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INVITED REVIEW

Phloroglucinol in plant tissue culture

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Abstract In plant tissue culture research, there is a constant need to search for novel substances that could result in better or more efficient growth *in vitro*. A relatively unknown compound, phloroglucinol (1,3,5-trihydroxybenzene), which is a degradation product of phloridzin, has growth-promoting properties. Phloroglucinol increases shoot formation and somatic embryogenesis in several horticultural and grain crops. When added to rooting media together with auxin, phloroglucinol further stimulates rooting, most likely because phloroglucinol and its homologues act as auxin synergists or auxin protectors. Of particular interest is the ability of phloroglucinol—a precursor in the lignin biosynthesis pathway—to effectively control hyperhydricity through the process of lignification, thus maximizing the multiplication rate of woody species and other species that are difficult to propagate. Phloroglucinol has also been used to improve the recovery of cryopreserved *Dendrobium* protocorms, increasing the potential of cryopreservation for application in ornamental biotechnology. Phloroglucinol demonstrates both cytokinin-like and auxin-like activity, much like thidiazuron, and thus has considerable potential for application in a wide range of plant tissue culture studies.

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Introduction

Rationale for the use of phloroglucinol in plant tissue culture. Phloroglucinol (PG, 1,3,5–trihydroxybenzene or phloroglucin (PG tautomer); Fig. 1), is not well-known to many plant tissue culture scientists. Although PG is often used as a supplement to other plant growth regulators (PGRs) *in vitro*, it has rarely been the focus of tissue culture or developmental studies simply because its true effect has usually been masked by the presence of other, more commonly used PGRs. However, a compilation of those studies in which PG has been used *in vitro* for inducing or improving different plant developmental events clearly shows PG to be a much more powerful and interesting compound than previously recognized (Table 1). Indeed, a wealth of PGRs is already used quite effectively in plant tissue culture for most plant species and the reader might pertinently ask, "Why is PG necessary or important"? However, there are still many plant species for which no effective *in vitro* propagation protocol has been described. Moreover, the ineffective recovery of cryopreserved tissue and the hyperhydricity of tissue cultures caused by poor lignification can often hamper the effectiveness of current protocols for hardwood species or even herbaceous plants that can easily become hyperhydric *in vitro*. There are other plausible solutions to such deficiencies in plant tissue culture, such as the use of gas-permeable vessels or CO_2 enrichment (Teixeira da Silva et al. 2005a, b), but the costs and technical requirements of such options still remain beyond the means of most plant tissue culture scientists. Thus, alternative chemical means of dealing with these issues are required. We have found that PG has much more far-reaching effects and wider

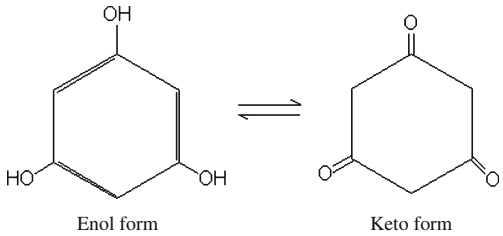

Figure 1. Equilibrium of enol and keto forms of phloroglucinol.

potential applications than simply serving as a PGR in *in vitro* growth and development.

By showing the reader how PG has been used *in vitro* and its effects on various tissue culture conditions and developmental stages, we hope to promote a new avenue of research in plant tissue culture. This research could provide solutions for several important problems that are currently bottlenecks to biotechnological approaches such as genetic transformation, growth in bioreactors, or *in vitro* molecular biology studies. For example, one of the problems underlying the rapid advance of transgenic studies in orchids (Teixeira da Silva et al. 2011) is the extremely sensitive nature of material to the PGRs currently used *in vitro*, and the use of PG could provide a viable alternative to current protocols that have either failed or have produced poor results. Moreover, the almost total lack of viable protocols in orchid for cryopreservation and successful regeneration of cryopreserved material could be reversed by the use of PG, as recently demonstrated for *Dendrobium* hybrid seeds (Galdiano et al. 2012). Thus, this review provides an overview of the literature on the use of PG in plant tissue culture and developmental studies and highlights areas in which PG presents a realistic alternative to more widely used PGRs.

Structure and function. PG, one of the degradation products of phloridzin (the 2′-glucoside of phloretic acid), is a phenolic compound known for its growth-regulating properties (James and Thurbon 1979; Kumar et al. 2005). PG crystallizes from water as the dihydrate, which has a melting point of 116–117°C, but the anhydrous form melts only at 218–220°C. The latter form sublimes but does not boil intact (http://pubchem.ncbi.nlm.nih.gov/). This makes PG resistant to autoclaving and thus highly applicable to plant tissue culture. The fact that PG does not need to be filter sterilized also simplifies its use *in vitro*. Knowledge about PG's physiological relevance *in planta* is limited, although apple plant tissues are known to accumulate high amounts of PG, possibly as a response to the invasion of various pathogens (Gosh et al. 2010); this suggests that PG is an important component of the response to plant pathogen attack.

PG is a benzenetriol (a trinitrobenzene derivative) that occurs in nature in the A-ring of flavonoid compounds and many other plant phenolic compounds. Naturally occurring bioactive PG compounds have been isolated from different sources such as plants, marine algae (families Phaeophyceae and Fucaceae), and microorganisms. PG derivatives are a major class of secondary metabolites that occur widely in the Myrtaceae as well as in several other families, including the Guttiferae, Euphorbiaceae, Aspidiaceae, Compositae, Rutaceae, Rosaceae, Clusiaceae, Lauraceae, Crassulaceae, Cannabinaceae, and Fagaceae (Pal Singh and Bharate 2006). The evidence suggests that naturally occurring or synthetically produced PG or PG derivatives may provide new classes of PGRs, opening up new avenues of research for plant tissue culture and development, much like the compounds in smoke water were classified into a new class of PGRs, the karrikins, just 3 yr ago (Chiwocha et al. 2009).

