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Somatic embryogenesis of Turkish *Cyclamen persicum* Mill

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ABSTRACT

Cyclamen sp. occupies a wide swathe of habitats across Turkey. Ten wild *Cyclamen* species grow naturally in Turkey, some being endemic. Due to this genetic variation, wild *Cyclamen* sp. with traits such as flower shape and colour, leaf shape and colour and disease resistance make these species important for cyclamen breeders. In this study, the potential of somatic embryogenesis from different explants (ovules, divided ovary parts, leaves and petiole segments) of 15 separate genotypes from one wild species (*Cyclamen persicum* Mill.) was studied. Explants were cultured on medium containing half-strength Murashige and Skoog macro- and micro-elements and 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.8 mg l^{-1} 6-(γ,γ-dimethylallylamino) purine for inducing embryogenic callus. Embryogenic potential differed significantly between explants and genotypes. Although callus was most prolific from petiole explants, somatic embryos formed most efficiently on ovary explants. The ability of petiole, ovary, ovule and leaf explants, when averaged for the 15 genotypes, to form callus, was 34.3, 30.16, 26.6 and 15.6%, respectively while the percentages of somatic embryos formed were 11.3, 8.00, 4.16 and 2.83% of ovary, petiole, leaf and ovule explants, respectively.

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1. Introduction

The genus *Cyclamen* (*Primulaceae* family) occupies an important position in the world pot plant industry, although current global market share is difficult to quantify. The genus *Cyclamen* includes about 20 species that are perennial and which originated from the Mediterranean coast. *Cyclamen persicum* Mill., which is the only economically important pot plant, has several colours: white, red, pink, purple (Terakawa et al., 2008) and yellow (Takamura et al., 2005).

Relative to *C. persicum*, little attention has been paid (in terms of economics, as ornamental resources, and in research) to *Cyclamen coum* Miller and other *Cyclamen* species. Cyclamen breeders are paying increasing attention and giving added importance to wild *Cyclamen* species because of their exclusive features such as attractive leaves and flower shapes, frost hardiness or disease resistance, and such species can be important material for use as perennials or to improve the genetic pool of commercial *C. persicum* (Prange et al., 2010a,b) through the introduction of novel traits.

Cyclamen habitats a wide range of areas of Turkey. Ten cyclamen species grow wild (*C. persicum* Miller, *C. coum* Miller, *C. hederifolium* Aiton, *C. graecum* Link, *C. mirabile* Hildebr., *C. pseud-ibericum* Hildebr., *C. trochopteranthum* O. Schwarz, *C. parviflorum* Pobed, *C. repandum* Sm. in Sibth & Sm and *C. cilicicum* Boiss & Heldr), the latter five being endemic to Turkey (Ekim et al., 1991).

Since cyclamen cannot be propagated from cuttings or tuber division, *in vitro* techniques have to be applied for vegetative propagation (Schwenkel and Winkelmann, 1998). Conventionally, cyclamen is propagated by seeds, which are produced by manual pollination and, therefore, are expensive (up to 0.20€ per seed). Moreover, since cyclamen suffers from inbreeding depression, the production and propagation of parental lines of F_1 hybrids is difficult. Thus, breeders are interested in the vegetative propagation of breeding lines and by having an economical form of vegetative multiplication that would allow breeders to produce clonal varieties by multiplying select elite plants. As far as *in vitro* techniques are concerned, conventional vegetative propagation and micropropagation through axillary or adventitious systems are inefficient and not economical (Geier et al., 1990; Kiviharju et al., 1992).

In cyclamen, the efficiency of organogenesis or somatic embryogenesis has already been widely reported from petiole, petal,

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peduncle, cotyledon, leaf, tuber and root explants (Wicart et al., 1984; Kiviharju et al., 1992; Kreuger et al., 1995; Takamura et al., 1995; Schwenkel and Winkelmann, 1998; Seyring et al., 2009; Jalali et al., 2010a,b; reviewed by Jalali et al., 2012). Somatic embryogenesis is advantageous since cyclamen somatic embryos are large relative to *Daucus carota* or *Arabidopsis thaliana* somatic embryos (Mordhorst et al., 1998). Somatic embryogenesis would allow for large-scale production in bioreactors, encapsulation, cryopreservation, genetic transformation and clonal propagation, although there are documented limitations such as genotype dependence (Takamura and Tanaka, 1996; Schwenkel and Winkelmann, 1998; Pueschel et al., 2003) of somatic embryo production and poor germination rate (Rout et al., 2006; Jalali et al., 2012; Sharma et al., 2013).

In cyclamen somatic embryogenesis, the first step is termed the induction phase, which includes embryogenic callus formation, usually under the influence of the strong synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Embryogenic cells that are cultured on 2,4-D-containing medium can divide and proliferate. The second step in somatic embryogenesis involves the differentiation of embryogenic callus and the development of somatic embryos with four characteristic stages, namely globular, heart, torpedo and cotyledon (Jalali et al., 2012). As for zygotic embryos, a somatic embryo gives rise to only one cotyledon in cyclamen because development of the second cotyledon is suppressed very early. Cyclamen needs darkness to germinate, and in fact all phases of somatic embryogenesis should be performed in darkness. In the final step, when somatic embryos germinate, they form plantlets that need to be adapted to light and transferred to soil (Winkelmann, 2010).

