JOHP
Journal of Ornamental Plants (Journal of Ornamental and Horticultural Plants)
Available online on: www.jornamental.com
ISSN (Print): 2251-6433
ISSN (Online): 2251-6441

Ammonium to Nitrate Ratio Affects Protocorm Like Bodies PLB Formation *In vitro* of Hybrid *Cymbidium*

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] Present address: P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799, Japan.

Received: 18 June 2013 Accepted: 15 August 2013
*Corresponding author's email: jaimetex@yahoo.com

Abstract

Carbon and nitrogen are the two elements that most affect plant organogenesis. *In vitro*, usually as part of the macronutrients. Some anecdotal evidence from the literature suggests that the ammonium (NH_4^+) to nitrate (NO_3^-) ratio may affect orchid organogenesis. In this study, to test this hypothesis, different NH_4^+: NO_3^- ratios were tested on the development of protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light', when modified in Teixeira *Cymbidium* (TC) No. 1 medium without plant growth regulators. More PLBs formed per half-PLB or per PLB tTCL when TC medium was used while TC from which PGRs or NO_3^- or NH_4^+ were removed (two controls) contained significantly less PLBs/explant. The same trend was observed for *neo*-PLB (i.e., newly formed PLB) fresh weight. A high NO_3^- ratio favoured *neo*-PLB formation, but always less than TC medium, but significantly higher than the two controls. tTCLs performed more poorly (i.e., fewer neo-PLBs/explant and lower fresh weight) than half-PLBs. The NH_4^+: NO_3^- ratio can be manipulated to increase the level of PLB production in the absence of plant growth regulators.

Keywords: Ammonium, Nitrate, PLB, Teixeira *Cymbidium* (TC) medium.

INTRODUCTION

Nitrogen (N) can be taken up by plants either as inorganic ions (ammonium, NH_4^+ or nitrate, NO_3^-), or as organic N (Barker, 1975). Among the essential nutrients, N has the greatest impact on plant growth, and tends to be species-specific (Marschner, 1995). Practical applications of this knowledge show that the yield of horticultural crops can be affected by the NH_4^+:NO_3^- ratio (Toor *et al.,* 2006). The slow growth of orchids was attributed to a sluggish N metabolism (Poddubnaya-Arnold, 1967). Oyamada (1989) showed that a high NO_3^-:NH_4^+ ratio resulted in as much as a 10-fold increase in fresh weight when *Cymbidium* PLBs were placed in liquid shake culture. The medium composition of several plant tissue culture media affected the organogenic outcome in hybrid *Cymbidium* protocorm-like body (PLB) formation and propagation (Teixeira da Silva *et al.,* 2005). Based on this knowledge, a new medium, Teixeira *Cymbidium* (TC) No. 1 medium was developed (Teixeira da Silva, 2012), favouring PLB production more than traditionally used Vacin and Went (VW; 1949) medium. In other orchids, high Cl^- and H^+ concentrations positively affected seedling regeneration in *Phalaenopsis* hybrid while increased NH_4^+ and NO_3^- concentrations promoted shoot growth (Hinnen *et al*., 1989). The supply of calcium nitrate to *Dendrobium wardianum* cultures resulted in direct shoot formation, surpassing the PLB formation stage, as would commonly occur with all the other nitrogen sources (Sharma and Tandon, 1992). Urea, although reported by Mariat (1948) to be an effective N source for *Cattleya* embryos, is a necrotic agent at higher concentrations (Sharma and Tandon, 1992). Concentrations and NH_4^+:NO_3^- ratio in the culture media have been known to affect the germination of orchid seeds and subsequent organogenesis considerably (for example, Shimasaki and Uemoto (1990) for *Cymbidium* and Zeng *et al*. (unpublished), for *Cypripedium*.

In vitro protocols for the induction and development of PLBs of hybrid *Cymbidium* are well established. Despite this, no study has yet examined the impact of the NH_4^+:NO_3^- ratio on organogenesis of *Cymbidium*. This is also the first study to examine the effect of the NH_4^+:NO_3^- ratio on PLB production in any orchid.

MATERIAL 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 almost identical to that described by Teixeira da Silva (2012). 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 every two months on TC medium (Teixeira da Silva, 2012), and was supplemented with two plant growth regulators, or PGRs (0.1 mg/l α-naphthaleneacetic acid and 0.1 mg/l kinetin), 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 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, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 $\mu mol\ m^{-2}\ s^{-1}$ provided by 40W fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally bisected PLBs (3-4 mm in diameter), referred throughout as half-PLBs, 10 per flask, were used as the explants for PLB induction and proliferation and as one of the two explants for all experiments. The second explant used was PLB transverse thin cell layers or tTCLs, prepared as explained in detail by Teixeira da Silva (2013). Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva *et al.*, 2005), biotic (Teixeira da Silva *et al.*, 2006b) and abiotic factors (Teixeira da Silva *et al.,* 2006a) for PLB induction, formation and proliferation.

