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In vitro Regeneration and Synthetic Seed Production of *Colchicum cilicicum* Grown Naturally in Turkey

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Abstract

Colchicum belongs to the *Colchicaceae* family and is naturally distributed in Turkey with 36 taxa. Colchicum species is known for both its medicinal aromatic and ornamental properties. In this study, *Colchicum cilicium* corms were cultured on RO medium including different dichlorophenoxyacetic acid (2,4- D) concentrations (2,3,4 mgL^{-1}) for *in vitro* regeneration and developing somatic embryogenesis protocol. Swelling of the corm explants, efficient embryogenic callus and somatic embryo were obtained at RO medium including 4 mgL^{-1} 2,4-D (30%). Embryogenic structures were histologically analyzed and globular, heart-like shape, torpedo stages were identified. Microcorm formation was obtained from the media with 2 mgL^{-1} 2,4-D + 0.05 mgL^{-1} 2iP as (81%) and germination of these microcorm were limited. Somatic embryos were encapsulated for germination and the highest germination rate was detected at 3% Na Alginate including RO medium+ 4mgL^{-1} 2,4-D as 27.8%.

Keywords: Somatic embryogenesis, Colchicum, encapsulation, RO, 2,4-D.

Türkiye'de Doğal Olarak Yayılış Gösteren *Colchicum cilicium*'un *In vitro* Rejenerasyonu ve Sentetik Tohum Üretimi

Öz

Colchicum Colchicaceae familyasına aittir ve Türkiye'de 36 tür ile doğal olarak yayılış göstermektedir. Colchicum türleri hem tıbbi aromatic bitki hem de süs bitkisi olarak bilinmektedir. Bu çalışmada Colchicum cilicium kormları farklı dichlorophenoxyacetic acid (2,4- D) konsantrasyonları içeren (2,3,4 mgL^{-1}) RO besi yerinde in vitro rejenerasyon ve somatic embriyogenesis protokolü geliştirmek için kültüre alınmıştır. Korm eksplantlarının şişmesi, etkili kallus oluşumu ve somatic embriyolar 4 mgL^{-1} 2,4-D içeren RO besi yerinde gözlemlenmiştir (30%). Embriyojenik yapılar histolojik olarak analiz edilmiş, globular aşama, kalp benzeri aşama ve torpedo aşaması tanımlanmıştır. Mikro korm oluşumları 2 mgL^{-1} 2,4-D + 0.05 mgL^{-1} 2iP içeren besi yerinde gözlenmiştir (%81)ve mikrokormların çimlenmesi sınırlı kalmıştır. Somatik embriyoların çimlenmesi için enkapsülasyon yapılmıştır ve en yüksek çimlenme oranı 3% Na Alginate RO + 4mgL^{-1} 2,4-D içeren besi yerinde 27% olarak belirlenmiştir.

Anahtar Kelimeler: Somatik embriyogenesis, Colchicum, enkapsülasyon, RO, 2,4-D.

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Introduction

Colchicum genus was formerly classified into Liliaceae family in the past and recently reclassified into the Colchicaceae family using different markers such as mitochondrial DNA (Fay et al., 2006) and plastids (Rudall et al., 2000; Vinnersten and Reeves, 2003; Jung et al., 2011).

This genus is separated into two groups according to flowering time. Leaves and fruits are formed in spring for autumn flowering species, and flowers occur together with leaves for spring flower species (Gülsoy Toplan et al., 2016). The flower colour of *Colchicum* ranges from white to purple. *Colchicum* sp. undergoes an annual cycle during the year and is dormant during summer. Some Colchicum species such as *C. autumnale* include an important alkoloid known as Colchicine. Colchicum consists of 99 species and these are perennial herbs containing rhizome and corm. Colchicum genus distributes from Northern Africa to Central Asia, including southern Europe, the Mediterranean region and the Middle East (Ellington et al., 1997; Jung et al., 2011). There are 50 Colchicum species naturally grown in different regions of Turkey and 22 of that endemic (Güner et al., 2000). *Colchicum cilicium* is one of the autumn flowering geophytes distributed in Pinus, Olea and Quercus forests at rocky and stony slopes. The corm of this species is a globe, elliptical and tunic colour ranges from light brown to dark brown. Leaves are rectangle or elliptical, undulated and smooth. Flowering occurs from August to November with light pink, purple and violet-purple flowers. This species is one of the important genetic resources due to its bioactive compounds such as alkaloids and attractive flowers for ornamental purposes.