Broader applications in plant biology. Although there are many uses of PG in the medical and pharmaceutical industries and research, their application in plant biology is still very limited. Phytochemical investigations of several species in the genus *Hypericum*, used in traditional medicine, have revealed the presence of PG derivatives, usually through the use of HPLC-MS (Kim et al. 2003), which possess cytotoxic, apoptotic, antibacterial, antioxidant, antidepressant, or anti-inflammatory bioactivities (Winkelmann et al. 2003; Athanasas et al. 2004; Ahn et al. 2007; Crockett et al. 2008; Kong et al. 2009; Saddique et al. 2010; Odabas and Çirak 2011; Stein et al. 2012).

PG isolated from lemon peel, a waste product in the citrus industry, was active against oral bacteria that cause dental caries and periodontitis, such as *Streptococcus mutans*, *Prevotella intermedia*, and *Porphyromonas gingivalis* (Miyake and Hiramitsu 2011). PG would thus provide potential application for use as an antimicrobial agent both *in vitro* and *in planta*. The half-life of PG and its derivatives is unknown, although its ability to be autoclaved makes it an attractive alternative to antibiotics. It might also serve to "sterilize" tissue culture media without the need for autoclaving, much like liquid chlorine dioxide (Cardoso and Teixeira da Silva 2012). PG is currently used in the Wiesner test (PG-HCl reagent), in which plant lignin-containing material stains red (Christiernin et al. 2005; Galla et al. 2011). If suitably modified, and if its toxicity in tissues could be avoided during plant growth, PG could serve as a potential *in situ* marker for identifying tissue lignification without the need to sacrifice tissue for histological analyses.

Applications of Phloroglucinol in Plant Tissue Culture and Micropropagation

Hyperhydricity: a root cause of poor plant development in vitro. Micropropagated shoots *in vitro* can become

Table 1. Studies in which phloroglucinol has been used in the effective tissue culture and micropropagation of different plant species

Genus, species and cultivar	Explant used (type, size, origin)	Basal medium	PGR (type and concentration)	Other culture conditions	Phloroglucinol concentration (μM)	Effect on tissue culture, development, etc.	Reference
Abies nordmanniana	Cell cultures	Half-strength BLG	40.07 μM ABA	131.46 μM maltose	39.65	Proliferation of embryos without maturation	Find et al. (2002)
Acca sellowiana (Berg.) Burret 'Guayabo del País'	Nodal segments	MS	BAP 0.44 μM + NAA 0.054 μM	3% sucrose, 0.7% agar, multiplication with 9.8 and 19.6 μM 2iP	317.2	Reduced hyperhydricity, increased lignification	Ross and Grasso (2010)
Achyrocline flaccida (Weinm.) D.C 'Marcela'	Nodal segments	MS	None	555 μM *myo*-inositol glycine, 2% sucrose	317.2	Reduced hyperhydricity, increased lignification, increased multiplication rate	Ross and Castillo (2010)
Arnica montana	Nodal segments	MS	5.3 mM NAA, 5.0 mM 2iP	0.2 mM adenine sulphate; 1.0 ml L^{-1} maize extract	600	Plant multiplication and root induction and growth	Buthuc-Keul and Deliu (2001)
Asparagus racemosus	Single node segments	Half-strength MS	1.61 μM NAA + 0.46 μM kinetin	98.91 μM adenine sulfate, 500 mg L^{-1} malt extract	79.3–198.25	Enhancement of rooting frequency	Bopana and Saxena (2008)
Bacopa monnieri L.	Leaf and internode explants	MS	4.9 μM IBA	Incubated in light (50 μmol m^{-2} s^{-1})	3.97	Induction of more and longer roots	Ceasar et al. (2010)
Camellia sinensis (L).O. Kuntze	Immature cotyledon explants	MS	1 μM NAA or 2,4-D, + 0.5 μM BA or kinetin	1 μM brassin	10	Improved conversion of callus to somatic embryos	Ponsamuel et al. (1996)
Capsicum annum L.	Seedlings	MS	26.63 μM BA, 2.28 μM IAA	Bud induction phase in continuous light	400	Enhancement of bud induction	Kumar et al. (2005)
Capsicum annum L.	Buds	MS	22.2 μM BA, 5.7 μM IAA	Shoot bud elongation with 0.87 μM GA_3	39.64	Shoot bud elongation	Bairwa et al. (2012)
Carthamus tinctorius, *C. arboresens*	Leaf and stem explants	MS	4.4 μM BA	IBA also required for rooting in light	634.4	Enhancement of rooting	Sujatha and Kumar (2007)
Citrus rootstocks: alemow, sour	Nodal explants	MS/DKW	4.4 μM BA	58.43 μM sucrose	80	Enhancement of rooting	Tallón et al. (2012)

Table 1. (continued)

Genus, species and cultivar	Explant used (type, size, origin)	Basal medium	PGR (type and concentration)	Other culture conditions	Phloroglucinol concentration (μM)	Effect on tissue culture, development, etc.	Reference
orange, and 'Cleopatra' mandarin	from mature trees						
Citrus sinensis L. Osbeck 'Pêra'	Internodal segments	MS	1.33 μM BA	555 μM *myo*-inositol; 500 mgL^{-1} malt extract; 87.65 μM sucrose	0, 237.9, 475.8, 713.7, 951.6	Enhancement of rooting	Romais et al. (2000)
Crataeva magna	Single node segments	Half-strength MS	11.42 μM IAA, 9.8 μM IBA, 0.46 μM kinetin	Incubated in the dark (2 d), then transferred to light for 20 d	198.25	Enhancement of rooting	Bopana and Saxena (2009)
Decalepis hamiltonii	Single node shoot tip	MS	1.1 μM BA, 5.8 μM GA_3	58.43 μM sucrose	800	Increased shoot proliferation	Gururaj et al. (2004); Giridhar et al. (2005)
			5.6 μM BA	58.43 μM sucrose, 0.011 μM triacontanol	200	Elongation of shoots	
			5.38 μM NAA	58.43 μM sucrose	400	Induction of rooting	
Dendrobium nobile	Cryopreserved protocorms	Half-strength MS	None	Darkness for 1 wk at 27°C, then transfer to light at same temperature, 120 $\mu molm^{-2}s^{-1}$; 16-h photoperiod	1% when combined with glycerol and PVS2 during cryopreservation	Improved recovery of cryopreserved protocorms	Vendrame and Faria (2011)
Feijoa sellowiana Berg	Zygotic embryos	MS	4.5 μM 2,4-D	Incubation in the dark	197.5	Embryogenesis induction	Reis et al. (2008)
Ficus carica L.	Bud and leaf segments	Half-strength MS	3 mM MES	1 wk in the dark	500	Diminished tissue-browning	Kim et al. (2007)
Ficus religiosa	Nodal segments	WPM	4.4 μM BAP, 2.28 μM IAA	Glutamine and adenosine sulphate essential	200	Improved shoot emergence; leaf senescence observed	Siwach and Gill (2011)
Garcinia indica	Apical and axillary buds	Half-strength WPM	24.60, 49, 98, 123 and 172.2 μM IBA	58.43 μM sucrose	79.3, 317.2, 793, 1,189.5, 1,586, 1,982.5 and 2,379	Did not further improve root induction	Chabukswar and Deodhar (2006)
Garcinia mangostana	Leaf with midrib	MS	2.22 μM BA, 2.25 μM TDZ	500 mg/l PVP, 3% sucrose	34.5	Enhancement of rooting	Te-chato and Lim (1999)
Jatropha curcas	Leaf disc	MS		N/A	200	Enhancement of rooting	

