- Short communication

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The Phloroglucinol Conundrum: Increase in Root Growth of Hybrid *Cymbidium* (Orchidaceae) with no Toxic Effect on Protocorm-like Body Formation

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Research on orchid roots is limited. Yet, the ability to artificially increase root mass could be a valuable technique for applied purposes in the future, for example in the production of root-specific secondary metabolites. In this study, phloroglucinol (PG), a known rooting enhancer in many plants and an inducer of phenolic substances, was used to assess the response of excised roots and also protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light'. When half-PLBs (i.e. transversally cut PLBs) were cultured on Teixeira *Cymbidium* (TC) No. 1 medium, the development of new PLBs or *neo*-PLBs was not enhanced by the addition of any concentration of PG. In fact, 1-8 mg/l PG was toxic to *neo*-PLB formation, i.e. the formation of new PLBs was reduced relative to the control. In contrast, PG at < 8 mg/l significantly enhanced the length and fresh weight of roots while 8 mg/l was toxic to root growth and development. When PG was applied at 2 mg/l in liquid TC medium, root fresh weight increased by 149%. Control roots not exposed to PG turned yellow and died. Although the applications of PG to orchid tissue culture is unexplored, there is a strong possibility for its application in orchid biotechnology where auxins or hairy roots induced by *Agrobacterium rhizogenes* might not work.

Most orchid biotechnologists would not pay much attention to roots, except for studies involving symbiotic interactions with microorganisms such as plant growth-promoting *Rhizobacteria*. Except for such cases, the roots of orchids remain relatively unexplored and unknown organs, despite the extent of biotechnological studies that exist on orchids (Hossain et al. 2013). Most root-related studies in orchids pertain to greenhouse studies related to irrigation or

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fertilization, phytopathology or cross sectional studies showing histological structure. One might ask, why might there be a need to mass produce orchid roots? At first, one immediate response could be that several orchids are medicinal and contain many important compounds and secondary metabolites (reviewed in part by Teixeira da Silva 2013a). As for other plants, different organs within the same plant have a different capacity to form metabolites, and even the same metabolite can be produced in vastly different quantities in aerial organs (e.g. leaves or flowers) *versus* roots, e.g., essential oils. Thus, even though the exact importance of increasing root mass might not be very apparent or evident at the moment, by creating protocols that can increase the mass production of roots could serve a future purpose with unknown applications. There are not many ways to generate roots *de novo* from roots or other organs, or to mass produce roots. Some possibilities include the use of auxins or the induction of hairy roots by *Agrobacterium rhizogenes*. A third option involves the use of chemical inducers using compounds that are not auxins. One such compound is phloroglucinol (1,3,5-trihydroxybenzene; PG), which is a degradation product of phloridzin, has growth-promoting properties (Teixeira da Silva et al. 2013). In this study, *Cymbidium* has been used as an emerging model plant due to its favorable response *in vitro* and clonal propagules, the protocorm-like body (PLBs), which are equivalent to orchid somatic embryos (Teixeira da Silva 2013b; Teixeira da Silva and Dobránszki 2013). When PLBs are encapsulated, the resulting synthetic seeds or synseed (Teixeira da Silva 2012a) can be useful for cryopreservation (Sharma et al. 2013).

PG has been used in some manner or form in the *in vitro* propagation of at least 33 plant genera (incl. about 40 - 50 species or cultivars in total) (Teixeira da Silva et al. 2013). In most of those studies, PG was not studied in isolation. Rather, it was added as an additive to a defined medium, usually in addition to other media additives. Approximately 60% of those studies resulted in improved rooting, about 30% in enhanced shoot induction or formation, while in the remaining 10% of studies, improvement in callus formation or somatic embryogenesis was observed. However, one study, and in fact the only one to date conducted thus far on an orchid, was the improved recovery of cryopreserved *Dendrobium nobile* seeds or protocorms (seed-derived propagules) when PG was added to the cryopreservation medium at 1% (Vendrame and Faria 2011; Galdiano et al. 2012).