Tissue culture technics are unique to preserving genetic resources that are under the extinction threat due to human activities such as industrialization and urbanization. Somatic embryogenesis is a way of obtaining bipolar embryogenic structures without vascular connections from different vegetative tissue. Somatic embryogenesis is affected by plant growth regulators, genotype, explant type, medium content and incubation conditions (İzgü et al., 2016). *In vitro* regeneration from various explants has been described in different Colchicum species such as organogenesis at *C. autumnale* (Ellington et al., 1997), callus induction and somatic embryogenesis at *C. hierosolymitanum* Feib. (Daradkeh et al., 2012), and callus induction at *C. calcedonium* (Karlik et al., 2020). Another important method for conserving and maintaining genetic resources is synthetic seed production. Somatic embryos are significant explants for synthetic seed production. Encapsulation with Na Alginate provides both easy conservation for the somatic embryos and clonal propagation of the genetic resources (Ara et al., 2000). This study is a comprehensive report on obtaining embryo-like structures and synthetic seed production of *Colchicum cilicium* endemic to Turkey. The corms were cultured on an RO (Ruggini Olive) medium including three concentrations of dichlorophenoxyacetic acid (2,4-D) and embryogenic structures were encapsulated with 3% Na Alginate to obtain somatic embryos.

Materials and Methods

Plant Material

Wild *C. cilicicum* (2n=2x=54) plants at the blooming stage were collected from rocky and stony woodlands of Meydan Castle, Aladağ, Adana. The collecting points and coordinates of the location are 37°30'37"N 35°22'06"E, 1316 m in November 2018. Plants were cultured in 13 cm diameter pots including peat and perlite mixture (1:1, v/v) in the polycarbonate greenhouse of the Horticulture Department, Çukurova University. A dark green net and temperature provided shading was adjusted by cooling pets in the greenhouse. Plants were cultured at approximately 20 °C. Plants were cultured in *in vitro* after 5 days of greenhouse cultivation.

Surface Sterilization

C. cilicicum corms were collected from nature during the vegetation period in November. Corms were sterilized before *in vitro* culture. Leaves, flowers and tunics were dissected from the corms and the remaining corms were used as explant. Corms were washed under tap water for 15 min., washed with liquid antibacterial soap (Activex ®, İstanbul/Turkey), and rinsed with distilled water. Explants were soaked in 70% ethanol for 3 min, 0.1% fungicide (Captan 50WP/Fruit and Ornamental, NY, USA) for 1 min and explants were rinsed with distilled water. Additionally, corms were kept in 0.1% $HgCl_2$ for 15 min and rinsed with distilled water. The corms were placed in a sterile laminar cabinet and soaked in 70% ethanol for 3 min, then rinsed with sterile distilled water once and then immersed into 30% NaOCl (4,5% active chlorine, v/v, NaOCl; Domestos®, Unilever, Turkey) containing 2 ml Tween-20 for 30 min. Explants

were washed with sterile distilled water four times.

Explant Preparation, *In vitro* Regeneration Medium and ELS Maturation Medium

Sterilized 1 cm diameter corms containing apical buds were cut into 0.5 cm^2 equal small square blocks with sterile forceps and scalpel. 15-20 explants were obtained from 1 corm. Explants were placed into the disposable plastic petri dishes (90x15 mm) including medium. Explants were cultured on *in vitro* regeneration medium (IRM). IRM consisted of full-strength Ruggini Olive medium (RO medium) and different concentrations (2, 3, 4 mgL^{-1}) of 2,4-Dichlorophenoxyacetic Acid (2,4-D). Hormone-free IRM medium was used as a control in the regeneration experiment. In addition, different concentrations of 2,4-D, 3% sucrose, 0.05% charcoal, and 3.5 g/l gelrite were added to the medium. pH was adjusted to 5.7-5.8 before autoclaving (121 °C for 15 min, 1.05 atm pressure) by 1N HCl and 1N KOH. Explants were cultured under dark conditions at 25±2°C for *in vitro* regeneration. Embryogenic callus (EC) and embryo-like structure (ELS) were sub-cultured every 4-6 weeks. Embryogenic calluses were transferred to RO medium including 2iP (0, 0.05 mgL^{-1}) or BA (0, 1 mgL^{-1}) combination of 2,4-D (0, 1, 2 mgL^{-1}) for maturation. After maturation, embryo-like structures were transferred to an MS medium containing 0.5 mg/l BA and 0.1 mgL^{-1} GA_3 for germination.

