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24-Epibrassinolide and 28-homobrassinolide, two brassinosteroids, inhibit protocorm-like body development in hybrid *Cymbidium* (Orchidaceae)

Jaime A. Teixeira da Silva[1,2]

[1] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa, 761-0795, Japan; [2] Current address: Miki cho post office, Ikenobe 3011-2, P. O. Box 7, Kagawa-Ken, 761-0799, Japan

*Corresponding author. Email: jaimetex@yahoo.com

Abstract Brassinolides, a sub-class of brassinosteroids, have demonstrated plant growth regulator (PGR)-like activity in other crops. Two brassinolides, 24-epibrassinolide (24-epiBL) and 28-homobrassinolide (28-homoBL), have shown some ability to induce somatic embryogenesis in a limited number of orchids, while they have primarily shown to mitigate the negative effects of biotic and abiotic stresses in several plants. In this study, the induction of new protocorm-like bodies (or *neo*-PLBs) in hybrid *Cymbidium*, an important ornamental, under the influence of 1, 2, 4, 8 or 16 μM of 24-epiBL and 28-homoBL (separately) was attempted using regular explants (half-PLBs) or PLB transverse thin cell layers (tTCLs) cultured on PGR-free Teixeira *Cymbidium* (TC) medium. tTCLs produced significantly fewer *neo*-PLBs than half-PLBs, which in turn produced significantly fewer PLBs on PGR-free medium supplemented with any concentration of 24-epiBL or 28-homoBL than on control TC medium with PGRs. Results of this study show that while brassinolides are unable to satisfactorily substitute for PGR-like activity, they can mitigate heavy metal- and salt-induced stress (unpublished data).

Key words

24-epiBL; 28-homoBL; brassinolide; brassinosteroid; PLB; Teixeira *Cymbidium* (TC) medium

Abbreviations: 24-epiBL, 24-epibrassinolide; 28-homoBL, 28-homobrassinolide; BL, brassinolide; BR, brassinosteroid; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; PLB, protocorm-like body; TC medium, Teixeira *Cymbidium* medium; tTCL, transverse thin cell layer

Brassinosteroids (BRs) are a group of polyhydroxy steroidal lactones of plant origin that regulate growth and development (Clouse and Sasse 1998), ubiquitous in plants, eliciting a wide spectrum of physiological responses and pleiotropic effects, including somatic embryogenesis, seed germination, rhizogenesis, growth, flowering and senescence (Choe 2006) as well as a wide range of physiological responses in plants, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth inhibition, induced synthesis of ethylene, activation of proton pump, xylem differentiation, synthesis of nucleic acids and proteins, activation of enzymes and photosynthesis (Yu *et al*. 2004; Hayat *et al*. 2007; Clouse 2008). BRs promoted adventitious shoot regeneration from segments of cauliflower hypocotyls (Sasaki 2002) and improve embryogenic tissue initiation in conifers and rice (Pullman *et al.* 2003). BRs are widespread in plants including dicots, monocots, gymnosperms, ferns and algae, and exist in all parts of the plant (Khripach *et al*. 2000; Rao *et al*. 2002). They are mainly produced in pollen but are also present in seeds, stems, young leaves and buds, but in lower amounts than pollen (Fujioka *et al*. 1998). BRs regulate cell elongation and divisional activities by activating cell wall-loosening enzymes, increasing thus the synthesis of cell wall and membrane materials (Khripach *et al.* 2000). The cell wall-loosening enzymes activate H^+-ATPases, which acidify the apoplast, and thereby possibly enhancing seedling growth (Haubrick and Assman 2006).

