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Influence of Salt Concentration of Media and Plant Growth Regulator Combination on Callus Formation and Somatic Embryogenesis of *Cyclamen persicum* Mill.

Neda Jalali[1*] • Rouhangiz Naderi[1] • Jaime A. Teixeira da Silva[2] • Mesbah Babalar[1] • Masoud Mirmasoumi[3]

[1] Department of Horticultural Sciences, Faculty of Agriculture, University of Tehran, Karaj 31587-77871, Iran
[2] Faculty of Agriculture and Graduate School of Agriculture, Kagawa University, Miki-cho, Ikenobe 2393, Kagawa-ken, 761-0795, Japan
[3] Department of Plant Sciences, Faculty of Biology, University of Tehran, Tehran, Iran

Corresponding author: * **ne.jalali@yahoo.com**

ABSTRACT

Cyclamen persicum Mill. (Primulaceae) is an important ornamental crop sold worldwide as pot flower for winter production. It is commercially propagated via seeds. Somatic embryogenesis which is an important requirement for breeding material and mass propagation, was induced through a callus phase from young leaf explants of an *ex vitro* mother plant. Somatic embryos were cultured on modified Murashige and Skoog (MS) medium to test the effect of half and full salt concentrations. In addition, embryogenic callus was induced on several media containing 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. Maximum somatic embryogenesis was possible on ½ MS medium containing 4 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin.

Keywords: callus induction, media, plant growth regulator, *Cyclamen persicum* Mill.
Abbreviations: 2,4-D, 2,4-diclorophenoxyacetic acid; **CRD**, completely randomized design; **DMRT**, Duncan's multiple range test; **Kin**, kinetin; **MS**, Murashige and Skoog medium; **PGR**, plant growth regulator

INTRODUCTION

Somatic embryogenesis is an asexual form of plant propagation in nature (Santacruz-Ruvalcaba *et al.* 1998) in which an embryo forms through the developmental pathway of zygotic embryogenesis and thus provides an excellent *in vitro* experimental system to study different aspects of plant embryo development (Fehér *et al.* 2001). *In vitro* somatic embryogenesis is also an important prerequisite for the use of many biotechnological tools for genetic improvement (Santacruz-Ruvalcaba *et al.* 1998), as well as an efficient vegetative propagation pathway (Winkelmann *et al.* 2006). There are advantages and disadvantages of somatic embryogenesis in large-scale plant multiplication. The major advantages are large-scale somatic embryo production in bioreactors, encapsulation, cryopreservation, genetic transformation and clonal propagation. The major limitations are genotypic dependence of somatic embryo production and poor germination rate (Rout *et al.* 2006).

Cyclamen persicum Mill. (Myrsinaceae, formerly Primulaceae) is a common geophyte growing in the East Mediterranean countries of Lebanon, Syria, Turkey, Cyprus, Greece, Israel and Iran with additional enclaves in North Africa (Dole and Wilkins 1999; Schwartz-Tzachor *et al.* 2008). Cyclamen is an ornamental species commercialized as a flowering pot plant (Ruffoni *et al.* 2000) of which annually more than 140 million plants are produced worldwide (Winkelmann *et al.* 2003). Cyclamen is traditionally propagated by seed despite the lack of homogenity of some cultivars, high costs of F_1-hybrid seed production, and inbreeding depression (Schwenkel and Winkelmann 1998). It is difficult to propagate cyclamens by division, cutting, and grafting (Bian *et al.* 2010) therefore vegetative propagation by tissue culture is an effective propagation method in cyclamen. Micropropagation of cyclamen though organogenesis (Hawkes and Wainwright 1987; Schwenkel and Grunewaldt 1988; Dillen *et al.* 1996; Karam and Al- Majathoub 2000) or via somatic embryogenesis (Kiviharju *et al.* 1992; Kreuger *et al.* 1995; Takamura *et al.* 1995; Schwenkel and Winkelmann 1998; Sugiyama *et al.* 2008) has already been reported. One aim of somatic embryogenesis is to find a protocol for the production of synthetic seeds that can replace the generative propagation technique used to date (Lyngved *et al.* 2008). Other more specialist applications include the production of high-value compounds by somatic embryos grown in bioreactors (Dunwell 2009). Consequently somatic embryogenesis has been deemed as an efficient system for *in vitro* clonal propagation (Bian *et al.* 2010).

Another aspect of breeding objectives is that conventional interspecific hybridization between *C. persicum* cultivars and other species originally failed due to cross-incompatibility (Ishizaka and Uematsu 1995) and further genetic enhancement was conceivable only via biotechnological applications such as micropropagation, cell fusion, induction of somaclonal variation, and genetic transformation (Terakawa *et al.* 2008). Therefore the study of somatic embryogenesis in cyclamen is vital for future biotechnological breakthroughs.

