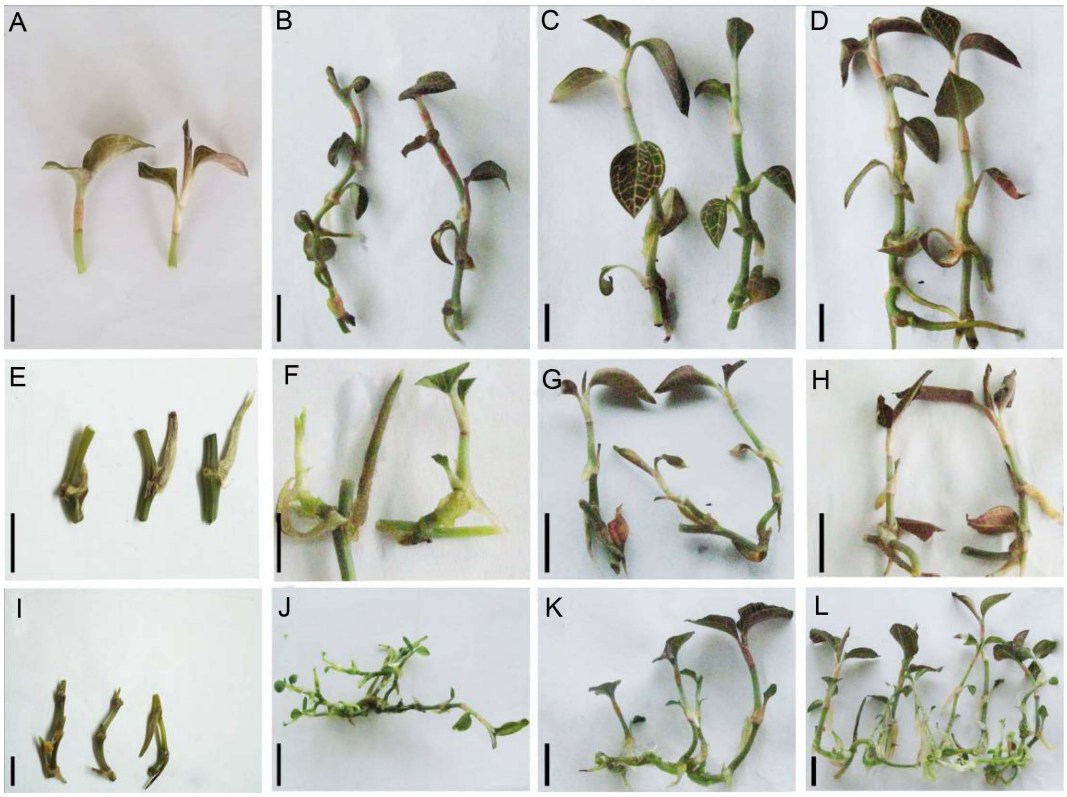

Fig. 2. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on plantlet regeneration from *Anoectochilus roxburghii* apical buds,mid-stem segments and basal rhizome segments. (A) Apical bud explants. (E) Mid-stem segment explants. (I) Basal rhizome segment explants. (B, F, J) Control group plantlets (no REE treatment) after culturing for 100 d. (C-D) Plantlets cultured for 100 d from apical buds [(C, 5.0 mg/L $La(NO_3)_3$; D, 2.0 mg/L $Ce(NO_3)_3$]. (G-H) Plantlets cultured for 100 d from mid-stem segments [(G, 1.0 mg/L $Ce(NO_3)_3$, H, 2.0 mg/L $Ce(NO_3)_3$)]. (K-L) Plantlets cultured for 100 d from basal rhizome segments [(K, 5.0 mg/L $La(NO_3)_3$, L, 3.0 mg/L $Ce(NO_3)_3$)]. Scale bars = 1 cm.