Synthetic Seed Production

The globular shape embryos were carefully selected at equal sizes to be homogeneous. 3% Na Alginate was including RO medium and 4 mgL^{-1} 2,4-D. 1.1% $CaCl_2$ was prepared, and pH adjusted to 5.8. Both 3% Na alginate and 1.1% $CaCl_2$ were autoclaved (121 °C for 15 min, 1.05 atm pressure). Embryos were soaked into the alginate with a scalpel and slowly dripped into a 1.1% calcium chloride ($CaCl_2$) solution with a micropipette. The container containing the $CaCl_2$ solution was shaken circularly during the dripping process to provide encapsulating and polarisation. Synthetic seeds were immersed in $CaCl_2$ solution for about 15 min. after dropping. Synthetic seeds were rinsed several times with sterile distilled water. Then synthetic seeds were transferred to the *in vitro* regeneration medium and cultured at 25±1 °C in dark conditions.

Histological Analysis

ELS samples were reamed by injector needle and explants were immersed in the formaldehyde-propionic acid-alcohol (FPA). Explants were submerged in the ethyl and tertiary butyl alcohol series for 4 hours to provide dehydration. Then explants were immersed into the paraffin. Samples were dissected as 10-12 µ with a rotary microtome (Leica RM2134) and stained with hematoxylin. Slides derived from explants were monitored under the light microscope (Olympus BX51) and photographed by the camera (Olympus DP72) (Karabıyık et al., 2017).

Experimental Design and Data Analysis

Colchicum cilicium plants in the nature were selected randomly and the genotype effect was ignored. Experiments were established in a completely randomized design and repeated three times with ten replicates for each treatment. *In vitro* regeneration process was investigated and morphologic differentiation of the corm explants such as puffing, ELS percentage and germination percentage of the embryos were examined. All data were subjected to arcsine transformation. Means were separated least significant differences test (LSD). Data were analyzed by JMP® program version 8. Significance was considered at $p< 0.05$.

Result and Discussion

***In vitro* Regeneration Results**

Differentiation of the explants started after 13 weeks of the culture depending on PGR concentrations. Embryo-like micro corms and shoot-like structures appeared after the corm explants' swelling period. These formations were proved with histological analysis. Bipolar structures and somatic embryos without vascular connections with explants were verified. However, acclimatization was restricted due to the limited tunic formation and root elongation. To regenerate the whole plant from corm explants by embryo induction, an RO medium containing different concentrations of 2,4-D (2, 3, 4 mgL^{-1}) was tested. As a result of the experiment, swelling and direct embryogenesis were obtained. There were no morphological differentiation and regeneration in the control group. The corm explants' highest morphological differentiation (Swelling) was determined at RO medium including 4 mgL^{-1} 2,4-D as 35% (Table

1). Somatic embryos were formed from these swelling corm explants after 16 weeks of the culture. Globular, heart shape and torpedo shape embryogenic structures were determined from the RO medium including 4 mgL^{-1} 2,4-D as 30% (Table 1). Embryos were bright and transparent at the beginning. Embryos were turned to opaque cream, yellow and light green after 5 weeks of the occurrence. Explants, including the different shapes of embryos, were transferred to the embryo maturation and germination medium. Embryos were maturated, and micro corms occurred on the embryo maturation medium (Figure 1). Although the best microcorm (%81) formed from the corm explants of the *C. cilicum* on the RO medium containing 2 mgL^{-1} 2,4-D + 0.05 mgL^{-1} 2iP after 25 weeks (Table 2), the differences between maturation medium were not significant statistically (Table 2). Maturated somatic embryos and micro corms were transferred to the MS medium containing 0.5 mgL^{-1} BA and 0.1 mgL^{-1} GA_3 for germination. Shoot-like and root-like structures were observed limitedly on the germination medium (Data not shown) (Figure 2).