Capitalizing upon this gap in information that exists with respect to orchids, and using the assumption that PG would have some effect on organogenesis, the effect of PG on PLB induction and proliferation as well as root growth was assessed. Interestingly, most of the studies that indicated an improved

organogenic response in fact did not include systematic studies to assess the optimal concentration of PG. Rather, most of them used a single concentration, usually based on a previously reported protocol and thus, most studies do not indicate what negative consequences, or toxic effects, that PG might have on plant growth. Many factors influence the outcome of the PLB developmental program, including the choice of plant growth regulator (PGR), culture conditions, or explants used, with new PLBs or *neo*-PLBs forming from PLBs (Teixeira da Silva and Tanaka 2006, 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, 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, Japan) originally developed spontaneously from shoot-tip culture on Vacin and Went (VW, 1949) agar medium without PGRs, were induced and subcultured (PLB induction and proliferation medium or VW_{PLB}) every two months on TC medium (Teixeira da Silva 2012b). TC, which contains unique levels of macro- and micronutrients, and which was supplemented with 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), according to procedures and advice outlined by Teixeira da Silva et al. (2005) and Teixeira da Silva and Tanaka (2006). All media were adjusted to pH 5.3 with 1N 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 aluminum foil, at 25°C, under a 16 hrs photoperiod with a light intensity of 45 μmol/m^2/s provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLB (3 - 4 mm in diameter) segments (hereafter termed half-PLBs), 15/flask, were used as explants for PLB induction and proliferation and for all experiments. Culture conditions and media followed the recommendations previously established for medium formulation, biotic and abiotic factors and for PLB induction, formation and proliferation (Teixeira da Silva 2012a, 2012b).

The effect of PG on *neo*-PLB induction from half-PLBs was assessed by adding 0 (control), 1, 2, 4 and 8 mg/l PG (Sigma-Aldrich) to TC medium. Similarly, and simultaneously, 5-mm long roots with intact root tips were excised with a feather blade from 6-month-old shoots growing on plant growth regulator-free Hyponex medium solidified with 7 g/l agar (Fig. 1, STEP 1). Using excised root tips (Fig. 1, STEP 2), the effect of PG 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-free 10 roots with intact root tips were cultured in 25 ml of liquid TC medium containing the same concentration of PG as the solid medium trials and placed on a shaker at constant 84 rpm under the same light and temperature conditions as solid TC medium (Fig. 1, STEP 3). 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). Plants and roots were photographed using stereo light microscopy and/or a digital camera. Experiments were statistically designed and data were analyzed as per Teixeira da Silva (2012a, b) and significant differences between means were assumed at $P \leq 0.05$.

