

Development of an efficient regeneration protocol for four *Cyclamen* species endemic to Turkey

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Abstract In this study, embryo-like structures (ELs) were induced in four endemic Turkish *Cyclamen* species (*C. cilicium* Boiss. et Heldr., *C. parviflorum* Pobed., *C. mirabile* Hildebr. and *C. pseudibericum* Hildebr.) in the presence of 13 combinations of two plant growth regulators (PGRs) (2,4-dichlorophenoxyacetic acid and 6-(γ,γ -dimethylallylamino)purine) and four explant types (ovules, ovaries, leaves and petioles). The ratio of callus induction, different stages of ELs formation and the conversion of ELs to plantlets were quantified. The most effective explant types for callus induction were leaves (56 % for *C. cilicium* and 59 % for *C. parviflorum*) and petioles (80 % for *C. mirabile* and 100 % for *C. pseudibericum*). Callus growth from the leaves and petioles of *C. cilicium* was 30 days earlier than that of *C. mirabile* and *C. pseudibericum*. In contrast, most callus formed from the petioles of *C. pseudibericum* (100 %) in medium with 2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP. The highest number of ELs was obtained successfully from petioles (2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP) and ovaries (2.5 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ 2iP) of *C. pseudibericum*, in 39 % and as 32 % of explants, respectively. The percentage conversion of ELs to plantlets was 38, 31, 16 and 15 % for *C. mirabile*, *C. cilicium*, *C. pseudibericum* and *C. parviflorum*, respectively. The plantlets were successfully acclimatized in the

greenhouse with 54, 70, 63 and 25 % of *C. cilicium*, *C. mirabile*, *C. pseudibericum* and *C. parviflorum* plantlets, respectively surviving after transfer to *ex vitro* conditions. This paper describes a unique, reliable and consistent protocol for the induction of ELs from four endangered endemic Turkish *Cyclamen* species, opening up the possibility of preserving these valuable genetic resources in vitro and also other applied biotechnologies that rely on a stable embryogenic or callus-based protocol.

Keywords *Cyclamen* · Endemic · Embryo-like structure · Callus · Regeneration

Introduction

The origin of 20 *Cyclamen* taxa belonging to the Myrsinaceae is the Mediterranean region (Compton et al. 2004; Yesson et al. 2009). These plants tend to grow under trees and bushes. Ten *Cyclamen* species grow naturally in Turkey. Of these, six are endemic (*C. pseudibericum* Hildebr., *C. trochopteranthum* O. Schwarz, *C. parviflorum* Pobed., *C. cilicium* Boiss. et Heldr., *C. cilicium* var. *intaminatum* Meikle, *C. mirabile* Hildebr.) (Grey-Wilson 2002; Takamura 2006). *Cyclamen* is an economically significant ornamental species used most commonly as a pot plant. Wild *Cyclamen* species possess a huge potential for the enhancement of *Cyclamen* as ornamental and medicinal plants (Seyring et al. 2009). The tubers of *C. repandum* are used in Sardinian folk medicine as a laxative and abortive (Speroni et al. 2007) while the tubers of *C. coum* var. *coum* are used to treat infertility in Turkish folk medicine (Calis et al. 1997a, b; Foubert et al. 2008). Extracts of *Cyclamen* spp. tubers exhibited in vitro cytotoxic (Kupchan et al. 1967), spermicidal (Primorac et al. 1985) and anti-

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microbial activities (Mahasneh and El-Oqlah 1999). Triterpenoid saponins were isolated from different Turkish *Cyclamen* species (Calis et al. 1997a, b; Yayli et al. 1998a, b).

Biotechnology, especially in vitro regeneration studies, are very important to preserve genetic resources that are under the threat of extinction for different reasons such as the destruction of nature, loss of tubers and irresponsible exploration of these resources. It is difficult to propagate cyclamens by division, cutting, and grafting (Takamura and Miyajima 1997). Organogenesis from somatic tissues has been widely described, mainly for *C. persicum* Mill. (Schwenkel and Grunewaldt 1988; Al-Majathoub 1999; Al-Majathoub and Karam 2000; Karam and Al-Majathoub 2000a, b; Abu-Qaoud 2004; Jalali et al. 2012; Nhut et al. 2012), but also for *C. mirabile* (Yamaner and Erdag 2008). Somatic embryogenesis has been found to be an efficient system for the in vitro propagation of *C. persicum* (Kiviharju et al. 1992; Motoyasu and Takiko 1991; Kreuger et al. 1995; Schwenkel and Winkelmann 1998; Ruffoni et al. 1998; Hohe et al. 1999a, b; Winkelmann et al. 2003; Winkelmann and Serek 2005; Winkelmann et al. 2006; Naderi et al. 2012; Kocak et al. 2014). Only few reports have dealt with somatic embryogenesis or the formation of embryo-like structures (ELSs) in wild *Cyclamen* species, namely *C. cilicium* and *C. mirabile* (Furukawa et al. 2001, 2002; Seyring et al. 2009; Prange et al. 2010a). Seyring et al. (2009) investigated ELS formation in *C. africanum* Boiss. and Reut., *C. cilicium* Boiss. and Heldr., *C. coum* Mill., *C. hederifolium* Ait., *C. persicum* and *C. purpurascens* Mill. Prange et al. (2010a) researched somatic embryogenesis of *C. coum*, *C. alpinum*, *C. mirabile* and *C. graecum*. Similar studies on other *Cyclamen* species investigated somatic embryogenesis in *C. cilicium*, *C. coum*, *C. graecum*, *C. hederifolium*, *C. persicum*, *C. purpurascens*, and *C. rohlfsianum* (Furukawa et al. 2001). Somatic embryogenesis in *Cyclamen* is affected by genotype, source of explant, medium content, PGRs, and their concentrations and culture conditions (Tagipur et al. 2016). The development of efficient somatic embryogenesis protocols in *C. persicum* may represent a useful method for the micropropagation of selected material, genetic transformation via *Agrobacterium tumefaciens* and cryopreservation (Aida et al. 1999; Boase et al. 2002; Winkelmann et al. 2004; Terakawa et al. 2008). Most of the previous research dealing with *Cyclamen* somatic embryogenesis included histological examination of the obtained structures which were classified as somatic embryos (Wicart et al. 1984; Kiviharju et al. 1992; Ishizaka and Uematsu 1995; Takamura et al. 1995; Ruffoni et al. 1998; Hoenemann et al. 2010). However, somatic embryos are defined bipolar structures, which do not have a vascular connection with the mother tissue at any time of their life and develop through characteristic embryological stages (Von Arnold et al. 2002; Fehér 2015). In some cases,

including in this study, clear classification of these structures was not possible without histological examination. These structures were designated as “embryo-like structures” (ELSs).

This paper provides a comprehensive report on the induction of ELSs in *C. cilicium*, *C. mirabile*, *C. pseudibericum* and *C. parviflorum* endemic to Turkey, and describes highly efficient and reproducible protocols for inducing ELSs generated from leaf-, petiole-, ovule- and ovary-derived callus using combinations of two PGRs, 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-(γ,γ -dimethylallylamino)purine (2iP).

Materials and methods

Plant material

Wild cyclamen plants at the flowering stage were collected from native habitats in different locations around Turkey between 2010 and 2013. *C. mirabile* (2n = 30) forms an inflorescence prior to leaf development (hysteranthly). The coordinates of the collecting points are: 38°01'00"N, 30°47'00"E, 1085 m for Barla/Isparta; 37°40'22"N, 28°40'57"E, 575 m for Karacasu/Aydin. *C. cilicium* (2n = 30) was collected from Pozantı/Adana and Aladağ (Kıçak köyü)/Adana, and the coordinates of the collecting points are: 37°28'24"N, 34°54'16"E, 1058 m in Pozantı/Adana; 37°32'47"N, 35°24'10"E, 782 m in Aladağ/Adana. *C. pseudibericum* (2n = 30) was collected from Aladağ/Adana, Feke/Adana and Kahramanmaraş, and the coordinates of the collecting points are: 37°35'33"N, 35°16'44"E, 917 m for Aladağ/Adana, 37°46'15"N, 35°54'19"E, 825 m for Feke/Adana, and 37°33'00"N, 36°35'00"E, 1320 m for Kahramanmaraş. *C. parviflorum* (2n = 30) was collected from Maçka, Meryemana/Trabzon and Samandra, Mas Irmağı Mevkii/Trabzon, with the coordinates of the collecting points being: 40°41'52"N, 39°38'51"E, 1215 m for Mas Irmağı Mevkii/Trabzon, and 40°41'51"N, 39°39'36"E, 1077 m for Maçka/Trabzon. Each plant was placed in 13-cm diameter pots in a polycarbonate greenhouse in a substrate mixture containing peat, sand and perlite (1:1:1, v/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. *Cyclamen* plants were irrigated once a week (300 ml/pot) and 50 % fungicide (Captan 50WP, Polsas, Chemical Co., Ankara, Turkey) was applied at 250 g/100 l when necessary.

Surface disinfection

Flower buds were harvested in September, October and November for *C. cilicium* and *C. mirabile*, and in

February, March and April for *C. pseudibericum* and *C. parviflorum* about 3–4 days before anthesis. Fresh leaves and petioles were collected from healthy plants in the flowering stage. Unopened flower buds were washed under tap water for 20 min, dipped in 70 % ethanol for 1 min, washed three times with sterile distilled water (SDW) and then immersed in 20 % sodium hypochlorite solution (commercial bleach solution with 4.5 % active chlorine, v/v, NaOCl; Domestos®) with 1–2 drops of Tween-20 for 20 min. They were washed three times with SDW. The leaves and petioles were also washed under tap water for 20 min, immersed in 0.1 % mercuric chloride solution for 10 min, washed with SDW three times, dipped in 70 % ethanol for 1 min, washed three times with SDW and in 20 % NaOCl solution with 1–2 drops of Tween-20 for 20 min. Finally, explants were rinsed three times with SDW.