Within this study the effect of media type and hormone composition in callus initiation and somatic embryogenesis was tested with *Cyclamen persicum* Mill. cv. 'Halios', a cultivar with high economic importance (Savona *et al.* 2007) in Iran and many countries and also an outstanding variety of cyclamen.

MATERIALS AND METHODS

Young leaf explants taken from an *ex vitro* plant of *Cyclamen persicum* Mill. cv. 'Halios' were used as starting material for callus initiation. They were disinfected with ethanol (70%, 60 s), followed by freshly prepared aqueous mercuric chloride solution

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(0.1%) for 9 min, and then by sodium hypochloride solution (commercial bleach; 1% active chlorine) with a few drops of Tween 20 for 8 min. Leaves were rinsed three times with sterile distilled water. The aseptic explants were cut into 1 cm × 1 cm segments and plated in plastic Petri dishes (100 mm × 16 mm) on callus induction medium. Murashige and Skoog (1962; MS) vitamins, macro- and micronutrients at full or half-strength were used and to which 30 g l^{-1} sucrose was added. All the media were gelled with 8 g l^{-1} agar (agar 1615, Merck, Darmstadt, Germany) and supplemented with different plant growth regulator (PGR) concentrations. 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma-Aldrich, St. Louis, MI, USA) served as auxin and kinetin (Kin; Merck, Germany) as cytokinin. Different ranges of 2,4-D and Kin concentrations were chosen based on previous studies of cyclamen somatic embryogenesis. 2,4-D at 1, 2 and 4 mgl^{-1} and Kin at 0, 0.1 and 0.5 mgl^{-1} in various combinations were selected to induce callogenesis. All other chemicals were purchased from Merck, of tissue-culture grade.

The pH of all medium was adjusted to 5.8 prior to autoclaving. All media were sterilized by autoclaving for 20 min at 121°C at 15 lbs pressure. The cultures were maintained at 24 ± 1°C, under continuous darkness and were subcultured on fresh nutrient medium every 30 days to increase and strengthen callus growth. The percentage of explants producing callus was scored after 8 weeks because during this period explants are capable of producing callus; remaining callus turned brown. Freshly formed embryogenic callus segments ~ 1 cm^3 were transferred to PGR-free medium with the same composition previously described, also maintained in the dark and at the same temperature. The embryogenic response of explants was recorded after 8 weeks of subculture.

A completely randomized design (CRD) was used for the experiments with 5 replicates (Petri dishes) and five explants (1 cm^3 callus clumps) per replicate. Treatment means were compared by analysis of variance (ANOVA) at P = 0.01 and P = 0.001, which was carried out using the SAS software (version 9.1). Percent data were arcsine transformed before performing ANOVA. Duncan's multiple range test (DMRT) at P = 0.05 was employed to compare treatment means.

RESULTS AND DISCUSSION

Using the sterilization protocol 100% sterile explants and cultures were obtained. Otani and Shimada (1991) used ethanol (70%, 30 s) and sodium hypochloride solution (5%, 5 min) for surfaced sterilization of leaf explants of cyclamen while Terakawa *et al.* (2008) utilized a different sterilization protocol for leaves: surface sterilization with ethyl alcohol (70%, 1 min) and sodium hypochlorite (1%, 30 min); however, these former protocols were not effective for our experiment and thus had to optimize and use another sterilization protocol. Takamura *et al.* (1995) and Kreuger *et al.* (1995) reported an effective system with aseptic seedling tissues for somatic embryogenesis to eliminate sterilization problems. However, we did not use their protocols since we wanted to apply tissue from adult plants in this study.