Effect of NH_4^+:NO_3^- ratio on PLB formation

Half-PLBs and PLB tTCLs were cultured on TC medium containing one of the following NH_4^+:NO_3^- ratios: 0:10, 2.5:7.5, 1:1, 7.5:2.5, 10:0, all at the same N molarity as TC. Two controls were used: C-PGR, which contained no PGRs but contained TC N levels and C-N, which contained TC PGRs but no NO_3^- or NH_4^+. The number of PLBs formed per PLB segment or PLB tTCL was measured. All measurements were made after 45 days in culture.

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 in triplicate (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). Significant differences between means were assumed at $p \leq 0.05$.

RESULTS AND DISCUSSION

TC medium resulted in the highest number of PLBs per half-PLB or per PLB tTCL while TC from which PGRs or NO_3^- or NH_4^+ were removed contained significantly less PLBs/explant (Table 1). The same trend was observed for *neo*-PLB fresh weight. A *neo*-PLB is a newly formed PLB. A high ratio of NO_3^- favoured *neo*-PLB formation, but never higher than TC medium, but significantly higher than both control treatments (Table 1). tTCLs performed more poorly (i.e., fewer *neo*-PLBs/explant and lower fresh weight) than half-PLBs, but this is understandable considering that tTCLs have a much lower surface area than half-PLBs (Teixeira da Silva and Dobránszki, 2013). Despite lower apparent, face-value productivity levels of tTCLs, it is likely that true productivity could exceed that of half-PLBs once a suitable conversion factor, the plant Growth Correction Factor, is applied (Teixeira da Silva and Dobránszki, 2011).

Kubota *et al*. (2000) noted that NH_4^+ antagonistically inhibited the absorption of P, K, Ca and Mg, but particularly divalent cations Ca^{2+} and Mg^{2+}; moreover, when NO_3^- was used as the only N source, or when the ratio of NO_3^- was high, then *Phalaenopsis* plant growth was favoured. In their study, Kubota *et al*. (2000) also noted that leaf number decreased at a 8:2 and a 10:0 ratio of NH_4^+:NO_3^-, leaf area increased when the ratio was 0:10 and 2:8, and root number and length increased linearly as the NH_4^+ concentration increased, but leaf, root and whole plant dry weight decreased as the NH_4^+ concentration increased. Hinnen *et al.* (1989) had recommended a 6:4 ratio of NH_4^+:NO_3^- for optimal growth of *Phalaenopsis* shoots, which contradicted the findings of Kubota et al. (2000). In the commercial production of orchids, Hyponex, a commercial liquid fertilizer, or in powder form, is used, with an N-P-K of 6.5-2.6-15.8, and this usually contains 14 mM of N, consisting of a 1.5:8.5 NH_4^+:NO_3^- ratio (Ichihashi, 1997), favouring the need for NO_3^-, as also presumed by Kubota *et al.* (2000). Ichihashi (1979) indicated that a 4:6 NH_4^+:NO_3^- ratio improved the *In vitro* growth of *Bletilla striata* seedling growth, but since molarities differed, those results were in fact flawed and/or inconclusive. Studying two epiphytic orchids, *Dendrobium nobile* and *Cattleya*, Uesato (1973, 1974) showed that a 8:2 or 7:3 ratio of NH_4^+:NO_3^-, respectively promoted *In vitro* shoot growth, but that shoot growth was depressed when the NH_4^+ ratio increased further. Shimasaki and Uemoto (1990) noted that high levels of $NH_4^+NO_3^-$ (ammonium nitrate) inhibited the growth of *Cymbidium kanran* (a terrestrial orchid) shoots *In vitro*, but that moderate levels of $NH_4^+NO_3^-$ together with moderate levels of potassium nitrate (KNO_3^-) increased shoot and root growth. Using nutrient solutions, Hew *et al*. (1992) showed that the growth of two terrestrial orchids, *Cymbidium* and *Bromheadia*, was not perturbed if only NH_4^+ was used. These studies that exist on the manipulation of the NH_4^+:NO_3^- ratio in orchids indicates that the optimal NH_4^+:NO_3^- ratio depends on the species and even on the genotype (cultivar).