BRs also confer resistance to plants against various biotic and abiotic stresses (Nunez *et al*. 2003; Sasse 2006; Ali *et al*. 2008; Kartal *et al*. 2009; Vardhini *et al.* 2010), including thermal stress (Kurepin *et al*. 2008), salinity (Ali *et al*. 2007), chilling injury (Liu *et al*. 2009) and heavy metal stress (Arora *et al*. 2008; Hayat *et al*. 2010), by modulating the activities of antioxidative enzymes involved in the Asada-Halliwell pathway (Bajguz and Hayat 2009; Arora *et al*. 2010). High concentrations of metals are toxic and severely interfere with physiological and biochemical functions of plants (Triantaphylidès and

Havaux 2009), and induce oxidative stress through formation of reactive oxygen species (ROS). BRs, which also regulate cell division and cell elongation, vascular differentiation, reproductive development and modulation of gene expression (Bajguz 2007; Park *et al*. 2010), are present in very low concentrations throughout the plant kingdom and are extensively disseminated in reproductive and vegetative tissues (Bajguz and Tryten 2003; Symons *et al.* 2007). Brassinolide (BL) is an important naturally occurring BR with strong biological activity that induces a large range of cellular responses, including plant growth, seed germination and nitrogen fixation (Fujioka 1999). 24-epiBrassinolide (24-epiBL) improves the resistance of plants towards cold, pathogens and salt stress (Kulaeva *et al.* 1991).

Inspired by studies on the use of 24-epiBL on *Pinus caribaea*, which could stimulate somatic embryogenesis in the presence of 2,4-dichlorophenoxy acetic acid (Malabadi *et al*. 2011), or of *Liparis elliptica* (Malabadi *et al*. 2009) or *Cymbidium bicolor* (Malabadi *et al*. 2008), this study was established since in orchids, protocorm-like bodies are considered to be somatic embryos (Teixeira da Silva and Tanaka 2006). 24-epiBL and 28-homobrassinolide (28-homoBL) could also modulate the toxic effects of chromium-, cadmium- and mercury-infected soil when radish (*Raphanus sativus*) seeds were sown, with an increase in the activity of several antioxidant enzymes (Anuradha and Rao 2007; Choudhary *et al*. 2009; Randhawa *et al*. 2010; Sharma *et al*. 2012). 28-homoBL enhanced protein content in *Brassica juncea* seedlings under Zn metal stress (Sharma *et al.* 2007) and enhanced protein content in *Oryza sativa* (Maheshwari and Dubey 2008) and *Vigna radiata* (Jaleel *et al.* 2007) under heavy metals stress. 24-epiBL and 28-homoBL enhanced the protein content in *O. sativa* (Anuradha and Rao 2003) and in wheat (Kulaeva *et al.* 1991), and improved the growth of Al-stressed mung bean seedlings by increasing the rate of photosynthesis and carbonic anhydrase activity (Ali *et al.* 2007). 24-epiBL reduced the effect of water stressed tomato plants (Yuan *et al*. 2010). Thus, in this study, 24-epiBL and 28-homoBL were employed (the latter differs from the former by the substitution at C-24 and its configuration at C-24; Fig. 1).

The use of BRs, in particular 24-epiBL and 28-homoBL, is rare in orchid tissue culture research (Hossain *et al*. 2013; Teixeira da Silva 2013a), and almost non-existent in *Cymbidium* research, except for *Cymbidium bicolor* (Malabadi *et al*. 2008). The protocorm-like body (PLB) is a somatic embryo in orchids (Teixeira da Silva and Tanaka 2006) whose neo-formation into new or *neo*-PLBs can be controlled through the use of thin cell layers, or TCLs (Teixeira da Silva 2013b), which are also used in this study in addition to standard explants, namely half-PLBs (i.e., transversally dissected PLBs after removing the shoot tip).

Materials and Methods

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

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) were cultured and propagated on TC medium as outlined in Teixeira da Silva (2012). TC medium contains 0.1 mg/l NAA, 0.1 mg/l Kn, 2 g/l tryptone and 20 g/l sucrose. TC medium was solidified with 8 g/l Bacto agar (Difco Labs., USA) and pH was adjusted to 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min (Teixeira da Silva *et al*. 2005; Teixeira da Silva and Tanaka 2006). Light 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 μmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Two types of explants (10/flask) were used for *neo*-PLB induction and proliferation in all experiments: a) longitudinally bisected PLB (3-4 mm in diameter) segments (hereafter termed half-PLBs), and b) transverse thin cell layers (tTCLs) prepared according to Teixeira da Silva (2013b). As indicated in other papers, recommendations related to culture conditions, media, and PLB induction, formation and proliferation were followed from the *Cymbidium* literature, specifically pertaining to medium formulation (Teixeira da Silva *et al*. 2005), abiotic factors (Teixeira da Silva *et al*. 2006a) and biotic factors (Teixeira da Silva *et al*. 2006b).