Histological Analysis Results

Histological analyses investigated embryogenic structures. The embryo formation process from the corm explants was presented in Figure 4. Proembryogenic structures and embedded globular embryos were determined in the presence of 2,4-D (4 mgL^{-1}). Embryos continued development and hearth shape and torpedo shape somatic embryos occurred at the embryo maturation was detected from the 27 weeks old explants. Vascular connection with the corm explant was not observed from all regenerated tissue and is one of the important proof for these structures to declare as the somatic embryo. Moreover bipolar vascular sections were determined at the torpedo stage of somatic embryos between two poles (Figure 3). Additionally, cell clumps, starch grains and cell nucleus were determined from the meristematic tissues. Somatic embryos were easily separated from the corm explant surface. Shoot and root-like structures were developed synchronously. Proliferation regions were easily monitored under a microscope.

Synthetic Seed Results

Somatic embryos obtained from RO medium containing 4 mgL^{-1} 2,4-D were encapsulated with 3% Na alginate including RO medium with 4 mgL^{-1} 2,4-D. The germination rate was determined as 27.8%. Germination and cormlet formation were observed synchronously after encapsulation. Micro corms and germinated embryos were transferred to the RO medium including 2 mgL^{-1} 2,4-D + 0,5 mgL^{-1} 2iP for maturation of these structures. Elongation microcorms were cultured on MS medium 0.5 mgL^{-1} BA + 0.1 mgL^{-1} GA3 but germination and shoot elongation were not observed (Figure 4).

Table 1. Swelling and embryo formation rates in *C. cilicicum*

	Content of RO Medium	Swelling (%)	Embryo Formation (%)
C	Control	0 (0) b	0 (0) b
RO1	2 mgL^{-1}2,4-D	10 (10,5) b	2,5 (3) b
RO2	3 mgL^{-1}2,4-D	5 (6) b	0 (0) b
RO3	4 mgL^{-1} 2,4-D	35 (31,5) a	30 (27) a

$LSD_{SWELLİNG}$= 18.66, LSD_{EMBRYO}= 16.25. All percentage values indicated in parentheses were arcsine transformed. Different letters within a column indicate significant differences (P<0.05)

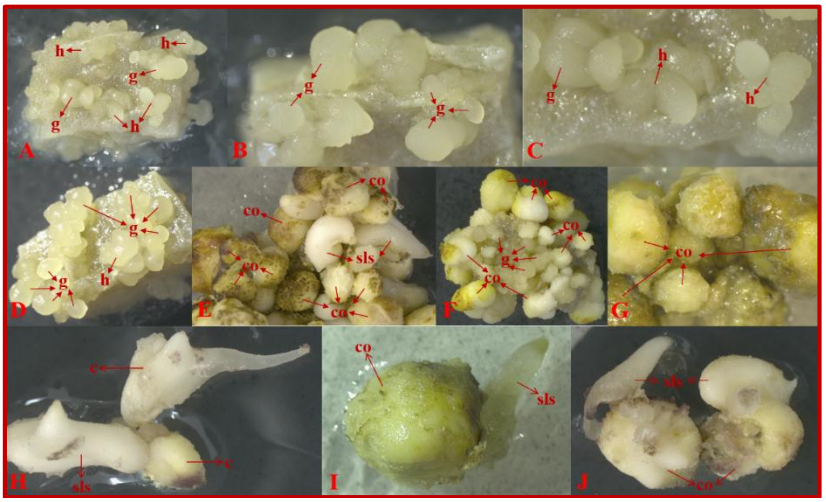

Figure 1. Structures obtained in somatic embryogenesis experiments A, B, C) Direct somatic embryos formed from swollen explants in RO medium containing 4 mg/l 2,4-D, D) Direct somatic embryos formed in RO medium containing 4 mgL^{-1} 2,4-D after 18 weeks, E, F, G) Cormlets obtained from somatic embryos, H, I, J) Shoot-like structures formed on cormlets in MS medium containing 0.5 mgL^{-1} BA and 0.1 mgL^{-1} GA_3 (g: globular, h: heart, c: cotyledon, co: cormlet, sls: shoot-like structure).

