THIN CELL LAYER TECHNOLOGY IN REGENERATION AND MICROPROPAGATION OF *CYCLAMEN PERSICUM* MILL.

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Abstract

In this study, the morphogenesis of *Cyclamen persicum* Mill. using the thin cell layer technology (TLC) was successfully established. The peduncles were sliced into thin cell layers (1 mm thick), which were then cultured on MS medium supplemented with NAA, IBA, 2,4-D at various concentrations (0.1; 0.3; 0.5; 0.7 or 1.0 mg l^{-1}) alone or in combination with TDZ (0.2 mg l^{-1}). The results showed that the presence of auxin alone in culture media was not sufficient for callus induction from peduncle TCL explants. MS medium supplemented with 0.2 mg l^{-1} TDZ and 1.0 mg l^{-1} 2,4-D was found to be the optimal medium for callus induction. Different sizes of thin cell layer explants (0.5; 1.0; 2.0 or 3.0 mm) from two positions of the peduncle (the upper or lower position of peduncle) were also studied. The 3 mm initiating explants at the upper position of the peduncle gave 100% callus induction rate and high callus fresh weight. After 8 weeks of culture, calli which were transferred onto MS medium supplemented with 0.5 mg l^{-1} BA and 0.7 mg l^{-1} IBA induced the highest number of shoots. Rooting was obtained by transferring 2-3 cm long shoots to MS medium supplemented with 1.0 mg l^{-1} IBA. The results indicated that different kinds of plant growth regulators, the explant size and position are important in determining the morphogenesis from peduncle TCL explants of *Cyclamen persicum* Mill.

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Keywords: *Cyclamen persicum Mill., thin cell layer, explant size, explant position, peduncle, TDZ.*

Running title: thin cell layer technology in *Cyclamen persicum* Mill.

INTRODUCTION

The thin cell layer (TCL) culture method was originally developed by Tran Thanh Van for inducing different patterns of morphogenesis in tobacco (Tran Thanh Van 1981). The thin cell layer consists of small size explants excised from different plant organs either longitudinally (lTCL, containing only a single tissue type such as a monolayer of epidermal cells) or transversally (tTCL, containing various tissue types: epidermal, cortical, cambium, perivascular and medullar tissue, parenchyma cells) (Tran Thanh Van 1981). The underlying concept has subsequently been successfully applied for somatic embryogenesis and shoot regeneration in many dicotyledonous and monocotyledonous plants, including a few orchid species and other horticultural crops, such as *Dendrobium* (Wang et al. 2007), *Lilium* (Nhut et al. 2001) and in the micropropagation of leguminous and medicinal plants, such as *Panax ginseng* (Ahn et al. 1996) and *Phaseolus vulgaris* (Cruz de Carvalho et al. 2000). It has also been used in *in vitro* regeneration systems for cereals and grasses, including *Digitaria sanguinalis* (Bui et al. 1997), *Oryza sativa* (Nhut et al. 2000), *Sorghum bicolor* (Baskaran et al. 2006); fruit crops, including *Musa* sp., *Cocos nucifera* (Nhut et al. 2003); and woody plants, including *Populus* spp. (Lee-Stadelmann et al. 1989), *Pinus radiata*, and *Sequidendron* spp. (Texeira da Silva 2003).

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Cyclamen is a genus of 23 species of perennials growing from tubers, valued for their flowers with upswept petals and variably patterned leaves. Cyclamen (*Cyclamen persicum* Mill) is one of the most important ornamental pot plants (Motoyasu and Takiko 1991). During the long period of cultivation, numerous cultivars have been created, differing from the others according to size of entire plant, petal shape, flower color and size (Jelena and Slobodan 2010). Over the

past 20 years, various studies on regeneration and micropropagation of cyclamen were performed: the effect of explant material on somatic embryogenesis (Kiviharju et al. 1991); shoot regeneration from mature tissue (Karam and Mohannad 2000); development of somatic embryos on solid media (Takamura and Tanaka 1996, Traud and Margrethe 2005; Claudia et al. 2010) and in liquid culture (Hohe et al. 2001); direct regeneration using seedling tissues (Hassan 2004); vegetative propagation via adventitious shoot induction from seedling tissue (Prange et al. 2008); callus production and plant regeneration from protoplast (Morgan 1999, Traud et al. 2006).