Table 1. (continued)

Genus, species and cultivar	Explant used (type, size, origin)	Basal medium	PGR (type and concentration)	Other culture conditions	Phloroglucinol concentration (μM)	Effect on tissue culture, development, etc.	Reference
			2 μM TDZ, 2 μM BAP, 1 μM IBA				Kumar et al. (2010)
Juglans regia (Sunland, Chandler and Kerman 120)	Microshoots	DKW	49 μM IBA	10 d in darkness	500 and 1,000	Enhancement of rooting	Sharifian et al. (2009)
Malus domestica	Shoots	Half-strength MS	1.4 μM IBA, 1.3 μM GA_3	1.2 μM thiamine-HCl, 0.56 mM *myo*-inositol	1,000	Enhancement of rooting of 'Delicious'; no effect on 'Golden Delicious'	Zimmerman (1984)
Minuartia valentina	Shoots	MS	4.4 μM BAP or 4.6 μM kinetin	555 μM *myo*-inositol, 33.24 μM thiamine, 8.12 μM nicotinic acid and 5.91 μM pyridoxine	634.4	Increased multiplication rate	Ibañez and Amo-Marco (1998)
Pelargonium graveolens	Leaf segments	MS	5 μM IBA	Liquid medium with filter paper support	10	Enhancement of rooting	Lakshmana Rao (1994)
Photinia × *fraseri* Dress.	Microshoots	MS	49.2, 147.6, 295.3, 442.9 μM IBA	N/A	634.4	Improved root length	Ramírez-Malagón et al. (1997)
Prunus armeniaca L.	Adventitious shoots	QL macronutrients, DKW micronutrients, and vitamins	1.12 μM BA-riboside, 0.05 μM IBA, 2.1 μM meta-topolin, 29.6 μM adenine	Initial explants in the dark for 1 wk at 23°C, then light (16-h photoperiod; 20–25 $\mu mol\,m^{-2}s^{-1}$)	800	Improved multiplication	Wang et al. (2011)
Prunus avium L.	Shoots	N/A	N/A	N/A	1,000	Enhancement of rooting	Hammatt (1994)
Prunus avium L.	Microshoots	MS	14.7 μM IBA	Including 30 μM $ZnS0_4{\cdot}7H_2O$ and 100 μM FeNaEDTA instead of $ZnSO_4{\cdot}4H_2O$, Na_2-EDTA and $FeSO_4{\cdot}7H_2O$	1,000	Enhancement of rooting	Hammatt and Grant (1997)
Prunus avium L. 'Bing', 'Sweetheart', 'Lapins'	Apical shoot tips isolated from orchard trees; and punctured	Half-strength MS, then WPM	3 μM BA	26.7 $\mu mol\,m^{-2}s^{-1}$ (shoot cultures), 18.5 $\mu mol\,m^{-2}s^{-1}$ (callus cultures); 4-wk sub-cultures	1.0	Enhancement of rooting	Feeney et al. (2007)

Table 1. (continued)

Genus, species and cultivar	Explant used (type, size, origin)	Basal medium	PGR (type and concentration)	Other culture conditions	Phloroglucinol concentration (μM)	Effect on tissue culture, development, etc.	Reference
	shoot tips, stem sections, and shoot bases of *in vitro* shoot cultures						
Prunus dulcis Mill.	Shoots	MS	1.0–4.4 μM BA, 0.05 μM IBA	Low light intensity (5 $\mu mol m^{-2} s^{-1}$), 4°C for 2–4 wk	100	Enhancement of rooting	Ainsley et al. (2001)
Prunus salicina Lindl 'Gulf Ruby'	Microshoots	Half-strength MS	0.76–1.89 μM IBA	43.83 μM sucrose	158.6–317.2	Induction of rooting	Zou (2010)
Prunus domestica 'Improved French'	Adventitious shoots	Half-strength MS	0.1 μM kinetin, 5.0 μM NAA	SGM organics (vitamins and sucrose); 0.7% (*w*/*v*) Bacto agar	793	Induction of rooting	Petri and Scorza (2010)
Pterocarpus marsupium Roxb.	Nodal segments	Half-strength MS	100 μM IBA	Pulse treatment for 7 d in liquid medium	15.84	Enhancement of rooting	Husain et al. (2007, 2008)
Pyrus calleryana Dcn.	Shoots	Half-strength MS	0–4.9 μM IBA, 2.69 μM NAA	43.83 μM sucrose	1,284.66	Enhancement of rooting	Berardi et al. (1993)
Pyrus communis L.	Shoots	Half-strength QL	9.8 μM IBA	Incubated in darkness	160.98 to 1,284.66	Enhancement of rooting	Wang (1991)
Rosa hybrida L. 'Arizona'	Somatic embryos	Half-strength MS	2.69 μM NAA	Continuous light	198.25, 396.5, 594.75, 793	Germination of mature embryos	Murali et al. (1996)
Rubus ursinus Cham and Schlect × *R. idaeus* L.	Shoots	MS	5 μM IBA	87.65 μM sucrose	1,000	Enhancement of rooting	James (1979)
Rubus idaeus 'Malling Jewel'	1-cm shoot tips	LS	4.4 μM BA, 0.49 μM IBA	None specified	1,284.66	Shoot proliferation, increased number of roots per rooted culture	James et al. (1980)
Solanum tuberosum L.	Shoot tips	MS	5.8 μM GA_3, 1.1 μM BA	8.39 μM D-calcium pantothenate; 0.1 or 0.2 M sucrose	80, 400, 800, 1,200, 1,600	Axillary shoot proliferation, induction of root formation	Sarkar and Naik (2000)
Solanum tuberosum L.	Shoot tips	MS	2.28 μM zeatin, 2.69 μM NAA	Stored at 25–26°C	793	Induction of *in vitro* tuberization	Agud et al. (2010)
Trapa japonica Flerov	Zygotic embryos	MS		108.0 μM casein hydrolysate	10.8		