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on Plantlet Regeneration from Mid-stem Segments

The effects of $La(NO_3)_3$ and $Ce(NO_3)_3$ treatments on shoot induction from mid-stem segments were shown in Table 2 and Fig. 2E-H. No significant variation in shoot formation rate or proliferation times was observed among different concentrations of $La(NO_3)_3$ (1.0-5.0 mg/L) and $Ce(NO_3)_3$

Table 2. Effect of 100-d treatment with $La(NO_3)_3$ and $Ce(NO_3)_3$ on plantlet regeneration from *Anoectochilus roxburghii* mid-stem segments

Number	Concentration(mg/L)		Percentage shooting response (%)[a]	Proliferation times[a]	Average shoot length (cm)[a]	Shoots quality
	$La(NO_3)_3$	$Ce(NO_3)_3$				
0	0	0	94±0.00a	1.0±0.00a	2.2±0.03a	+
1	1.0		95±0.03ab	1.0±0.02a	2.4±0.03ab	+
2	2.0		97±0.02abc	1.3±0.16cd	2.4±0.03ab	+
3	3.0		100±0.00c	1.4±0.09cd	2.5±0.03b	+
4	4.0		100±0.00c	1.4±0.11cd	2.5±0.03b	++
5	5.0		100±0.00c	1.5±0.04d	2.5±0.06b	++
6		1.0	100±0.00c	1.5±0.04d	3.0±0.06d	+++
7		2.0	100±0.00c	1.3±0.00cd	2.9±0.03d	+++
8		3.0	98±0.02abc	1.2±0.04bc	2.9±0.12d	+++
9		4.0	98±0.02bc	1.0±0.02ab	2.7±0.03c	++
10		5.0	100±0.00c	1.0±0.00a	2.7±0.03c	++

Mean values within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).
[a]Values are means ± SE of three independent experiments. + induced shoots were small, with light-green leaves; ++ induced shoots were medium-sized, with green leaves and strong stems; +++ induced shoots were large, with dark-green leaves and thick, sturdy stems

(1.0-5.0 mg/L) after 30 or 60 d of culturing. As shown in the Table 2, shoots were induced from almost all mid-stem segments by day 100. Compared with the control group, the better shoot proliferation times of 1.5-fold and the average shoot length of 2.5 cm for $La(NO_3)_3$ was at its concentration of 5.0 mg/L. The optimum concentration of $Ce(NO_3)_3$ was 1.0 mg/L; this concentration yielded the best-quality shoots, which had a better proliferation times of 1.5-fold and an average length of 3.0 cm (Fig. 2G) compared with 1.0-fold and 2.2 cm (Fig. 2F) in the control group. On the whole, the optimum concentration for middle stem segments was 1.0 mg/L of $Ce(NO_3)_3$ (Fig. 2G). The greater sensitivity of *A. roxburghii* mid-stem segments to $Ce(NO_3)_3$ than to $La(NO_3)_3$ contrasts with experimental results previously reported for *Cistanche deserticola* cells (Peng et al. 2013).

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on Plantlet Regeneration from Basal Rhizome Segments

Varied results were observed when basal rhizome segments were inoculated onto media supplemented with different concentrations (1.0-5.0 mg/L) of $La(NO_3)_3$ and $Ce(NO_3)_3$ (Fig. 2I-L, Table 3). By day 100, shoots were induced from almost all rhizome stem segments regardless of treatment. The indicator data were increased within the range of increase in concentration of $La(NO_3)_3$ (0-5.0 mg/L), whereas first increased with 0-3.0 mg/L $Ce(NO_3)_3$ and then decreased with 3.0-5.0 mg/L $Ce(NO_3)_3$ (Table 3). Comparison of the proliferation times and the average shoot length in the Table 3 revealed that groups treated with different concentrations of $Ce(NO_3)_3$ had better proliferation times than those subjected to similar concentrations of $La(NO_3)_3$. Similarly, shoots treated with $Ce(NO_3)_3$ grew better than those subjected to $La(NO_3)_3$ (Table 3). Taking all factors into consideration, optimum conditions for plantlet growth from basal rhizome segments was 3.0 mg/L $Ce(NO_3)_3$ which provided a better proliferation times of 5.1-fold and an average shoot length of 4.5 cm (Table 3, Fig. 2L).

