

The effect of ethylene inhibitors (AgNO_3 , AVG), an ethylene-liberating compound (CEPA) and aeration on the formation of protocorm-like bodies of hybrid *Cymbidium* (Orchidaceae)

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Abstract Protocorm-like bodies (PLBs) or thin cell layers (TCLs) derived from PLBs of hybrid *Cymbidium* Twilight Moon ‘Day Light’ can induce new or *neo*-PLBs on Teixeira *Cymbidium* (TC) medium, which contains 0.1 mg/L α -naphthaleneacetic acid, 0.1 mg/L kinetin, 2 g/L tryptone and 20 g/L sucrose, and is solidified with 8 g/L Bacto agar. This study aimed to assess the response of *neo*-PLB formation to an ethylene-liberating compound (2-chloroethylphosphonic acid (CEPA)), to two ethylene inhibitors (silver nitrate (AgNO_3) and aminoethoxyvinylglycine (AVG)), and to aeration (made possible by using Milliseal® or autoclaved filter paper). AgNO_3 at 1 or 2 mg/L in TC medium significantly increased the fresh weight of PLBs while 1 mg/L of AgNO_3 also showed a significant increase in the number of *neo*-PLB from both half-PLBs and from tTCLs. In contrast, AVG and CEPA inhibited *neo*-PLB formation. *Neo*-PLB formation from half-PLB or TCL explants in the presence of aeration resulted in significantly lower *neo*-PLB weight. The use of AgNO_3 and aeration are alternative means to mass produce *neo*-PLBs for micropropagation purposes.

Keywords AgNO_3 , AVG, CEPA, PLB, Teixeira *Cymbidium* (TC) medium

Introduction

Ethylene inhibitors are most commonly used in post-harvest research and industry to limit the negative impacts of ethylene on senescence, ripening and other post harvest degeneration of fresh produce induced by ethylene build up (Teixeira da Silva, 2006; van Doorn and Woltering 2008).

Silver nitrate (AgNO_3) and aminoethoxyvinylglycine (AVG) are ethylene inhibitors that have been shown to enhance shoot regeneration in pomegranate (*Punica granatum* L.) (Naik and Chand, 2003; Serek et al., 2006). AgNO_3 also stimulated callus formation in date palm (*Phoenix dactylifera* L.) (Al-Khayri and Al-Bahrani, 2004) but has also shown negative impacts such as inhibited shoot growth in sugarcane (*Saccharum* sp. hybrids) (Taylor et al., 1994).

Although *in vitro* protocols for the induction and development of protocorm-like bodies (PLBs) of hybrid *Cymbidium*

are well established (Hossain et al., 2013), to the best of the author’s knowledge, no study has yet assessed the impact of ethylene inhibitors or ethylene-liberating compounds such as chloroethylphosphonic acid (CEPA) on *in vitro* morphogenesis of this orchid. Using a recently developed optimized *Cymbidium* PLB regeneration medium, Teixeira *Cymbidium* (TC) medium (Teixeira da Silva, 2012), the effects of AgNO_3 , AVG and CEPA on new or *neo*-PLB formation were assessed with the expectation of increasing *neo*-PLB formation. Were there to be ethylene build-up in the growth vessel, then ethylene inhibitors would theoretically reduce ethylene within the vessel and thus stimulate growth and development of *neo*-PLBs while CEPA would inhibit these processes. PLB thin cell layers (TCLs), which are considered to be developmentally sensitive explants (Teixeira da Silva, 2013a, 2013b; Teixeira da Silva and Dobránszki, 2013a), and were thus also used in this study. To date, only one other study by Cruz de Carvalho et al. (2000) has examined the effect of AgNO_3 on the growth of epicotyl transverse TCLs (tTCLs) on shoot formation. The term plant growth regulator (PGR) in this paper refers to all phytohormones except for AgNO_3 and AVG, even though they are considered to be PGRs.

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Materials and methods

Chemicals and reagents

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

Plant material and culture conditions

The protocol is similar to that described by Teixeira da Silva (2012), with minor modifications. PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Japan) developed spontaneously from shoot-tip culture on Vacin and Went (1949) agar medium without PGRs. These PLBs were subcultured every two months on TC medium No. 1 (Teixeira da Silva, 2012), which contains 0.1 mg/L α -naphthaleneacetic acid (NAA) and 0.1 mg/L kinetin (Kin), 2 g/L tryptone and 20 g/L sucrose. Medium was solidified with 8 g/L Bacto agar (Difco Laboratories., USA). All media were adjusted to pH 5.3 with 1 N NaOH or HCL prior to autoclaving at 100 kPa for 17 min. Cultures were maintained on 40 mL medium in 100-mL Erlenmeyer flasks that were double-capped with aluminum foil. Flasks were placed at 25°C, under a 16-h photoperiod with a light intensity of 45 $\mu\text{mol}/\text{m}^2/\text{s}$ provided by plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Half-PLBs, 3–4 mm in diameter, which are longitudinally bisected PLBs without the growing tip, were used as explants for PLB induction and proliferation. PLBs were cultured at 10/flask. Culture conditions, media and procedures for PLB induction, formation and proliferation, followed the advice outlined for medium formulation (Teixeira da Silva et al., 2005; Teixeira da Silva and Tanaka, 2006), biotic (Teixeira da Silva et al., 2006c) and abiotic factors (Teixeira da Silva et al., 2006a). tTCLs, as prepared in Teixeira da Silva (2013b), were also used as explants for all treatments.