Table 1. (continued)

Genus, species and cultivar	Explant used (type, size, origin)	Basal medium	PGR (type and concentration)	Other culture conditions	Phloroglucinol concentration (μM)	Effect on tissue culture, development, etc.	Reference
			0, 0.27, 0.54, 2.7 and 5.5 μM 2,4-D; 0.27 and 0.54 μM BA			Callus induction, reduced accumulation of phenolics	Hoque and Arima (2002)
Vaccinium corymbosum L.	Nodal segments	WPM	20 μM 2iP	0.01 mM ascorbic acid, 0.22 mM adenine sulfate, 58.43 μM sucrose	317.2	Reduced hyperhydricity, increased lignification, increased multiplication rate	Ross and Castillo (2009)
Vitex negundo L.	Nodal segments	MS	4.4 μM BA	117.74 μM $AgNO_3$	793	Early bud break, enhancement of axillary shoot proliferation	Steephen et al. (2010)
Withania coagulans (Stock) Dunal	Leaf explants	Half-strength MS	1.2 μM IBA, 3.6 μM PAA, 14.3 μM CC	7-d pulse treatment with PG for rooting	3.9	Elongation of shoots, induction of rooting	Jain et al. (2009)
Wrightia tomentosa	Axillary nodes	MS	10 mM TDZ	5 sub-cultures required	400	Enhancement of axillary shoot proliferation	Purohit et al. (2005)

2,4-D 2,4-dichlorophenoxyacetic acid, *2iP* 6-γ-γ-dimethylamino purine, *ABA* abscisic acid, *BA* 6-benzyladenine, *BAP* 6-benzylaminopurine, *BLG* Verhagen and Wann (1989), *CC* choline chloride, *DKW* Driver and Kuniyuki (1984), *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *LS* Linsmaier and Skoog (1965), *MES* 2-(*N*-morpholino) ethanesulfonic acid, *meta-topolin* 6-(3-hydroxybenzylamino) purine, *MS* Murashige and Skoog (1962), *NAA* 1-naphthaleneacetic acid, *PAA* phenylacetic acid, *PG* phloroglucinol, *PVS2* plant vitrification solution no. 2 (Sakai et al. 1990) , *QL* Quoirin and Lepoivre (1977), SGM organics (Gonzalez-Padilla et al. 2003) , *TDZ* thidiazuron, *WPM* Woody Plant Medium (Lloyd and McCown 1981) , *N/A* information not available

Table 2. The effects of phloroglucinol on apple *in vitro* shoot formation

Cultivar	Treatment			Rooting percentage	Root number per shoot	Reference
M.9	PG (1284.66 µM)		For 38 d	0%	0	James and Thurbon (1979)
	IBA (9.8 µM)			8%	2.2	
	PG (1284.66 µM) + IBA (9.8 µM)			62%	3.6	
	PG (1284.66 µM)		For 4 d prior to culture on hormone-free medium	5.5%	1.8	
	IBA (9.8 µM)			30%	4.4	
	PG (1284.66 µM) + IBA (9.8 µM)			69%	12.0	
Delicious	With PG (1 mM) *vs.* no PG			31% *vs.* 74%	N/A	Zimmerman and Fordham (1985)
Royal Red Delicious	With PG (1 mM) *vs.* no PG			15% *vs.* 45%	N/A	
Vermont Spur Delicious	With PG (1 mM) *vs.* no PG			20% *vs.* 60%	N/A	
Spartan	With PG (1 mM) *vs.* no PG			66% *vs.* 80%	N/A	
Redchief Delicious	With PG (1 mM) *vs.* no PG			0% *vs.* 12%	N/A	
Redspur Delicious	With PG (1 mM) *vs.* no PG			90% *vs.* 98%	N/A	
Gala	With PG (1 mM) *vs.* no PG			97% *vs.* 100%	N/A	
Golden Delicious	With PG (1 mM) *vs.* no PG			92% *vs.* 95%	N/A	
McIntosh	With PG (1 mM) *vs.* no PG			68% *vs.* 85%	N/A	
Spur McIntosh	With PG (1 mM) *vs.* no PG			82% *vs.* 87%	N/A	
Mutsu	With PG (1 mM) *vs.* no PG			70% *vs.* 50%	N/A	
York Imperial	With PG (1 mM) *vs.* no PG			15% *vs.* 15%	N/A	
M.9[a]	PG (1284.66 µM)	In shooting medium	In rooting medium			Webster and Jones (1989)
		−	−	77%	N/A	
		−	+	93%	N/A	
		+	−	69%	N/A	
		+	+	93%	N/A	
Tydeman Early Worcester[b]	PG (793 µM)	In liquid medium	In solid medium			Modgil et al. (1999)
		−	−	50%	N/A	
		−	+	68%	N/A	
		+	+	64%	N/A	
MM.106	PG (793 µM)	In shooting medium	In rooting medium			Sharma et al. (2000)
		−	−	66%	N/A	
		+	−	81%	N/A	
		−	+	53%	N/A	
		+	+	60%	N/A	