Explant preparation and callus induction

Ovules and ovaries were removed from flowers under a stereo binocular microscope (Leitz, Weitzlar, Germany) at 5× magnification. Ovaries were divided into four equally sized pieces and ovules were removed with a surgical blade from ovaries and placed onto callus induction medium (CIM) [25 ovules/Petri dish (90 × 15 mm), 15 leaf segments and petiole segments/Petri dish, 8 pieces of ovary parts/Petri dishes and five replicates of each explant type], as described by Schwenkel and Winkelmann (1998) and Kocak et al. (2014). Leaf (approx. 50 mm²) and petiole (approx. 7 mm length) segments were sectioned aseptically. CIM served as the basal medium to induce callus from ovules, ovary pieces, leaves—which were placed abaxial side down on medium—and petioles. CIM consisted of half-strength Murashige and Skoog (Murashige and Skoog 1962) (½ MS) 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, 1 g l⁻¹ MES, 3.7 g l⁻¹ gelrite (all Sigma-Aldrich, St. Louis, USA) with different combinations of 2iP (0, 0.5, 1, 1.5 and 2 mg l⁻¹) and 2,4-D (0, 1, 2 and 2.5 mg l⁻¹) for 16 weeks in constant darkness at 25 ± 1 °C and then subcultured every 8 weeks until sufficient callus formed (Kocak et al. 2014). The pH of the medium was adjusted to 5.7 prior to autoclaving (20 min at 121 °C). Two different callus sizes were observed: 3–4 mm diameter and/or larger than 4 mm (data not shown). The callus that developed was subcultured onto fresh CIM in Petri dishes and cultured for an additional 8 weeks. Thereafter, callus formation and regeneration of ELSs were recorded.

Differentiation and germination of ELSs

Callus was transferred to PGR-free CIM when callus structures turned into ELSs that were globular- and torpedo-shaped and when callus was 3–4 mm in diameter. Callus was incubated at 25 ± 2 °C in constant darkness and subcultured every 4 weeks, as described by Kocak et al. (2014). To convert ELSs into plantlets, mature ELSs from different explant types were transferred to magenta boxes (76 × 76 × 100 mm) containing PGR-free CIM, 30 % sucrose, 3.5 g l⁻¹ gelrite, at pH 5.7, and maintained for 2 months under a 16-h photoperiod and 75 μmol m⁻² s⁻¹ provided by cool white fluorescent lamps at 24 ± 1 °C.

Acclimatization and ex vitro growth

Plantlets with well-developed shoots and roots, having formed 2–3 leaves within about 180 days, were removed from magenta boxes and the roots were washed gently under running tap water and dipped in a solution containing 50 % (w/v) of a 2.5 g l⁻¹ fungicide (Captan 50WP, Fruit and Ornamental, NY, USA) for 10 s then transferred to plastic pots (7 cm × 7 cm width and length) containing autoclaved peat (Klasmann, KTS-1) and sand (2:1, v/v). The potted plants were placed in a greenhouse under natural light at 95–98 % relative humidity and 22–24 °C.

Experimental design and statistical analysis

The explants (ovules, ovaries, leaves and petioles) in the greenhouse were selected randomly by ignoring the genotype effect. All experiments were set up in a completely randomized factorial design and repeated twice with five replicates per treatment. Callus percentage was calculated as: number of explants on which callus formed/total number of explants. The number of ELSs/explant was calculated by dividing the number of ELSs by the number of leaf, petiole, ovule and ovary explants. All quantitative data expressed as percentages were subjected to arcsine transformation. Means were separated by analysis of variance and the least significant difference (LSD) test was performed to examine differences in callus formation and ELS production among different explants in each *Cyclamen* species. All data analysis was examined using the JMP® program (SAS Institute, Cary, NC) ver. 5.00 and significance was considered at $p < 0.01$. The results of number ELSs per explants were expressed as mean ± SD (standard deviation).

Results

After 2–6 months, structures began to differentiate depending on the species, type of explant and PGR used. Some shoots with abnormal leaves appeared, resembling

somatic embryos. Since no histological analysis was performed to verify the origin of different issues, these structures were designated as ELSs in this paper.

Callus induction

Leaves, petioles, ovules and ovaries were transferred to CIM supplemented with 2 mg l^{-1} of an auxin (2,4-D) and 1.5 mg l^{-1} of a cytokinin (2iP). After 6 weeks, CIM induced light or dark brown friable callus from leaves (Fig. 1g) or yellow–brown compact callus (Fig. 1j, k) from petioles while yellow–dark brown compact callus formed from ovules and ovaries (Fig. 1a, d, e) in *C. cilicium*. In addition, dark-brown compact callus was induced from

ovules (Fig. 1a) while 2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP produced friable callus from petioles (Fig. 1g). Even though all four *C. cilicium* explants could form regenerative and nonregenerative callus together on CIM, leaves (56 %) and petioles (48 %) formed significantly more callus than ovules (12 %) and ovaries (10 %) (Table 1). The best callus induction (56 %) was from *C. cilicium* leaf explants on the medium containing 2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP. Callus was generally dark-brown (Fig. 1a), yellowish (Fig. 1k), soft, friable and separable (Fig. 1d, g). Even though callus formed from leaves and petioles by the third week (Figs. 3a, b, 4b), it formed only 3–4 weeks later from ovaries and ovules (Figs. 1a, 2b). Leaves and petioles produced more fresh callus mass than ovule and ovary

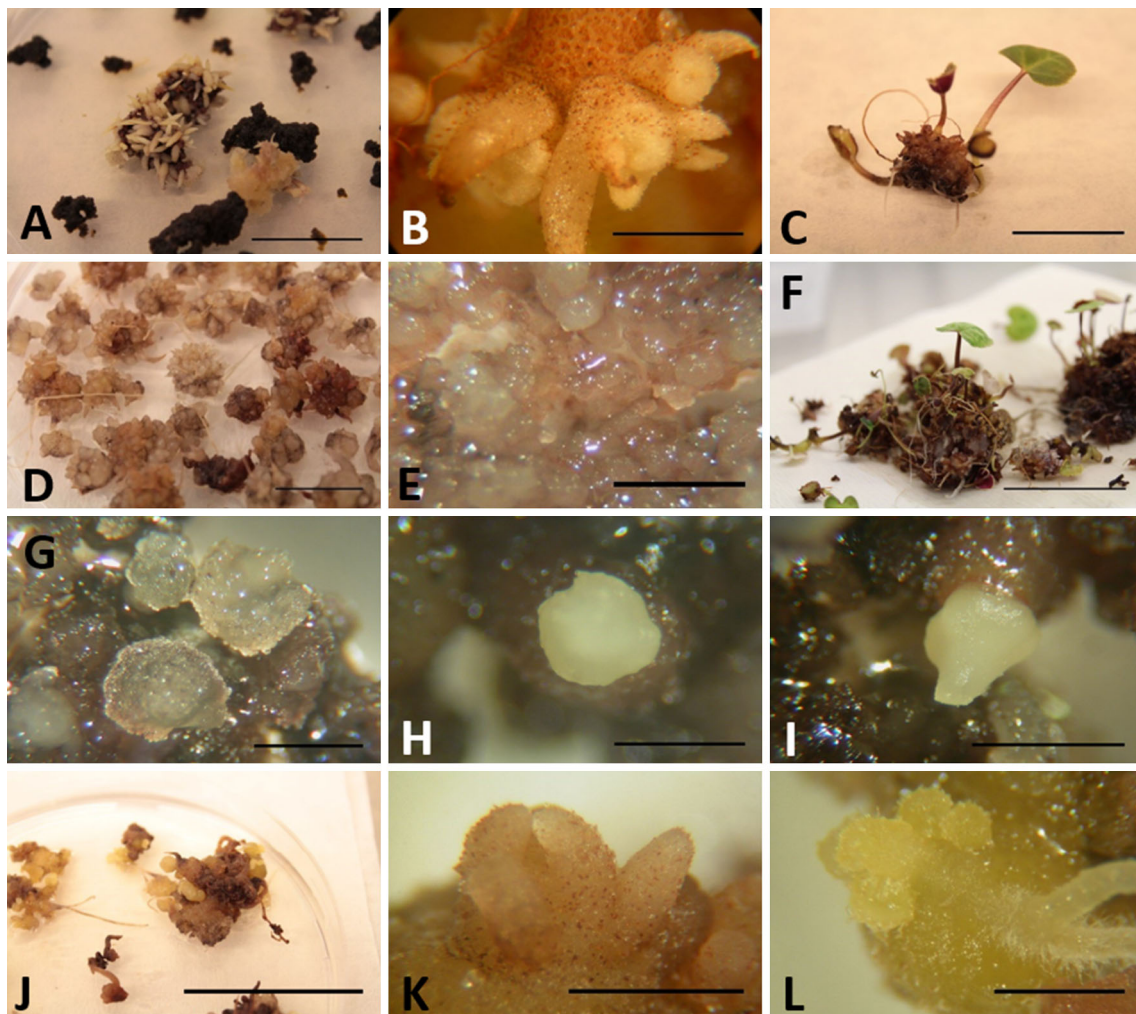


Fig. 1 Embryo-like structures (ELs) from different explant types to plants in *C. cilicium*. **a** Preparation of ovules then callus induction after 8 weeks of culture (2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP). **b** Differentiation of ELs after 4 weeks on PGR-free medium, typical developmental stage of ELs. **c** Young plants ready for acclimatization. **d, e** Differentiation of ELs on callus derived from ovaries (2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP as well as 2 mg l^{-1} 2,4-D and 1 mg l^{-1} 2iP). **f** Formation of shoots from ovaries. **g** Formation of

callus from leaves after 8 weeks (2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP). **h, i** Callus growth and microtuber-like structure from leaves on CIM containing 2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP. **j–l** Development of ELs starting with a microtuber and skipping the globular-embryonal stages from petioles (2 mg l^{-1} 2,4-D and 1.5 mg l^{-1} 2iP). Scale bars 3 cm (**a, d, j**); 4 mm (**b**); 5 cm (**c, f**); 3 mm (**e, g, k**); 5 mm (**h, i, l**)

Table 1 The effect of PGR concentration and explant types on callus formation in *C. cilicium* (%)