To TC medium free of PGRs, the following concentrations of 24-epiBL and 28-homoBL (Fig. 1) were added: 1, 2, 4, 8, 16 μM. Sincere preliminary trials indicated that 0.01-0.5 μM of both BLs had no effect on *neo*-PLB formation, and that concentrations in excess of 50 μM had a negative effect on *neo*-PLB formation (data not shown), the five tested concentrations within the range of 1-16 μM was selected. The number of *neo*-PLBs per half-PLB or per PLB tTCL were measured after 45 days in culture. Usually, 30 days would only allow for the observation of premature *neo*-PLBs, 60 days would already result in the formation of shoot tips from *neo*-PLBs while by 120 days shoots and roots will have fully developed, hence the choice of 45 days (Teixeira da Silva and Dobránszki 2013).

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

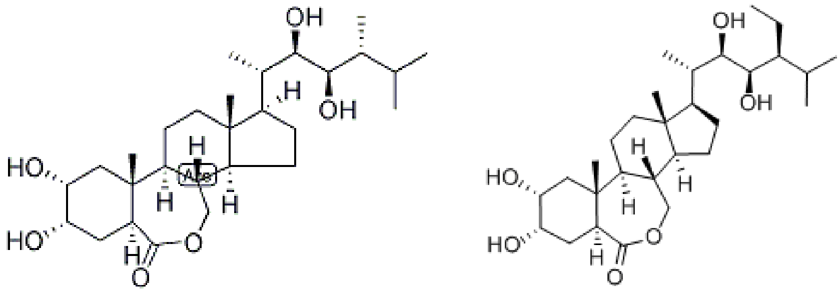

Fig. 1 Chemical structure of 24-epibrassinolide (24-epiBL; left; www.chemexper.com) and 28-homobrassinolide (28-homoBL; bottom; www.chemicalbook.com) used in this study.

Results and Discussion

tTCLs produced significantly fewer *neo*-PLBs than half-PLBs and half-PLBs produced significantly fewer PLBs on PGR-free medium supplemented with any concentration of 24-epiBL or 28-homoBL than on control TC medium with PGRs (Table 1; Fig. 2). In this study, while BRs were unable to satisfactorily substitute for PGR-like activity (Table 1), they could still induce *neo*-PLB formation in the absence of PGRs, and can mitigate heavy metal- and salt-induced stress (unpublished data).

Table 1

Effect of plant growth regulators on *neo*-PLB formation from half-PLB or PLB tTCL culture of hybrid *Cymbidium* Twilight Moon 'Day Light'

Medium composition	Concentration (µM)	Explants forming *neo*-PLBs (%)	Number of *neo*-PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
Half-PLBs on: TC (control)		100 a	8.3 a	526 a
PLB tTCLs on: TC (control)		100 a	2.1 e	188 e
Half-PLBs on: TC minus PGRs		100 a	1.2 f	321 d
PLB tTCLs on: TC minus PGRs		100 a	0.3 g	81 fg
Half-PLBs on: TC + 24-epiBL	1	100 a	6.7 b	483 b
	2	91 b	6.3 b	437 bc
	4	88 b	5.8 bc	401 c
	8	71 bc	5.1 c	356 cd
	16	33 d	2.3 e	193 e
Half-PLBs on: TC + 28-homoBL	1	94 ab	6.2 b	468 b
	2	86 b	5.8 bc	416 c
	4	83 b	4.1 d	325 d
	8	36 d	1.3 f	127 ef
	16	14 e	0.4 g	77 g
PLB tTCLs on: TC + 24-epiBL	1	96 ab	1.9 ef	173 e
	2	84 b	1.4 f	153 ef
	4	64 c	0.9 fg	106 f
	8	19 e	0.3 g	74 g
	16	3 f	0.1 g	60 g
PLB tTCLs on: TC + 28-homoBL	1	89 b	1.4 f	154 ef
	2	74 bc	1.1 fg	121 ef
	4	42 cd	0.5 fg	88 fg
	8	11 ef	0.1 g	62 g
	16	0 f	0 g	54 g*

