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Full Length Research Paper

Investigations of different strategies for high frequency regeneration of *Dendrobium malones* 'Victory'

Saima Anjum, Muhammad Zia and M. Fayyaz Chaudhary*

Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

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Regeneration of orchid, *Dendrobium malones* "Victory", has been established by direct differentiation and through callus formation. With direct thin leaf section differentiation method, the number of protocorm like bodies (PLBs) formed from Thin Section (TS) explants obtained from a single leaf was 10 times more than that from a single whole leaf, in a short time period. This therefore suggested that the proliferation potential is distributed all along the seedling leaves and morphogenically competent cells were not limited in the basal ends or the tips of the leaves in TS of orchid leaf cultures. Full MS media supplemented with auxins/cytokinins either in single or in combination along with peptone, yeast extract, casein hydrolysate (each at 100 mg/l), banana powder (40 g/l), 15% coconut water and 2% sucrose have shown excellent results for high frequency regeneration in orchid cultures. In many higher plants, regenerating leaves responded by developing proliferative loci in some "Predetermined Regenerative" cells in the dermal layers by tissue culture methods.

Key words: *Dendrobium malones,* protocorm, callus, organogenesis.

INTRODUCTION

Orchids (*Dendrobium malones* "Victory") are one of the evolved plants in the plant kingdom with high academic and commercial value. They are generally difficult to grow from seeds because in nature they require symbiotic fungus. Beyrle et al. (1985) described that *in vitro* mycorrhiza formation for *D. maculata* and other *Dendrobium* sp. protocorms development took place within 2 weeks while further development required a cold treatment of 12 weeks. Ye et al. (1988) studied immature seed morphology and the development of *D. candidum.* It was found that *in vitro* germination rate was 95% and the protocorm arose form embryos and could form calli or plantlets. Devi et al. (1990) compared different media for germination and growth of *Dendrobium* sp. They found that germination was 50-60% higher in Vacin and Went (VW) (1949) medium and addition of 15% coconut milk, 5% banana extract to VW medium further enhanced germination and resulted in accelerated seedling, leaf and root growth. Liquid VW medium was effective for callus initiation but protocorms failed to develop further unless transferred to agar-based medium supplemented with 15% coconut milk. Sharon et al. (1990) cultured the shoot meristem of the cut flower orchids *D. joanmie* on liquid and solid VW medium. Green protocorms were differentiated within four weeks which however failed to develop further unless transferred to agar based medium. Xiulin and Ye (1995) reported regeneration potential of isolated organs of *Dendrobium* hybrid *in vitro* with callus forming at the base of shoots, apical meristem, young leaves and nodes. The callus gave rise to protocorms like bodies and adventitious buds that could be used for mass clonal propagation.

*Corresponding author: fayyazchaudhary@yahoo.com. Tel: 92-51-2873641.

This study was conducted to analyze hormonal effect of seed germination of *D. malones* and direct and indirect organogenesis through apical meristem thin section of leaf explants.

MATERIALS AND METHODS

The method illustrated by Knudsen (1974) was used for seed sterilization. Orchid's seeds were sterilized in a solution of calcium hypochlorite (1 mg/10 ml). The test tube containing seeds and sterilant solution was vigorously shaken for 15 min in a rotary shaker. After thorough washing on filter paper, seeds were collected and inoculated over the surface of the Murashige and Shoog (MS, 1962) medium containing different concentrations / combinations of growth hormones.

Shoot apical meristems of *in vitro* grown seedlings served as explant source for callus initiation. Top portions of shoots were cut and leaves were removed without damaging the tips to prepare 0.5 cm explants. MS medium was used throughout the entire studies with peptone, casein hydrolysate, yeast extract (100 mg/l each), banana powder (40 gm/l), 15% coconut water and 2% sucrose in addition to various growth regulators. The media was solidified with 0.8% (w/v) agar and the cultures were incubated under fluorescent white light, 16/8 h light/dark cycle. The temperature was regulated at 25 ± 1°C and relative humidity was maintained at 70%, approximately.