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1)	0 c (0)	0 c (0)	0 c (0)	10 c (6)	0.7 C (0.5)
(2 + 1.5)	56 a (48)	48 a (40)	12 c (9)	10 c (6)	26 A (17)
(2 + 2)	28 b (28)	0 c (0)	0 c (0)	0 c (0)	5 B (5)
(2.5 + 1)	0 c (0)	24 b (23)	0 c (0)	0 c (0)	4 B (4)
Control	0 c (0)	0 c (0)	0 c (0)	0 c (0)	0 C (0)
Means of explants	7 A (6)	6 A (5)	0.7 C (0.6)	0.8 C (0.7)	

$p_{0.05}$ Explants: <0.0001; $p_{0.05}$ PGRs: <0.0001; $p_{0.05}$ Explants × PGRs: <0.0001

LSD_{0.05} Explants: 2.723; LSD_{0.05} PGRs: 4.390; LSD_{0.05} Explants × PGRs: 9.816

The numbers in parenthesis represent the arcsine transformed value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

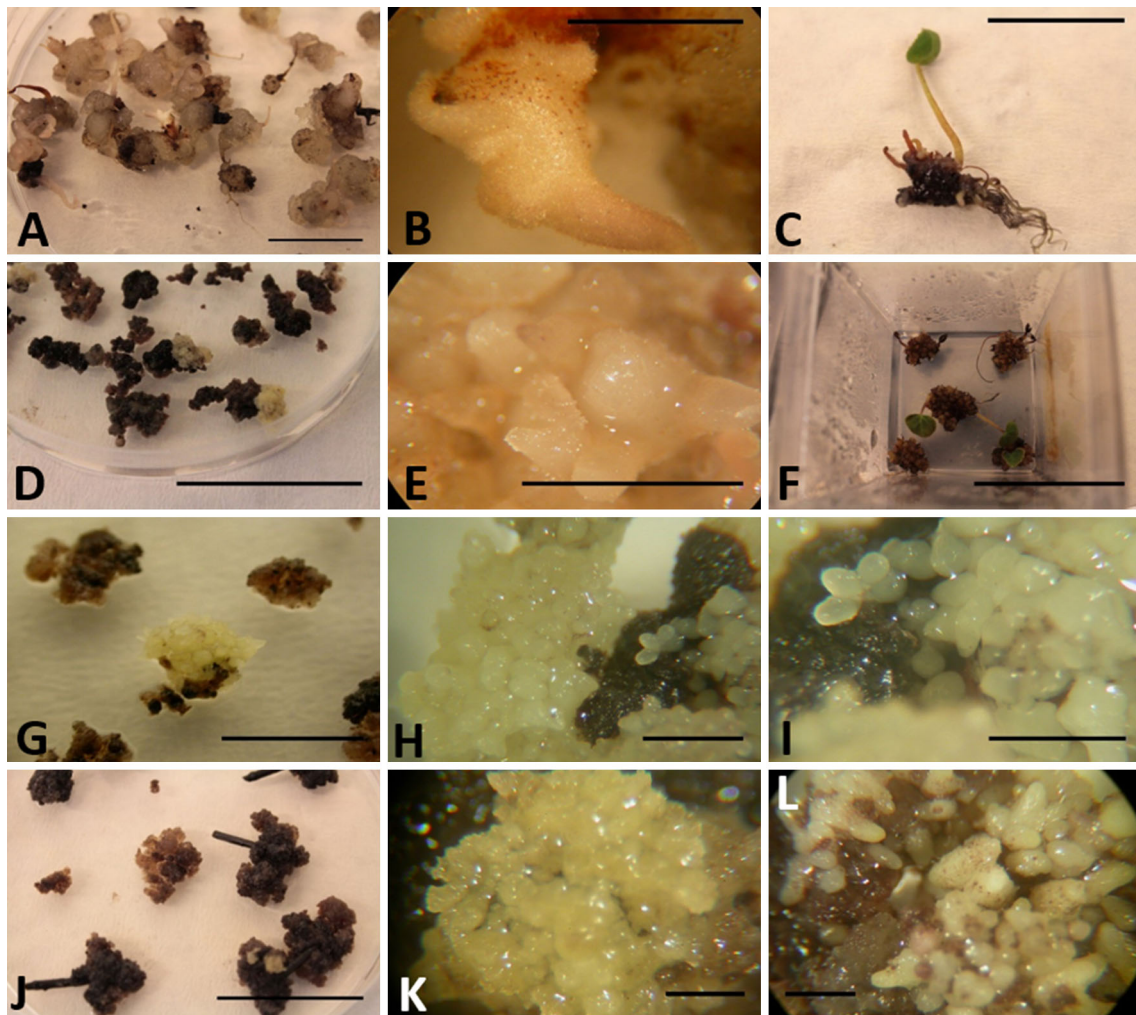


Fig. 2 Embryo-like structures (ELs) from different explant types to plants in *C. mirabile*. **a** Callus growth and development of ELs from ovules on CIM with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. **b**, **c** Development of ELs beginning with a microtuber stages and plantlets formation. **d**, **e** ELS clusters from small-sized globular and torpedo stages on PGR free-CIM for 12 weeks (ovaries). **f** Plantlets

on ½ MS PGR-free medium for 180 days. **g–i** Globular and torpedo stages from leaves after 8 weeks. **j–l** Callus induction from petioles exhibiting browning and small-sized globular ELs after 160 days. Scale bars 3 mm (**b**, **e**, **h**, **i**, **k**, **l**); 3 cm (**c**); 2 cm (**a**, **g**); 5 cm (**d**, **j**); 7 cm (**f**)

explants (data not shown). Callus that formed from petioles and leaves was homogenous and friable (Fig. 1g, k).

In *C. mirabile*, callus started to form within 4–5 weeks following culture initiation (Fig. 2a, d, g, j). Petioles and leaves formed callus within 4 weeks, the former forming dark-brown, compact callus at the cut ends (Fig. 2h, k). The efficiency of callus induction was dependent on explant type and the 2,4-D and 2iP concentration (Table 2). Least callus formed from leaves (4 %), ovules (15 %) and ovaries (12 %) on CIM with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. However, callus formed on all explant types, in 80 % of petioles with 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP and in 36 % of petioles in 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. Callus induction from petioles was significantly higher than that from leaves, ovules and ovaries (Table 2). The highest concentration of 2,4-D (2.5 mg l⁻¹) inhibited callus formation in all four explants. Callus formed in 66 % of petioles and 5 % of leaves on CIM with 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP. Callus growth from *C. mirabile* leaves was slower than from the leaves of other species. Explants started to turn brown after transfer to regeneration medium. After 160 days, explants began to lose their regeneration capacity, but callus continued to grow (data not shown) but turned dark brown. All *C. mirabile* explants showed callus expansion after 150 days but from *C. cilicium* leaves and petioles after 130 days and from petioles after 60 days. In all cases, callus was compact and less than 3 mm in diameter (data not shown). Initially, callus was dark brown but turned light brown over time.

In *C. pseudibericum*, the first morphogenic changes in petioles were observed after 4 weeks of cultivation on CIM supplemented with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP (Fig. 3m, n). Yellow–brown callus at the cut ends of petioles was visible after 2 weeks (Fig. 3J). There were no visible changes on the surface of petioles. During the next 2–3 weeks of cultivation, the consistency of callus changed into more compact callus from petioles (Fig. 3j). There were significant effects of explant type, PGR concentration, and their interaction on callus formation and the

percentage of explants forming callus (Table 3). 2,4-D and 2iP concentration had a highly significant effect on callus formation from petioles in 100 % of explants, followed by leaves (92 %) in CIM containing 2.5 mg l⁻¹ 2,4-D + 1 mg l⁻¹ 2iP and 2 mg l⁻¹ 2,4-D + 2 mg l⁻¹ 2iP, respectively (Table 3). Ovules and ovaries cultured on CIM supplemented with 2.5 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ 2iP produced the least callus (20 % and 60 %, respectively) (Table 3; Fig. 3a, d). Callus also formed on all types of explants (Fig. 3a, d, g, j). Callus growth, as indicated by changes in color and size, was dependent on explant type and PGR concentration (Fig. 3a, d, g, j, h). The structure of callus growth obtained from the leaf explant of *C. pseudibericum* was different from the other species. The colour of the callus changed from brown to yellow after 160 days. Callus was obtained from ovaries after 140 days. Callus (1.5–2 mm in diameter) first turned brown then dark yellow, and new callus formed from brown callus.

In *C. parviflorum*, callus formed in CIM supplemented with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ from leaves (59 %) and petioles (49 %), in 12 % of ovules when 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ was used, or in 100 % of ovaries in the presence of 2 mg l⁻¹ 2,4-D and 1 mg l⁻¹ (Table 4). Even though all explants produced callus on CIM containing different concentration of PGRs (Fig. 4a, c, e, g), least callus (10 %) was obtained from ovaries on CIM containing 2 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP (Table 4; Fig. 4a). Callus was generally dark brown, hard and compact (Fig. 4a, e, g). Differences in callus induction were significantly affected by the 2,4-D and 2iP concentration, and explant type (Table 4). Higher concentrations of 2,4-D caused explants to swell without inducing callus (Table 4). Callus was generally light brown, soft, friable and separable. Different types of callus formed from different explant types: yellowish-white (Fig. 4b, c), brown (Fig. 4e), dark-brown (Fig. 4a, e), friable and compact (Fig. 4g).

