In Vitro Young Leaf Culture of *Doritis Pulcherrima* Var. *Buyssoniana*

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Abstract

In vitro culture of young leaf segments from *in vitro* seedlings of *Doritis pulcherrima* var. *buyssoniana* were examined in three studies. The first study examined leaf segment parts (basal and tip segments) and effects of positioning (vertical or horizontal) explants on the new Dogashima medium (NDM). Three months after culture, tip segments showed no growth, but the basal segments developed differently depending on how they were positioned on the medium. Basal segments placed vertically produced plantlets or shoots at their bases. Those placed horizontally produced whole plantlets, shoots, multiple shoots, callus and protocorm-like bodies (PLBs). The second study examined the effect of the plant growth substances, α–naphthaleneacetic acid (NAA) at 0, 0.1, 0.5, 1 and 5 mg/l in combination with benzyladenine (BA) at 0, 0.1, 0.5, 1 and 5 mg/l in the NDM. The best results for further micropropagation were obtained from 0.1 mg/l NAA and 1 mg/l BA in the NDM which produced PLBs and callus from basal segments upto 70%. In the third study, the effect of 4 basal media: modified Vacin and Went medium (VW), NDM, Murashige and Skoog medium (MS) and Ichihachi and Yamashita medium (IY) were examined when supplemented with 0.1 mg/l NAA and 1 mg/l BA. The best medium was NDM that enabled *D. pulcherrima* var. *buyssoniana* leaf to produce 26% multiple shoots, 17% callus and 18% PLBs.

Keywords : basal medium, *Doritis*, young leaf, micropropagation, plant growth regulator

1. Introduction

Doritis pulcherrima var. *buyssoniana* is a monopodial lithophytic species distributed only in Thailand and Indochaina (Christenson, 2001). It is known as Daeng Ubon, an endemic orchid in Ubon Ratchathani province, north-east Thailand (Rakpaibul-sombat, 1992). With larger flowers, larger leaves and longer inflorescences than *Doritis pulcherrima*, its attractiveness creates a huge demand of plant materials. It is thus necessary to find the most suitable method for mass clonal propagation.

Tissue culture techniques using shoot tips and axillary buds as explants are used widely in commercial orchid micropropagation. However, *Phalaenopsis* and *Doritis* have short stems. *In vitro* culture using shoot tips may lead to the loss of the mother plant. The culture of young leaves is a technique that has used in orchids since 1965 (Arditti, 1977a). Under appropriate conditions, these explants may produce protocorm-like-bodies (PLBs) that develop into complete plants, either through direct organogenesis or callus formation (Arditti, 1977b). Propagation of orchids through young leaf culture has been successful in *Aranda, Cattleya, Cymbidium, Dendrobium, Epidendrum, Laeliocattleya, Oncidium, Paphiopedilum, Phalaenopsis, Rhynchostylis* and *Vanda* (Arditti, 1977a; Vij, 1984; Chen, 2001; Chung, 2005; Kuo., 2005; Chen, 2006). A few genus such as *Oncidium* (Chen, 2001), *Phalaenopsis* (Chen, 2006) and *Doritanopsis* (Park, 2002) are reported to be the best orchids in inducing PLBs by leaf culture. The aim of this experiment was to establish suitable techniques for producing PLBs from leaf segments of *Doritis*.

2. Material and methods

2.1 Plant material

Three-month-old capsule was collected from *Doritis pulcherrima* var. *buyssoniana* plants cultured in a shade house. The capsule was surface sterilized with 70% alcohol for 2 minutes and direct flame for 30 seconds. Seeds from the capsule were sown on modified Vacin and Went medium (VW). Young leaves, 1-2 cm. in length, were taken from six-month-old seedlings by cutting at their base and then crossly divided into two explants (basal and tip segments). Micropropagation experiments were carried out with these explants. A completely randomized design (CRD) was used in all experiments

2.2 Induction of PLBs from leaf segment parts and method of placement on the medium

Explants (basal and tip segments) were placed on new Dogashima medium (NDM) containing 1 mg/l BA with two placements: insert in vertically or lay horizontally on, the media. Five replicates of eight explants were used for each treatment. Treatment means were compared by The Least Significant Difference.