Taking the extensive literature on cyclamen somatic embryogenesis into consideration, and assuming that wild and endemic Turkish *Cyclamen* species contain potentially valuable genetic resources, an experiment was established to induce somatic embryogenesis from 15 different genotypes of *C. persicum* growing wild in Turkey.

2. Materials and methods

2.1. Plant material

C. persicum plants with tubers were collected from the natural flora of Cukurova University campus area in Balcali, Adana, Turkey in January, 2010. Fifteen cyclamen genotypes were randomly selected from a collection of 110 cultured *C. persicum* species in the greenhouse. *C. persicum* is the parent species of florist's cyclamen but the description which follows is concerned with the natural species as found in the wild (Wilson and Grey, 1997). The locations of plants are shown in Table 1. Each plant was placed in 13 cm diameter pots in a polycarbonate greenhouse in a substrate mixture contained turf, sand and perlite (1:1:1, v/v). Since cyclamen plants grow naturally under trees and bushes, the plants in the greenhouse were cultured to mimic their natural habitat conditions, under half shade and natural temperature. Plants were irrigated once a week (300 ml/pot) and a 50% concentration of fungicide (Captan 50WP, Polsaş Chemical Co., Ankara, Turkey) was applied at 250 g/100 l when necessary. No pesticides were used. Four different explants (ovule, ovary, leaf and petiole) from the 15 genotypes collected from the natural flora were used in this experiment.

2.2. Surface sterilization and explant preparation

Flowers from 15 genotypes that had been randomly selected were separately harvested in February, March and April, about two days before anthesis. They were washed under tap water for 20 min. Under sterile conditions, flowers were sterilized in 70% ethanol for 1 min and rinsed three times in sterile distilled water (SDW), then in 30% Domestos® (NaOCl, 4.5% v/v) for 20 min. After treatment with NaOCl, flowers were rinsed 5 times and maintained in SDW until explant preparation. Working under a stereo binocular microscope (Leitz, Weitzlar, Germany) at 5× magnification, whorls were carefully excised from the outer to the inner, central whorl until the ovary was reached. Due to the limited number of flowers, each ovary was cut into four equal parts and transferred to callus induction medium (CIM; see medium details next). Ovules were isolated from ovaries and transferred to CIM. Fresh leaves and petioles, which were collected from healthy plants in the flowering stage, were washed under tap water for 20 min, then placed in 0.1% mercuric chloride ($HgCl_2$) for 5 min, sterilized in 70% ethanol for 1 min, rinsed in SDW, then dipped in 30% Domestos® for 20 min. After rinsing a half-dozen times with SDW, explants were maintained in SDW until further preparation. Leaves and petioles were cut into small parts (approx. 3 mm^2) and placed on CIM. Leaves were placed abaxial side down on medium.

Table 1
The locations of *Cyclamen* plants.

Plant codes	Geographic coordinates	Altitude (m above sea level)
C-1	37°03′05″N 035°20′44″E	75
C-2	37°03′31″N 035°21′07″E	149
C-3	37°03′06″N 035°20′46″E	74
C-4	37°03′11″N 035°20′39″E	119
C-5	37°03′11″N 035°20′39″E	120
C-6	37°03′11″N 035°20′38″E	113
C-7	37°03′11″N 035°20′40″E	128
C-8	37°03′09″N 035°20′28″E	101
C-9	37°03′06″N 035°20′44″E	80
C-10	37°03′33″N 035°21′07″E	147
C-11	37°03′06″N 035°20′46″E	57
C-12	37°03′26″N 035°21′09″E	106
C-13	37°03′29″N 035°21′05″E	150
C-14	37°03′01″N 035°20′44″E	73
C-15	37°03′06″N 035°20′46″E	77

2.3. Callus induction culture

Cultures for inducing embryogenic callus were established and maintained as described by Schwenkel and Winkelmann (1998). CIM, which served as the basal medium to induce embryogenic callus from ovules, ovaries, leaves and petioles, consisted of half-strength Murashige and Skoog (1962) macro- and micronutrients, except for full strength Fe–EDTA, 250 mg l^{-1} peptone, 100 mg l^{-1} *myo*-inositol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} nicotinic acid, 0.1 mg l^{-1} thiamine HCl, 0.5 mg l^{-1} pyridoxin-HCl, 30 g l^{-1} sucrose, 2 g l^{-1} glucose, 3.7 g l^{-1} gelrite (all Sigma-Aldrich, St. Louis, USA), 2.0 mg l^{-1} 2,4-D and 0.8 mg l^{-1} 6-(γ,γ-dimethylallylamino) purine (2iP). All explants were cultured at 22–25 °C in constant darkness for 8 weeks to induce and propagate callus and then subcultured every 8 weeks until sufficient embryogenic callus formed (Winkelmann, 2010).