Table 2 The effect of PGR concentration and explant types on callus formation in *C. mirabile* (%)

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1.5)	0 c (0)	36 b (30)	15 c (8)	12 c (7)	7 B (6)
(2 + 2)	4 c (5)	80 a (66)	0c (0)	0 c (0)	16 A (14)
Control	0 c (0)	0c (0)	0c (0)	0 c (0)	0 C (0)
Means of explants	0.3 C (0.4)	9A (8)	0.8 B (0.9)	0.7 B (0.8)	

*p*_{0.05 Explants}: <0.0001; *p*_{0.05 PGRs}: <0.0001; *p*_{0.05 Explants × PGRs}: <0.0001

LSD_{0.05 Explants}: 2.093; LSD_{0.05 PGRs}: 3.375; LSD_{0.05 Explants × PGRs}: 7.547

The numbers in parenthesis represent the arcsine transformed value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

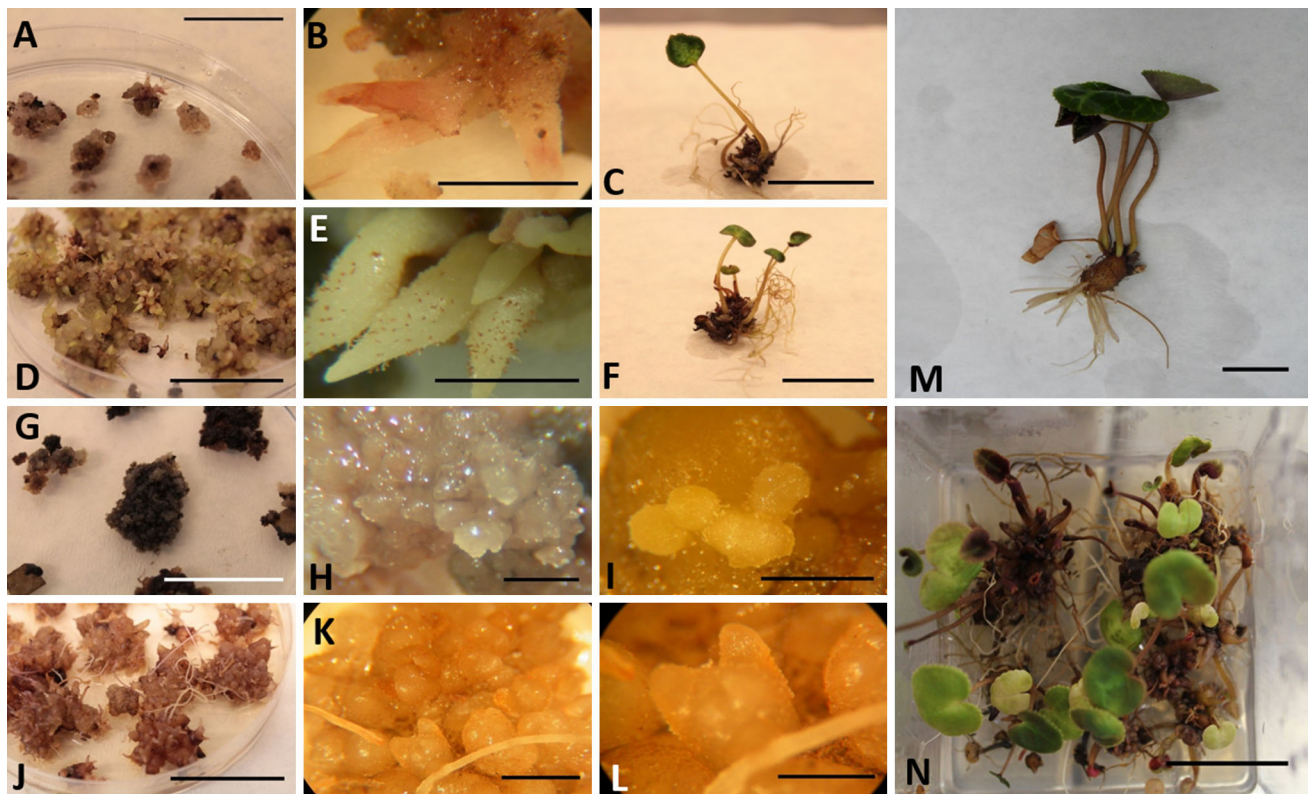


Fig. 3 Embryo-like structures (ELSs) from different explant types to plants in *C. pseudibericum*. **a, b** Callus with many torpedo-shaped ELSs from ovules. **c** Young plantlet from an ovule after 90 days. **d, e** ELSs in clusters from large-sized torpedo stages from ovaries on ½ MS medium after 8 weeks. **f** Plantlet derived from ovary explant after 160 days. **g–i** Callus induction from leaves exhibiting browning and

germination of ELSs. **j** Callus with proembryonic-like structures on ½ MS medium after about 8 weeks (petioles). **k, l** ELS in the torpedo-shaped stage developed from petioles. **m, n** Plantlet conversion after 190 days in culture showing shoot and root development, ready for acclimatization. *Scale bars* 3 cm (**a, c, d, f, g, j, m**); 5 cm (**n**); 3 mm (**b, e, h, i, l**); 2 mm (**k**)

Table 3 The effect of PGR concentration and explant types on callus formation in *C. pseudibericum* (%)

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1.5)	56 cd (48)	80 bc (63)	0 g (0)	0 g (0)	27 BC (24)
(2 + 2)	92 ab (79)	44 cde (41)	0 g (0)	0 g (0)	27 BC (22)
(2.5 + 0.5)	16 fg (12)	60 bcd (54)	20 efg (18)	60 bcd (54)	31 B (27)
(2.5 + 1)	52 cd (46)	100 a (90)	0 g (0)	52 cde (43)	49 A (44)
(2.5 + 1.5)	32 def (30)	0 g (0)	12 fg (10)	0 g (0)	12 D (11)
(2.5 + 2)	20 efg (18)	8 fg (7)	0 g (0)	36 def (30)	16 CD (14)
Control	0 g (0)	0 g (0)	0 g (0)	0 g (0)	0 G (0)
Mean of explants	22 A (19)	24 A (21)	6 B (5)	9 B (7)	

*p*_{0.05} Explants: <0.0001; *p*_{0.05} PGRs: <0.0001; *p*_{0.05} Explants × PGRs: <0.0001

LSD_{0.05} Explants: 7.199; LSD_{0.05} PGRs: 11.608; LSD_{0.05} Explants × PGRs: 25.956

The numbers in parenthesis represent the arcsine transformed value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

Table 4 The effect of PGR concentration and explant types on callus formation in *C. parviflorum* (%)

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1)	0 c (0)	0 c (0)	0 c (0)	10 c (4)	0 C (0)
(2 + 1.5)	59 a (48)	49 a (40)	12 c (6)	0 c (0)	24 A (17)
(2 + 2)	28 b (28)	0 c (0)	0 c (0)	0 c (0)	5 B (5)
(2.5 + 1)	0 c (0)	24 b (23)	0 c (0)	0 c (0)	4 B (4)
Control	0 c (0)	0 c (0)	0 c (0)	0 c (0)	0 C (0)
Mean of explants	7 A (6)	6 A (5)	0.7 C (0.8)	0.6 C (0.7)	

$p_{0.05}$ Explants: <0.0001; $p_{0.05}$ PGRs: <0.0001; $p_{0.05}$ Explants × PGRs: <0.0001

LSD_{0.05} Explants: 2.723; LSD_{0.05} PGRs: 4.390; LSD_{0.05} Explants × PGRs: 9.816

The numbers in parenthesis represent the arcsine transformed value of percentages. Different letters within a column indicate significantly different means (LSD test). Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

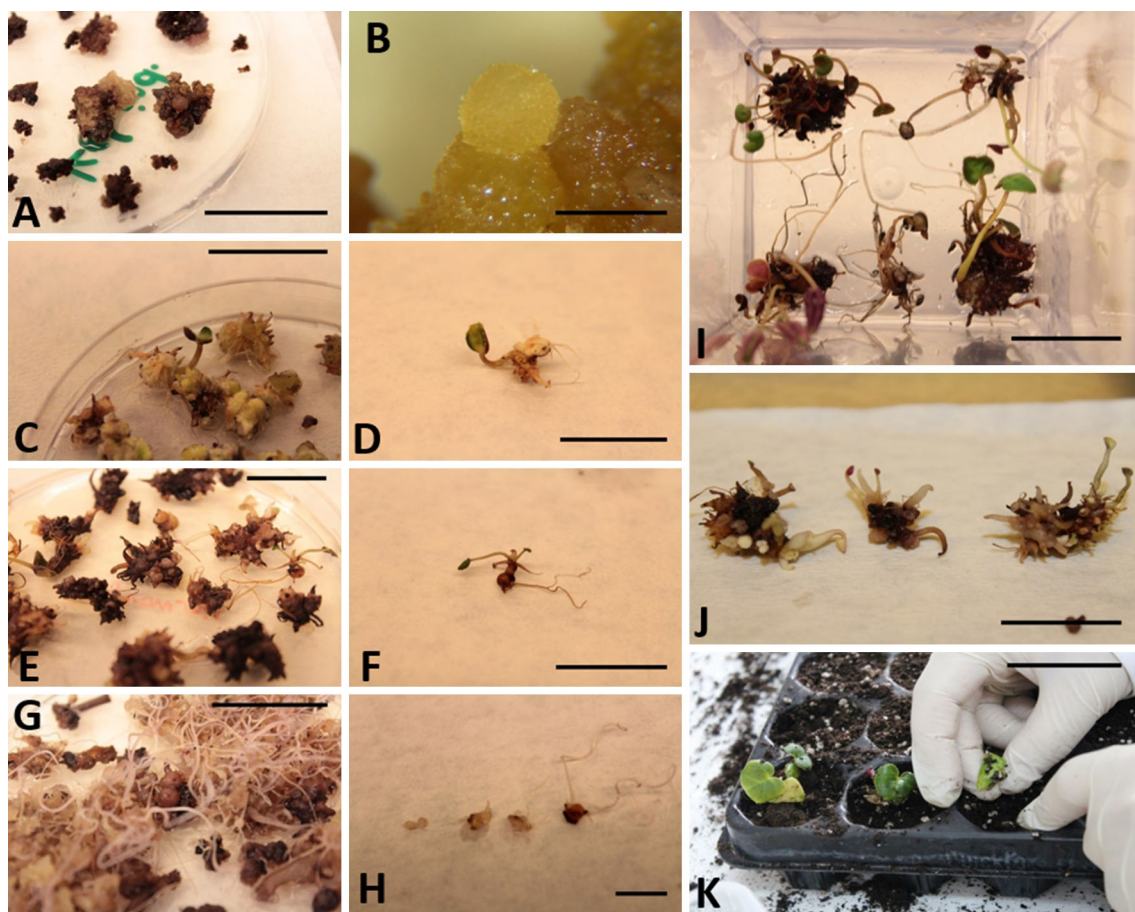


Fig. 4 Embryo-like structures (ELSs) from different explant types to plants in *C. parviflorum*. **a, b** Formation of ELSs from ovules after 8 weeks [callus-induction medium (CIM) containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP]. **c, d** Callus growth and development of ELSs from ovary explants on CIM. **e, f** ELS formation from a leaf on CIM

containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. **g, h** Development of ELSs from petioles on CIM supplemented with 2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP. **i, j, k** Plantlets about 190 days after transfer of ELSs to PGR-free CIM prior to the acclimatization in the greenhouse. Scale bars 3 cm (**a, c–f, g, i**); 3 mm (**b**); 1 cm (**l**); 4 cm (**h**); 5 cm (**k**)