2.3 Determining the appropriate concentration of plant growth substances

Explants (basal and tip segments) were laid horizontally on NDM with 0, 0.1, 0.5, 1 and 5 mg/l α–naphthaleneacetic acid (NAA) in combination with 0, 0.1, 0.5, 1 and 5 mg/l benzyladenine (BA). Twenty explants were used in each treatment.

2.4 Determining the appropriate basal media on young leaf culture

The best results of the first two studies were used as the basis for studies of four media. Basal leaf segments were laid horizontally on four basal media; VW, NDM, Murashige and Skoog (MS) and Ichihashi and Yamashita (IY) (Table 1) supplemented with 0.1 mg/l NAA and 1 mg/l BA. Five replicates with eight explants were used for each treatment. Treatment means were compared by The Least Significant Difference.

2.5 Culture condition

Cultures were maintained at 25 $\pm$ 2 ^{o}C under 14 hr photoperiods at irradiance of 37.6 μmol $m^{-2}s^{-1}$ (daylight fluorescent lamp TL-D, 36W/54, Philips Electronic N.V. Thailand)

Table 1 Components of four basal media (mg/l) used for young leaf culture

Components	Murashige and Skoog (MS)	Ichihashi and Yamashita (IY)	new Dogashima medium (NDM)	mod-Vacin and Went (VW)
KH_2PO_4	170	-	550	250
$Ca(NO_3)_2 \cdot 4H_2O$	-	826	470	-
KNO_3	1900	747	200	525
$MgSO_4 \cdot 7H_2O$	370	172	250	250
$(NH_4)_2SO_4$	-	-	-	500
NH_4NO_3	1650	-	480	-
$CaCl_2 \cdot 2H_2O$	440	-	-	-
Ca_3PO_4	-	-	-	200
$NH_4H_2PO_2$	-	391	-	-
KCl	-	-	150	-
$FeSO_4 \cdot 7H_2O$	27.9	-	-	27.9
Na_2EDTA	37.3	-	-	37.3
Fe_2EDTA	-	25	21	-
$CoCl_2 \cdot 6H_2O$	0.025	-	0.025	0.025

Components	Murashige and Skoog (MS)	Ichihashi and Yamashita (IY)	new Dogashima medium (NDM)	mod-Vacin and Went (VW)
$CuSO_4 \cdot 5H_2O$	0.025	0.03	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.025	0.25
KI	0.83	0.01	-	0.83
$ZnSO_4 \cdot 7H_2O$	8.6	7	0.5	8.6
$MnSO_4 \cdot 4H_2O$	22.3	0.01	3	16.9
H_3BO_3	6.2	1.0	0.5	6.2
$NiCl_2$	-	0.03	-	-
conc.H_2SO_4	-	-	0.5 ml	-
Nicotinic acid	0.5	-	1.0	0.5
Pyridoxine	0.5	-	1.0	0.5
Thiamine	0.1	-	1.0	0.1
Glycine	2.0	-	-	2.0
Inositol	100	-	100	100
Biotin	-	-	0.1	-
Calcium pantothenate	-	-	1.0	-
Adenine	-	-	1.0	-
l-Cystein	-	-	1.0	-
Sucrose	20	20	20	20
Agar	6	6	6	6
pH	5.6	5.2	5.4	5.0

3. Results

3.1 Induction of PLBs from leaf segment parts and method of placement on the medium

The tip segments did not form PLBs regardless of how they were placed on the medium. The basal segments placed either vertically or horizontally, produced shoots at their bases (Table 2). Development pattern of basal segments depended on how they were placed on the medium. Of basal segments placed vertically on the medium, 17.5% developed whole plantlets, 2.5% produced roots and 10% formed shoots.

Table 2 Developmental percentage of leaf segments of *Doritis pulcherrima* var *buyssoniana* placed vertically or horizontally on new Dogashima medium with 1 mg/l BA after three months of culture.