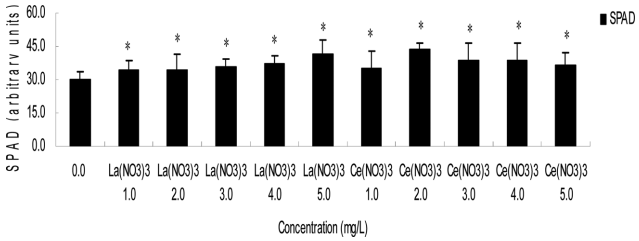

Fig. 3. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on chlorophyll content of regenerated *Anoectochilus roxburghii* plantlets. Values are means ± SD (n=20). *Symbols over bars indicate significant differences compared with the control (no REE treatment) according to LSD multiple range test ($P < 0.05$).

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on Chlorophyll Contents

$La(NO_3)_3$ and $Ce(NO_3)_3$ could enhance chlorophyll contents according to the SPAD values (Fig. 3). The chlorophyll contents of *A. roxburghii* significantly increased within the range of increase in concentration of $La(NO_3)_3$ (0-5.0 mg/L), while the chlorophyll contents first increased with 0-2.0 mg/L $Ce(NO_3)_3$ and then decreased with 2.0-5.0 mg/L $Ce(NO_3)_3$. The experimental results showed that the highest chlorophyll contents of *A. roxburghii* increased by 38.5% in 5.0 mg/L $La(NO_3)_3$ solution and 44.2% in 2.0 mg/L $Ce(NO_3)_3$ solution compared with that in non-REE solution.

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on SOD, CAT, and POD Activity

Table 3. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on plantlet regeneration from *Anoectochilus roxburghii* basal rhizome segments

No.	Concentration (mg/L)		Proliferation times[a]			Percentage shooting response (%)[a]	Average shoot length(cm)[a]	Shoots quality
	$La(NO_3)_3$	$Ce(NO_3)_3$	30d	60d	100d	100d	100d	
0	0	0	1.2±0.03a	2.1±0.03a	2.0±0.08a	100±0.00c	3.5±0.18a	+
1	1.0		1.4±0.07abc	2.2±0.01a	2.8±0.02b	100±0.00c	3.8±0.03ab	+
2	2.0		1.4±0.08abc	2.2±0.02a	3.2±0.07bcd	100±0.00c	3.8±0.10ab	+
3	3.0		1.6±0.03bcd	2.3±0.18a	3.3±0.02cd	100±0.00c	3.8±0.09ab	+
4	4.0		1.8±0.07de	2.3±0.18a	3.4±0.12cde	100±0.00c	3.9±0.06b	++
5	5.0		1.8±0.14de	2.4±0.04a	3.8±0.37efg	100±0.00c	4.0±0.03bc	++
6		1.0	1.3±0.13ab	2.1±0.02a	3.6±0.07def	100±0.02ab	3.8±0.06ab	++
7		2.0	1.6±0.13cde	3.0±0.06b	4.1±0.07g	100±0.00c	4.0±0.03bc	++
8		3.0	1.9±0.06e	3.4±0.16c	5.1±0.12h	100±0.00c	4.5±0.06d	+++
9		4.0	1.8±0.13de	2.9±0.03b	4.0±0.12fg	98±0.02bc	4.2±0.12c	++
10		5.0	1.4±0.10abc	2.2±0.04a	3.1±0.04bc	100±0.00a	3.9±0.06b	++

Mean values within a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).
[a]Values are means ± SE of three independent experiments. + induced shoots were small, with light-green leaves; ++ induced shoots were medium-sized, with green leaves and strong stems; +++ induced shoots were large, with dark-green leaves and thick, sturdy stems

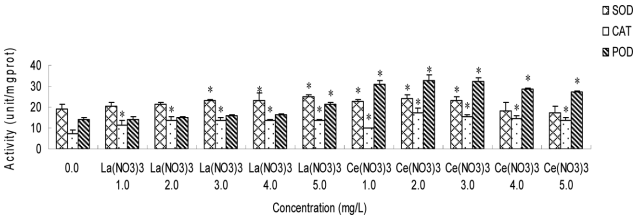

Fig. 4. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on SOD, CAT and POD activity of regenerated *Anoectochilus roxburghii* plantlets. Values are means ± SD (n=3). *Symbols over bars indicate significant differences compared with the control (no REE treatment) according to LSD multiple range test ($P < 0.05$).