2.4. Differentiation and germination of somatic embryos

After embryogenic callus formed, it was transferred to differentiation medium that was CIM free of plant growth regulators, *i.e.*, 2,4-D and 2iP. Callus was cultured at 22–25 °C in constant darkness and subcultured every 4 weeks as described by Winkelmann (2010). At this stage, callus, which was soft and friable, was homogenously spread on the same medium. Somatic embryos germinated spontaneously and developed a small tuber, cotyledons and roots, which were individually subcultured. The

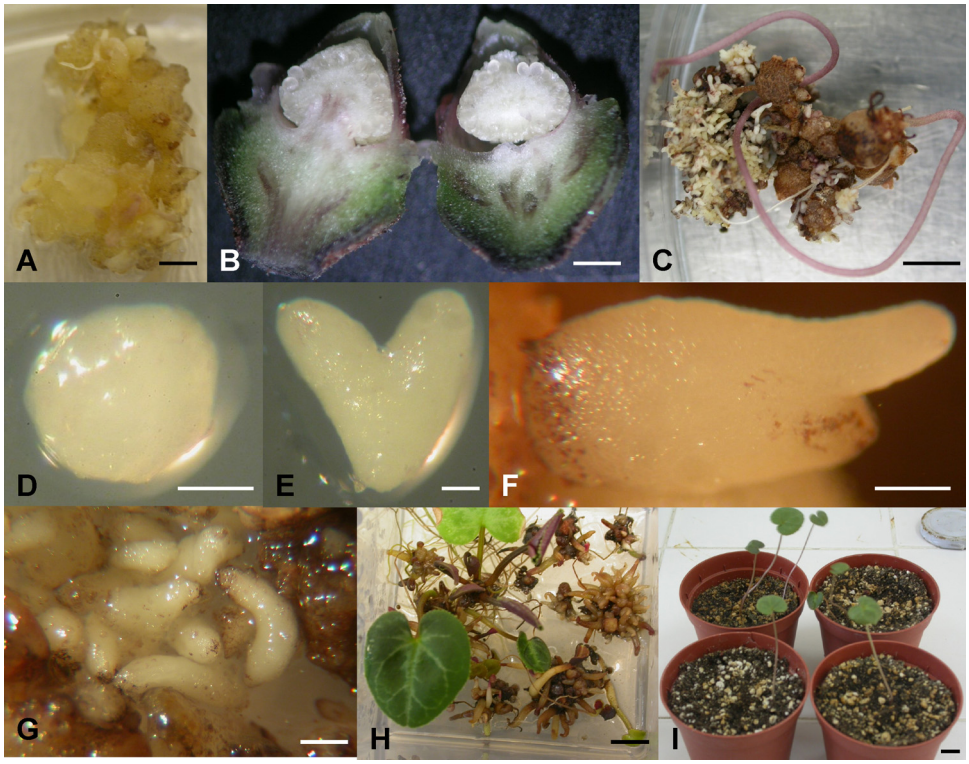


Fig. 1. Developmental stages of somatic embryogenesis in ovary explants (from C-9 plants). (A) Ovary at the beginning of culture; (B) embryogenic callus formed from ovary after 10 weeks in culture; (C) somatic embryo masses at 10th week after transfer to plant growth regulator (PGR)-free medium (30th week); (D) globular stage somatic embryo at 10th week after transfer to PGR-free medium (30th week); (E) heart stage somatic embryo at 10th week after transfer to PGR-free medium (30th week); (F) torpedo or cotyledonary stage somatic embryo at 10th week after transfer to PGR-free medium (30th week); (G) somatic embryos at 10th week after transfer to PGR-free medium (30th week); (H) plants derived from somatic embryos on PGR-free medium after 30 (in total 50 weeks) weeks in culture; (I) acclimatized plants formed from somatic embryos. Scale bars: 1 mm (I); 5 mm (A, B, D, E, G); 10 mm (C, F, H).

further cultivation of somatic embryos and development into plantlets was performed at 25 ± 2 °C under a 16-h photoperiod under 75 μmol m^{-2} s^{-1} cool white fluorescent lamps. Petri dishes (90 × 15 mm) containing 30 ml of medium were used for the first four subculture stages then magenta bottless (76 × 76 × 100 mm) containing 40 ml of medium were used in the last stage so as to accommodate developed plantlets.

2.5. Acclimatization and ex vitro growth conditions

Plantlets were removed from the culture medium. The roots were washed carefully under tap water to remove adhering medium and dipped in a solution containing 50% (w/v) fungicide (Captan 50WP) at 250 g/100 l for 10 s. The size of plantlets derived from somatic embryos was different: some were well developed while others were small and undeveloped, but all were planted into 5-cm high plastic pots filled with peat (Klasmann, KTS-1). The pots were placed in a greenhouse under natural light at 95–98% relative humidity and 22–24 °C.

2.6. Experimental design and statistical analysis

The experimental design was a completed randomized plot (10 replicates × 4 explants × 15 genotypes). The experiment was repeated twice. Means were separated by analysis of variance and the LSD test was performed to examine significant differences ($P < 0.01$) in callus formation, somatic embryo production, tuber formation and acclimatization between different explant types and genotypes. Percentage data was arcsine-transformed. All data analyses were carried out with the JMP® program (SAS Institute, Cary, NC) ver. 5.00.