Although extensive works have been carried out on this explant, there is no report on the morphological generation of peduncle thin cell layers. Our previous research reported that the size and the position of tTCL explants from the stem of *Lilium longiflorum* influenced callus induction and shoot regeneration (Nhut et al. 2001). In this study, an efficient procedure has been established for micropropagation of *Cyclamen persicum* Mill. using peduncle TCLs.

MATERIALS AND METHODS

Plant materials and initial culture

The peduncles of *Cyclamen persicum* Mill. flower buds (Figure 2a) were washed carefully with ethanol (70%) for 30 s, then soaked with 0.1% $HgCl_2$ for 7 mins, and rinsed 5 times in sterile distilled water. These peduncles were cut into cylindrical slices (0.5-3.0 mm thickness transverse slices). Culture media were MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose, 8 g l^{-1} agar (Hai Phong, Vietnam) and different kinds of plant growth regulators. The pH of the media was adjusted to 5.7 before autoclaving at 121°C for 20 min.

Callus induction from TCL explants of Cyclamen peduncles

In order to study the optimal medium for callus induction, peduncle TCLs (1 mm thick) were placed on MS medium and ..... (2,4-D), ... (IBA) or ..... (NAA) at various

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concentrations (0.1, 0.3, 0.5, 0.7 or 1.0 mg l^{-1}) alone or in combination with 0.2 mg l^{-1} ..... (TDZ). Callus induction and callus fresh weight were scored after 6 weeks of culture.

In a second trial, different TCL sizes (0.5, 1.0, 2.0 or 3.0 mm thick) from two positions of the peduncle (position 1, from the top to the middle and position 2, from the middle to the bottom; see Figure 1) were studied to find out the optimal size and position of TCL explants on callus induction capacity. The sections were cultured on optimal medium for callus induction from peduncle TCL explants, as evidenced by the previous experiment. Data were recorded after 6 weeks of culture (Figure 1).

After 6 weeks of culture, calli were transferred to MS medium supplemented with 0.5 mg l^{-1} ..... (BA) or 0.5 mg l^{-1} kinetin in combination with IBA or NAA at various concentrations (0.1, 0.3, 0.5 or 0.7 mg l^{-1}) for shoot regeneration. The percentage of shoot regeneration from callus and average number of shoots were recorded 8 weeks after the transfer of callus to shoot regeneration media.

As a final step of micropopagation, in order to indicate the most suitable medium for root induction and proliferation, elongated healthy shoots (2-3 cm) were excised and cultured on root induction media, consisting of MS medium supplemented with IBA or NAA (0.5 or 1.0 mg l^{-1}). Percentage of rooting, number of roots and root length were recorded after 6 weeks of culture.

Culture conditions and data analysis

All cultures were incubated at 25±2ºC with a 16-h photoperiod at a light intensity of 60 µmol s^{-1} m^{-2} fluorescent light. Data were analyzed for significance by analysis of variance with mean separation by Duncan's multiple range test (Duncan 1995) using Statgraphics Centurion XV (StatPoint Technologies Inc., Warrenton, VA, USA).