Formation of ELSs

Torpedo- and globular-shaped embryos were observed on CIM (Fig. 1a), but further embryo development was not observed on the same medium. In many *Cyclamen* somatic embryogenic systems, the transfer of ELSs to a culture medium free of PGRs enhanced the development of ELSs and/or somatic embryos and their conversion to plantlets. One of the reasons for the low rates of ELS conversion to plantlets was associated with the residual effects of 2,4-D and 2iP. After 10 weeks, characteristic microtuber-like structures arose from the callus (Fig. 1g–i) on CIM with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. The addition of 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP in maturation medium increased the percentage of ELSs significantly (23) (Table 5). All explants first produced callus then ELSs in *C. cilicium*. The highest conversion of callus to ELSs (i.e., number of ELSs/explant) was observed in leaves and petioles (23 ± 3 and for leaves and petioles in 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP). The number of ELSs/explant was calculated as 15 ± 3 for petioles, 23 for leaves, 7 for ovules, and 5 for ovaries when all PGR concentrations were compared (Table 5). A small number of ELSs/explant were produced on CIM containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP from ovules (7) and ovaries (5). ELSs that regenerated from leaves (Fig. 1g–i) petioles (Fig. 1k, l), ovules and ovaries (Fig. 1b, e) were easily separated and subcultured individually (Fig. 1i, l).

The most effective CIM contained 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP, inducing the intensive development of ELSs for all four explant types compared to all other PGR concentrations (Table 6). The color and structure of ELSs

varied depending on the choice of PGR and explant type in *C. mirabile* (Fig. 2b, e, i, l). Explants cultured on CIM containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP increased the number of torpedo-shaped ELSs, which were yellow and compact (Fig. 2b, e, i, l). The multiplication of callus and the formation of ELSs was possible when the same PGRs used to induce callus were used. ELSs, which developed on CIM containing 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP (petioles) (Fig. 2l) or 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP (ovaries) (Fig. 2e), were characterized by their embryo-like shapes. On the other hand, the highest number of ELSs/explant (16) developed from callus cultured with 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP derived from petioles. ELSs also formed on CIM containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP from ovules (9 ELSs/explant) (Fig. 2b) while 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ 2iP was most effective for *C. mirabile* (Table 2).

In *C. pseudibericum*, small torpedo (approximately 0.5 mm²)- and globular-shaped (approx. 0.5 × 1.5 mm) structures on the surface appeared after 8 weeks in culture. The former were very abundant after 6–8 weeks of culture (Fig. 3b, e, h). Most globular-shaped ELSs were white or yellow and were concentrically arranged (Fig. 3k, l). ELSs were also successfully induced in all *C. pseudibericum* explants (Table 4). Leaf explants cultured on CIM supplemented with different concentrations of 2,4-D and 2iP became swollen, mainly at the cut end of explants and produced yellow nodular ELSs within 4 weeks of culture (Fig. 3h, i). Four to five weeks after ELS initiation, torpedo-shaped ELSs were obtained by continuous cultivation on solid CIM. CIM containing 2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP produced most ELSs/explant from petioles (39) (Table 7). There were highly

Table 5 Number of embryo-like structures formation in *C. cilicium*

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction of number of ELSs ^x				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1)	0 ± 0 ^z f	0 ± 0 f	0 ± 0 f	5 ± 1 e	2 ± 1 C
(2 + 1.5)	23 ± 3 a	15 ± 3 b	7 ± 2 d	5 ± 1 e	12 ± 7 A
(2 + 2)	9 ± 2 c	0 ± 0 f	0 ± 0 f	0 ± 0 f	4 ± 2 B
(2.5 + 1)	0 ± 0 f	10 ± 1 c	0 ± 0 f	0 ± 0 f	4 ± 2 B
Control ^y	0 ± 0 f	0 ± 0 f	0 ± 0 f	0 ± 0 f	0 ± 0 D
Mean of explants	6 ± 2 A	4 ± 1 B	1 ± 0.5 C	1 ± 0.7 C	

*p*_{0.05 Explants}: <0.0001; *p*_{0.05 PGRs}: <0.0001; *p*_{0.05 Explants × PGRs}: <0.0001

LSD_{0.05 Explants}: 0.71 LSD_{0.05 PGRs}: 0.41 LSD_{0.05 Explants × PGRs}: 1.43

^x Values are mean ± standard deviation of 2 separate experiments

^z Means in each column followed by different letters are different according to LSD test (*p* = 0.01)

^y Control = 1/2 MS medium free of PGRs

The number of ELSs per explants (mean and SD) were calculated and differences between means were tested for significance using ANOVA and LSD test at the level of *p* ≤ 0.01

Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

Table 6 Number of embryo-like structures formation in *C. mirabile*

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction of number of ELSs ^x				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1.5)	0 ± 0 ^z e	12 ± 2 b	9 ± 1 d	12 ± 2 b	8 ± 5 A
(2 + 2)	10 ± 2 c	16 ± 2 a	0 ± 0 e	0 ± 0 e	7 ± 6 B
Control ^y	0 ± 0 e	0 ± 0 e	0 ± 0 e	0 ± 0 e	0 ± 0 C
Mean of explants	0.8 ± 0.8 B	5 ± 2 A	2 ± 0.7 B	3 ± 1 B	

$p_{0.05}$ Explants: <0.0001; $p_{0.05}$ PGRs: <0.0001; $p_{0.05}$ Explants × PGRs: <0.0001

LSD_{0.05} Explants: 0.34 LSD_{0.05} PGRs: 0.59 LSD_{0.05} Explants × PGRs: 1.19

^x Values are mean ± standard deviation of 2 separate experiments

^z Means in each column followed by different letters are different according to LSD test ($p = 0.05$)

^y Control = 1/2 MS medium free of PGRs

The number of ELSs per explants (mean and SD) were calculated and differences between means were tested for significance using ANOVA and LSD test at the level of $p \leq 0.01$

Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

Table 7 Number of embryo-like structures formation in *C. pseudibericum*

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction of number of ELSs ^x				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1.5)	17 ± 1 ^z ef	21 ± 2 d	0 ± 0 i	0 ± i	10 ± 9 D
(2 + 2)	31 ± 2 b	22 ± 3 d	0 ± 0 i	0 ± 0 i	14 ± 13 C
(2.5 + 0.5)	16 ± 3 f	26 ± 4 c	26 ± 2 c	32 ± 1 b	25 ± 6 A
(2.5 + 1)	20 ± 2 d	39 ± 4 a	0 ± 0 i	26 ± 2 c	21 ± 1 B
(2.5 + 1.5)	19 ± 2 de	0 ± 0 i	17 ± 2 f	0 ± 0 i	9 ± 9 D
(2.5 + 2)	12 ± 4 g	5 ± 3 h	0 ± 0 i	15 ± 2 fg	8 ± 6 D
Control ^y	0 ± 0 i	0 ± 0 i	0 ± 0 i	0 ± 0 i	0 ± 0 E
Mean of explants	10 ± 9 A	13 ± 9 A	8 ± 3 C	11 ± 6 B	

$p_{0.05}$ Explants: <0.0001; $p_{0.05}$ PGRs: <0.0001; $p_{0.05}$ Explants × PGRs: <0.0001

LSD_{0.05} Explants: 0.77 LSD_{0.05} PGRs: 1.35 LSD_{0.05} Explants × PGRs: 2.70

^x Values are mean ± standard deviation of 2 separate experiments

^z Means in each column followed by different letters are different according to LSD test ($p = 0.05$)

^y Control = 1/2 MS medium free of PGRs

The number of ELSs per explants (mean and SD) were calculated and differences between means were tested for significance using ANOVA and LSD test at the level of $p \leq 0.01$

Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

significant differences between explant types when ELSs were cultured on medium with different 2,4-D and 2iP combinations (Table 7). The addition of 2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP as well as 2.5 mg l⁻¹ 2,4-D and 0.5 mg l⁻¹ 2iP in CIM resulted in the highest number of ELSs/explant, ranging from 32 to 39 for ovaries and petioles, respectively (Table 7). The highest mean number of ELSs/explant from leaves was 10.7 (Table 7).

ELSs formed from all four explant types in *C. parviflorum*. ELSs formed from leaves within 7–8 weeks of

culture and from petioles within 7 weeks. The most regenerative zones were at the cut ends of petioles (Fig. 4g). Different types of ELSs formed in all explant types, including dark yellow globular-shaped ELSs from ovules (Fig. 4b), light-yellow torpedo-shaped ELSs from ovaries, hard and dark brown torpedo- and globular-shaped ELSs from leaves, and yellow and compact torpedo-shaped ELSs from petioles (Fig. 4b, c, e, g). A significant difference in ELS induction was observed between leaves and petioles. Leaves produced significantly more ELSs than the

Table 8 Number of embryo-like structures formation in *C. parviflorum*

PGR concentration (2,4-D + 2iP) (mg l ⁻¹)	Effects of explants and PGRs interaction of number of ELSs ^x				Mean of PGRs
	Leaf	Petiole	Ovule	Ovary	
(2 + 1)	0 ± 0 ^z f	0 ± 0 f	0 ± 0 f	4 ± 1 e	2 ± 1 C
(2 + 1.5)	19 ± 1 a	17 ± 1 b	8 ± 2 cd	0 ± 0 f	11 ± 8 A
(2 + 2)	7 ± 4 d	0 ± 0 f	0 ± 0 f	0 ± 0 f	1 ± 3 B
(2.5 + 1)	0 ± 0 f	9 ± 3 c	0 ± 0 f	0 ± 0 f	4 ± 2 B
Control ^y	0 ± 0 f	0 ± 0 f	0 ± 0 f	0 ± 0 f	0 ± 0 D
Mean of explants	5 ± 2 A	5 ± 2 A	2 ± 0.6 B	1 ± 0.3 B	

$p_{0.05}$ Explants: <0.0001; $p_{0.05}$ PGRs: <0.0001; $p_{0.05}$ Explants × PGRs: <0.0001

LSD_{0.05} Explants: 0.42; LSD_{0.05} PGRs: 0.73 LSD_{0.05} Explants × PGRs: 1.46

^x Values are mean ± standard deviation of 2 separate experiments

^z Means in each column followed by different letters are different according to LSD test ($p = 0.01$)

^y Control = 1/2 MS medium free of PGRs

The number of ELSs per explants (mean and SD) were calculated and differences between means were tested for significance using ANOVA and LSD test at the level of $p \leq 0.01$

Lower case letters indicate significant differences between PGRs and explants combinations. Upper case letters indicate significant differences among total explants and mean of PGRs values

remaining three explant types. The efficiency of ELS formation depended on PGR concentration and explant type, and their interaction (Table 8). The highest number of ELSs/explant induction in leaves (19) and petioles (17) was on CIM containing 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP. The ELSs developed from a globular to a microtuberization stage (Fig. 4h), then developed into plantlets without any observable morphological aberrations (Fig. 4d, f, h–j).

