Black Sea Journal of Agriculture

PRODUCTION OF MICROSPORE-DERIVED PLANTS BY ANTHER CULTURE OF *Cyclamen coum*

Mahdi AL-KHAFAJI[1]*, Mehtap YILDIZ[1], Metin KOÇAK[1], Mehmet TÜTÜNCÜ[2]

[1]*Van Yüzüncü Yıl University, Faculty of Agriculture, Department of Agricultural Biotechnology, 65080, Tuşba, Van, Türkiye*
[2]*Ondokuz Mayıs University, Faculty of Agriculture, Department of Horticulture, 55139, Samsun, Türkiye*

Abstract: Cyclamen is one of the most important ornamental crops sold worldwide as a potted flower for winter production. *Cyclamen* species take up a wide swathe of habitats across Türkiye. Ten wild *Cyclamen* species grow naturally in Türkiye and some of them are endemic. This study aimed to produce haploid plants of *C. coum* using anther culture. The microspore developmental stage was evaluated by staining anther with acetocarmine (%2), and then the stage was correlated with bud size. It was determined that the buds between 7.64 and 8.23 mm had the appropriate bud size for the late uninuclear stage. Anthers were cultured in B5 medium containing different levels of 1-Naphthaleneacetic acid (NAA) (0.1, 1, 2 mgL^{-1}), 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.1, 1, 2 mgL^{-1}), and kinetin (0, 1 mgL^{-1}), 90 gL^{-1} sucrose and 3 gL^{-1} gelrite for haploid embryo production. Anthers were kept at 4°C for 4 days after culture. The explants were incubated at 24°C in a completely dark condition until the embryo was formed, then embryos were transferred to hormone-free media in 16:8 hours (light (75 µmolm^{-2}s^{-1}): dark) photoperiod. The experiment was carried out for two years. In the first year, 12 different media were examined in view of regeneration and the experiments were continued with selected 7 media in the second year. The highest callus regeneration rates were %5.71 and 14.5% and the highest embryo induction rates varied between 8.57% and 4.0% in the first and second year respectively. Embryo/callus formation was observed in 7 of a total of 12 different media tested for haploid plant production, and the best media were kinetin (1 mgL^{-1}) + NAA (1, 2 mgL^{-1}) and kinetin (1 mgL^{-1}) +2,4-D (2 mgL^{-1}). Our findings indicated that cold pre-treated anther explants collected at appropriate flower bud size resulted in embryo production. Additionally, B5 medium supplemented with NAA and kinetin ensured successful embryo regeneration from anther explants in wild *C. coum*.

Keywords: Androgenesis, Anther culture, *Cyclamen coum*, Haploid plants

1. Introduction

The geographical landscape of Türkiye displays remarkable diversity, leading to distinct climatic attributes. Additionally, Türkiye occupies a significant juncture where three prominent botanical regions intersect: Iran-Turanian, Mediterranean, and Euro-Siberian. Furthermore, Türkiye boasts a reputation as one of the world's most botanically affluent nations (Atalay, 1994; Yilmaz et al., 2003). Among the prized selections within the horticultural realm, cyclamen stands out as a preeminent and economically pivotal plant. Annually, the United States witnesses the sale of over five million potted cyclamen plants, while Japan produces in excess of 17 million potted specimens (Jalali et al., 2012). While traditionally belonging to the Primulaceae family, the *Cyclamen* genus underwent a recent reclassification into the Myrsinaceae family (Jalali et al., 2012). Comprising over ten perennial species, *Cyclamen* captivates with its vibrant, diverse flowers encompassing shades of pink, white, yellow, purple, and

red, widely cultivated in gardens (Yesson and Culham, 2006). The climatic preferences of various *Cyclamen* species differ, except for certain expansive geographic variants (Yesson and Culham, 2006). These species, adapted to cold climates, thrive within the range of 60-80% humidity, along with daytime temperatures of 16 °C and nocturnal temperatures of 14 °C, fostering robust breeding and quality (Çürük, 2013). Despite displaying resistance to photoperiodic effects in terms of light demand, heightened light intensity is acknowledged to augment the production of flower buds (Çürük, 2013). *Cyclamen*'s habitat spans the Mediterranean region (including Israel, Syria, Greece, Lebanon, Iran, Cyprus, and Türkiye) as well as northern Africa (Dole and Wilkins, 1999; Schwartz et al., 2008). The versatile *Cyclamen* species find utility in folk medicine and alternative remedies across various nations, capitalizing on their cosmetic, medicinal, and aromatic attributes. Harnessing the potential of wild *Cyclamen* tubers could elevate their application as medicinal and ornamental