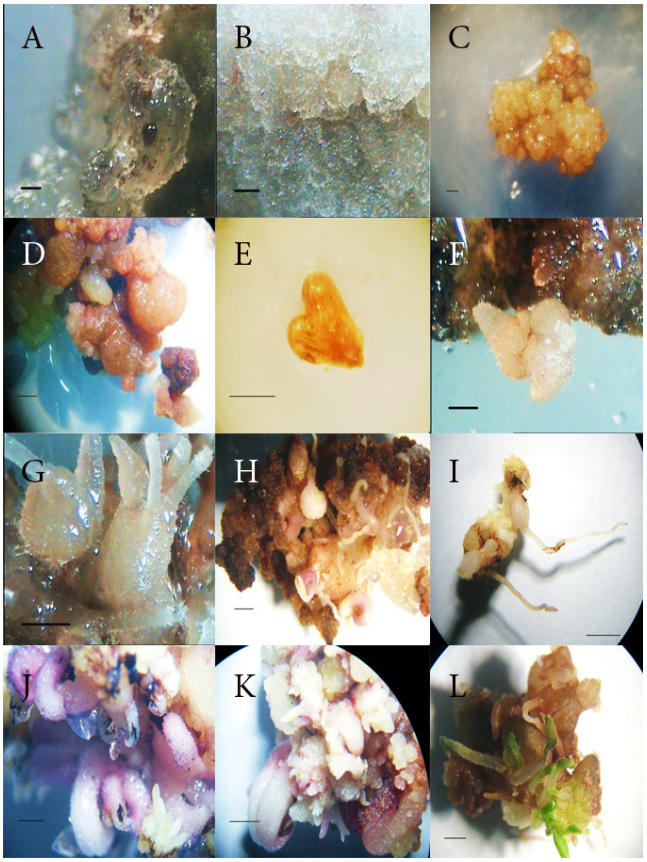

Fig. 1 (**A**) Transparent and watery callus (bar = 1 mm) (**B**) Opaque, white and compact callus (bar = 1 mm). (**C**) Embryogenic callus (bar = 1 mm). (**D**) Globular stage (bar = 1 mm). (**E**) Heart shaped embryo (bar = 1 mm). (**F**) Elongation of embryos (bar = 1 mm). (**G**) Torpedo stage (bar = 1 mm). (**H**) Germination of somatic embryos (bar = 2 mm). (**I**) Germinated embryos with root (bar = 2 mm). (**J**) Different stages of somatic embryos (bar = 2 mm). (**K**) Growth of embryos (bar = 2 mm). (**L**) Plant regenerated from somatic embryos (bar = 5 mm).

Table 1 Percentage callus formation and somatic embryogenesis in *Cyclamen persicum* Mill. cultures as influenced by medium type and PGR composition (in mg l^{-1}).

Treatment	Callus formation (%)	Number of somatic embryos/explant
Media type (M)		
MS	51.0 a	6.4 b
1/2MS	51.6 a	6.6 a
PGRs in half-strength (P)		
2,4-D 1 Kin 0	44.7 c	8.3 c
2,4-D 1 Kin 0.1	53.8 b	6.8e
2,4-D 1 Kin 0.5	64.5 a	7.2 d
2,4-D 2 Kin 0	28.7 d	6.5 f
2,4-D 2 Kin 0.1	62.0 a	7.2 d
2,4-D 2 Kin 0.5	61.7 a	7.3 d
2,4-D 4 Kin 0	41.5 c	6.5 f
2,4-D 4 Kin 0.1	53.0 b	9.1 a
2,4-D 4 Kin 0.5	51.0 b	8.7 b
Significance[a]		
M	NS	**
P	***	***
M × P	***	***

[a] NS,**,*** - non-significant or significant at P≤ 0.01 or 0.001, respectively

Callus was induced on leaf segments 15 days after culture on all media. Two types of callus formed: one was transparent and watery (**Fig. 1A**) while the other was opaque, white and compact (**Fig. 1B**). Similar observations were reported by Otani and Shimada (1991). The effects of two media on the percentage of callus formation from leaf explants are presented in **Table 1**. In this study we found no differences between two concentrations of media on callus formation. Mean callus induction percentage ranged between 50.96 and 51.64% depending on the medium. There was no statistical difference between MS and ½-MS medium. Significant differences were observed in callus initiation percentage among the PGR treatments. The highest percentage callus formation was in three media: 1) 1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin (65.3%), 2) 2 mg l^{-1} 2,4-D + 0.1 mg l^{-1} Kin (65.8%), 3) 2 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin (64.0%).

Callus induction percentage was affected by the type of medium used and the PGR concentration, ranging from 21.5 to 65.8% (**Table 1**). Callus formed best from leaf explants on ½-MS medium containing 2 mg l^{-1} 2,4-D + 0.1 mg l^{-1} Kin. However, this was not statistically significant from 1 mg l^{-1} 2,4-D + 0.5 Kin on ½-MS medium. Broadly, callus formation was promoted by combining an intermediate auxin and a low cytokinin concentration, and suppressed by combining higher auxin and cytokinin concentrations. The variation in callus formation may be explained by degree of cell sensitivity towards exogenously-applied PGRs

Table 2 Compare our protocol and data with the results by other studies on somatic embyogenesis of *Cyclamen persicum* Mill.