Leaf segment part	Placement on media	Survival	No response	Plantlets	Roots	Shoots	Multiple shoots	C*	PLB+C*
Basal	Vertical	70 c	40 b	17.5 a	2.5 a	10 b	0 b	0 b	0 a
Tip	Vertical	82.5 bc	82.5 a	0 b	0 a	0 c	0 b	0 b	0 a
Basal	Horizontal	100 a	12.5 c	17.5 a	0 a	30 a	17.5 a	20 a	2.5 a
Tip	Horizontal	87.5 ab	87.5 a	0 b	0 a	0 c	0 b	0 b	0 a

* C, callus ; PLB+C, protocorm-like body and callus

Means followed by same letters in a column are not significantly different at $P = 0.05$ by LSD

Among basal segments placed horizontally on the medium, 17.5% produced whole plantlets, 30% formed shoots, 17.5% grew multiple shoots, 20% gave rise to callus and 2.5% generated PLBs with callus (Table 2 and Fig. 1). Horizontal placement of basal segments was thus the most suitable for mass propagation from young leaf segments of *Doritis pulcherrima* var. *buyssoniana*.

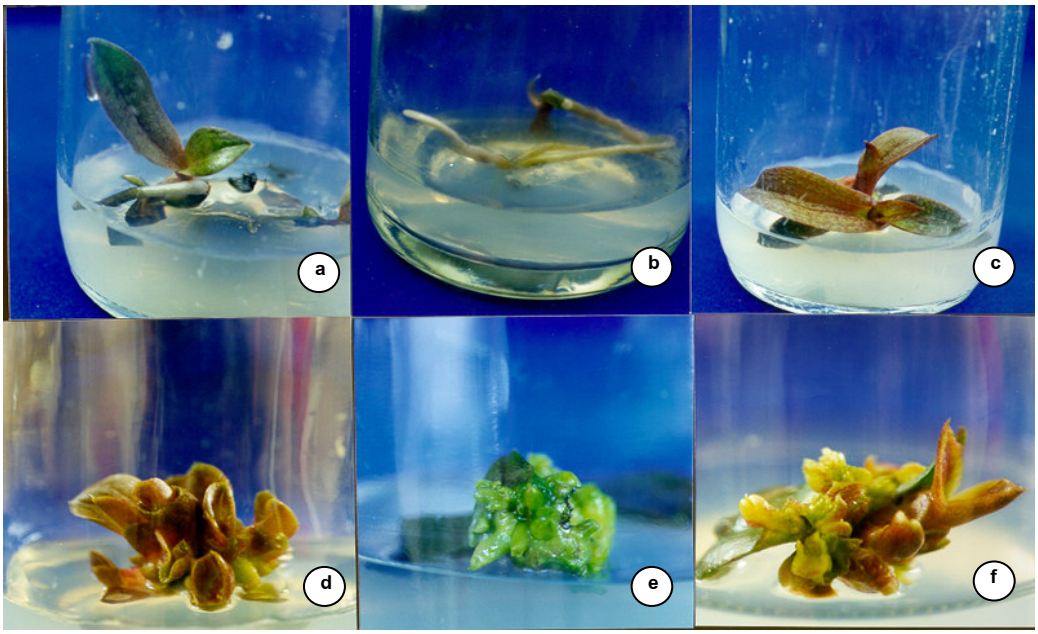

Fig. 1 Development of basal leaf segments of *Doritis pulcherrima* var. *buyssoniana*
a. plantlet b. root c. shoot d. multiple shoots e. PLBs with callus f. PLBs developing shoots.

3.2 Determining the appropriate concentration of plant growth substances

Leaf tip segments cultured in every medium had no response (data not shown). The basal explants responsed in different patterns of development (Table 3). A high percentage of plantlets (60%) developed on NDM in absence of NAA and BA. Propagation of *Doritis pulcherrima* var. *buyssoniana* from young leaf base explants was successful in the presence of 0-1 mg/l NAA in combination with 0.1-5 mg/l BA. However, the presence of 0.1 mg/l NAA and 1 mg/l BA was the best for inducing PLBs in mass propagation.

Table 3 Effect of naphthaleneacetic acid and benzyladenine on differentiation of basal leaf segments after three months in culture.