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RESULTS AND DISCUSSIONS

Effects of plant growth regulators on callus induction

The effect of TDZ as a cytokinin-like substance (Mok et al. 1987), as well as its effects on shoot regeneration in *in vitro* cultures (Hosokawa et al. 1996) were demonstrated. Other authors also reported TDZ effects on organogenesis of peanut embryo sections and hypocotyl (Saxena et al. 1992) and on *Geranium* seedlings (Gill et al. 1993). When studying the effects of TDZ on callus induction of rose leaf explants, Canli et al. (2003) found that there were significant differences among various TDZ concentrations on induction of callus, and callus was not induced without TDZ. In this research, we obtained high callus induction frequency by employing TCL method with the utilization of TDZ in combination with 2,4-D on *Cyclamen persicum* Mill.

When using 0.2 mg l^{-1} TDZ in combination with 2,4-D at various concentrations (0.1, 0.3, 0.5, 0.7 or 1.0 mg l^{-1}), high frequency of callus induction (78.9 - 88.9 %) and high fresh weight of callus (0.92 - 2.09 g) were obtained (Table 1). TDZ (0.2 mg l^{-1}) in combination with IBA (1.0 mg l^{-1}) or NAA (0.1 or 0.3 mg l^{-1}) also induced high callus induction frequency (more than 80 %) but the callus fresh weight was low (less than 0.4 g). MS medium supplemented with 0.2 mg l^{-1} TDZ and 1.0 mg l^{-1} 2,4-D was found to be the optimal medium for callus induction from peduncle TCL explants of *Cyclamen persicum* Mill. (Figure 2b).

Morgan (1999) studied the effects of TDZ, BA, NAA and 2,4-D on callus induction from protoplast of *Cyclamen*. The results showed that there were variations of callus growth among MS media supplemented with different growth regulators. White and solid calli were produced on media containing TDZ, while friable calli were formed on media supplemented with BA and NAA. In another study, ovules of *Cyclamen* were cultured on half-strength MS medium supplemented with 9.05 µM 2,4-D and 3.94 µM 2iP for callus induction. High rates of callus

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123 induction were observed for most cultivars. Callus varied in color and consistency (Traud and
124 Margrethe 2005).
125 ***Effects of sizes and positions of TCL explants on callus induction***
126 The peduncles of Cyclamen flower buds were sliced into thin cell layers with various
127 thickness and were cultured on optimal medium of previous experiment (MS medium containing
128 0.2 mg l^{-1} TDZ and 1.0 mg l^{-1} 2,4-D). After 6 weeks of culture, the results showed that the
129 thickness of TCL explants had an important influence on callus induction. The percentage of
130 explants forming callus decreased when the explant size decreased: the high percentage of callus
131 iduction was obtained at 3.0 mm thick explant which showed absolute callus induction rate and
132 high callus fresh weight (Table 2). These results were different from those of Gendy et al. (1996)
133 which concluded that the percentage of explants forming callus from tTCLs of *Sorghum bicolor*
134 decreased when the explant size increased, with 0.3 mm explants inducing the highest callus
135 induction.
136 Explants of small size have a specific, larger surface in contact with the culture medium,
137 which might promote a better response of those with growth regulators (Fehér et al. 2003).
138 Bui et al. (1999) cultured tTCLs (0.2-0.4 mm thick) excised from *in vitro Lilium*
139 *longiflorum* pseudo-bulblets on MS media supplemented with 0.12-10 mM forclofenuron and
140 obtained bud primoridia initiating from the surface area of tTCLs without intermediate callus.
141 Nhut et al. (2001) tested different tTCL sizes (0.5, 1.0, 2.0 or 3.0 mm thick) in *Lilium longiflorum*
142 and found that 1.0, 2.0 or 3.0 mm thick tTCLs produced the highest number of shoots. tTCLs of
143 0.5 mm thickness exhibited necrosis in 90% of explants and thus had a much lower shoot
144 initiation.
145 Here, further experiments were carried out in an attempt to study the optimal callus
146 induction by testing the explant position along the peduncles of cyclamen. The TCL explants

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from two positions of the peduncle were cultured on the same medium with the same experimental conditions. The efficiency of callus induction decreased with an increase in distance from the proximal end of the peduncle tip (Table 2). After 6 weeks of culture, TCL explants from position 1 (Figure 2c) showed higher frequency of callus initiation (100%) and callus fresh weight (3.29 g) than those from position 2 (Figure 2d) (14.3%, 0.28 g, respectively).