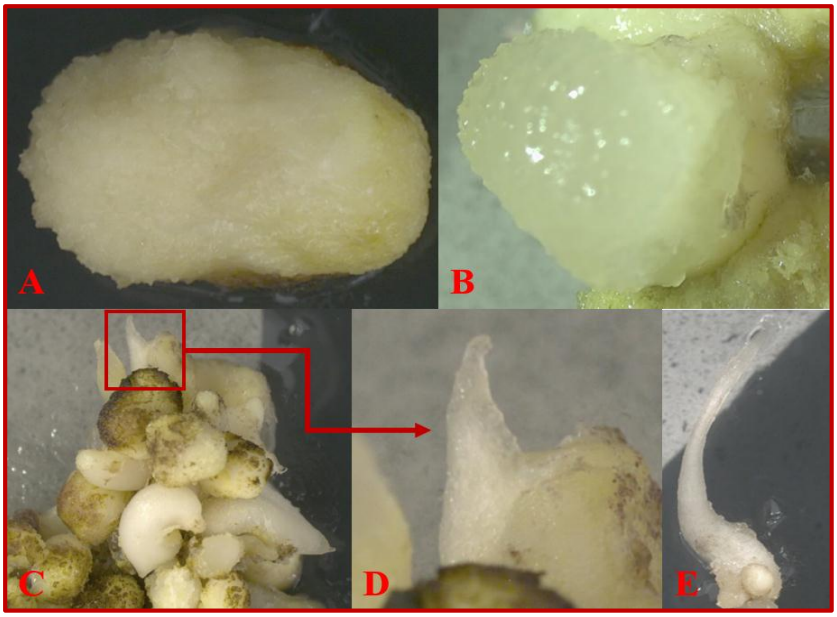

Figure 2. Different developmental stages of somatic embryos occurring in *Colchicum cilicicum* species A) Globular stage, B) Heart stage, C) Torpedo stage, D) Close view of torpedo stage, E) Cotyledon stage.

Table 2. Cormlet formation rates in *C. cilicicum*

	Content of RO Medium	Cormlet Formation (%)
G1	PRG free RO	45,5 (40,9) a
G2	1 mgL^{-1}2,4-D + 1 mgL^{-1}BA	45,5 (40,9) a
G3	2 mgL^{-1}2,4-D + 0.05 mgL^{-1} 2iP	81,8 (73,6) a

$LSD_{CORMLET}$= 42,33 All percentage values, indicated in parentheses, were arcsine transformed. Different letters within a column indicate significant differences (P<0.05)

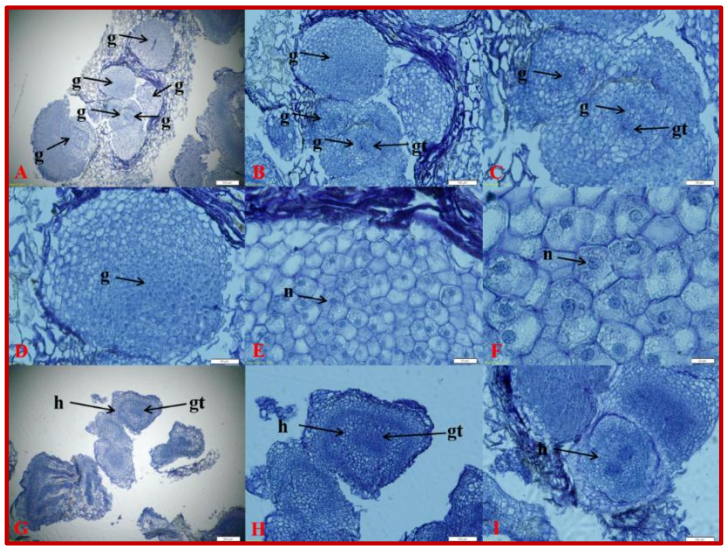

Figure 3. Images obtained as a result of histological analysis A, B) Cross-section image of globular embryos occurring in RO medium containing 4 mgL^{-1} 2,4-D, C, D) Close-up view of the cross-section of globular stage embryos, E, F) Nuclei that appear prominently in the cells of the globular embryo, G) Image of embryo transitioning to the heart stage, H) Close-up view of the embryo entering the heart stage, I) Image of a heart stage embryo (g: globular, h: heart, n: nucleus, gt: growth tip).