Effect of ethylene inhibitors and aeration

Half-PLBs and tTCLs (Fig. 1A, left and right, respectively) were cultured on PGR-free TC or on TC medium with PGRs, at 40 mL/100-mL Erlenmeyer flask, in the presence of 1, 2, 4, 8, or 16 mg/L AgNO_3 , AVG or CEPA. Neither control contained AgNO_3 , AVG or CEPA. Solutions of all three compounds were made fresh and were filtered (Dismic-25, mixed cellulose ester, 0.2 μm , Advantec) prior to the addition to cooled TC medium. One separate treatment contained a single Milliseal® ring in the center of the aluminum foil cap covering the 100-mL Erlenmeyer flask or a single sheet of autoclaved filter paper (Whatman No. 1, GE Healthcare) covering the entire mouth of the 100-mL Erlenmeyer flask.

Morphological parameters assessed

Three parameters were measured: the percentage of explants forming *neo*-PLBs, the number of *neo*-PLBs that formed per PLB segment, and the fresh weight of the explant + *neo*-PLBs. All measurements were made after 60 days in culture according to Teixeira da Silva and Dobránszki (2013b).

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment. All experiments were repeated three times ($n = 90$, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's new multiple range test (DNMRT) using SAS® version 6.12 (SAS Institute, Cary, NC, USA) with significant differences between means indicated at $P \leq 0.05$.

Results and discussion

The addition of 1 or 2 mg/L of AgNO_3 to PGR-free TC



Figure 1 (A) Protocorm-like body (PLB)-derived transverse thin cell layer (tTCL) (left) or half-PLB (right) forming PLBs 60 days after culture on Teixeira *Cymbidium* medium No. 1 (TC; Teixeira da Silva, 2012) containing 0.1 mg/L α -naphthaleneacetic acid and 0.1 mg/L kinetin. (B) PLB formation on TC without plant growth regulators but supplemented with 2.0 mg/L AgNO_3 . (C) PLB formation with excessive trichomes on TC without plant growth regulators but supplemented with 2.0 mg/L CEPA. Bars = 1 mm (A, for left and right), 5 mm (B, C). (A) modified from Teixeira da Silva (2012). Indentations (top and bottom) on *neo*-PLB in C indicate pincer marks.

medium significantly increased the fresh weight of PLBs while 1 mg/L of AgNO₃ also showed a significant increase in the number of *neo*-PLB (Table 1) from both half-PLBs and from tTCLs (Fig. 1). Although the percentage of explants forming *neo*-PLBs in the presence of aeration (induced by the use of a Milliseal[®]-capped flask) was comparable to *neo*-PLB formation in the presence of AgNO₃ or AVG, PLB fresh weight was significantly lower. The impact of a double layer of filter paper on *neo*-PLB formation was significantly worse

than when Milliseal[®] was used. Hyperhydricity was not observed (data not shown). Aeration through the use of autoclaved filter paper, presumably causing excessive drying of plant material, resulted in lower fresh weight. Ethylene inhibitors are fairly expensive chemicals (e.g., approx. \$US 210 for 1 g of ethephon, Sigma-Aldrich), but Milliseal[®]-derived aeration is a simple way to produce *neo*-PLBs for micropropagation purposes. The use of an aerated vessel, such as the Vitron, is also an excellent way to micropropagate

Table 1 Effect of ethylene inhibitors, an ethylene-liberating compound and aeration on *neo*-PLB formation from half-PLBs or PLB tTCLs 60 days after culture of hybrid *Cymbidium* Twilight Moon 'Day Light'