3. Results

3.1. Callus induction and formation

Each explant from 15 *C. persicum* genotypes (C-1 to C-15) had a variable capacity to form callus *in vitro* on CIM, petioles forming statistically more callus than ovule and leaf explants (Table 2) (Fig. 1A). The highest callus formation of C-15 was obtained from ovules (100%), ovaries (90%) and petioles (90%). C-14 also resulted in the highest callus induction percentage from leaves (95%), followed by ovules (80%). Only petioles resulted in callus production (100% and 82.5%, respectively) in C4 and C-12. The remaining explants did not show any growth response. When averaged over all explants, the most prolific callus-inducing genotype was C-15 (74.38%), while the least prolific were C-8, C-2 and C-6 (1.25%, 3.13% and 4.38%, respectively). When callus formation across all 15 genotypes was averaged for each explant type, the following was observed: 34.3%, 30%.16, 26.6% and 15.6%, respectively from petioles, ovaries, ovules and leaves. All explants formed embryogenic and nonembryogenic callus. Embryogenic callus was generally light brown, soft, friable and separable (Figs. 1B, 2C, 3B and C, 4B and C). Although callus formed from leaf and petiole explants in the first week (Figs. 3B and C, 4B and C), it formed 1–2 weeks later from ovary and ovule explants (Figs. 1B and 2C). In addition, petiole and leaf explants produced more callus mass than ovule and ovary explants (data not shown). Callus that formed from petiole and leaf explants was very homogenous and friable (Figs. 1B, 2D, 3B and C, 4B and C).

Table 2
Percentage of callus formation from 15 *Cyclamen persicum* genotypes and explants and assessment of the genotype-explant interaction.

Cyclamen persicum genotypes numbers	Ovules	Ovaries	Leaves	Petioles	Mean of genotypes
C-1	25 (24)ijklm	30 (27)hijkl	0 (0)n	0 (0)n	13.75 (12.75)ef
C-2	0 (0)n	12.5 (12)klmn	0 (0)n	0 (0)n	3.13 (3.00)gh
C-3	0 (0)n	50 (45)efgh	0 (0)n	0 (0)n	12.50 (11.25)efg
C-4	0 (0)n	0 (0)n	0 (0)n	100 (90)a	25.00 (22.50)cd
C-5	50 (45)efgh	0 (0)n	0 (0)n	0 (0)n	12.50 (11.25)efg
C-6	0 (0)n	17.5 (16.5)jklmn	0 (0)n	0 (0)n	4.38 (4.13)fgh
C-7	50 (45)efgh	5 (4.5)n	67.5 (57)defg	70 (63)cde	48.13 (42.38)b
C-8	0 (0)n	5 (6)mn	0 (0)n	0 (0)n	1.25 (1.50)h
C-9	0 (0)n	80 (70.5)bcd	0 (0)n	0 (0)n	20.00 (17.63)de
C-10	7.5 (9)lmn	0 (0)n	35 (31.5)hij	35 (30)hijk	19.38 (17.63)de
C-11	45 (39)ef	45 (39)ghi	20 (18)jklmn	20 (18)jklmn	32.50 (28.50)c
C-12	0 (0)n	0 (0)n	0 (0)n	82.5 (73.5)abcd	20.63 (18.38)de
C-13	42.5 (37.5)hi	87.5 (78)abc	0 (0)n	50 (43.5)fgh	45.00 (39.75)b
C-14	80 (72)abcd	30 (27)hijkl	95 (84)ab	67.5 (58.5)def	68.13 (60.38)a
C-15	100 (90)a	90 (81)abc	17.5 (16.5)jklmn	90 (81)abc	74.38 (67.13)a
Mean of explants	26.66 (24.1)B	30.16 (27.1)AB	15.66 (13.8)C	34.33 (30.5)A	

$LSD_{explant}$ = 4.8244, $LSD_{genotype}$ = 9.3467, $LSD_{explant \times genotype}$ = 18.6848 ($P < 0.01$). All percentage values, indicated in parentheses, were arcsine transformed. Different letters within a column (for each explant type) or within a row (for mean of explants) indicate significant differences ($P < 0.01$)

3.2. Differentiation and germination of somatic embryos

Although all explants from all 15 genotypes formed callus from at least one explant type (Table 2), this did not translate into somatic embryogenesis in all cases and considerable genotype- and explant-dependent variation was observed (Table 3). However, highest callus formation was observed in C-14 and C-15, although this callus could not convert to somatic embryos (5% and 0% for C-14 and C-15, respectively). Somatic embryos were obtained in 2.83 to 11.3% of all explant types, the highest being from ovary explants. Although more somatic embryos formed from ovary culture (Fig. 1C–G) than from any other explant, the number of somatic embryos per explant in leaf and petiole culture (Figs. 3D–H and 4D–G) was higher than from all other explants. Only 5–50 somatic embryos/Petri dish formed from ovule and ovary explants, but more than 100 somatic embryos/Petri dish were obtained from leaf and petiole (data not shown). A small number of embryos were produced on CIM containing 2 mg l^{-1} 2,4-D and 0.8 mg l^{-1} 2iP. Embryos regenerated from leaf and petiole explants were separated easily and subcultured individually (Figs. 4E–F and 5D–F).

3.3. Tuber formation, acclimatization and ex vitro growth

Developed somatic embryos produced small cotyledons or small microtubers. Despite the development of somatic embryos with cotyledons that then developed spontaneously into plantlets. The plantlets obtained from somatic embryos could be acclimatized in the greenhouse and the relationships between explant type, genotype and acclimatization were statistically analyzed. The percentage of plantlet formation from different explants and the