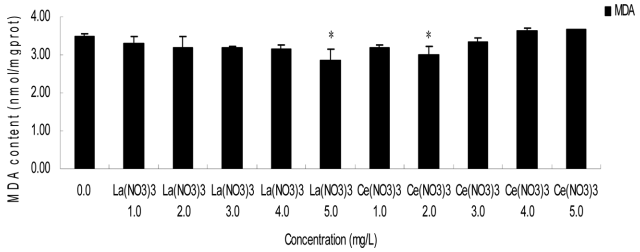

Fig. 5. Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on MDA content of regenerated *Anoectochilus roxburghii* plantlets. Values are means ± SD (n=3). *Symbols over bars indicate significant differences compared with the control (no REE treatment) according to LSD multiple range test ($P < 0.05$).

As shown in Fig. 4, the activities of SOD, CAT, and POD of regenerated *A. roxburghii* substantially increased as the increase of the concentration of $La(NO_3)_3$ within the range of 0-5.0 mg/L. The optimum concentration of 5.0 mg/L $La(NO_3)_3$ caused the highest enzymes activity, such as SOD (25.13 unit/mgprot), CAT (13.73 unit/mgprot), and POD (21.22 unit/mgprot), which increased respectively by 31.4%, 83.1%, and 51.6% as compared with those in control group. Fig. 4 also showed that the effect of $Ce(NO_3)_3$ on SOD, CAT, and POD activity was consistent with the hormesis effect (low-dose stimulatory and high-dose inhibitory response). Those enzymes activity increased as the $Ce(NO_3)_3$ concentration increased within the range of 0-2.0 mg/L. However, SOD, CAT, and POD activity decreased when the $Ce(NO_3)_3$ concentration was higher than 2.0 mg/L, and SOD activity dropped below that of control when the $Ce(NO_3)_3$ concentration was higher than 4.0 mg/L, which indicated that $Ce(NO_3)_3$ could inhibit the enzyme system of plant when their concentration is beyond the critical value. The highest SOD (23.88 unit/mgprot), CAT (17.07 unit/mgprot), and POD (32.89 unit/mgprot) activity obtained upon treatment with 2.0 mg/L $Ce(NO_3)_3$, which were 1.25-fold, 2.28-fold, and 2.35-fold of those in control group. Furthermore, the change of enzymes activity (SOD, CAT, and POD) was fundamentally in agreement with the change of chlorophyll contents (SPAD).

Effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on MDA Content

The effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on peroxidation of membrane lipid (MDA contents) was presented in Fig. 5; it can be seen that $La(NO_3)_3$ and $Ce(NO_3)_3$ had influence on MDA contents of regenerated *A. roxburghii*. With 1.0-5.0 mg/L $La(NO_3)_3$ treatments, the MDA contents were all lower than the control group (3.48 nmol/mgprot). It showed that $La(NO_3)_3$ could weaken the peroxidation of membrane lipid of *A. roxburghii*. With 0-5.0 mg/L $Ce(NO_3)_3$ treatments, the MDA contents decreased at 0-2.0 mg/L $Ce(NO_3)_3$, especially

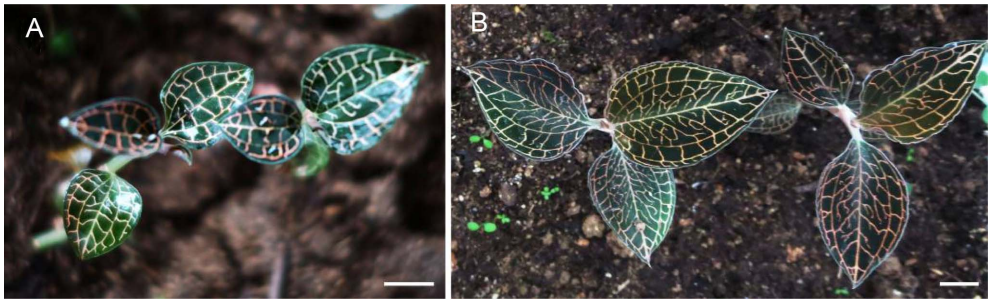

Fig. 6. Regenerated *Anoectochilus roxburghii* plantlets after acclimatization and transplantation into soil. (A) Control group plantlets (no REE treatment) were transplanted into soil after six weeks. (B) Plantlets treated with 2.0 mg/L $Ce(NO_3)_3$ were transplanted into soil after six weeks. Scale bars = 1 cm.