Cultivar	Type of explant	Sterilization protocol	Callus induction media	Somatic embryogenesis media	Callus formation	Somatic embryo formation	Study
Table mini lilac rose	Leaf	Ethanol 70% (30 s) + NaOCl 5% (5 min)	LS + 1 mg l^{-1} 2,4-D + 0.1 mg l^{-1} Kin + 0.2% Gelrite in dark and 26°C	PGR-free media (LS)	60% callus	7 (46%) number of embryogenic calli	Otani and Shimada 1991
Anneke	Cotyledon, petiole, tuber and root	Aseptic seedlings were used	MS + 5 µM 2,4-D + 0.5 µM Kin + 0.2% gellan gum (dark and light)	PGR-free media (MS)	80% cotyledon, 65% petiole, 40% tuber, 35% root	Number of somatic embryos per organ: 200/cotyledon, 100/petiole, 60/tuber, 200/root	Takamura and Miyajima 1997
Purple flamed	Ovule	Ethanol 70% (30 s) + NaOCl 2.6% (20 min)	½-MS + 2 mg l^{-1} 2,4-D + 0.8 mg l^{-1} 2iP+ 3.7 g l^{-1} Gelrite in dark and 22-25°C	PGR-free media (1/2-MS)	51% ovule with callus	21% callus with somatic embryos	Schwenkel and Winkelmann 1998
Halios	Ovule	Ethanol 70% + NaOCl 1.2% (20 min)	½-MS + 2 mg l^{-1} 2,4-D + 0.8 mg l^{-1} 2ip+ 4 g l^{-1} Phytagel in dark and 24°C	PGR-free media (1/2-MS)	_	7 somatic embryos from callus induced from two ovule	Savona *et al.* 2007
Halios	Leaf	Ethanol 70% (60 s) + $HgCl_2$ 1% (9 min) + NaOCl 1% (8 min)	½ MS + 4 mg l^{-1} 2,4-D + 0.1 mg l^{-1} Kin + 8 g l^{-1} agar in dark and 25 °C	PGR-free media (1/2 MS)	65% callus	10 somatic embryos per 1 cm^3 callus	This study

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6-(γ,γ-dimethylallyamino) purine; Kin, kinetin; LS, Linsmaier and Skoog (1964) medium; MS, Murashige and Skoog (1962) medium; PGR, plant growth regulator

which might be more important than their actual concentration due to the levels of endogenous PGRs (such as IAA) and activity of natural auxin and cytokinin oxidases in the tissue (Yamaner and Erdag 2008). During reactivation, 2,4-D (at relatively high concentrations) acts at the same time as an inducer and as an inhibitor of cell division and result of cellular adaptation is induced the formation of meristematic/embryogenic cells which maybe due to their increased endogenous IAA content (Fehér *et al.* 2001). Cells, in the presence of continuous high level of 2,4-D (>2 mg l^{-1}) enter a dormant stage and show no significant change in size and do not divide furthr (Fehér *et al.* 2001). A negative consequence of a high auxin concentration (5 mg l^{-1} 2,4-D) was the weak differentiation of the apical meristem in flax (Preťová and Obert 2006). So, excessively high levels of 2,4-D inhibit callus from forming.

The callus clusters were subcultured to PGR-free medium for somatic embryo development. Transparent and watery callus formed somatic embryos earlier and at higher numbers than the other callus type. All the different stages of somatic embryogenesis were easily observed after 30 days in PGR-free medium (from globular to mature somatic embryos; **Fig. 1C-H**). After approximately 30-40 days somatic embryos germinated and regenerated to plantlet in the same media (**Fig. 1 I-L**).

Table 1 shows the embryogenic response of leaf explants on different media and with different PGR combinations scored after 8 weeks. More somatic embryos differentiated when explants were cultured on ½-MS. Most somatic embryos formed in 4 mg l^{-1} 2,4-D + 0.1 mg l^{-1} Kin.

In this study we found that media with half salt concentration as better than full salt concentration. Culture on media with increased salt concentration increases the osmotic concentration of the media; optimization of the salt concentration of media is an important factor in tissue culture (Groll *et al.* 2002). Kruger (1996) suggested that B5 media for the initiation of an embryogenic cell line of *Cyclamen persicum* Mill. is better than KK media, directly in liquid medium.

Number of somatic embryos was least in media without cytokinin. In contrast, Iantcheva *et al.* (2006) reported that somatic embryo formation was enhanced in *Medicago truncatula* and *M. sativa* when the induction medium was supplemented with BAP. The effect of cytokinin appears to be more promotive in indirect somatic embryogenesis systems. In addition, Sharry and Teixeira da Silva (2006) found that cytokinin concentration is very important for somatic embryo development in *Melia azedarach* L. They reported an inductive effect of cytokinins in somatic embryogenesis.

In this study, media salt level and PGR concentration played a critical role in callus induction and somatic embryogenesis of cyclamen. Similar findings are also observed on *C. persicum* by Terakawa *et al.* (2008) and Otani and Shimada (1991) but they did not compare salt levels of media and a broad range of PGRs. In our work an acceptable yield of somatic embryos in comparison with other studies was found, presented in **Table 2**.

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