NAA mg/l	BA mg/l	No. of explant	Survival (%)	No. explant responding (%)	Percent explants responding						
					Plantlet	Roots	Shoots	Multiple shoots	C+ *	C++ *	PLB +C *
0	0	20	95	25	60	5	5	-	-	-	-
	0.1	20	70	25	35	-	5	5	-	-	-
	0.5	20	85	20	10	-	5	20	15	15	-
	1	20	60	-	5	-	-	20	20	15	-

NAA mg/l	BA mg/l	No. of explant	Survival (%)	No. explant responding (%)	Percent explants responding						
					Plantlet	Roots	Shoots	Multiple shoots	C+ *	C++ *	PLB +C *
	5	20	50	10	-	-	-	20	10	10	-
0.1	0	20	75	35	40	-	-	-	-	-	-
	0.1	20	85	30	25	-	-	10	-	-	20
	0.5	20	95	15	10	-	-	10	20	-	40
	1	20	100	10	5	-	10	5	-	-	70
	5	20	80	25	-	-	15	10	-	-	30
0.5	0	20	90	25	30	-	15	15	5	-	-
	0.1	20	95	20	40	-	15	15	5	-	-
	0.5	20	95	40	15	-	25	-	-	10	5
	1	20	90	25	-	-	10	45	10	-	-
	5	20	50	10	-	-	-	-	15	-	25
1	0	20	95	20	10	-	5	5	-	-	55
	0.1	20	85	20	-	-	15	15	5	-	30
	0.5	20	80	45	5	-	5	5	-	-	20
	1	20	75	15	10	-	15	5	20	-	10
	5	20	55	40	5	-	5	-	-	-	5
5	0	20	65	55	-	-	10	-	-	-	-
	0.1	16	81.25	81.25	-	-	-	-	-	-	-
	0.5	20	70	50	-	-	15	-	5	-	-
	1	20	35	20	-	-	5	-	10	-	-
	5	18	66.66	22.22	-	-	11.11	-	33.3	-	-

*C+, small callus; C++, some callus; PLB+C; protocorm-like bodies with callus

3.3 Determining the appropriate basal media on young leaf culture.

Effects of NDM, VW, MS and IY supplement with 0.1 mg/l NAA and 1 mg/l BA on the induction of multiple shoots, callus and PLBs are presented in Table 4. Explants on NDM medium had 85% survival and produced the best result with 27.5% multiple shoots, 20% callus and 22.5% PLBs development. Survival on MS medium was only 32.5%. However, surviving explants produced a high percentage of multiple shoots (Table 4). Explants on the IY medium had high survival (88%) but low induction (15%). Shoot formation from explants on the IY medium was only 5% whilst 10% of explants produced callus (Table 4).

4. Discussion

Basal segments of *D. pulcherrima* var. *buyssoniana* induced shoot, callus and PLBs at their base. Development at the base of leaves was similar to that in *Cattleya*. In *Cattleya*, the meristematic area which forms callus and PLBs are in the epidermal cells of the basal region (Arditti, 1977b; Pierik, 1989). The same is true for *Aranda* (Loh, 1975). In *Rhynchostylis retusa*, the initiation of PLBs formation was in the upper and lower epidermal cells near the cut ends of the explants. The entire surface of the juvenile leaf is potentially meristematic in *Rhynchostylis retusa* and *Phalaenopsis amabilis* (Vij, 1984; Chen, 2006). The restriction of such an activity in the leaf tip or leaf base may be associated with the genetic makeup and physiological age of the explant, and/or the medium being employed (Vij, 1984).

Table 4 Survival and developmental percentage of basal leaf segment of *Doritis pulcherrima* var. *buyssoniana* on four basal media supplemented with 0.1 mg/l naphthaleneacetic acid and 1 mg/l benzyladenine after three months in culture.