Shoot regeneration from callus

The above calli were transferred to MS media supplemented with 0.5 mg l^{-1} BA or 0.5 mg l^{-1} kinetin in combination with IBA or NAA at various concentrations. Shoot regeneration was obtained from all the calli (100%) after 8 weeks of culture (Figure 2e1, 2e2). The number of shoots regenerated on MS media containing BA and IBA or NAA was higher than those from MS media comprising kinetin and IBA or NAA (Table 3).

Hassan (2004) found that BA in culture medium was responsible for direct shoot regeneration from seedling tissues of *Cyclamen persicum* Mill. Direct regeneration occurred from tuber and leaf explants taken from aseptic seedlings. The highest shoot number was obtained from tuber explants with BA at 1 µM. In another study, Nabila and Mohannad (2000) obtained 88% shoot regeneration from leaf tissues on half-strength MS medium supplemented with 0.1 mg l^{-1} NAA and 0.22 mg l^{-1} TDZ.

The most important point in plant propagation was the evaluation of the regeneration ability. Auxin/cytokinin ratio during *in vitro* tissue culture played a critical role to induce the regenerative response of callus (García et al. 2008). Current results showed that alteration in exogenous auxin (IBA or NAA) and cytokinin (BA or kinetin) ratio strongly influenced shoot development under *in vitro* conditions. Supplementing the medium with 0.5 mg l^{-1} BA and 0.7 mg l^{-1} IBA induced the higher average number of shoots from callus of Cyclamen (39.4 shoots per initial callus mass) than those from other media (Figure 2f1, Table 3).

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Rooting of in vitro shoots

In vitro shoots (up to 2-3 cm long, Figure 2f2) were transferred to MS medium supplemented with 0.5 or 0.1 mg l^{-1} of NAA or IBA (Table 4). Root induction occurred at all media (Figure 2g), however, the highest frequency of rooting was obtained in MS medium supplemented with 1.0 mg l^{-1} IBA (100%). Primary root number and primary root length on medium containing 1.0 mg l^{-1} IBA were higher than those from other media. NAA in culture media was not suitable for rooting in *Cyclamen persicum* Mill. because of the low percentage of root induction and proliferation.

In conclusion, best micropropagation protocol using peduncles of cyclamen consisted of.....

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Table 1. Effects of plant growth regulators on callus induction from peduncle TCL explants

TDZ (mg l^{-1})	2,4-D (mg l^{-1})	IBA (mg l^{-1})	NAA (mg l^{-1})	Frequency of callus induction (%)	Fresh weight of callus (g)	Morphology of callus
0.2	0.1			78.9	0.92 e*	Small, light yellow
	0.3			80.0	0.97 d	Small, ivory-white
	0.5			87.6	1.13 c	Small, ivory-white
	0.7			87.6	1.30 b	Large, ivory-white
	1.0			88.9	2.09 a	Large, ivory-white
0.2		0.1		20	0.25 k	Small, yellowish brown
		0.3		37.6	0.29 j	Small, yellowish brown
		0.5		77.8	0.40 h	Small, yellowish brown
		0.7		77.9	0.35 i	Small, yellowish brown
		1.0		87.6	0.33 i	Small, yellowish brown
0.2			0.1	83.4	0.24 k	Small, yellowish brown
			0.3	80	0.25 k	Small, yellowish brown
			0.5	75	0.20 l	Small, yellowish brown
			0.7	55	0.19 l	Small, milky white
			1.0	25	0.18 l	Small, milky white
	0.1			13.8	0.20 l	Small, ivory-white
	0.3			80	0.64 f	Small, ivory-white
	0.5			16.7	0.53 g	Small, ivory-white
	0.7			50	0.27 jk	Small, ivory-white
	1.0			25	0.16 l	Small, ivory-white
		0.1		0.0		Necrosis
		0.3		0	0	Necrosis
		0.5		0	0	Necrosis
		0.7		0	0	Necrosis
		1.0		0	0	Necrosis
			0.1	0	0	Necrosis
			0.3	0	0	Necrosis
			0.5	0	0	Necrosis
			0.7	0	0	Necrosis
			1.0	0	0	Necrosis