RESULTS AND DISCUSSION

Seed germination in *D. malones* plantlet development following greening the seeds and protocorm formation was determined. BAP (6-benzylamino purine) and NAA (α-nephthalene acetic acid) in combination at all concentrations proved the best (100%) for *D. malones* seed germination (Table 1) where protocorm formation was excellent and assimilatory roots also developed on protocorms. Seed germination was only 10% at hormone free MS medium and it took 190 days. Callus formation from green seed repressed the development of plantlets. Effect of other cytokinins and auxins in MS medium on germination of *D. malones* seeds was also observed but with these hormones seed germination was low, protocorm formation was less and it took many days for plantlet development. MS medium supplemented with 2 mg/l NAA proved the best in single where seed germination was 100% with excellent protocorm formation within 4 days. 100% seed germination was also observed at BAP (2 and 3 mg/l) but root development on protocorm was suppressed. De-Pauw et al. (1995) also concluded that BAP at various concentrations was best for seed germination; however higher concentrations delayed root development.

2,4-Dicholorophenoxy acetic acid (2,4-D), an effective auxin for callus induction was analyzed from apical meristem explants of *D. malones*. The results are summarized in Table 2. Soft, friable and green callus were produced on MS medium supplemented with 1.0 mg/l 2,4-D. The callus turned to embryogenic and differentiated into protocorm like bodies that were swollen at the base and pointed at the tips. Some of these subsequently regenerated shoots into matured swollen structures called pseudo-bulbs (characteristics of some epiphytic orchids). Nearly same results were observed at 2.0 mg/l 2,4-D. At low concentration of hormone, explant did not show prominent callus formation while at higher concentrations necrosis of explants was observed. Ye et al. (1988) also reported that high nitrogen and minor nutrient content of MS medium are responsible for callus induction and their subsequent proliferation into embryogenic calli and PLBs, finally leading to plantlet formation.

Callus differentiation into shooting response depends upon cytokinin and auxin ratios in combination. In *D. malones* callus differentiation was analyzed as formation of plantlets or production of PLBs which subsequently produced shooting (Figure 1). Vij and Kaur (1992) stated that regeneration in orchids is usually accomplished through somatic embryos which are referred as PLBs in orchids. PLBs formation was observed at all combination (Table 3) but with Kinetin (Kin) and NAA at all combinations in MS medium, plantlet development was also excellent within minimum time period. At these combinations, percentage age of plantlet development was only 50-60 where buds matured into PLB's which subsequently developed into plantlets. At other combination of Kin and BAP with auxins, 80-90% relative PLB's formation were observed but most of these PLB's failed to develop plantlets and took long time period. Similar results have been reported by Kusomoto (1997) in *Cattleya,* Sobhana and Rajeevan (1993) and Mujib and Jana (1994) in *Dendrobium*.

Direct organogenesis through explant is a better approach to develop plantlet in short time period. There is a direct correlation of regeneration potential with physiological age and source of leaf explant (Vij et al., 1986). In the present study, callus formation was observed at all combinations but formation of PLB's and plantlet development were key points to be studied. Kin (3 mg/l) with indol acetic acid (IAA, 1 mg/l) or indol-3-butyric acid (IBA, 1 mg/l) were best from all combinations (Table 4). But with Kin and IBA, secondary PLB's formation from calli was higher then other one. Same results were also observed at BAP (5.0 mg/l) with IAA (2.0 mg/l). At this combination, high callus mass forma-tion was also observed. Table 3 and 4 describes high number of PLB's formation and plantlet development through direct organogenesis, which was 10 times higher than through callus in short time period. Lakshmanan et al. (1995) also reported same results during direct organogenesis from leaf section explants.

Roots were also developed on protocorm due to presence of auxins in the medium during direct or indirect organogenesis, but experiments were also conducted for better root growth and to develop rooting on rootless protocorms. Table 5 shows that NAA in MS medium at 0.5 and 1.0 mg/l was best for root development, root thickness and root length as well as for better plantlet

Table 1. Effect of cytokinins and auxins in MS medium on the *in vitro* seed germination of *D. malones*.