+, presence; −, absence

IBA indole-3-butyric acid, *PG* phloroglucinol, *N/A* information not available

[a] After 21 subcultures

[b] Shoots were rooted in liquid medium for 9 d followed by on agar-solidified medium

hyperhydric as a result of growth and culture conditions that act as stress factors. Hyperhydric tissues show several biochemical characteristics that explain their morphological abnormalities such as reduced lignin and oxidative stress (Rogers and Campbell 2004). Hypolignification is associated with lower activities of enzymes involved in the synthesis of lignin precursors and their polymerization and can arise from *in vitro* conditions such as high humidity, high PGR levels, gas accumulation, and high light intensity (Kevers et al. 2004; Saher et al. 2004). These stress conditions could mediate a rapid endogenous ethylene burst, which decreases peroxidase activity and lignification (Al-Maarri and Al-Ghamdi 2000). One of the greatest hindrances to successful plant tissue culture protocols, other than somaclonal variation, is

hyperhydricity. Although there are several methods for reducing hyperhydricity in plant tissue culture, such as manipulating the levels of PGRs or ions in the medium (Wu et al. 2011), manipulation of light conditions or choice of gelling agent (Ascencio-Cabral et al. 2008), or choice of culture vessel or CO_2-enrichment (Teixeira da Silva et al. 2005a, b), there are few chemical means for reducing hyperhydricity.

PG is used to prevent hyperhydricity in micropropagation by providing precursors which normally are synthesized at low levels or not synthesized at all in hyperhydric tissues. Certain enzymes, particularly *p*-coumarate/CoA ligase, show significantly less activity in hyperhydric explants (Phan and Hegedus 1985). Adding phloridzin or its precursor PG to the culture medium for apple and sunflower shoots prevented hyperhydricity by increasing the activity of enzymes involved in lignin synthesis (Phan and Hegedus 1985). In that study, the activities of some enzymes involved in the synthesis of lignin were found to be consistently lower in hyperhydric plants than in normal plants.

The addition of PG as a precursor in the lignin biosynthesis pathway resulted in the effective control of hyperhydricity and maximized the multiplication rate of *Vaccinium corymbosum* cultured in liquid medium. When stained with toluidine blue (O'Brien et al. 1964), cross-sections of shoots displayed more lignified tissues than the non-PG control, with xylem development similar to shoots from semisolid medium (Ross and Castillo 2009). Similar results were obtained with *Achyrocline flaccida*, in which shoots from liquid medium with PG had a narrower pith, better xylem development, and increased lignification (Ross and Castillo 2010). Preliminary results obtained with *Acca sellowiana* cultured in permanent immersion bioreactors showed similar results: the addition of PG to the medium prevented the occurrence of hyperhydric shoots (Ross and Grasso 2010). PG has also been successfully employed to prevent hyperhydricity in *A. flaccida* and *V. corymbosum* (Ross 2006).

Phan and Letouze (1982) observed that phenolic compounds were more abundant in normal *Prunus avium* plants than in hyperhydric plants. Phenolic production is directly associated with the C/N ratio and since phenolics influence lignification, this would explain how the addition of PG and phloridzin to culture media could help plants undergoing hyperhydricity to return to a normal state, i.e., reverse hyperhydricity (Al-Maarri and Al-Ghamdi 2000).

Even though bioreactors have shown potential for the micropropagation of many plants, the greatest hindrance to their use is hyperhydricity of the resultant regenerants. The ability to use PG as an additive to liquid medium in a bioreactor to increase lignification, improve rooting, and remove (i.e., reverse or prevent) hyperhydricity would have massive implications for the mass production of plants.

PG in plant growth and development: possible contrasting roles. The plant tissue culture literature contains numerous protocols that describe, in considerable detail, the effective micropropagation of plant tissues *in vitro*. That success is most frequently attributed to the use of specific combinations of PGRs, gelling agents, light conditions, and a wealth of other abiotic and biotic factors. Chemical means of manipulating plant growth *in vitro*, however, remains the most popular method, and any new resources or chemicals that could serve the same purpose as the currently used PGRs, would be advantageous for both industrial and research use. This section explores how PG could be used in different capacities to manipulate growth and development of plant cells or tissues *in vitro*, providing an alternative to current methods. Both positive and negative effects have been described so that the reader is aware that not all outcomes are necessarily beneficial.

Various phenols are typically added to tissue culture media mainly to enhance callus growth, form adventitious shoots more effectively, improve rooting, and increase the rate of shoot proliferation in certain shoot cultures. Most plant responses to phenols involve a synergism with auxins, particularly indole-3-acetic acid (IAA), so it is likely that the mode of action is dependent on the regulation of internal IAA levels. Oxidative catabolism of IAA is the chemical modification of the indole nucleus or side chain that results in the loss of auxin activity (Normanly et al. 2004). As far as we know, this is the only truly irreversible step regulating IAA levels. Many mono-, di-, and tri-hydroxyphenols and their more complex derivatives found naturally in plant cells are strong reducing agents that can serve as substrates for oxidative enzymes. This has led to the following two hypotheses as to their growth regulatory activity (George et al. 2010):

1. When added exogenously, hydroxyphenols act as alternative substrates for oxidative enzymes and may protect auxin from oxidative breakdown.
2. Morphogenic activity is induced by the products formed when phenolic compounds such as PG and phloridzin are oxidized. This hypothesis was advanced by Gur et al. (1988), who found that PG only promoted rooting in apple clones with sufficient polyphenol oxidase activity to cause significant oxidation of the compound.

Phenolic compounds constitute a wide range of plant substances that are normally viewed as deleterious during *in vitro* culture, since their exudation and oxidation cause browning and necrosis, especially when mature explants of woody plants are used (Benson 2000; Martin and Madassery 2005; Reis et al. 2008). However, in several cases, phenolic compounds seem to be essential for the control of some morphogenic processes occurring *in vitro*, indicating that their role is far from being understood. Examples of the positive effect of phenolic compounds on morphogenic processes occurring *in*

vitro are the stimulation of root formation (Hammatt and Grant 1997; Romais et al. 2000) and elongation (Ceasar et al. 2010), shoot proliferation (Sarkar and Naik 2000), shoot organogenesis (Lorenzo et al. 2001), androgenesis (Delalonde et al. 1996), and somatic embryogenesis (Hanower and Hanower 1984; Find et al. 2002).