at 2.0 mg/L, it declined by 14.1% as compared with that in control group. At high $Ce(NO_3)_3$ concentrations (2.0-5.0 mg/L), the MDA contents increased in comparison with those of lower concentrations (0-2.0 mg/L). But, at 4.0 mg/L and 5.0 mg/L $Ce(NO_3)_3$, the MDA contents increased higher than that of control. Thus, it should be noted that the level of lipid peroxidation remained lowest at the most effective concentrations (5.0 mg/L $La(NO_3)_3$ and 2.0 mg/L $Ce(NO_3)_3$) than the level found in the control group.

Acclimatization

Through six weeks after acclimatization and transplantation into garden soil (Fig. 6), the rooted plantlets had a survival frequency of 90% in REE treatment group as well as 65% in non-REE group. The transplants treated with $La(NO_3)_3$ and $Ce(NO_3)_3$ showed good growth and had attractive leaves with golden-yellow venation (Fig. 6B).

Discussion

$La(NO_3)_3$ and $Ce(NO_3)_3$ at concentrations ranging from 1.0 to 5.0 mg/L showed positive effect on plantlet growth. This result is in agreement with previous reports that REEs have numerous biological effects on plant growth and development (d'Aquino et al. 2009), such as improvement of cell growth and enhancement of secondary metabolite synthesis (Yuan et al. 2002; Tyler 2004; Yuan et al. 2005; Feng et al. 2006). $La(NO_3)_3$ and $Ce(NO_3)_3$ also showed varied eects among apical buds, mid-stem segments and basal rhizome segments because absorption of specific REEs varies according to plant organ and growth environment (Jing et al. 2007; Peng et al. 2013). And the degree of growth stimulation also depends on cell type and culture growth stage (Wu et al. 2001; Peng et al. 2013). There are some possible reasons for the positive eect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on *A. roxburghii* shoot induction and plantlet growth. First, the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ is similar to the hormesis effect which can regulate the plant growth. Second, the positive eect is probably associated with an increase in the cell membrane permeability due to interactions of cells with $La(NO_3)_3$ and $Ce(NO_3)_3$. With the increase of membrane permeability, the cellular uptake, utilization and transformation of nutrients is enhanced (Feng et al. 1999; Wu et al. 2001), which thus promotes the growth of regenerated *A. roxburghii*. Third, due to their trivalent charges and thus higher charge density, REEs (coined the term "super-calcium"), can likely displace the divalent Ca, which has a lower charge density, at Ca-binding sites in biological molecules. The effects of REEs on the various calcium-mediated biological processes in plants have been investigated (Brown et al.1990; Burda et al. 1995). In their study, the authors have concluded that many enzymes, other functional proteins and cell membrane are affected by $La(NO_3)_3$ and $Ce(NO_3)_3$ at binding sites in cell membrane, thereby lead to the fast growth of plant.

Additionally, the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on regenerated *A. roxburghii* is probably due to the influence on chlorophyll contents. Some studies have shown that REEs could improve growth by enhancing photosynthetic rate of plants (Brown et al. 1990). It also has been reported that $Ce(NO_3)_3$ can catalyze the change of proto-chlorophyll into chlorophyll, and chlorophyll contents enhance over that of control (Ni 1995). The other study have suggested that REE in chlorophyll contents of spinach could increase absorption of nitrogen and phosphorus, and induce great synthesis of pre-compounds of chlorophyll (Liao et al. 1994). Our experimental results showed that $La(NO_3)_3$ could accelerate synthesis of chlorophyll, which further supported the view that REEs are some catalysts and play an indirect role in chlorophyll formation (Hong et al. 2002a, 2002b). In our study, the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on chlorophyll contents may lead to a series of physiological effects on growth of *A. roxburghii*.