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Table 2. Effects of peduncle TCL sizes and positions on callus induction

Position	Size of explants (mm)	Percentage of callus induction (%)	Fresh weight of callus (g)
1	0.5	20.0	0.03 d*
	1.0	40.0	0.98 b
	2.0	90.0	0.34 c
	3.0	100.0	3.29 a
2	0.5	0.0	0.00 d
	1.0	0.0	0.00 d
	2.0	14.3	0.23 c
	3.0	14.3	0.28 c

* Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ according to Duncan multiple range test

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Table 3. Effects of BA or kinetin in combination with IBA or NAA on shoot regeneration from callus. For all the treatments, percentage of shoot regeneration was always 100%.

Concentration of plant growth regulators (mg l^{-1})				Number of shoots	Percentage of shoot regeneration (%)
BA	kinetin	IBA	NAA		
0.5		0.1		25.6 d*	100
		0.3		26.2 cd	100
		0.5		26.7 bcd	100
		0.7		39.4 a	100
0.5			0.1	30.8 b	100
			0.3	30.0 bc	100
			0.5	28.7 bcd	100
			0.7	25.5 d	100
	0.5	0.1		15.2 g	100
		0.3		16.4 fg	100
		0.5		17.1 fg	100
		0.7		19.4 ef	100
	0.5		0.1	26.6 cd	100
			0.3	21.4 e	100
			0.5	18.8 efg	100
			0.7	9.6 h	100

* Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ according to Duncan multiple range test

Commento [n°22]: Delete this column.

Commento [n°23]: Delete this column.

Eliminato: =

Table 4. Effects of NAA and IBA on rooting

NAA (mg l^{-1})	IBA (mg l^{-1})	Percentage of rooting (%)	Number of roots	Root length (cm)
0.5	-	33.3	12.0 a*	1.87 c
1.0	-	46.8	13.7 ab	1.45 d
-	0.5	54.2	15.3 b	5.29 b
-	1.0	100	20.0 c	6.43 a

Commento [n°24]: Insert statistical analysis of percentages or, at least, ± Standard Errors

* Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ according to Duncan multiple range test

Figure 1. Diagram of TCL explants preparation from peduncles of *Cyclamen persicum* flower buds

Figure 2. The morphogenesis and plantlet formation of *Cyclamen persicum* using the thin cell layer technology: a) The peduncles of *Cyclamen persicum* flower buds used as culture explants; b) Callus induction from various thickness TCL explants (0.5, 1.0, 2.0 or 3.0 mm) excised from the top to the middle of peduncle or from the middle to the bottom of peduncle; c1) Shoots regenerated from callus; c2) Particular of c1; d) Shoots elongated after 8 weeks of culture; e) Plantlets with full developed root system **(bars, ...)**.

Eliminato: ing

Eliminato: b) Callus induction from peduncle TCL explants on MS medium containing 0.2 mg l^{-1} TDZ and 1 mg l^{-1} 2,4-D;

Eliminato: c,d

Eliminato: position 1 (from

Eliminato:) and position 2 (

Eliminato:)

Eliminato: e

Eliminato: ,e2

Eliminato: f1,f2

Eliminato: g

- Reduce Figure 2 to a 6-picture plate by eliminating pictures previously b, d and f1 which are useless.

- Re-number accordingly the citations of Figure 2 along the text.

- Insert bars in the pictures and report the size in the caption.