The structurally related glycoside phloridzin has the same effect as PG but is heat labile and more expensive. Phloridzin is metabolized into PG and phloretic acid. Phloretic acid increased the proportion of apple shoots which could root (Jones and Hatfield 1976) and led to elongation of papaya roots *in vitro* (Ascencio-Cabral et al. 2008), but it was less active than PG, which has occasionally been found to have inhibitory effects (Snir 1983; Kooi et al. 1999, cited by George et al. 2010).

Consequently, PG and its precursors (such as phloridzin) or its products of metabolism (such as phloretic acid) have the potential to influence a wide range of plant growth processes and development, although it is expected, as for most other phenolic compounds and PGRs, that excessively high concentrations would have an inhibitory effect.

1. *Somatic embryogenesis.* Somatic embryogenesis is often desirable as somatic embryos represent true clones of single-cell origin. It is not always possible to obtain somatic embryogenesis using conventional PGRs or even manipulation of media or culture-room conditions. The possibility of utilizing PG as an alternative for induction of somatic embryogenesis would strongly benefit plant tissue culture. Adding phloridzin or PG to medium increased the number of somatic embryos produced from embryogenic callus of oil palm (*Elaeis guineensis*) (Hanower and Hanower 1984, cited by George et al. 2010). Reis et al. (2008) found that the inclusion of PG at specific concentrations in the induction medium increased the levels of somatic embryogenic induction in *Feijoa sellowiana*. When 197.5 μM PG was added to the culture medium, 94.7% of explants differentiated embryos, but higher concentrations strongly inhibited somatic embryo formation. However, the exact mechanism by which PG promotes somatic embryogenesis is unclear. According to Reis et al. (2008), some of the phenolic compounds produced in the presence of PG might promote somatic embryogenesis. Murali et al. (1996) report enhanced somatic embryogenesis in callus cultures of rose (*Rosa hybrida* 'Arizona') petal explants. The addition of PG strongly promoted the development of somatic embryos into plantlets: 93% of somatic embryos formed plantlets with 0.79 mM PG in medium containing 2.69 μM α-naphthaleneacetic acid (NAA). In a study to improve the transition from proliferation to maturation in embryogenic cultures of *Abies nordmanniana*, the addition of 40 μM PG increased the rate of proliferation in most cases, but embryo maturation and development was limited with this treatment (Find et al. 2002). Manjula et al. (1997) claimed that the addition of PG to medium controlled the formation of polyphenolics *in vitro*, leading to improved callus formation of *Aristolochia indica*.

   Occasionally, PG has shown inhibitory effects. A 50% inhibition of rooting of sour cherry shoots (*Prunus cerasus*) grown *in vitro* occurred when 1.28 mM PG was added to the medium (Snir 1983). In sentag shoots (*Azadirachta excelsa*), addition of 1.28 mM PG to the control medium significantly reduced the rooting percentage from 55.5% to 29.4% (Kooi et al. 1999).

   PG may act as a bactericide, increasing shoot regeneration only in shoot cultures carrying concealed bacterial infections. However, the stimulatory effect of the compound is now thought to be largely independent of this effect (Jones and Hopgood 1979; Jones and James 1979).
2. *Shoot proliferation.* Shoot induction and proliferation is one of the most common means of mass-producing plants due to its ease, and it can be achieved through elongation of either the apical meristem or axillary buds. Improved shoot proliferation *in vitro* has been reported for several species following the inclusion of PG in medium. Sarkar and Naik (2000) studied the efficacy of PG in promoting growth and development of *in vitro*–derived shoot tips in six potato (*Solanum tuberosum* L.) genotypes. They found a significant PG×sucrose interaction for number of shoots developed per shoot tip (1 shoot per shoot tip in the control medium without PG, 5.8–8.2 shoots per shoot tip with PG, depending on genotype), shoot tip fresh weight, and number of roots induced/shoot tip (from 0 to 0.3–6.1, depending on genotype). Optimum shoot tip growth was possible in a medium containing 0.8 mM PG and 0.2 M sucrose. Early bud break and enhanced shoot regeneration in nodal explants of *Vitex negundo* was achieved by supplementing the medium with 0.79 mM PG and 0.12 mM silver nitrate in addition to 4.44 μM BA (from 1 shoot/explant in the control to 15 shoots/explant) (Steephen et al. 2010). Supplementation in the bud induction medium of *Capsicum annuum* with 400 μM PG increased the bud induction response by 17–18% (Kumar et al. 2005). In these cases, the presence of PG in the medium allowed for shoot induction and proliferation without the need for any additional PGRs.

   Plant multiplication of the medicinal plant *Arnica montana* was improved (from 1.2 to 3.6 plantlets/explant) on MS medium containing 0.6 mM PG and 0.2 mM adenine sulfate (Buthuc-Keul and Deliu 2001). Successful shoot multiplication of *Minuartia valentina*, an endangered and endemic Spanish plant, was achieved on MS medium with 0.63 mM PG in combination with either 4.44 μM 6-benzyladenine (BA) or 4.65 μM kinetin. PG improved

the number of shoots/explant from 1.2 to 2.6 and from 1.4 to 2.5 (for BA and kinetin respectively) and increased shoot length from 12.4 to 24.1 mm with BA (Ibañez and Amo-Marco 1998). It seems that PG in combination with a cytokinin serves to further enhance shoot development and proliferation. This synergistic effect that PG had on shoot multiplication was observed by Gururaj et al. (2004), who found that multiple shoots formed from single-node explants of *Decalepis hamiltonii* when BA and gibberellic acid (GA_3) were added to PG-containing medium. The maximum number of shoots/culture was observed on medium containing 1.1 μM BA, 5.8 μM GA_3, and 800 μM PG. Subculturing of the shoots onto MS medium containing 5.6 μM BA, 200 μM PG, and 0.011 μM triacontanol produced elongated shoots and secondary shoots. The long shoots could be rooted on medium containing 5.38 μM NAA and 400 μM PG. Here, too, PG promoted shoot induction and subsequent rooting when used synergistically with other shoot- or root-inducing PGRs. A marked stimulatory effect of 1.28 mM PG on shoot number in red raspberry (*Rubus idaeus*) 'Malling Jewel' was found with all auxin and cytokinin concentrations evaluated. The medium for optimum shoot production contained 4.44 μM BAP and 0.5 μM IBA in the presence of 1.28 mM PG. On average, the rate of shoot multiplication for all genotypes was about 6-fold/month when PG was present and about 4-fold/month in its absence (James et al. 1980).