S/N	Conc. of hormone (mg/l)		Av. days to germinate	Growth rate of protocorms	Germination (%)
	Cytokinins	Auxins			
1	-	-	190	±	10
2	BA				
i	0.5	-	14	±	80
ii	1.0	-	24	++	30
iii	2.0	-	17	++++	100
iv	3.0	-	23	++++	100
3	Kn				
i	1.0	-	15	+	50
ii	2.0	-	14	++	70
iii	3.0	-	15	+	80
4		NAA			
i	-	0.5	19	++	80
ii	-	1.0	7	+++	100
iii	-	2.0	4	++++	100
iv	-	3.0	5	+++	100
5		2,4-D			
i	-	1.0	14	++	50
ii	-	2.0	10	+	50
iii	-	3.0	10	+	10
6	BA	NAA			
i	0.2	0.1	7	++++	100
ii	1.0	1.0	17	++++	100
iii	2.0	1.0	19	++++	100

Table 2. Effect of 2,4-D on callogenesis in apical meristem explants on MS medium.

2,4-D Mg/l	Intensity of type of neo-formation induced					Callogenic Response
	GC	PLB	R	Psu	Pn	
0.1	-	-	-	-	-	No response
0.5	-	++	-	-	+	Negligible callus
1.0	++++	+++	+++	++++	++	Prolific callus with morphogenesis
2.0	++++	++++	+++	+++	++++	Prolific callus with morphogenesis
3.0	++	+	+++	+++	+++	Satisfactory callus and high pseudobulb induction
5.0	-	-	-	-	-	Necrosis of explant
7.0	-	-	-	-	-	Necrosis of explant

GC, Greening of callus; PLBs, protocorm like bodies; R, roots; Psu, pseudopulbs; Pn, plantlet development.

growth. At other hormonal concentrations, root development was absent or negligible.

All the data makes it tempting to believe that, in orchids, the inherent meristematic potential of foliar explants, controlled by some factors emanating from the leaf margins, is markedly influenced by the nutritional regime *in vitro* and it diminishes with maturity.

Figure 1. Callus differentiation and protocorm formation in *Dandrobium malones*.

Table 3. Effect of MS medium supplemented with cytokinin in combination with auxin on the organogenetic capacity of callus cultured on MS medium with 2.0 mg/l 2,4-D.

Hormones	Conc. (mg/l)		Callus Proliferation	Intensity of type of neo-formation induced						Culture period (weeks)
				GC	PLB	R	Psu	Pn	%	
Kn + IAA	0.5	0.5	-	+++	-	-	-	-	40	3
	1.0	0.5	-	+++	+	+	-	+	60	6
	2.0	1.0	+	+++	+++	+++	-	++	50	8
	3.0	1.0	+	++	++++	+	-	++	80	8
	5.0	2.0	+++	+++	+++	++++	-	+++	40	7
Kn + IBA	2.0	.05	+++	+++	++++	+	-	+++	60	3
	3.0	1.0	++++	++++	++++	++	-	+++	80	6
	5.0	2.0	++++	++++	++++	++	-	+++	80	5
Kn +NAA	0.5	0.5	+	+	+++	++	-	+	20	8
	2.0	1.0	+++	++++	++++	+	-	++++	60	5
	3.0	1.0	++++	++++	++++	+	-	++++	50	3
	5.0	2.0	++++	++++	++++	++	-	++++	60	2
BA +NAA	1.0	0.5	-	+	+	++	-	+	30	4
	2.0	1.0	++++	++	++	++	-	+	50	8
	3.0	1.0	++++	+++	+++	++++	-	++	40	3
	5.0	2.0	+	+++	++++	+++	++	+++	90	4
BA +2,4-D	1.0	0.5	++	+++	+++	+	-	++	70	8
	1.0	1.0	+++	+++	+++	++	-	++++	50	3
	3.0	1.0	++++	+++	+	-	+	-	70	4
BA + IAA	0.5	0.5	+	+++	+++	+++	-	+++	40	7
	1.0	1.0	++++	++++	++++	-	-	+++	80	8
	2.0	1.0	+++	++++	+++	+	-	++	80	7
	5.0	2.0	++	+++	+++	-	-	++	60	3

GC, Greening of callus; PLBs, protocorm like bodies; R, roots; Psu, pseudopulbs; Pn, plantlet development.