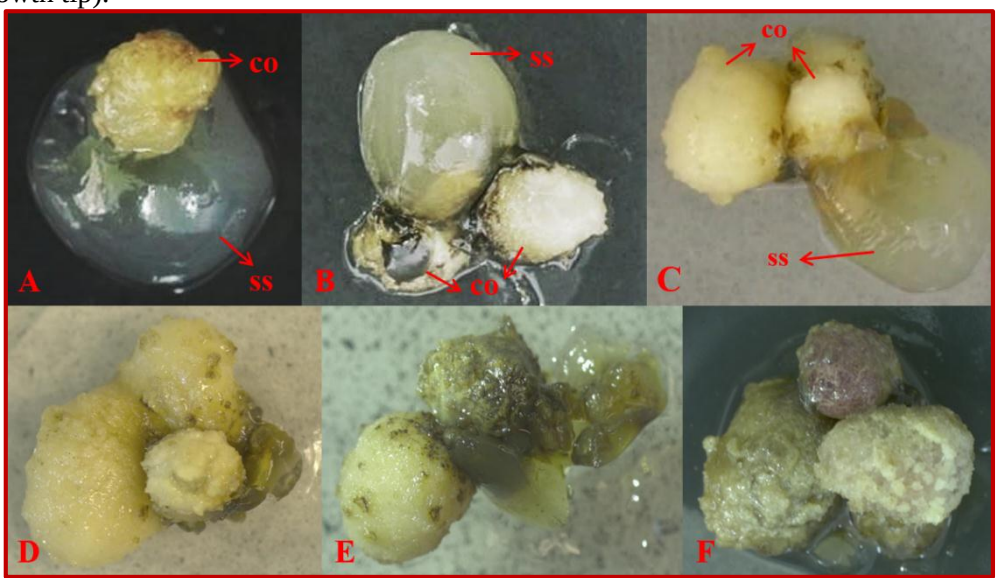

Figure 4. A) The swollen view of the synthetic seed coated with 3% Na alginate containing 4 mgL^{-1} 2,4-D and RO medium before germination, B, C) Cormlets formed from synthetic seed, D, E) Cormlets developing in the maturation medium, F) Cormlets with getting dark in the germination medium (ss: synthetic seed, co: cormlet).

Discussion

Somatic embryogenesis is one of the important methods for *in vitro* clonal propagation of the important endemic species and naturally grown genetic resources (Koçak et al., 2014). *In vitro* regeneration studies were limited for the Colchicum species (Hayashi et al., 1988; Yoshida et al., 1988; Daradkeh et al., 2012; Wagh et al., 2015; Karlık et al., 2020, Sarı, 2020, Özkaya, 2021). To produce somatic embryo three different PGR concentrations were tested for *C. cilicicium*. Histological analyses were performed to observe the developmental stage of somatic embryos and microcorm formation. Direct somatic embryos were obtained from the corm