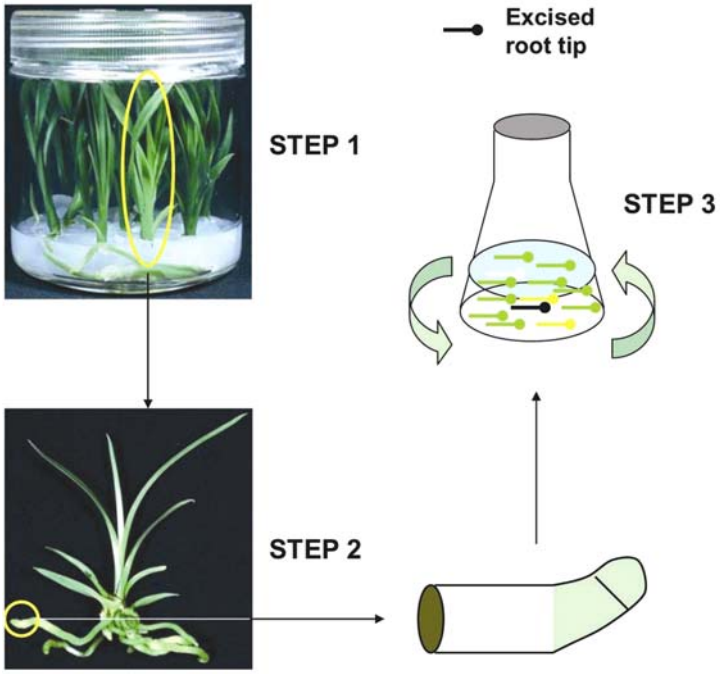

Fig. 1. Experimental design for assessing the effect of phloroglucinol (PG) on root growth and development. In STEP 1, mature plants, usually of 6 - 8 cm in height, following culture on plant growth regulator-free Hyponex medium solidified with 7 g/l agar to stimulate thick roots, serve as the donor plants for this experiment. In STEP 2, mature plants are gently removed from the agar, remaining agar is washed off gently with sterile distilled water, and roots, including the intact root tip, are excised under sterile conditions on a clean bench. This process should be conducted rapidly to avoid oxidation of root tissue and subsequent browning in response to tissue damage. Excised roots (15/flask) are placed in 25 ml of TC (Teixeira da Silva 2012b) liquid medium containing the same concentration of PG as the solid medium trials and placed on a shaker at constant 84 rpm under the same light and temperature as solid TC medium, i.e., 25°C, 16 hrs photoperiod, light intensity = 45 μmol/m^2/s. 100 ml Erlenmeyer flasks double-capped with aluminum foil are used. In this experiment, shaking took place for 3 months.

The key finding of this paper is that PG reduced *neo*-PLB formation but increased root growth. All concentrations of PG significantly decreased the number of *neo*-PLBs that formed on the surface of half-PLBs, the percentage of half-PLBs forming *neo*-PLBs and the fresh weight (mg) of half-PLB + *neo*-PLBs relative to the control (PG-free TC medium) (Table 1). In contrast, PG between 1 and 8 mg/l significantly increased root weight and root length relative to the control after culture for 60 days but this increase was particularly acute in liquid

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

Treatment	% of half-PLBs forming *neo*-PLBs	Number of *neo*-PLBs formed per half-PLB	Fresh weight (mg) of half-PLB + *neo*-PLBs	Hyperhydricity[2]
TC + 0 mg/l PG[1]	100 a	8.3 a	526 a	-
TC + 1 mg/l PG	67 b	6.1 b	328 b	-
TC + 2 mg/l PG	23 c	3.2 c	181 c	-
TC + 4 mg/l PG	6 d	0.3 d	71 d	-
TC + 8 mg/l PG	0 d	0 d	56 d[3]	-

Mean values followed by the same letter in the same column are not significantly different based on DMRT (P = 0.05). n = 90 (10 × 3 × 3). PG = Phloroglucinol; PLB = Protocorm-like body; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012b). [1] control with no PG. [2] Hyperhydricity: - = No occurrence; + = occurrence. [3] In fact, the average fresh weight of initial half-PLB explants is 54 mg.

medium (Table 2, Fig. 2), with as much as a 149% increase in fresh weight when culture in liquid TC with 2 mg/l PG. Even though the secondary metabolites or potential nutritional qualities of *Cymbidium* roots have not been explored, this protocol could serve as one method for mass producing roots (or for increasing the fresh weight of roots) of this or other orchids for which valuable compounds have been identified. It is important to emphasize that only the terminal 5-mm of roots was used to avoid possible interference from shoot-derived reserves, i.e. root development in Fig. 2 (right) is derived exclusively from medium composition, with enhanced growth and biomass resulting directly as a consequence of PG.