Plantlet formation and acclimatization

Plantlets derived from different PGR treatments, explant types and species were evaluated separately (Tables 9, 10). Plantlets with clearly formed shoots and roots that developed from ELSs were easily separated into individual seedlings that germinated further on PGR-free CIM. After 2 weeks in culture, well developed plantlets were transferred to plastic pots containing peat and sand (2:1, v/v) (Figs. 3n, 4k). Highest plantlet formation was observed in *C. mirabile* (38 %), *C. cilicium* (31 %), *C. pseudibericum* (16 %), then *C. parviflorum* (15 %) across all explant types. Leaves were the most efficient explant (29 %) across all genotypes (Table 9). The percentage of plantlet formation from different explants and the explant × genotype interaction showed no significant difference but highest plantlet formation was observed from *C. mirabile* petioles (43 %) followed by *C. cilicium* leaves (31 %) (Table 9). Highest productivity was displayed by *C. mirabile* plantlets derived from petioles (43 %). Plants were successfully acclimatized in a greenhouse with 70, 63, 54 and 25 %

survival for *C. mirabile* (Fig. 1c, f), *C. pseudibericum* (Fig. 2d, h), *C. cilicium* (Fig. 3c, f, m, n) and *C. parviflorum* (Fig. 4d, f, i, j, k), respectively (Table 10).

Discussion

To increase the amount of callus and the number of ELSs that formed from the four explant types tested in four endemic Turkish *Cyclamen* species, all explants were transferred to 13 different PGR combinations to evaluate their effects on ELS formation and conversion to plantlets. To better understand the development of ELSs, a comparison of in vitro culture of these *Cyclamen* species was also performed by comparing different explant types and combinations of 2,4-D and 2iP. The ideal PGR combinations for each species and each explant type are indicated in Fig. 5.

Callus induction from different explant types and PGRs of endemic cyclamens

Callus formation in *Cyclamen* has been investigated for the production of organs or somatic embryos. Regeneration from callus derived from different explants, such as tubers (Loewenberg 1969; Geier 1977; Bian et al. 2008, 2010), leaves (Wicart et al. 1984; Otani and Shimada 1991; Dillen et al. 1996; Terakawa et al. 2008; Gou et al. 2008; You et al. 2008; Prange et al. 2010b; Naderi et al. 2012; Kocak et al. 2014), petioles (Wicart et al. 1984; Kiviharju et al. 1992; You et al. 2008; Naderi et al. 2012; Kocak et al.

Table 9 Plantlet formation in four endemic Turkish *Cyclamen* species

Genotypes	Effects of explants and genotype interaction on plantlet formation (%)				Mean of genotypes
	Leaf	Petiole	Ovule	Ovary	
<i>C. cilicium</i>	41 (40) ⁿ	37 (37)	35 (32)	11 (14)	31 (31) a***
<i>C. mirabile</i>	42 (40)	43 (41)	29 (32)	37 (37)	38 (37) a***
<i>C. pseudibericum</i>	21 (27)	14 (22)	15 (22)	16 (23)	16 (23) b***
<i>C. parviflorum</i>	14 (19)	14 (21)	14 (19)	18 (22)	15 (20) b***
Means of explants	29 (31)	27 (30)	23 (26)	20 (24)	

*** $p_{0.05}$ Genotypes: <0.0001; $LSD_{0.05}$ Explants: not significant; $LSD_{0.05}$ Genotypes: 7.46; $LSD_{0.05}$ Explants \times Genotypes: not significant

ⁿ Sample size of 15 plantlet formation

The numbers in parenthesis represent the arcsine-transformed value of percentages

Explants and explant-genotype means are not significantly different at $p < 0.05$ according to LSD test

Lower case letters indicate significant differences mean of genotypes

Table 10 Survival rate of four endemic Turkish *Cyclamen* species

Genotypes	Effects of explants and genotype interaction on survival (%)				Mean of genotypes
	Leaf	Petiole	Ovule	Ovary	
<i>C. cilicium</i>	60 (51) ⁿ	73 (58)	45 (42)	37 (33)	54 (46) a***
<i>C. mirabile</i>	38 (34)	79 (63)	70 (61)	93 (82)	70 (60) a***
<i>C. pseudibericum</i>	66 (54)	60 (51)	59 (50)	68 (56)	63 (53) a***
<i>C. parviflorum</i>	40 (35)	29 (28)	33 (31)	0 (0)	25 (24) b***
Means of explants	51 (44)	60 (50)	52 (46)	49 (43)	

*** $p_{0.05}$ Genotypes: <0.0001, $LSD_{0.05}$ Explants: not significant; $LSD_{0.05}$ Genotypes: 15.53, $LSD_{0.05}$ Explants \times Genotypes: not significant

ⁿ Sample size of 15 plantlet formation

The numbers in parenthesis represent the arcsine-transformed value of percentages

Explants and explant-genotype means are not significantly different at $p < 0.05$ according to LSD test

Lower case letters indicate significant differences mean of genotypes

2014), anthers (Geier 1977; Kiviharju et al. 1992), peduncles (Kiviharju et al. 1992; Gou et al. 2008; Nhut et al. 2012), ovaries (Wicart et al. 1984; Kiviharju et al. 1992; Kocak et al. 2014), ovules (Ishizaka and Uematsu 1992, 1995; Schwenkel and Winkelmann 1998; Winkelmann et al. 1998a, b; Winkelmann et al. 2006; Ishizaka 2008; Lyngved et al. 2008; Prange et al. 2010a; Rode et al. 2012; Kocak et al. 2014), roots (Oohashi et al. 1992), and aseptical seedlings (Takamura et al. 1995; Karam and Al-Majathoub 2000a, Prange et al. 2008) has been reported. Previous studies demonstrated that in vitro regeneration from tubers, petioles, peduncles, leaves and ovules of *C. persicum* (Hawkes and Wainwright 1987; Schwenkel and Grunewaldt 1988; Kiviharju et al. 1992; Kreuger et al. 1995; Takamura et al. 1995; Schwenkel and Winkelmann 1998; Winkelmann et al. 1998a, b; Al-Majathoub 1999; Karam and Al-Majathoub 2000b; Savona et al. 2007; Borchert et al. 2007; Seyring et al. 2009; Jalali et al. 2010a, b; Yamaner and Erdag 2008; Rode et al. 2011, 2012; Naderi et al. 2012; Nhut et al. 2012; Kocak et al. 2014) was possible using CIM with 2 mg l⁻¹ 2,4-

D + 1.5 mg l⁻¹ 2iP (Savona et al. 2007) and 4 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ Kin (Jalali et al. 2010b). In our present study, most callus was induced on CIM supplemented with 2 mg l⁻¹ 2,4-D + 1.0 mg l⁻¹ 2iP from *C. pseudibericum* petioles (100 %) but much less on CIM supplemented with 2.5 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ 2iP from ovules (20 %) and ovaries (60 %) (Table 3). The best explant types to induce callus (in descending order) were petioles, leaves, ovaries then ovules from *C. pseudibericum* (Table 3). Wicart et al. (1984) first obtained callus then adventitious shoots of *C. persicum* in MS basal medium with 0.5 mg l⁻¹ NAA + 2.5 mg l⁻¹ BA, 60 g l⁻¹ sucrose, 10 g l⁻¹ agar and different explant types (leaf blades, leaf stalks and ovaries). They also obtained unipolar and bipolar tubers on MS medium with 0.1 mg l⁻¹ NAA while 0.1 mg l⁻¹ 2,4-D + 0.5–2.5 mg l⁻¹ Kin formed ELSs. Our study showed that the effect of auxin at low concentrations (1–2 mg l⁻¹ 2,4-D) with a cytokinin (1–1.5 mg l⁻¹ 2iP) increased callus induction, but concentrations higher than 2 mg l⁻¹ 2,4-D were often inhibitory, which may be due to an increase in ethylene production at higher auxin concentrations