Basal media	Survival	Shoots	Multiple shoots	Callus	PLB
NDM	85 a	0 a	27.5 a	20 a	22.5 a
VW	47.5 b	5 a	7.5 b	10 ab	2.5 b
MS	32.5 c	0 a	22.5 a	5 b	0 b
IY	87.5 a	5* a	0 b	10 ab	0 b

Means followed by same letters in a column are not significantly different at $P = 0.05$ by LSD

*Shoot in the IY medium showing one small leaf with 0.3-0.5 cm

Table 5 Concentration and ratio of NH_4^+ and NO_3^- in four basal media

Media	NH_4^+ (mM)	NO_3^- (mM)	NH_4^+ / NO_3^- ratio
NDM	6.0	11.96	0.50
VW	7.57	5.19	1.46
MS	20.63	39.42	0.52
IY	3.40	14.39	0.24

Placement of explants on the medium, either vertically or horizontally, resulted in different patterns of development. Horizontal orientation of *D. pulcherrima* var. *buyssoniana* not only produced more shoots but also induced multiple shoots, callus and PLBs. This induction was performed by the appropriate ratio of auxin and cytokinin in explants. *Fraxinus angustifolia* and *Quercus robur* explants were found to have better induction by horizontal placement (Parez-Parron, 1994; Vieitez, 1994). On the other hand, vertical orientation of *Wrightia tomentosa* was found better than horizontal orientation (Purohit, 2004). The distinctive property auxin movement is known as basipetal transport (from tip to base). Vertically placed explants of *D. pulcherrima* var. *buyssoniana* might accumulate auxin at the base of base leaf segments, causing a high ratio of auxin: cytokinin, promoting plantlets, shoots and roots.

The most suitable concentration of NAA and BA for inducing PLBs in mass clonal propagation was 0.1 mg/l NAA and 1 mg/l BA. The concentration of plant growth substances in this experiment was the same concentration used to induce PLBs from shoot tips of flower stalk buds in *Phalaenopsis* and *Doritaenopsis* (Tokuhara, 1993). A high percentage of plantlets (60%) developed on NDM in absence of NAA and BA suggests that endogenous auxins and cytokinins in young leaves of *Doritis pulcherrima* var. *buyssoniana* at an appropriate ratio for plantlet induction.

The four basal media used in this experiment have been used successfully in other studies of orchid leaf culture. VW was used to induce PLBs from young leaves of *Phalaenopsis* (Tanaka, 1980; Chen, 1998) and *Dendrobium* leaf bases (Arditti, 1993). MS was used to culture leaves of *Phalaenopsis* (Tanaka and Sakanishi, 1980; Hass-Von Schmude, 1984; Kuo, 2005; Chen, 2006) and *Dendrobium* (Arditti and Ernst, 1993). IY was used to culture leaves of *Acampe rigida* (Yam, 1991). NDM was used in mass micropropagation, cell suspension culture and leaf culture of *Phalaenopsis* and *Doritanopsis* (Tokuhara, 2003; Tsukazaki, 2000; Tokuhara, 1993). The culture of *D. pulcherrima* var. *buyssoniana* leaf explant found that survival on MS medium gave only 33% survival

(Table 4). This was probably due to the high ionic concentration in the MS medium. In each of the basal media, there are several nitrogen sources. It is possible that the form, concentration and ratios of inorganic nitrogen (NH_4^+ and NO_3^-) influence the differentiation and growth of plantlets. Some orchid tissue such as *Vanda* are sensitive to nitrogen content, others like *Cattleya* are not (Arditti, 1977b). In case of *Doritis*, form and concentration of nitrogen might not serious effect on the differentiation but ammonium/nitrate ratio did. The ammonium/nitrate ratio of the four basal media in Table 5 showed that NDM and MS had an ammonium/nitrate ratio of 0.50 and 0.52 respectively. It is possible that this ratio is optimal for high formation of multiple shoots. However, the reason why NDM medium is suitable for mass rapid propagation from leaf explant of *D. pulcherrima* var. *buyssoniana* is probably due to the presence of adenine (Table 1). Adenine has some cytokinin activity (Arditti, 1993). Chen. (1998) and Tanaka and Sakanishi (1980) added adenine to the culture medium to induce PLBs from *Phalaenopsis* young leaves.

5. Conclusion

Micropropagation of *D. pulcherrima* var. *buyssoniana* by young leaf segment cultures could be effectively establish when basal segments were placed horizontally on the NDM medium with 0.1 mg/l NAA and 1 mg/l BA.

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