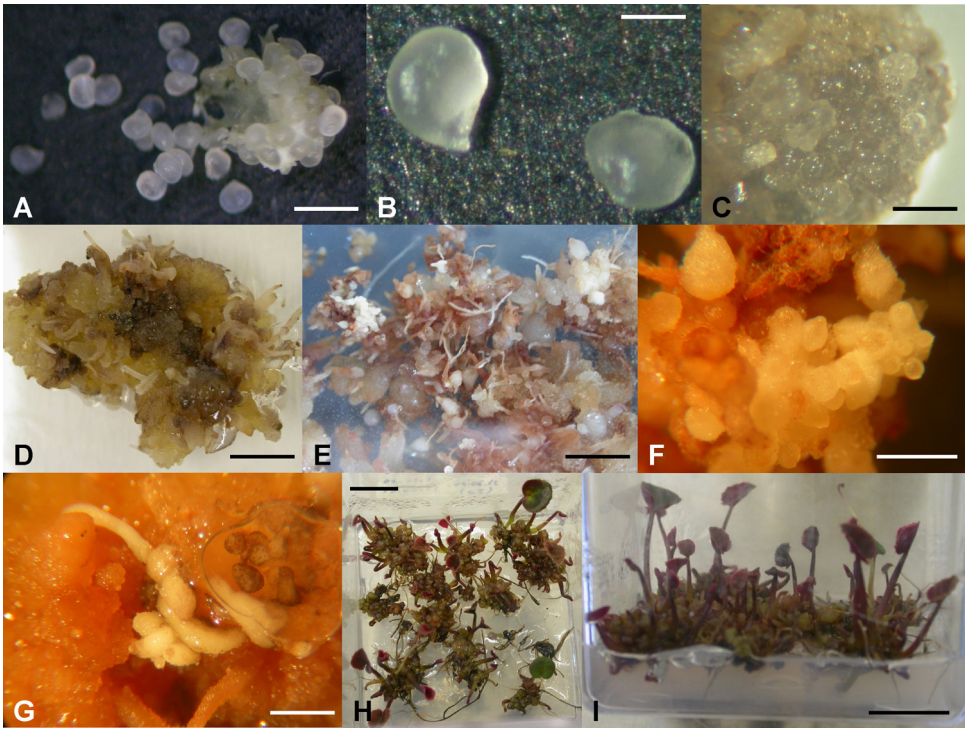


Fig. 2. Developmental stages of somatic embryogenesis in ovule explant (from C-13 plants). (A, B) Ovule explants at the begining of culture; (C, D) callus formed from ovule explants after 6 and 16 weeks of culture; (E, F, G) somatic embryos at 10th week of culture on PGR-free medium (total of 30 weeks); (H, I) plants regenerated from somatic embryos on PGR-free medium after 50 weeks. Scale bars: 10 mm (A, B, C, D, F, G); 15 mm (E); 20 mm (H, I).

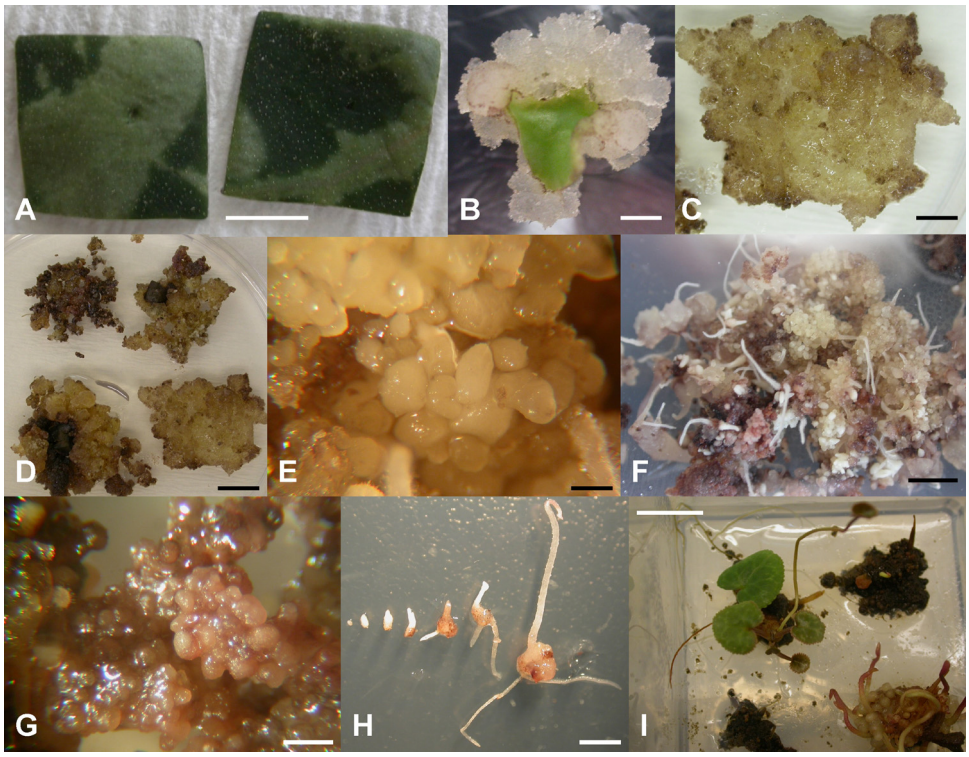


Fig. 3. Developmental stages of somatic embryogenesis from leaf explants (from C-7 plants). (A) Leaf explant at the start of culture; (B, C, D) callus formed from leaf explant after 4, 8 and 12 weeks of culture, respectively; (E–G) somatic embryos on plant growth regulator (PGR)-free medium after 10 weeks subsequently transferred to PGR-free medium for a total of 30 weeks; (H) typical developmental stages of somatic embryos after 10 weeks following transfer to PGR-free medium (total of 30 weeks); (I) plants regenerated from somatic embryos after 30 weeks following transfer to PGR-free medium (total of 50 weeks). Scale bars: 5 mm (B, C, D, E); 10 mm (A, F, G, H); 20 mm (I).

explant × genotype interaction showed that the most efficient regeneration was from leaf and petiole explants of C-7 (28.33% and 26.66%, respectively). The ovary was the best explant for plantlet formation (7.77%). No plantlets were obtained from any explant of C-5, C-10 or C-15 (Table 4). The percentage survival of regenerated plantlets from different genotypes during acclimatization was 9.58%, 6.25% and 5.41% for C-7, C-13 and C-9, respectively (Table 5).