Table 4. Direct organogenetic capacity through thin layer leaf section of *D. malones*.

Hormones	Conc. (mg/l)		Intensity of type of neo-formation induced						Culture period (weeks)	%
			PLBs	CF	Splb	R	Pn	Psu		
Kn + IAA	0.5	0.5	++	++	+++	+++	++	-	8	50
	1.0	0.5	+++	+++	++	++	+++	-	8	70
	2.0	1.0	+++	++	++	++	+++	-	9	100
	3.0	1.0	++++	+++	++	++	++++	-	8	100
	5.0	2.0	+	++++	+++	++	+++	-	7	30
Kn + IBA	2.0	0.5	++	++++	++++	++	++++	-	8	40
	3.0	1.0	++++	++	++++	++	++++	-	7	100
	5.0	2.0	+++	+++	++++	+++	++++	-	5	90
Kn +NAA	0.5	0.5	+++	+	+	+++	++	-	8	70
	1.0	0.5	+++	+++	++	+	++++	-	12	60
	2.0	5.0	++	+	-	++	++	-	8	30
	3.0	1.0	+++	++	-	+++	++++	-	6.5	80
	5.0	2.0	++	+++	++	+++	++++	-	6	50
BA +NAA	1.0	0.5	+++	+++	+++	++	++++	-	9	80
	2.0	1.0	++	++++	++++	+++	++++	-	8	100
	3.0	1.0	++	+++	++	++	+++	-	7.5	100
	3.0	2.0	+	+	+++	++++	+	-	7	80
	5.0	2.0	+	++++	++++	+++	++++	-	5	60
BA +IBA	1.0	0.5	++	++++	++++	+	++++	-	8	60
	1.0	1.0	+	+++	++	-	+	-	7	40
	3.0	1.0	++	++++	-	-	-	+++	8	75
BA + IAA	0.5	0.5	+++	+	+	+++	++	-	8	70
	1.0	0.5	+++	+++	++	+	++++	-	12	85
	3.0	1.0	+++	++	-	+++	++++	-	6.5	95
	5.0	2.0	++++	++++	++	+++	++++	-	6	100

PLBs, Protocorm-like bodies; CF, callus formation; Splb, secondary PLBs formation on calli; R, roots; Pn, plantlet development; Psu, pseudobulbs.

Table 5. Influence of auxins on rooting of microshoots and PLB's of *D. malones*.

Auxin	Conc. (mg/l)	Intensity of type of neo-formation					Period (weeks)	Response
		Rf	RT	RL	PG	%		
IAA	0.1	-	++	++++	+	60	10	+ive geotropism, pale semituberous
	0.5	-	+	++	++	40	8	+ive geotropism, pale semi fine roots
	1.0	-	+	+++	+++	90	6	+ive geotropism, pale semi fine roots
IBA	0.1	+	+++	++	+++	50	10	+ive and–ive geotropism, semi tubers
	0.5	-	±	++++	++++	80	8	-ive geotropism, subterranean
	1.0	-	±	++++	++++	80	8	+ive and -ive geotropism
NAA	0.1	++	++	++	+++	50	8	Tuberous
	0.5	+++	+++	++	+++	70	9	Tuberous assimilatory roots
	1.0	+++	+++	+++	++	90	5	Palmately tuberous, convoluted fine roots

Rf, Root formation; RT, root thickness; RL, root length; PG, plantlet growth.

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