Furthermore, SOD which can scavenge the organism harmful substances O_2, produced during metabolism, plays a vital role on the body antioxidant balance; CAT and POD could break down the body H_2O_2, providing antioxidant

defense mechanism for the plant. Therefore, SOD, CAT, and POD maintain a low level of free radicals and prevent free-radical toxicity. They participate in many important physiological activities and play a major role in plant metabolism regulation and environmental stress response (Peng et al. 2013). Previous studies have shown that REEs could stimulate POD and SOD activity (Guo et al. 2012; Peng et al. 2013), and reduce MDA contents, thus alleviating the oxidative damage (Ippolito et al. 2007; Wang et al. 2009). In our report, it was shown that $La(NO_3)_3$ treatment in suitable concentration could significantly enhance the SOD, CAT and POD activity, and reduce MDA contents of regenerated *A. roxburghii* seedlings. It suggested that suitable $La(NO_3)_3$ treatment could protect the cells from active oxygen damage, which was similar to the point that REEs could affect the function of superoxide dismutase by scavenging O_2 (Zeng et al. 2003; Wang et al. 2007; Ippolito et al. 2010), thereby protecting cell organelles and promoting plant cell growth (Wang et al. 2007). Results of $Ce(NO_3)_3$ treatment showed that low concentrations of $Ce(NO_3)_3$ (0-2.0 mg/L) promoted plant growth from apical buds of *A. roxburghii*, stimulated the activity of antioxidant enzymes (SOD, CAT and POD), and decreased MDA contents, whereas higher concentrations (2.0–5.0 mg/L) suppressed plant growth, inhibited those enzymes activity, and increased MDA contents. In general, it suggested that $Ce(NO_3)_3$ may stimulate plant growth and enzyme activity at lower concentrations but become toxic to the plants at higher concentrations. In conclusion, an appropriate concentration of $La(NO_3)_3$ or $Ce(NO_3)_3$ may enhance growth by reacting with certain enzymes in cells and cell membranes, thereby affecting enzyme function and altering membrane permeability. These changes in turn may enhance nutrient uptake and use and promote rapid growth of plants (Hong et al. 1999; Hong et al. 2000; Chen et al. 2003; Diatloff et al. 2008; Olivares et al. 2011; Huang et al. 2012).

Conclusions

Our study results revealed the effect of $La(NO_3)_3$ and $Ce(NO_3)_3$ on *A. roxburghii* shoot induction and plantlet growth. Better plantlet growth was obtained using 5.0 mg/L $La(NO_3)_3$ of culturing apical buds, with optimum $Ce(NO_3)_3$ concentrations of 2.0, 1.0, and 3.0 mg/L determined for treatment of apical buds, mid-stem segments, and basal stem segments, respectively. And the experimental results showed that the highest chlorophyll contents, enzymes activity (SOD, CAT and POD) and the lowest MDA contents of *A. roxburghii* were caused by the 5.0 mg/L $La(NO_3)_3$ solution and 2.0 mg/L $Ce(NO_3)_3$ solution from apical buds. Our results showed that the mechanism on promoting *A. roxburghii* regeneration and growth by adding an appropriate concentration of $La(NO_3)_3$ and $Ce(NO_3)_3$ was probably due to their effect on chlorophyll contents, enzymes activity (SOD, CAT and POD) and MDA contents. But, a difficult study on the accumulation rules of $La(NO_3)_3$ and $Ce(NO_3)_3$ inside plants and on how to keep balance among soil, moisture and plants after transplanting has not been made, affected by the rebellious and complicated factors such as soil ingredients, moisture, illumination, environment and so forth after transplanting. Further research is required to clarify the clear regulation mechanism of $La(NO_3)_3$ and $Ce(NO_3)_3$ on promoting plants regeneration and growth.

Materials and Methods

Explant Materials

Wild-collected seeds of *A. roxburghii* from Wuzhishan, Hainan Province, China were used to establish a sterile breeding system. All experiments were conducted on apical buds, mid-stem segments, and basal rhizome segments obtained from the resulting subcultured aseptic seedlings.

Lanthanum nitrate and Cesium Nitrate

Two REE compounds were used in this study: $La(NO_3)_3$ and $Ce(NO_3)_3$ obtained from Tianjin Kermel Chemical Reagent Co. Nitrate solutions were prepared in distilled water and added to the culture media before pH adjustment. All agents used were analytical grade.