4. Discussion

Turkey is divided into three regions corresponding to main climatic types and three transitional regions according to at least two transitional climatic types. Each region is divided into subregions based on topographic properties such as elevation and orographic ranges, with exposure forming the orobiomes (Atalay, 2002). Turkey is also one of the most important biodiversity centres in the world. It contains more than 30% endemics out of nearly 12,000 natural vascular plant taxa. The reasons for this rich biodiversity in Turkey are climatic differences, geological and geomorphologic diversity, the presence of seas, streams and lakes, and varied elevation. Turkey has varied topographies and soil, as well as fresh, salty and alkaline water types (Kahraman et al., 2012). *Cyclamen* is a widespread genus of flowering plants with various species and subspecies that grow naturally in and are endemic to Turkey. *C.*

Table 3
Percentage somatic embryo formation from 15 *Cyclamen persicum* genotypes and explants and assessment of the genotype-explant interaction.

Cyclamen persicum genotype numbers	Ovules	Ovaries	Leaves	Petioles	Mean of genotypes
C-1	15 (13.50)efg	5.00 (4.50)gh	0 (0)h	0 (0)h	5.00 (4.50)def
C-2	0 (0)h	10.0 (9.00)fgh	0 (0)h	0 (0)h	2.5 (2.25)ef
C-3	0 (0)h	45.0 (40.5)c	0 (0)h	0 (0)h	11.25 (10.13)c
C-4	0 (0)h	0 (0)h	0 (0)h	35 (31.50)cd	8.750 (7.88)cd
C-5	2.50 (3.00)gh	0 (0)h	0 (0)h	0 (0)h	0.63 (0.75)ef
C-6	0 (0)h	17.5 (16.50)ef	0 (0)h	0 (0)h	4.38 (4.13)def
C-7	2.50 (3.00)gh	0 (0)h	62.5 (52.50)b	62.5 (55.50)b	31.88 (27.75)a
C-8	0 (0)h	5.00 (6.00)fgh	0 (0)h	0 (0)h	1.25 (1.5)ef
C-9	0 (0)h	77.5 (67.5)a	0 (0)h	0 (0)h	19.38 (16.88)b
C-10	0 (0)h	0 (0)h	0 (0)h	0 (0)h	0 (0)f
C-11	7.5 (6.00)fgh	0 (0)h	0 (0)h	0 (0)h	1.88 (1.5)ef
C-12	0 (0)h	0 (0)h	0 (0)h	22.5 (22.50)de	5.63 (5.63)cde
C-13	10.0 (6.00)fgh	10.0 (10.50)fgh	0 (0)h	0 (0)h	5.00 (4.88)cdef
C-14	5.00 (6.00)fgh	0 (0)h	0 (0)h	0 (0)h	1.25 (1.5)ef
C-15	0 (0)h	0 (0)h	0 (0)h	0 (0)h	0 (0)f
Mean of explants	2.83 (2.7)C	11.3 (10.3)A	4.16 (3.5)C	8.00 (7.3)B	

$LSD_{explant}$ = 2.8833, $LSD_{genotype}$ = 5.5836, $LSD_{explant \times genotype}$ = 11.16723 ($P < 0.01$). All percentage values, indicated in parentheses, were arcsine transformed. Different letters within a column (for each explant type) or within a row (for mean of explants) indicate significant differences ($P < 0.01$)

