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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.

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