PG has had in most plants studied to date (Teixeira da Silva et al. 2013), a positive effect on rooting, but almost invariably, this effect has been synergistic in the presence of other auxins or PGRs, and rarely has the effect of PG exclusively been studied on root growth or development. The only study on an orchid, *Dendrobium nobile*, using PG indicated the improved recovery of cryopreserved encapsulated seed or protocorms (Vendrame and Faria 2011, Galdiano et al. 2012). PG, a precursor in the lignin biosynthesis pathway, was

used to prevent hyperhydricity in micropropagation by providing precursors which normally are synthesized at low levels or not synthesized at all in hyperhydric tissues and by increasing the activity of enzymes involved in lignin synthesis (Phan and Hegedus 1985, Ross and Grasso 2010). Most plant responses

Table 2. Root growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to phloroglucinol after 60 days in culture.

Treatment	Length of roots (mm) [Increase in root length (%)]	Fresh root weight (mg) [Increase in root weight (%)]	Hyperhydricity[2]
Initial roots (day 0 of culture)	5.0 c [0][3]	103 e [0][4]	-
TC_S + 0 mg/l PG[1]	5.3 c (6)	147 cd (43)	-
TC_S + 1 mg/l PG	5.9 b (18)	163 c (58)	-
TC_S + 2 mg/l PG	7.8 a (56)	181 bc (76)	-
TC_S + 4 mg/l PG	5.6 bc (12)	126 d (22)	-
TC_S + 8 mg/l PG	5.3 c (6)	118 d (15)	-
TC_L + 0 mg/l PG[1]	5.4 bc (8)	192 bc (86)	+
TC_L + 1 mg/l PG	5.6 bc (12)	201 b (95)	+
TC_L + 2 mg/l PG	6.2 b (24)	256 a (149)	+
TC_L + 4 mg/l PG	6.0 b (20)	237 ab (130)	+
TC_L + 8 mg/l PG	5.8 b (16)	179 bc (74)	+

Mean values followed by the same letter in the same column are not significantly different based on DMRT (P = 0.05) when compared to initial length[3] and fresh weight[4] of roots at day 0 of culture. n = 90 (10 × 3 × 3). PG = phloroglucinol; PLB = Protocorm-like body; TC = Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva 2012b); TC_S = solid (8 g/l Bacto agar) TC medium; TC_L = Liquid TC medium. [1]control with no PG. [2]Hyperhydricity: - = No occurrence; + = Occurrence

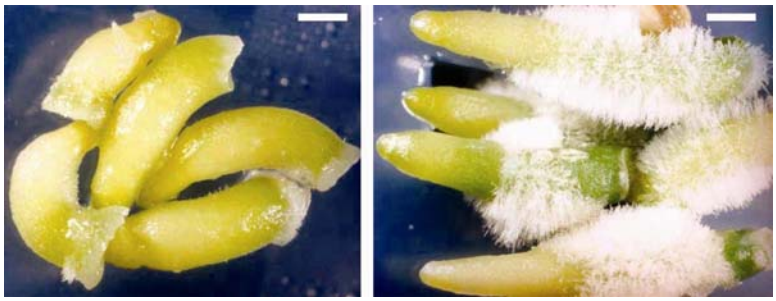

Fig. 2. Root growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' was enhanced following the addition of 2 mg/l phloroglucinol (PG) (right) relative to (left) no PG in solid basal TC (Teixeira da Silva 2012b) medium. Bars = 1 mm.

(particularly those studies involving enhanced rooting in the presence of an auxin; Teixeira da Silva et al. 2013) to phenols, including PG, involve a synergism with auxins, particularly IAA, thus the mode of action is likely dependent on the

regulation of internal IAA levels in which oxidative catabolism of IAA results in the loss of auxin activity (Normanly et al. 2004). PG has occasionally been found to have inhibitory effects (George et al. 2010) although is difficult to propagate hardwood species like apple, PG is essential for root formation (Dobránszki and Teixeira da Silva 2010, Magyar-Tábori et al. 2010). These apparently contradictory effects of PG on plant organogenesis *in vitro* are understandable if we observe the results of this study. At only limited concentrations was PG stimulatory to root growth, while PG was, overall, toxic to *neo*-PLB formation (Tables 1, 2). This study confirms that PG may be considered as a new class of plant growth regulator.

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