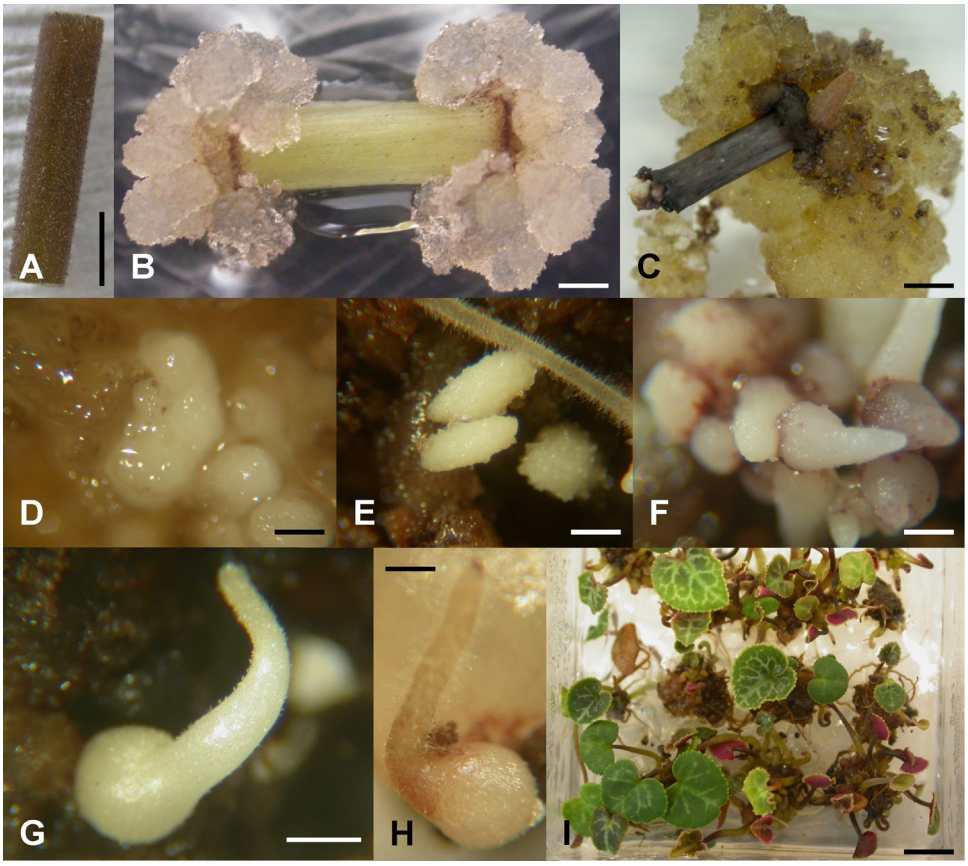


Fig. 4. Developmental stages of somatic embryogenesis from petiole explants (from C-7 plants). (A) Petiole explant at the beginning of culture; (B, C) callus forming from petiole after 4 and 12 weeks of culture, respectively; (D-F) somatic embryos on plant growth regulator (PGR)-free medium after 10 weeks subsequently transferred to PGR-free medium for a total of 30 weeks; (G, H) small tubers regenerated from somatic embryos 10 weeks after transfer to PGR-free medium (total of 30 weeks); (I) plants regenerated from somatic embryos on PGR-free medium after 30 weeks on PGR-free medium (total of 50 weeks). Scale bars: 2 mm (B); 5 mm (D, E, H); 10 mm (A, C, F, G, I).

persicum is native to the eastern Mediterranean region from Libya to Lebanon, Turkey and Greece. This genetic variation and distinctive features of this pool of wild Turkish *Cyclamen* species would be valuable for breeders as they can serve as a base for new genetic traits related to flower colour, resistance to diseases or other horticulturally beneficial characteristics.

Somatic embryogenesis is important for the clonal propagation of higher plants, and when integrated with conventional breeding programs and molecular and cell biological techniques, can provide a valuable tool for the genetic improvement of commercial crops (Stasolla and Yeung, 2003). Somatic embryogenesis was thus considered to be a possible viable method for clonal propagation of Turkish endemic wild species.

In this study, somatic embryogenic potential was investigated using four explants derived from ovules, ovaries, leaves and petioles from 15 *C. persicum* genotypes collected from the

Table 4
Plantlet formation percentage from four different explants and the explant × genotype interaction.

Cyclamen persicum genotype number	Effects of explants and genotype interaction				Mean of genotypes
	Ovules	Ovary	Leaves	Petioles	
C-1	20(26.45)cd	5(10.45)hi	0(0)j	0(0)j	6.25 (9.22)c
C-2	0(0)j	26.66 (30.99)ab	0(0)j	0(0)j	6.66 (7.74)cde
C-3	0(0)j	8.33 (16.59)fg	0(0)j	0(0)j	2.08 (4.14)g
C-4	0(0)j	0(0)j	0(0)j	16.67(24.04)de	4.16 (6.01)ef
C-5	0(0)j	0(0)j	0(0)j	0(0)j	0(0)h
C-6	0(0)j	23.33 (28.66)bc	0(0)j	0(0)j	5.83 (7.16)de
C-7	3.33 (8,61)i	0(0)j	28.33 (32.14)ab	26.66 (30.94)ab	14.58 (17.92)a
C-8	0(0)j	5(12.92)gh	0(0)j	0(0)j	1.25 (3.23)g
C-9	0(0)j	31.66 (34.23)a	0(0)j	0(0)j	7.91 (8.55)cd
C-10	0(0)j	0(0)j	0(0)j	0(0)j	0(0)h
C-11	8.33 (16,59)fg	0(0)j	0(0)j	0(0)j	2.08 (4.14)g
C-12	0(0)j	0(0)j	0(0)j	25(29.92)bc	6.25 (7.48)cde
C-13	15(22.59)e	16.66 (24.04)de	0(0)j	0(0)j	7.91 (11.66)b
C-14	10(18.04)f	0(0)j	0(0)j	0(0)j	2.5 (4.51)fg
C-15	0(0)j	0(0)j	0(0)j	0(0)j	0(0)h
Mean of explants	3.77 (6.15)B	7.77 (10.52)A	1.88 (2.14)C	4.55 (5.66)B	

$LSD_{explant}$ = 0.984, $LSD_{genotype}$ = 1.905, $LSD_{explant \times genotype}$ = 3.81 ($P < 0.01$). All percentage values, indicated in parentheses, were arcsine transformed. Different letters within a column (for each explant type) or within a row (for mean of explants) indicate significant differences ($P < 0.01$)

Table 5
Survival percentage of regenerated plantlets from 15 wild Turkish *Cyclamen persicum* genotypes during acclimatization.