In other crops *In vitro*, a high NH_4^+:NO_3^- ratio improved garlic (*Allium sativum*) micropropagation (Luciani *et al.,* 2001). By changing the NH_4^+:NO_3^- ratio from 39.5 mM + 20.5 mM to 15.8 mM + 20.5 mM, Bensaddek *et al*. (2001) could improve the growth, alkaloid production and scopolamine/hyoscyamine ratio of two clones of belladonna (*Atropa belladonna*) hairy roots. By exposing plants to a high level of ammonium nitrate, Greer *et al.* (2009) were able to improve the frequency of wheat (*Triticum aestivum*) transformation. Garnica *et al*. (2010) showed that NO_3^- reduces the negative effects caused by NH_4^+ and promotes plant growth by enhancing the levels of cytokinins and indole-3-acetic acid, and lowering the levels of abscisic acid. There are many other studies that have examined the effect of the NH_4^+:NO_3^- ratio in greenhouse or field production, but those studies lie beyond the scope of this study.

PLBs and somatic embryos are synonymous in orchids (Teixeira da Silva and Tanaka, 2006), and thus this protocol represents another simple way of producing PLBs, which are essential to orchid biotechnology (Hossain *et al*., 2013).

ACKNOWLEDGEMENTS AND CONFLICT OF INTEREST

The author thanks Prof. Michio Tanaka for research support. The author declares no conflicts of interest.

Litrature Cited

Barker, A.V. 1975. Organic vs. inorganic nutrition and horticultural crop quality. HortScience 10: 50-53.

Bensaddek, L., Gillet, F., Saucedo, J.E.N., Fliniaux, M-A. 2001. The effect of nitrate and ammonium concentrations on growth and alkaloid accumulation of *Atropa belladonna* hairy roots. J. Biotechnol. 85: 35-40.

Garnica, M., Houdusse, F., Zamarreño, A.M., Garcia-Mina, J.M. 2010. The signal effect of nitrate supply enhances active forms of cytokinins and indole acetic content and reduces abscisic acid in wheat plants grown with ammonium. J. Plant Physiol. 167: 1264-1272.

Greer, M.S., Kovalchuk, I., Eudes, F. 2009. Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. New Biotechnology 26: 44-52

Hew, C.S., Lim, L.Y. and Low, C.N. 1992. Nitrogen uptake by tropical orchids. Environ. Exp. Bot. 33: 273-281.

Hinnen, M.G.J., Pierik, R.L.M., and Bronsema, F.B.F. 1989. The influence of macronutrients and some other factors on growth of *Phalaenopsis* hybrid seedlings *In vitro*. Sci. Hort. 41: 105-116.

Hossain, M.M., Kant, R., Van, P.T., Winarto, B., Zeng, S.J., and Teixeira da Silva J.A. 2013. The application of biotechnology to orchids. Critical Rev. Plant Sci. 32(2): 69-139.

Ichihashi, S. 1979. Studies on the media for orchid seed germination. III. The effects of total ionic concentration, cation/anion ratio, NH_4^+/NO_3^- ratio, and minor elements on the growth of *Bletilla striata.* J. Jpn. Soc. Hort. Sci. 47: 524-536.

Ichihashi, S. 1997. Research on micropropagation of *Cymbidium*, nobile-type Dendrobium, and *Phalaenopsis* in Japan. In: Orchid Biology: Review and Perspectives, VII, Arditti, J., Pridgeon, A.M. (eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 285-316.

Kubota, S., Yoneda, K., Suzuki, Y. 2000. Effects of ammonium to nitrate ratio in culture medium on growth and nutrient absorption of *Phalaenopsis* seedlings *In vitro*. Environ. Control Biol. 38(4): 281-284.

Luciani, G.F., Marinangeli, P.A., Curvetto, N.R. 2001. Increasing nitrate/ammonium ratio for improvement of garlic micropropagation. Sci. Hortic. 87: 11-20.

Mariat, F. 1948. Influence des facteurs de croissance sur le dévelopment et la differenciation des