Establishment of *in vitro* Cultures

Intact, ripe capsules of *A. roxburghii* were rinsed in water containing dilute detergent for 1 to 2 min. After trimming the carpopodia, capsules were rinsed with running tap water for 20 min. The capsules were then surface-sterilized under a laminar airflow cabinet by dipping into 70% ethanol for 30 s and 0.5% sodium hypochlorite for 20 min, followed by four to five washes with sterile water. The surface-sterilized capsules were slit longitudinally; the seeds were scooped out and thinly sown over the surface of half-strength Murashige–Skoog (MS) medium. The germinated seedlings were subcultured every few weeks for use in a series of experiments.

Shoot Induction and Seedling Growth

This portion of the study was conducted using apical buds, mid-stem segments, and basal rhizome segments obtained from the above-mentioned *A. roxburghii* aseptic seedlings. Apical bud portions consisted of 2-cm sections including two leaves and a single node; 1-cm mid-stem segments including a single node, and 3-cm basal rhizome segments including two nodes. Following collection, the apical buds, mid-stem segments, and basal rhizome segments were perpendicularly inoculated onto various formulations of half-strength MS media supplemented with 2 g/L activated charcoal and various concentrations (0-5.0 mg/L) of $La(NO_3)_3$ or $Ce(NO_3)_3$. Three apical bud portions or two stem segments were inoculated into each bottle, with three replicates consisting of ten bottles each performed per treatment.

Culture Conditions

Prior to inoculation, all media were supplemented with 30 g/L sucrose, solidified with 8 g/L agar, pH-adjusted to 5.8, and autoclaved

at 121°C and 120 kPa for 20 min. All cultures were incubated in a culture room at 25 ± 2°C and 55–60% relative humidity under 1,500-2,000 lux light intensity with a 12/12-h (day/night) photoperiod provided by cool white fluorescent lamps.

Chlorophyll, Enzymes and MDA Assay

The chlorophyll contents of regenerated *A. roxburghii* leaves (85 d) from apical buds were measured by SPAD-502Plus (made in Japan), and the SPAD values were collected. Also, leaves (1.0 g) of regenerated *A. roxburghii* (90 d) from apical buds were homogenized in 9 mL of ice-cold phosphate buer (0.1 mol/L, pH 7.4). The homogenate was centrifuged at 4000 rpm/min for 10 min at 2°C, and the supernatant was transferred for enzymes activity assay. SOD, CAT, POD activity and MDA contents were measured in strict accordance with the MDA, SOD, POD, and CAT kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) instructions.

Acclimatization and Field Transfer

Approximately 5-6 cm long, well-rooted plantlets were removed from the culture bottles and gently washed under running tap water to remove adhered medium. The plantlets were then transferred to rectangular baskets (40 cm × 30 cm) containing autoclaved wood chips, peat moss, and garden soil (2:3:1 v/v/v), pH 6.5-6.8, and immediately enclosed in transparent polythene bags (40 × 30 × 10 cm^3) to maintain high relative humidity (90%) and to prevent desiccation. The plantlets were irrigated daily, and initially maintained under culture room conditions of 25 ± 2°C. After 6 weeks, the acclimatized plants were transferred outdoors and placed under shade cloth. Fully grown, hardened plants (6-8 cm) were planted in the field under natural sunlight.

Data Collection and Statistical Analysis

After culturing of explant material for 30, 60, and 100 d, the following indicator data were collected: average height (cm) of apical buds, percentage shooting responses, proliferation times, and average shoot lengths (cm) of stem segments. These above data were expressed as the mean ± SE of three experiments, and significance of differences among means was assessed using Duncan's multiple range test ($P < 0.05$). Other data of chlorophyll contents, antioxidase enzymes activity and MDA contents were expressed as the mean ± SD, and significance of differences among means was assessed according to LSD multiple range test ($P < 0.05$). Data all were analyzed by using IBM SPSS version 21 statistics software.

Author's Contributions

YX designed experiments, performed lab work, analyzed data and drafted manuscript. YW and GG assisted to proofread manuscript. GZ helped to design experiments, revised manuscript and was the project leader. All authors have agreed to the contents of the manuscript and declare no conflicting interests.

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