explants. The corm has been used as a significant explant type for the *in vitr*o propagation of geophytes such as Colchicum, (Karlık, 2020; Sarı, 2020; Özkaya, 2021). Karlık et al. (2020) used three different explants of *Colchicum chalcedonicum* and they reported that callus was formed only from corm explants. Additionally, *in vitro* regeneration was obtained from the corm explants of *C. szovitsii, C. burtii, C. serpentinum, C. figlalii, C. atticum, C. triphyllum, C. sobolifera, C. speciosum* (Sarı, 2020; Özkaya, 2021), *Colchicum luteum* (Wagh et al., 2015). On the other hand, seeds (Daradkeh et al., 2012) and flowering shoots (Hayahsi et al., 1988; Yoshida et al., 1988) were used as explant for in vitro regeneration and cell regeneration suspension cultures of Colchicum sp. The success of the *in vitro* regeneration is dependent on medium selection. In this study, RO mediums including 2, 3, and 4 mgL^{-1} 2,4-D were tested for *in vitro* regeneration and the highest regeneration was obtained from RO medium including 4 mgL^{-1} 2,4-D. Daradkeh et al. (2012) cultured the callus induced from *C. hierosolymitanum* seeds, on MS medium including 0.45 µM 2,4-D, then transferred the callus to MS medium including 4.52 µM 2,4-D for *in vitro* colchicine production. Wagh et al. (2015) used chitosan for *in vitro* germination of dormant *C. luteum*. They determined that 0.015 and 0.5 $gL^{-1}$of chitosan concentration positively affected the shoot length and dry weight of *in vitro* plantlet. Karlık et al. (2020) cultured *C. chalcedonium* corms on MS medium including NAA, BAP, ZEA, 2iP and 2,4-D with different sucrose concentrations to obtain callus, and the highest callus initiation was reported as 75% from the ½ MS medium including 3% sucrose, 0.05 % active carbon and 2 mgL^{-1} 2,4-D+0.5 mgL^{-1} 2iP. Sarı (2020) obtained the highest somatic embryos as 74% from the corms explants cultured on MS medium including 2 mgL^{-1} 2,4-D for *C. figlalii*. Özkaya (2021) obtained somatic embryos from the corm explants from the MS medium including 2 mgL^{-1} NAA for *C. triphyllum*, and 6 mgL^{-1} 2,4-D for *C. speciosum*, 0.5 mgL^{-1} 2,4-D +0.5 mgL^{-1} 2iP *C. burtii* and *C. sobolifera*. On the other hand, Hayashi et al. (1988) cultured the *Colchicum autumnale* on modified MS containing 10^{-5} M 2,4-D+ 10^{-6} M Kin. They then transferred the tissues to the medium with 0.5µM IBA + 0.5µM Kin to produce colchicine in cell suspension culture. Ellington et al. (1997) reported that the application of paclobutrazol to the cormlet occurring in *Colchicum autumnale* positively affected the development of cormlet. Literature review shows that 2,4-D is an efficient plant growth regulator for *in vitro* regeneration of *Colchicum*. Regeneration is highly dependent on plant growth regulator types and dosages. 2,4-D is known as a significant promoter for *in vitro* regeneration, especially in monocots (Ullah and Khan, 2022), and positive effects of 2,4-D and combinations with different cytokinin on *in vitro* regeneration have been proved for different species such as crocus (Sevindik et al., 2018), lillium (Karalija et al., 2013). Additionally, the medium is another significant factor in inducing regeneration. In the regeneration studies of Colchicum, MS medium was used distinctly in our study.

Somatic embryos were analyzed histologically and different tissues, embryo stages such as globular, heart-like stage, torpedo-like stage and coleoptile, bipolar structures, vascular polarization, and proliferation regions were detected. Blazquez et al. (2009) investigated nodular callus and embryos of saffron obtained from MS medium including 0.1 mgL^{-1} 2,4-D histologically. Blazquez et al. (2009) showed bipolar structures, mini corms, apical meristems and vascular connections between two poles similar to our study. Colchicum sp. is monocotyledon and somatic embryo formation showed differentiation dependent on its monocotyledon or dicotyledon. There have been numerous studies on the somatic embryogenesis of monocotyledon plants such as banana, rice, wheat, lillium, etc. However, embryo stages of the monocotyledon plants are identified as globular, scutellar, and coleoptile. In this study, globular, heart like and torpedo-like or scutellar, cotyledonary or coleoptillar embryogenic stages were observed with histological analyses. Similar to our observations, Additionally, Natarajan et al. (2020) described the embryogenic stage globular, heart, late heart and torpedo stage at Musa sp. Synthetic seed application provides germination of somatic embryos under *in vitro* and *ex-situ* conditions for clonal propagation (Aitken-Christie et al., 1995; İpekçi and Gözükırmızı, 2003). To our knowledge, no prior studies have examined synthetic seed production of Colchicum species. In our study, somatic embryos were encapsulated with 3%Na Alginate

including RO medium +4 mgL^{-1} 2,4-D. Embryos were germinated at 27.8% and cormlet was formed after encapsulation.

Conclusion

In conclusion, *in vitro* regeneration and somatic embryogenesis protocol were investigated using corm explants from wild *Colchicum cilicium* naturally grown in Turkey. Although acclimatization was limited, this is the first original paper that shows callus induction and embryogenic development process for *Colchicum cilicium*. It was concluded that a higher auxin level stimulates the embryogenic development of *C. cilicium* corm explants. This study has potential for *in vitro* propagation, synthetic seed production and conservation of the naturally grown Colchicum species.

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