Mean values followed by the same letter in the same column (i.e., across explants and chemical compounds) are not significantly different based on DMRT (P = 0.05). See text for media constituents. n = 90 (9 (3 × 3 blocks) Petri dishes × 10 for each treatment).
24-epiBL, 24-epibrassinolide; 28-homoBL, 28-homobrassinolide; PGR, plant growth regulator (in this table refers to NAA and kinetin); PLB, protocorm-like body; TC, Teixeira *Cymbidium* medium (Teixeira da Silva 2012), includes 0.1 mg/l α-naphthaleneacetic acid (NAA) and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose (see reference for modified micro- and macro-nutrients); tTCL, transverse thin cell layer.
* Vestigial mass of original explant, thus the value would never be 0, even though no *neo*-PLB formation.

24-epiBL has been shown to have various effects on plant growth and amelioration of plant stress. 24-epiBL improved *Brassica juncea* growth following the activation of antioxidant enzymes (Arora et al. 2011), possibly due to an increase in transcription and/or translation processes of specific genes related to stress tolerance (Fariduddin *et al*. 2004; Kagale *et al.* 2007). BRs increased DNA, RNA and protein contents of *Chlorella vulgaris* (Bajguz 2000) and in *Brassica napus* and tomato, 24-epiBL improved thermotolerance and accumulated heat-shock proteins (Dhaubhadel *et al*. 1999, 2002).

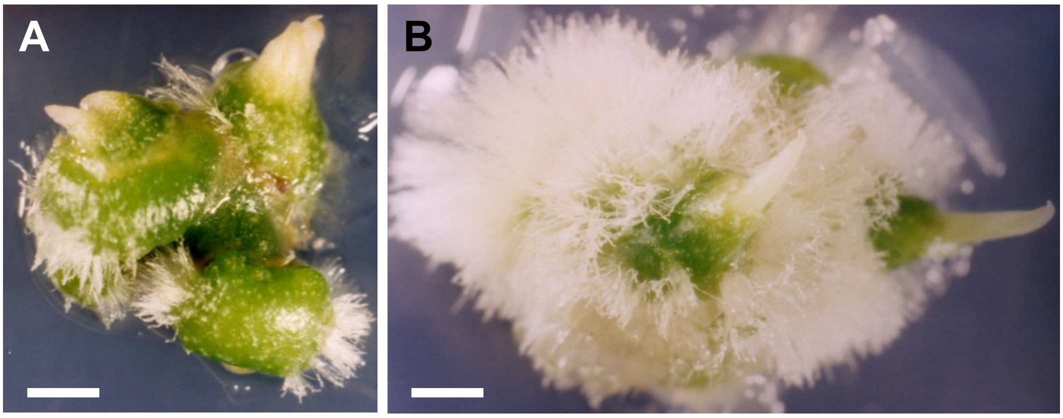

Fig. 2 Growth of *neo*-PLBs on control TC medium 45 days after culture in the presence of PGRs (A) or in the presence of 4 µM 24-epiBL showing the excessive formation of hairy trichomes on the surface of *neo*-PLBs (B). Scale bars = 1 mm (A); 2 mm (B).

Mazorra *et al*. (2002) found that CAT activity in rice was enhanced under the influence of BRs. ROS generated by heavy metals could be alleviated by BR treatments (Almeida *et al*. 2005; Hayat *et al*. 2007). Liu *et al*. (2009) demonstrated that epi-BR treatment significantly enhanced antioxidant enzyme activity and antioxidant content in *Chorispora bungeana* under chilling stress. One possible mechanism involved in reducing toxicity is the chelation of metal ions by ligands, including amino acids, organic acids, peptides or polypeptides (e.g., Arora *et al.* 2008). 28-HomoBL ameliorated Ni toxicity by increasing the activities of several antioxidant enzymes: superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase and glutathione reductase (Bhardwaj *et al.* 2008).

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