Medium composition	Concentration (mg/L)	Percentage of explants forming <i>neo</i> -PLBs (%)	Number of <i>neo</i> -PLBs per explant	Fresh weight (mg) of PLB explant + <i>neo</i> -PLBs
Half-PLBs on: TC (control)*		100 a	8.3 ab	526 b
PLB tTCLs on: TC (control)		100 a	2.1 d	188 f
Half-PLBs on: TC minus PGRs		100 a	1.2 de	321 de
PLB tTCLs on: TC minus PGRs		100 a	0.3 e	81 gh
Aeration (Milliseal [®])		100 a	7.9 ab	481 c
Aeration (filter paper)		100 a	6.2 b	386 d
Half-PLBs on: TC + AgNO ₃	1	100 a	8.7 a	576 ab
	2	100 a	9.1 a	601 a
	4	100 a	8.6 a	546 b
	8	67 bc	4.2 c	301 e
	16	18 d	0.8 e	183 f
Half-PLBs on: TC + AVG	1	100 a	8.2 a	581 ab
	2	100 a	6.8 b	315 de
	4	81 b	1.4 de	285 e
	8	46 c	0.9 de	191 f
	16	0 e	0 e	56 h
Half-PLBs on: TC + CEPA	1	88 ab	4.6 c	281 e
	2	61 bc	2.2 d	126 g
	4	23 d	0.6 e	38 h
	8	3 e	0.1 e	9 i
	16	0 e	0 e	0 i
PLB tTCLs on: TC + AgNO ₃	1	100 a	2.4 d	186 f
	2	100 a	3.1 cd	202 ef
	4	100 a	1.8 de	196 f
	8	46 c	1.2 de	124 g
	16	18 d	0.3 e	61 h
PLB tTCLs on: TC + AVG	1	100 a	1.8 de	168 g
	2	100 a	1.7 de	173 g
	4	88 ab	1.2 de	98 gh
	8	16 d	0.3 e	66 h
	16	0 e	0 e	0 i
PLB tTCLs on: TC + CEPA	1	79 b	0.2 e	48 h
	2	21 d	0 e	0 i
	4	7 e	0 e	0 i
	8	0 e	0 e	0 i
	16	0 e	0 e	0 i

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($P \leq 0.05$). See text for media constituents. $n = 90$ (9 (3 × 3 blocks) Petri dishes × 10 for each treatment). * Treatment against which all other treatments are compared.

plantlets without negative side-effects such as hyperhydricity or ethylene-induced senescence (Teixeira da Silva et al., 2006b). CEPA decreased all parameters related to *neo*-PLB formation (Table 1). The number of *neo*-PLBs formed by PLB-derived tTCLs is admittedly significantly less than control PLBs (Table 1), but the actual value, once adjusted to take into account total surface area and volume of the explant, using the Plant Conversion Factor (Teixeira da Silva and Dobránszki, 2011), is estimated to exceed the control values 3- to 4-fold (Teixeira da Silva and Dobránszki, 2013b). tTCLs also stimulated the growth of whole bean plants more than controls after induction by thidiazuron (Zuily-Fodil et al., 2000).

Ethylene build-up in culture vessels is a serious concern in plant tissue culture. The presence of PGRs (Gude and van der Plaas, 1985) or agar (Mensuari-Sodi et al., 1992) in medium can enhance the production of ethylene, negatively impacting plant cultures *in vitro* by stimulating senescence and by, in most cases, inhibiting regeneration. AgNO₃ and AVG only enhanced shoot regeneration in pomegranate when added to medium containing other PGRs, namely 6-benzyladenine and NAA (Naik and Chand, 2003). In that study, shoot regeneration decreased when CEPA was added to the medium. AgNO₃ inhibits ethylene action by competing with ethylene for binding sites in plant cell membranes while AVG inhibits ethylene biosynthesis (Yang and Hoffman, 1984). Several ethylene inhibitors were shown to improve Chinese cabbage *in vitro* shoot formation (Chi and Pua, 1989) while AgNO₃ and AVG stimulated shoot regeneration in 18 cultivars of cabbage, cauliflower and broccoli (Pua et al., 1999). Using tobacco TCLs, AVG inhibited ethylene production, presumably by inhibiting the activity of 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) genes, while simulating putrescine (a polyamine) synthesis (Torrighiani et al., 2003). This generally also leads to a reduction in endogenous ethylene production which in turn leads to a reduction of ACS and ACO gene expression (Ebrahimzadeh et al., 2008).

In orchids, PLBs are in fact considered to be somatic embryos (Teixeira da Silva and Tanaka, 2006), and thus this simple protocol (i.e., the use of ethylene inhibitors or aeration) represents an alternative means of producing *neo*-PLBs where a bioreactor is not available. This is relevant since AgNO₃ was also shown to enhance somatic embryogenesis in coffee (*Coffea canephora*) (Fuentes et al., 2000). The ability of ethylene inhibitors to reduce or eliminate the negative impacts of ethylene over quite a wide range of cellular and developmental stages could, in part, explain the apparent contradictions between the use of ethylene inhibitors and ventilated culture (Table 1), although ethylene itself may, depending on its concentration, also have a growth-stimulatory ability (Serek et al., 2006; Dugardeyn and Van Der Straeten, 2008; Muday et al., 2012).

Abbreviations

AgNO₃, silver nitrate; AVG, aminoethoxyvinylglycine; CEPA, 2-chloroethylphosphonic acid (or ethephon); NAA, α -naphthaleneacetic acid; PLB, protocorm-like body; PGR, plant growth regulator; TC, Teixeira Cymbidium medium (Teixeira da Silva, 2012); TCL, thin cell layer; tTCL, transverse TCL; TDZ, thidiazuron (*N*-phenyl-*N*-1,2,3-thidiazuron-5'-ylurea)

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Compliance with ethics guidelines

The author declares no conflicts of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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