As indicated by these reports, PG, whether present alone or combined with other PGRs, overwhelmingly stimulates shoot induction and subsequent development.

3. *Root initiation and development.* It is common to find protocols in which somatic embryos are successfully induced, but root elongation does not occur. Moreover, it is not uncommon, especially in woody plants and hardwood species such as forestry or fruit trees, where callus tends to form at the base of explants but where roots are never formed. As indicated above, methods such as aerated vessels or inclusion of activated charcoal in medium have been used to stimulate rooting, but these methods are not always effective. Here again, it would be advantageous to have another alternative to evaluate, and PG should be considered. PG added to rooting media together with auxin will sometimes stimulate rooting. This synergistic effect has been reported for several ornamental, fruit, and other species. Hammatt (1994) found that the proportion of shoots rooting in media containing auxin, or auxin plus PG, increased with the number of successive subcultures, but the proportion that rooted with PG alone was unaffected by the number of subcultures. Before the shoots had become responsive to auxin, 1 mM PG was more effective than auxin in inducing roots. PG seems to have a synergistic effect not only during shoot formation, but also during subsequent root development.

De Klerk et al. (2011) evaluated the effect of phenolic compounds on root formation from apple stem slices. The phenolics were added to the rooting medium along with suboptimal concentrations of IAA (3 μM) or NAA (0.3 μM). When IAA was used, most phenolics (including 1 mM PG) increased rooting. With NAA, rooting was far less promoted or even inhibited. The logic of the experiment was to use PG to "compensate" for the suboptimal levels of auxins by assuming that PG protects auxins from oxidation. The effects of PG and ferulic acid on a dose–response curve of IAA and the timing of their action indicated that both acted as antioxidants, protecting IAA from decarboxylation and the tissue from oxidative stress. A 5-fold increase in the maximum number of roots was achieved with 3 μM IAA and 1 mM PG; IAA oxidation was reduced from 100% in the control to 21% in the treatment, resulting in a 44% increase in IAA uptake by the explants. These authors did not report the effect of PG used alone in the medium, but the results suggest that IAA plays a much stronger role than PG in root induction, although PG serves to enhance the effect of IAA (Dobránszki and Teixeira da Silva 2010). Indeed, PG is essential for enhancing root initiation and subsequent development in the presence of an auxin (IAA or indole-3-butyric acid [IBA]), confirming the notion that PG is an auxin promoter, although its effectiveness is strongly dependent on genotype (Zimmerman 1984; Magyar-Tábori et al. 2010).

The presence of PG in the rooting medium exerted a positive effect on rooting of the pear (*Pyrus communis*) rootstock BP10030. The magnitude of the effect was dependent on PG concentration, with the effective range (0.16–1.28 mM) resulting in 100% rooting. PG concentrations outside this range decreased rooting (Wang 1991). Berardi et al. (1993) found that PG alone did not affect rooting of *Pyrus calleryana*; however, PG in combination with NAA or IBA promoted the greatest increase in rooting throughout the acclimatization period.

Sharifian et al. (2009) evaluated the effect of PG on rooting parameters with micropropagated shoots of three cultivars of *Juglans regia*. PG at 0.5 and 1.0 mM increased the rooting percentage, but 1.6- to 10-fold differences among cultivars were obtained. Ainsley et al. (2001) found that the effect of PG on *in vitro* rooting of almond (*Prunus dulcis* Mill.) varied depending on the auxin used for root induction. When shoots were subjected to PG following a 12-h treatment in water–agar medium containing 1.0 mM NAA, the number of explants that developed roots decreased significantly (from 40% to 20%), as did the mean root length. Comparatively, changes in the rooting frequency of shoots cultured for

12 h in water–agar containing only 1.0 mM IBA (i.e., no PG) were not significant. Maximum rooting was achieved by inserting shoots for 12 h into water–agar containing 1.0 mM IBA, followed by 2 wk in half-strength MS supplemented with 100 μM PG. Under these conditions, 60% of the shoots developed multiple roots.

Rooting of British wild cherry (*P. avium* L.) was also improved by the addition of 1 mM PG to the rooting medium (Hammatt and Grant 1997). With red raspberry, 1.28 mM PG in the presence of IBA synergistically promoted the number of roots per rooted culture but did not significantly increase the frequency of rooted cultures (James et al. 1980). An auxin–PG synergism promoted the rooting of a *Rubus* hybrid (*R. ursinus* Cham. and Schlect×*R. idaeus* L.) but not the perpetually fruiting strawberry 'Gento Hummell' (a hybrid of *Fragaria virginiana* Duchesne×*Fragaria chiloensis* Duchesne). In strawberry, IBA could be successfully replaced by PG, but the presence of both IBA and PG reduced rooting compared with other treatments (James and Thurbon 1979). This indicates that the root-inducing ability of PG is not universal; rather, it is an experimental parameter that needs to be tested for individual genotypes.

Root induction in *Arnica montana* was stimulated by the addition of maize extract (crushed kernels) (12.2 roots/explant, 3.58 cm in length), PG (10.2 roots/explant, 2.44 cm in length), or PG plus adenine sulfate (13 roots/explant, 2.94 cm length), compared to the control (2.4 roots/explant, 1.94 cm in length) (Buthuc-Keul and Deliu 2001). For *Arnica montana*, PG was not the best enhancer of rooting, but it was still better than the control (no additives), supporting the notion that PG is an additional alternative rather than a substitute for other auxins or auxin-like substances.