embryons d'orchidées. Rev. Générale Bot. 55: 229-243.
Marschner, H. 1995. Mineral nutrition of higher plants (2nd Edn), Academic Press, London.
Oyamada, T. 1989. Effects of mineral nutrient composition on the proliferation of *Cymbidium* PLBs cultures *In vitro*. Scientific Rep. Fac. Agric., Meijo Univ. 25: 27-34.
Poddubnaya-Arnold, V.A. 1967. Comparative embryology of the Orchidaceae. Phytomorphology 17: 312-320.
Sharma, A., and Tandon, P. 1992. *In vitro* culture of *Dendrobium wardianum* Warner: morphogenetic effects of some nitrogenous adjuvants. Indian J. Plant Physiol. 35: 80-85.
Shimasaki, K., and Uemoto, S. 1990. Micropropagation of a terrestrial *Cymbidium* species using rhizomes developed from seeds and pseudobulbs. Plant Cell, Tiss. Organ Cult. 22: 237-244.
Teixeira da Silva, J.A. 2012. New basal media for protocorm-like body and callus induction of hybrid *Cymbidium*. J. Fruit Ornamental Plant Res. 20(2): 127-133.
Teixeira da Silva, J.A. 2013. The role of thin cell layers in regeneration and transformation in orchids. Plant Cell, Tiss. Organ Cult. 113(2): 149-161.
Teixeira da Silva, J.A., Chan, M.T., Sanjaya Chai, M.L. and Tanaka, M. 2006a. Priming abiotic factors for optimal hybrid *Cymbidium* (Orchidaceae) PLB and callus induction, plantlet formation, and their subsequent cytogenetic stability analysis. Sci. Hort. 109(4): 368-378.
Teixeira da Silva, J.A., and Dobránszki, J. 2011. The plant Growth Correction Factor. I. The hypothetical and philosophical basis. Int. J. Plant Dev. Biol. 5(1): 73-74.
Teixeira da Silva, J.A., and Dobránszki, J. 2013. How timing of sampling can affect the outcome of the quantitative assessment of plant organogenesis. Sci. Hort. 159: 59-66.
Teixeira da Silva, J.A., Singh, N., and Tanaka, M. 2006b. Priming biotic factors for optimal protocorm-like body and callus induction in hybrid *Cymbidium* (Orchidaceae), and assessment of cytogenetic stability in regenerated plantlets. Plant Cell, Tissue Organ Cult. 84(2): 119-128.
Teixeira da Silva, J.A., and Tanaka, M. 2006. Embryogenic callus, PLB and TCL paths to regeneration in hybrid *Cymbidium* (Orchidaceae). J. Plant Growth Reg. 25(3): 203-210.
Teixeira da Silva, J.A., Yam, T., Fukai, S., Nayak, N., and Tanaka, M. 2005. Establishment of optimum nutrient media for *In vitro* propagation of *Cymbidium* Sw. (Orchidaceae) using protocorm-like body segments. Prop. Orn. Plant 5(3): 129-136.
Toor, R.K., Savage, G.P., and Hee, A. 2006. Influence of different types of fertilisers on the major antioxidant components of tomatoes. J. Food Composition Anal. 19: 20-27.
Uesato, K. 1973. Effects of different forms of nitrogen sources in the culture media on the growth of *Cattleya* young seedlings. Sci. Bull. Coll. Agric. Univ. Ryukyus 20: 1-12.
Uesato, K. 1974. Effects of different forms of nitrogen sources in the culture media on the growth of *Dendrobium nobile* seedling. Sci. Bull. Coll. Agric. Univ. Ryukyus 21: 73-81.
Vacin, E., and Went, F.W. 1949. Some pH changes in nutrient solutions. Bot. Gaz. 110: 605-613.
Zeng, S.J., Teixerita da Silva, J. A., Wu, K. L., Zhang, J.X. and Duan, J. 2013. Cypripedium culture *In vitro*-A review. In review.

Tables

Table 1. Effect of NH_4^+:NO_3^- ratio on PLB formation from half-PLB culture of hybrid *Cymbidium Twilight* Moon 'Day Light'.

Medium composition	NH_4^+:NO_3^- ratio (all identical molarity)	Percentage of explants forming *neo*-PLBs (%)	Number of PLBs per explant	Fresh weight (mg) of PLB explant + *neo*-PLBs
TC medium*		100 a	8.3 a	526 a
TC medium**		100 a	1.6 de	103 e
C-PGR (control 1)*		47 c	1.4 de	108 e
C-N (control 2)*		23 a	0.8 e	86 f
Half-PLBs	0:10	96 a	7.1 b	418 b
	2.5:7.5	91 ab	6.3 c	381 c
	1:1	76 b	2.6 d	196 d
	7.5:2.5	27 d	1.1 e	83 f
	10:0	0 f	0 f	0 h
PLB tTCLs	0:10	88 ab	1.4 de	74 fg
	2.5:7.5	61 bc	1.1 e	56 g
	1:1	45 c	0.6 ef	48 g
	7.5:2.5	13 e	0.1 f	26 gh
	10:0	0 f	0 f	0 h

Mean values followed by the same letter in the same column are not significantly different based on DMRT ($P = 0.05$). n = 90 (9 Petri dishes × 10 for each treatment). * = half-PLBs; ** = tTCLs. C-PGR: contains no PGRs but contains TC N levels; C-N, contains TC PGRs but no NO_3^- or NH_4^+. General abbreviations: PGR, plant growth regulator; PLB, protocorm-like body; TC, Teixeira *Cymbidium* No. 1 medium (Teixeira da Silva 2012), includes 0.1 mg/l α-naphthaleneacetic acid 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.