Survival ratio from different genotypes															
C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12	C-13	C-14	C-15
3.75 (11.47)bcd	4.58 (12.45)bcd	1.25 (5.23)f	2.91(9.54)cde	0 (0)g	5 (12.87)bc	9.58 (18.09)a	0 (0)g	5.41 (13.62)b	0 (0)g	1.66 (7.1)ef	5 (12.87)bc	6.25 (14.51)ab	2.5 (9.08)ef	0 (0)g

$LSD_{genotype}$ = 1.915, ($P < 0.01$). All percentage values, indicated in parentheses, were arcsine transformed. Different letters within a column indicate significant differences ($P < 0.01$).

natural flora of Turkey. Petiole explants followed by ovary explants formed the highest amount of callus on CIM. However, ovary and ovule explants tended to take longer to induce callus than leaf and petiole tissue. Most somatic embryos formed from ovary explants. Kiviharju et al. (1992) showed that petioles and peduncles produced very homogenous callus (in terms of colour and morphology). Karam and Al-Majathaub (2002) noted that peduncle and petal explants on half-strength MS medium containing 0.22 mg l^{-1} thidiazuron formed most callus after 8 weeks of culture. Jalali et al. (2010b) showed that most callus formed from leaf explants on half-strength MS medium containing 2 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin.

In this study, most somatic embryos formed on differentiation medium, but a small amount formed on CIM. Kiviharju et al. (1992) observed that morphologically mature somatic embryos developed on CIM. In addition, somatic embryos were not produced after transfer to differentiation medium. Thus, 2,4-D did not inhibit somatic embryo development nor was differentiation medium necessary for somatic embryo development.

Winkelmann and Serek (2005) evaluated genotypic differences in callus formation and regeneration of *C. persicum* somatic embryos. They monitored callus induction after 8 weeks, showing that 42 and 85% of callus-derived somatic embryos regenerated on CIM after 8 and 16 weeks, respectively. They rarely observed somatic embryo formation in several genotypes during primary culture on CIM.

Naderi et al. (2012) reported that callus could be obtained about 15 days after culture in leaf explants and after about 20 days in petiole explants from inflicted wounds. Media and explants showed significant interaction effects on callus percentage. Somatic embryos were produced after callus was transferred to medium free of plant growth regulators.

In this experiment, embryogenic callus was generally light brown, soft, friable and separable. Other studies showed callus to be transparent and watery or white and compact (Otani and Shimada, 1991; Takamura and Tanaka, 1996; Jalali et al., 2010b). Takamura et al. (1995) noted that embryoids (somatic embryo-like structures) usually formed from transparent and separable callus. In this study, however, somatic embryos from ovule and ovary explants were generally interconnected and could not be easily separated. Prange et al. (2010a) mentioned that one out of 10 genotypes formed friable callus that was easily subcultured, brown embryogenic cultures consisted of undifferentiated cells, but proembryogenic masses developed into somatic embryos when transferred to medium without plant growth regulators. Schwenkel and Winkelmann (1998) reported yellow and friable or brown and soft callus from ovule explants. Savona et al. (2007) induced soft, friable and yellowish callus from *C. persicum* cv. 'Halios' ovule explants. Naderi et al. (2012), investigating differences between the response of leaf and petiole explants and MS and half-strength MS on callus percentage and somatic embryo number, observed two callus types: white, opaque and compact as well as soft, watery and transparent.

Acclimatization is one of the most important processes in tissue culture. In this study, plantlets obtained from somatic embryos were successfully transferred to the greenhouse (Fig. 1I). The most efficient acclimatization in this study resulted in a survival percentage of 38.8% in C-7, the highest percentage of regenerated plantlets was from ovaries (31.66%) in C-9, and the highest percentage of acclimatized plantlets was observed for C-7 (Tables 4 and 5). Kiviharju et al. (1992) transferred *in vitro*-derived plantlets both to an acclimatization room and directly to a greenhouse, but no difference in the survival of plantlets was observed. Schwenkel and Winkelmann (1998) acclimatized 70–95% of plants regenerated from 5000 somatic embryos. Karam and Al-Majathaub, 2002 showed that *in vitro* rooted shoots acclimatized successfully, and

that the plants appeared normal and did not show morphological abnormalities. Winkelmann et al. (2006) observed a 41% acclimatization frequency, lower than the 80–95% frequency normally obtained for somatic embryo-derived cyclamen plants. Savona et al. (2007) transferred the tubers of plantlets derived from somatic embryogenesis that had formed 2–3 leaves to a greenhouse, and were able to acclimatize them under ambient light. Prange et al. (2010a) reported almost 100% acclimatization of plants with three or more leaves but even 35% of plants regenerated from protoplasts, when acclimatized, could survive; 60% of control plants survived. Prange et al. (2010b) observed that somatic embryos developed in two different ways, either as small white cotyledons or frequently with small, hairy roots on microtubers before cotyledon development.

5. Conclusion

In this study, a somatic embryogenesis protocol was optimized using four explants (ovule, ovary, leaf and petiole) from 15 *C. persicum* genotypes collected from the natural flora of Turkey. Such somatic embryos would serve for further cryopreservation, encapsulation of embryos or vegetative propagation. Best callus formation was possible from petiole explants, while ovary explants formed most somatic embryos. Embryogenic callus that formed was generally light brown, soft and separable.

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