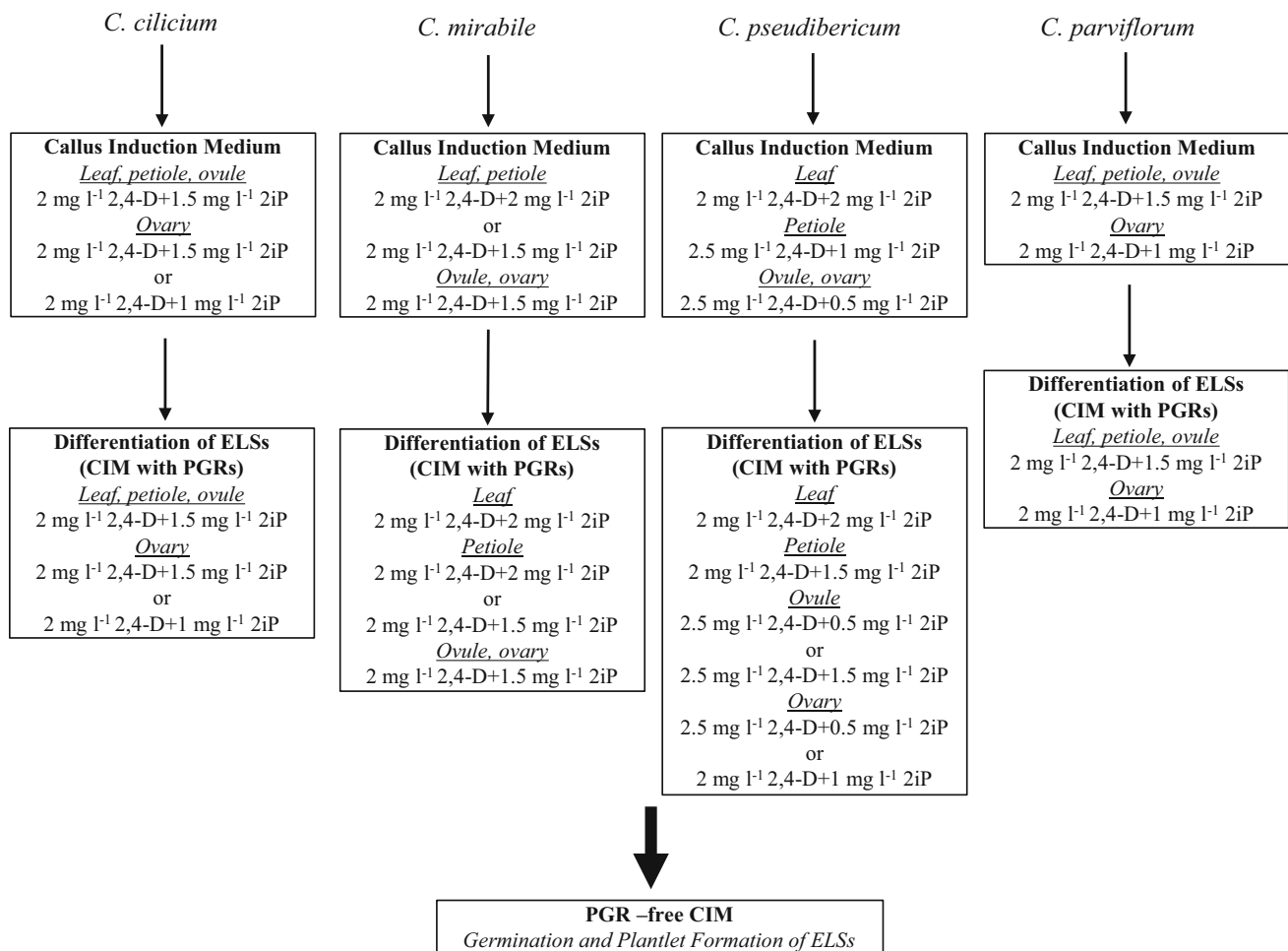


Fig. 5 Flow diagram of ideal regeneration pathways in four endemic *Cyclamen* species from Turkey for four explant types

(George et al. 2008). Takamura et al. (1995) reported highest callus formation and somatic embryogenesis at an intermediate level of 2,4-D (5.0 μ M) and also noted that a high concentration of auxin (50 μ M 2,4-D) delayed or inhibited embryogenesis (embryoid differentiation). They demonstrated that the relative ratio of auxin (5.0 μ M 2,4-D) to cytokinin (0.5 μ M Kin) was an important factor for ELS formation in *C. persicum* ‘Anneke’. Winkelmann and Serek (2005) investigated the ability of somatic embryogenesis in 32 F₁ hybrid *Cyclamen* cultivars obtained from two cyclamen breeders (Goldsmith Seeds Europe BV, Andijk, The Netherlands and S.A.S. Morel Diffusion, Frejus, France). Embrogenic callus and somatic embryos were induced from ovule explants according to the Schwenkel and Winkelmann (1998) protocol. They showed a wide range of callus formation ability for Giganteum, Midi, and Mini types. The mean callus induction percentages for Giganteum-type cultivars from Goldsmith and Morel were 61 and 49 %, respectively indicating that all commercial F₁ *Cyclamen* cultivars formed callus from ovules although that callus varied in colour and consistency.

In our study, we obtained callus from ovule explants (mean of ovule explants \times PGR interaction was 12 % for *C. cilicium*, 15 % for *C. mirabile*, 20 % for *C. pseudibericum* and 12 % for *C. parviflorum*) of all endemic *Cyclamen* species (Tables 1, 2, 3, 4, respectively). There was no significant difference between callus formation among all four wild species. Comparing our study with that of Winkelmann and Serek (2005), commercial *Cyclamen* cultivars induce more callus (41–85 % range) from ovule culture than Turkish endemic species (12–20 % range). One possible reason for low callus induction may be the recalcitrance of wild or endemic species, supported by evidence in the literature, as follows next. For example, You et al. (2011) noted that some *C. persicum* cultivars were recalcitrant to somatic embryogenesis. *C. persicum* showed very low levels of somatic embryogenesis when mature tissues or organs were used as explants (Seyring et al. 2009; Schmidt et al. 2006). In addition, there was a considerable decrease in the embryogenic competence of *C. persicum* callus as subcultures increased (Kiviharju et al. 1992; Winkelmann et al. 2004). Borchert et al. (2007)

also reported a low conversion of SEs into plantlets and the genetic instability of SEs. In this study, callus from the leaves and petioles of *C. cilicium* formed earlier than from *C. mirabile* and *C. pseudibericum*. However, all *C. mirabile* and *C. pseudibericum* explants formed callus after 150 days, and after 130 days from *C. cilicium* leaves and petioles. However, callus structures 3–4 mm in diameter, callus structures derived from ovary (after 10 weeks), ovule (after 4 weeks), leaf (after 4 weeks) and petiole (after 4 weeks) in culture were transferred to the fresh CIM in *C. persicum* (Kocak et al. 2014). In our present study, it was similarly determined that the calluses grew as a size of 3–4 mm. Naderi et al. (2012) investigated difference between response of two explant types leaf and petiole and two different medium MS and ½ MS supplemented with 4 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ Kin, 30 and 8 g l⁻¹ agar on callus percentage and somatic embryo number in *C. persicum* ‘Halios’. They showed highest percentage of callus formation (51 %) from leaf explant on full strength MS medium. They also obtained two type of callus produced: white, opaque and compact and other one was soft, watery and transparent. In this study we obtained different ratio of callus formation from leaf explant in all endemic species (92 % for *C. pseudibericum*, 56 % for *C. cilicium*, 4 % for *C. mirabile*, 59 % for *C. parviflorum*; Tables 1, 2, 3 and 4, respectively). Kocak et al. (2014) investigated the potential of somatic embryogenesis from different explants (ovules, divided ovary parts, leaves and petiole segments) of 15 distinct wild *C. persicum* genotypes. Explants were cultured on CIM containing 2.0 mg l⁻¹ 2,4-D and 0.8 mg l⁻¹ 2iP to induce callus: all explants formed regenerative and nonregenerative callus, and the former was generally light brown, soft, friable and separable. In our study, different types of callus formed: yellowish-white (Fig. 1d, g), brown (Fig. 3d, j), dark-brown (Fig. 2d, j), friable and compact (Fig. 4e, g) from different explant types and from different *Cyclamen* species. Winkelmann (2010) obtained rigid and dark callus from *C. persicum* ovules, similar to the callus obtained in this study from from *C. mirabile* petioles. However, the combination of 2.0 mg l⁻¹ 2,4-D + 0.8 mg l⁻¹ 2iP used by Winkelmann (2010) for *C. persicum* was not effective for the *Cyclamen* species tested in our study. Our results showed that the induction of callus from leaves, petioles, ovules and ovaries in *C. cilicium* (6, 6, 12 and 10th weeks, respectively) *C. mirabile* (6, 6, 10 and 12th week, respectively), *C. pseudibericum* (6, 6, 12 and 10th week, respectively) and *C. parviflorum* (8, 8, 12 and 14th week, respectively) formed more slowly than the same explants (leaves: 3rd week; petioles: 4th week; ovules: 6th week; ovaries: 10th week) from wild *C. persicum* (Kocak et al. 2014). Kiviharju et al. (1992) reported that different explant types (anthers, ovaries, peduncles, petioles, zygotic embryos) of *C. persicum* cv. ‘Rosamunde’

produced embryogenic callus on ½ MS basal medium containing 1.0 mg l⁻¹ 2,4-D with or without 10 % coconut milk. All explants produced slow-growing callus but best growth was observed in the dark and callus cultures were pale or brownish and hard regardless of the PGR applied. When cultured alone with auxin, callus growth was poor and the explants died soon. In our study, we observed different types of callus from various explant types.

Jalali et al. (2010b) indicated that two callus types of callus formed from *C. persicum* cv. ‘Halios’ leaves: the first was transparent and watery while the second was opaque, white and compact. The effects of different PGR combinations (2,4-D and Kin) on the percentage of callus formation from leaves were assessed. They highest percentage of callus formation depended on the medium (in descending order): 2 mg l⁻¹ 2,4-D + 0.1 mg l⁻¹ Kin (65.8 %); 1 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ Kin (65.3 %); 2 mg l⁻¹ 2,4-D + 0.5 mg l⁻¹ Kin (64.0 %). Callus formation was promoted by combining 1–2 mg l⁻¹ 2,4-D with a low cytokinin concentration (0.1–0.5 mg l⁻¹ Kin) but suppressed by combining a higher concentration of auxin (2–4 mg l⁻¹ 2,4-D) and cytokinin (>0.5 mg l⁻¹ Kin) (Jalali et al. 2010b, 2012). The variation in callus formation might be explained by the degree of cellular sensitivity towards exogenously-applied PGRs 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 (Van and Trinh 1990; Yamaner and Erdag 2008; Jalali et al. 2012). During dedifferentiation, 2,4-D at relatively high concentrations acts simultaneously as an inducer and as an inhibitor of cell division so the process can be suppressed and cells enter a dormant stage (Fehér et al. 2001; Jalali et al. 2012).