PG was a critical ingredient for the rooting of *Asparagus racemosus*, a medicinal plant of high value. The inclusion of 198.25 μM PG enhanced the rooting frequency from 41.37% (in the absence of PG) to 85% (Bopana and Saxena 2008). The same authors achieved 96% rooting within 22 d by culturing the *in vitro*–formed shoots of *Crataeva magna* (a high-value medicinal tree) on half-strength MS medium with 11.42 μM IAA, 9.8 μM IBA, 0.46 μM kinetin, and 198.25 μM PG (Bopana and Saxena 2009).

Rooting of *Pterocarpus marsupium* was most effectively induced using microshoots excised from proliferating shoot cultures on semisolid hormone-free half-strength MS medium, after a pulse (dip) treatment for 7 d in half-strength MS liquid medium containing 100 μM IBA and 15.84 μM PG. A maximum frequency of root formation (70%), highest number (3.8±0.37) of roots, and maximum root length (3.9±0.05 cm) were achieved by using the IBA–PG dip (Husain et al. 2008).

Petri and Scorza (2010) enhanced the rooting of regenerated shoots of plum cultivar 'Improved French' by 53% by adding 0.79 mM PG to the rooting medium. A stimulating effect of PG on the growth and proliferation of *in vitro* 'M.26' apple shoots was reported by Jones et al. (1977); however, James and Thurbon (1981) found that PG did not increase the number of 'M.9' shoots in any of 12 combinations of PGRs evaluated, and with one combination, PG had decreased the number of shoots formed. Webster and Jones (1989) noted differences in shoot production on four apple rootstocks. Shoot production was readily achieved with 'P.22' and 'Ottawa 3', although shoot production was less effective with 'P.2' and 'B.9'. In the case of these latter rootstocks, some improvement in shoot production was achieved on a medium containing 1.28 mM PG, when BA was increased from 4.44 to 8.88 μM, and with a long subculture period. The maximum shoot number (6.66) and shoot length (2.73 cm) of 'MM.106' was observed on shoot proliferation media supplemented with 0.79 mM PG. Taken together, it seems that the usefulness of PG for improvement of shoot proliferation depends on genotype, PGR content of the medium, and presumably on the number of subcultures (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010). PG treatment (1.0 mM) during rooting caused different rooting responses in scions from 12 apple cultivars (Zimmerman and Fordham 1985). Systematic studies were conducted regarding the effect of PG on *in vitro* rooting to enhance the rooting process. PG was able to increase the rooting percentage (James and Thurbon 1979, 1981; Webster and Jones 1989; Modgil et al. 1999) and also the number of roots/shoots in some but not in all cultivars (Zimmerman and Fordham 1985; Sharma et al. 2000). PG also enhanced the effects of IBA in the rooting medium (James and Thurbon 1979). Moreover, Sharma et al. (2000) showed the preconditioning effect of PG on shoots for subsequent rooting. The results of studies on the effects of PG on shoot formation and rooting in apple are summarized in Table 2.

Other growth and developmental responses. PG added at 1% enhanced the recovery and survival of cryopreserved *Dendrobium nobile* protocorms when it was used as a cryoprotectant additive. A 2-fold increase in protocorm survival was achieved when PG was used in combination with 2 M glycerol and PVS2 (plant vitrification solution; Sakai et al. 1990; Vendrame and Faria 2011). Interestingly, hyperforin and adhyperforin, which are PG derivatives found in *Hypericum* spp., may have played a part in the success of recovery of *Hypericum perforatum* shoot tips from cryopreservation (Bruňáková et al. 2011).

Agud et al. (2010) evaluated the effect of PG, either alone or in combination with PGRs (BA, 2iP, zeatin, NAA), to

induce tuberization of potato *in vitro*. They found that PG in a moderate dose (0.79 mM) in combination with any of the cytokinins (0.5 mgL^{-1}) resulted in 100% regeneration. Prevention or reduction of tissue browning by PG in culture was tested by Kim et al. (2007) with leaf segments of fig tree (*Ficus carica*). PG had a beneficial effect, ultimately resulting in an increased survival rate and morphogenesis.

Hammond and Mahlberg (1994) reported PG to be the only detectable phenolic component in the glandular trichomes in *Cannabis*, suggesting that it may play an important role in the *in vivo* enzymatically regulated biogenesis of cannabinoids.

Future Prospects

Plant tissue culture is one of the bulwarks of plant biotechnology. As with most fields in this area of study, it is faced with several limitations and drawbacks that require the constant search for solutions. In fact, there are very few plant species for which we can truly say that an excellent, repeatable *in vitro* tissue culture protocol exists across different genotypes and for a wide range of explants. Most plant species require very specific protocols that target specific genotypes, tissues, or organs. That said, the search for suitable alternatives to current protocols is one of the main driving forces in plant tissue culture research. One way of improving different aspects of organogenesis or morphogenesis would be to include a new growth-promoting substance. Despite being tested in a number of studies, PG is relatively unknown as a plant growth regulator, and its use in plant tissue culture has increased only recently (Table 1). The effects of PG on plant organogenesis have not been uniform; indeed, there are several exceptions to the overall trends observed. Overall, however, PG has shown the ability to stimulate rooting, shoot formation, and somatic embryogenesis, sometimes alone and sometimes in combination with other PGRs. This phenolic compound opens up new avenues for use as a plant growth regulator in clonal propagation of shoots for short-, medium-, or long-term storage, as an inducer of rooting to speed up plantlet growth and development, and as a means to reduce hyperhydricity and fortify tissue through lignification in bioreactor systems. Improved somatic embryogenesis in PG-supplemented medium could allow for a steady supply of robust somatic embryos for mass production in bioreactors or for synthetic seed technology (Sharma et al. 2013), and would also provide a stable supply for genetic transformation studies. Moreover, transgenic material would have a greater chance of survival if improved shoot and root formation could be achieved by the inclusion of PG in regeneration medium. Finally, with a view to the future, the fact that PG has already shown success in improving the recovery of cryopreserved tissue provides hope for long-term storage of useful, rare, and important germplasm. Although the road to fully understanding the mechanism of PG in all of these developmental events is still in its infancy, this compilation of information and assessment of trends will allow for more plant tissue culture scientists to better direct their research efforts, perhaps now considering PG as an important component in their experimental designs.

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