Karam and Al-Majathoub (2000b) used leaf discs, petioles, petals and peduncles from wild Jordanian *C. persicum* plants for in vitro culture. All explants were cultured on MS medium supplemented with different concentrations of BA or TDZ. In addition, etiolated and non-etiolated petioles were excised from the tubers and cultured on media with the same composition as for callus culture. Callus formed on all explant types. Peduncle and petal explants on medium containing 0.22 mg l⁻¹ TDZ exhibited most callus growth after 8 weeks of culture. Callus growth was significantly greater on etiolated than on non-etiolated explants after 8 weeks of culture, which is in agreement with the observations of Ando and Murasaki (1983). Some of the tissues were dark red. Rigid structures similar to leaf structures were obtained from these tissues at the end of 180 days. Winkelmann (2010) reported that embryogenic callus formed from ovules after 56 days in *C. persicum*. In our study, callus growth of *C. pseudibericum*, *C. mirabile*, *C. cilicium* and *C. parviflorum* was not as fast as *C. persicum* callus, taking 150 days in *C.*

pseudibericum. The growth of callus from petioles in *C. pseudibericum* was similar to that from ovules and petioles (light brown, soft, friable and separable) in *C. persicum* (Winkelmann et al. 1998a, b; Winkelmann 2010; Kocak et al. 2014).

Effect of different explant types and PGRs on ELSs formation

Takamura and Miyajima (1997) induced somatic embryos in different organs: 200/cotyledon, 100/petiole, 60/tuber and 200/root. Seyring et al. (2009) also reported ELSs in *C. africanum* (50 % in peduncles), *C. cilicium* Boiss. and Heldr. (15 % in petioles), *C. coum* (15.8 % in petioles), *C. hederifolium* ssp *confusum* (25 % in leaves), *C. persicum* (50 % in peduncles), *C. purpurascens* (25 % in leaves and petioles). After an 8–12 week period, callus was observed on all explant types (young leaves, petioles, flower buds and peduncles) and after 3–7 months structures formed, some of which were ELSs (Seyring et al. 2009). In the present study, ELSs developed in all species, but fewer ELSs were obtained from some explants of all species such as from *C. cilicium* ovaries (5.0) and *C. parviflorum* ovaries (4.3). Naderi et al. (2012) reported that explant type (leaf and petiole) and basal medium (MS and ½ MS) had a significant interaction effect on somatic embryo number. They showed highest number of somatic embryos per explant (mean of 7.5) on ½ MS medium using leaf explants. We also found highest number of ELSs per explant from leaves (23.0 for *C. cilicium*, 10.7 for *C. mirabile*, 31.0 for *C. pseudibericum*, 19.3 for *C. parviflorum*) in all endemic species. Nhut et al. (2012) investigated the use of thin cell layers (TCLs) in *C. persicum*. They used peduncles explants sliced into TCLs (0.5, 1.0, 2.0 or 3.0 mm thick), which were then cultured on MS medium supplemented with NAA, IBA or 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). They showed highest rates of callus formation using 3 mm TCLs from the upper position of the peduncle, resulting in 100 % callus induction in MS medium with 0.2 mg l⁻¹ TDZ and 1.0 mg l⁻¹ 2,4-D. Similarly, in our study, we obtained 100 % callus induction from petioles in *C. pseudibericum* after exposure to 2.5 mg l⁻¹ 2,4-D and 1 mg l⁻¹ 2iP. Prange et al. (2010a, b) obtained somatic embryos from leaf explants of *C. coum*, *C. alpinum*, *C. mirabile* and *C. graecum* using the protocol devised by Schwenkel and Winkelmann (1998). In our study, matured ELSs were cultured on CIM with different concentrations of 2,4-D and 2iP. The highest frequency of germination (42.7 %) was achieved when ELSs were cultured on CIM supplemented with 2 mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ 2iP after transfer to PGR-free CIM (Table 9). The combination of 2,4-D and 2iP appeared to

be necessary for the maturation of ELS. The number of ELSs per explant varied considerably, ranging from only a few to more than 70 and ELS formation was highly asynchronous with somatic embryos at different stages of development being observed from the same explant (data not shown). By the end of the culture period (160 days), the explants had induced ELSs at four main phases of development: globular, torpedo-shaped, and microtuber-like structures. The morphology of ELSs was highly variable, including color, structure and developmental stage (Fig. 2a). Kocak et al. (2014) reported that all explants (ovules, ovaries, leaves and petioles) formed embryogenic and nonembryogenic callus from *C. persicum*. Winkelmann et al. (2015) investigated the metabolite profile of SEs (torpedo stages), zygotic embryos (torpedo stages), endosperm and testa of *C. persicum* cv. 'Maxora Light Purple'. They indicated different levels of metabolites in SEs (tryptophan, galactose, adenosine, gluconate-1.5-lactone, shikimate, and especially ethanolamine, arabinose, fructose, citric acid, glucose, *myo*-inositol) versus zygotic embryos [γ -aminobutyric acid (GABA), glutamate, proline, sucrose and raffinose]. In our study, we observed the torpedo-like stages of ELSs of callus derived from *C. cilicium* ovules (Fig. 1a), *C. mirabile* ovules, ovaries, leaves and petioles (Fig. 2b, e, i, l), *C. pseudibericum* ovules, ovaries and leaves (Fig. 3b, e, k) and *C. parviflorum* ovaries and leaves (Fig. 4c, e) but some ELS torpedo stages did not transform into plantlets. Several problems were observed during somatic embryogenesis in *Cyclamen*, such as asynchronous development (Rode et al. 2011), precocious germination, lack of desiccation tolerance, or the absence of growth arrest associated with maturation (Schmidt et al. 2006). Rode et al. (2012) indicated that physiological disorders associated with a large portion of SEs as well as asynchronous development limit commercial application. To better control these factors, profound knowledge of the physiology of embryogenesis is essential. In endemic cyclamens, in order to overcome these limitations (arrested torpedo stages or germination problems), an approach to optimize somatic embryogenesis or the formation of ELSs is to modify the maturation medium, which could lead to a clear separation of the differentiation phase and the later germination phases, and could include the addition of sucrose, raffinose, proline, GABA, and/or glutamate (Winkelmann et al. 2015).

Our findings showed that the efficiency of microtuber-like organ formation in endemic *Cyclamen* species was dependent on explant type, genotype and PGRs. Yamaner and Erdag (2008) indicated the importance of PGRs on microtuberization from intact tubers of *C. mirabile*. In our work we observed that some microtuber-like organs formed from *C. parviflorum* leaves and petioles (Fig. 4e, g, h), *C. mirabile* leaves and petioles (Fig. 2b, e), and *C.*

pseudibericum ovules, ovaries and petioles (Fig. 3c, g, o). In other study, Nhut et al. (2012) showed a higher average number of shoots (39.4 shoots and 2–3 cm long shoots) from callus (induced from peduncles) of *C. persicum* TCLs cultured in MS medium containing 0.5 mg l⁻¹ BA and 0.7 mg l⁻¹ IBA. The exogenous auxin (IBA or NAA) to cytokinin (BA or Kin) ratio strongly influenced shoot development in vitro.

Evaluation of plantlet formation and acclimatization

ELs with normal shoots and roots were germinated 3 months after culturing on PGR-free CIM. Four to five months later, chlorophyllous plantlets having 2–3 leaves were removed from culture vessels. In the present study, the highest percentage of regenerated plantlets was from leaves (42 %) in *C. mirabile*. Murasaki and Tsurushima (1988) reported the highest plantlet formation (38 %) from *C. mirabile* ELs. Our study showed that 30.0 % of explants formed plantlets in *C. cilicium* (Table 9). Winkelmann et al. (1998b) produced 90,000 plantlets from 1 l of embryogenic cell suspension culture. Prange et al. (2010a) reported conversion percentages of plantlets from somatic embryos as 0–2 % for *C. alpinum*, 3–40 % for *C. mirabile* and 11–60 % for *C. persicum*. Nhut et al. (2012) obtained most *C. persicum* plantlets with fully developed roots from shoots derived from peduncle TCLs that were cultured on MS medium containing 1 mg l⁻¹ IBA, resulting in 100 % survival. Kocak et al. (2014) reported that the highest percentage of regenerated plantlets was from ovaries (31 %) and the highest percentage (9 %) of acclimatized plantlets was observed in wild *C. persicum*.

Acclimatization is one of the most important processes in tissue culture. In our study, plantlets obtained from ELs were successfully transferred to a greenhouse. The most efficient acclimatization resulted in a survival percentage of 70 % in *C. mirabile*. Rooted plantlets, which were successfully adapted to *ex vitro* conditions (Table 9), were very uniform and continued to grow, developing new leaves. Kiviharju et al. (1992) acclimatized plantlets derived from somatic embryos by either transferring plantlets directly into a greenhouse (37.27 %) or incubating them first in an acclimatization chamber then transferring them to a greenhouse (43 %). Schwenkel and Winkelmann (1998) acclimatized plants (70–95 % survival) regenerated from 5000 somatic embryos. Savona et al. (2007) found that the tubers of 35 % of plantlets derived from somatic embryogenesis formed 2–5 leaves, and these were transferred to a greenhouse for acclimatization under ambient light. Kocak et al. (2014) reported the most efficient acclimatization resulted in a survival percentage of 38.8 % in wild *C. persicum*. Winkelmann et al. (2006) observed 41 % acclimatization frequency, lower than 80–95 %

frequency obtained from ovule of somatic embryo-derived *C. persicum* plants. The survival ratio of plantlet was reported by other researchers: 42 % (Geier 1977), 95 % (Murasaki and Tsurushima 1988), 90 % (Otani and Shimada 1991). Prange et al. (2010a) used plantlets with at least one leaf for acclimatization: 88 % of 169 *C. mirabile* plants survived.

Conclusion

In conclusion, a highly efficient embryogenic-like structure system for endemic *C. cilicium*, *C. mirabile*, *C. pseudibericum* and *C. parviflorum* by using different explant types and PGRs were developed successfully (Fig. 5). This is the first original paper which shows the comparison of callus formation and ELS conversion to plantlets of four endemic cyclamen species. It was concluded that the explants originating from the part of the somatic tissues were characterized by a higher capability of regenerative callus formation than the ovule and ovary explants isolated from the flower. This protocol could be of great potential for in vitro micropropagation, genetic transformation, mutagenesis programs, cryopreservation or synthetic seed production in *Cyclamen* plants.

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