resources (Seyring et al., 2009). Among the *Cyclamen* species naturally occurring in Türkiye, ten varieties, such as *C. coum* Miller, *C. persicum* Miller, and *C. cilicicum* Boiss and Heldr, thrive, with six of them being endemic (Ekim et al., 1991). Blooming periods vary, with some like *C. coum*, *C. parviflorum*, *C. pseudibericum*, *C. trochopteranthum*, and *C. persicum* flowering in spring, while others like *C. graecum*, *C. hederifolium*, *C. mirabile*, *C. repandum*, and *C. cilicium* bloom in autumn (Grey-Wilson, 1988). Chromosome counts display diversity, ranging from 2n=48 in *C. persicum* to 2n=20 in *C. repandum* (URL1). Traditionally, *Cyclamen* reproduction in its natural habitats occurs through costly manual pollination and seed production (up to 0.20D per seed). Furthermore, propagation via division, cutting, and grafting poses challenges. Biotechnology, particularly *in vitro* regeneration research, plays a pivotal role in preserving genetic resources endangered by factors like environmental degradation and unsustainable resource utilization (Takamura and Miyajima, 1997). Haploids serve as a valuable tool for delving into genetic transformation and induced mutagenesis studies (Folling, 2002). Haploid is a widely used term encompassing all sporophytes with either diploid or polyploid gametic chromosomes. Specifically, a haploid plant derived from a diploid species, possessing only one set of chromosomes, is more accurately described as a monoploid, signifying a singular genome (Heyne, 1987; Fehr, 1993). Haploid plants manifest distinct gametic chromosome numbers. The replication of a haploid's chromosomes, whether by natural or artificial means, gives rise to a doubled haploid (DH) plant. Diploid plants (2n=2x) trace their origins to autotetraploid (4x) haploid plants (Kasha and Maluszynski, 2003). Gynogenesis, due to its modest efficacy, currently occupies a lesser role among techniques, yet it finds utility in species unresponsive to more potent approaches (Forster et al., 2007). The acquisition of haploids and DHs, entailing the regulation and reprogramming of pollen production and function, represents a paramount application of pollen biotechnology in the realms of plant breeding and genetics (Testillano et al., 2000). Anther or isolated microspore cultures serve as the prevailing methods for inducing pollen embryogenesis. The straightforward nature of this methodology lends itself to large-scale anther culture, adaptable to a broad spectrum of genotypes. Anther culture stands as a frequent practice for DH generation in numerous crops. Haploid production holds immense significance for enhancing crops, eliminating pathogens, and substantially augmenting yield (Jain et al., 1996). Isolated microspore culture, involving the extraction of somatic anther tissue, demands more sophisticated equipment and expertise compared to anther culture. Nonetheless, the former method offers an optimal approach for scrutinizing the cellular, physiological, biochemical, and molecular mechanisms inherent in pollen embryogenesis (Pelletier and Ilami, 1972; Reinert and Bajaj, 1977). The concept of

the "Wall Factor," introduced by Heberle-Bors (1985) and Pulido et al. (2005), postulates the pivotal role played by the somatic tissues of the anther in stimulating sporophytic divisions within pollen. Nutrient diffusion through anther walls is also a determinant of microspore embryogenesis, with research revealing the anther wall's dual function of facilitating and inhibiting nutrient flow (Heberle-Bors, 1985; Pulido et al., 2005). Over the past decades, researchers have achieved callus induction from cultivated anthers, subsequently leading to the development of haploid plants from these calli (Wehr, 1976; Finnie et al., 1989). Chromosome doubling in haploid plants offers a rapid means of generating complete homozygous lines, significantly expediting and enhancing selection efficiency (Snape, 1989). Anther culture, initially a pioneering *in vitro* technique for haploid induction, proved successful for plant breeding purposes (Reed, 2005). Haploid plant induction from *in vitro* anther culture involves excising anthers from unopened flower buds at the appropriate developmental stage, corresponding to the first pollen mitosis. Staining methods like acetocarmine and DAPI aid in determining the developmental stage based on post-staining examination of pollen grains under a microscope (Germana, 2011). To enhance the response of *in vitro* anther culture for haploidy induction, specific physical or chemical pre-treatments are applied to flower buds. Isolated anthers from these treated buds are then incubated in diverse *in vitro* culture media under aseptic conditions (Olsen, 1992; Ferrie and Caswell, 2011). Although the use of microspore cultivation in ornamental plants lags behind field crops and vegetables, efforts to obtain haploid plants in ornamentals have been escalating. Regeneration from the anther wall is feasible in distinct cultures, yielding haploid plantlets from a single microspore cell. Thus, meticulous determination of the microspore's developmental stage and precise anther isolation are pivotal for a successful microspore culture (Olsen, 1992; Ferrie and Caswell, 2011). In anther culture, a similar approach is followed to ascertain the appropriate developmental stage of microspores, with subsequent steps favored for anthers containing microspores at the uninucleate to the early binucleate stage. Mechanical isolation of microspores from surface-sterilized anthers is performed using a mortar, pestle, blender, or collected anthers immersed in a liquid medium to facilitate microspore dehiscence (Amaury, 1997). Additionally, anther culture serves as a widely employed technique in practical breeding. However, its application is constrained by a multitude of factors influencing cultural performance, encompassing explant genotype, donor plant conditions, microspore growth, light exposure, media composition, and pre-treatment temperature (Amaury, 1997; Zhang et al., 2009). Anther and microspore cultures represent highly effective methods for generating substantial haploid plants (Amssa et al., 1980), proving invaluable in the realm of plant breeding and genetic research (Custódio et al.,

2005). This study's objectives revolve around identifying the most efficacious haploid production protocol in *C. coum* and contributing insights to future breeding endeavors.

2. Materials and Methods

2.1. Plant Material and Anther Culture

For this study, a total of 50 pots of wild *Cyclamen coum* plants were utilized and grown within a greenhouse setting. Anther extraction from the buds was conducted using forceps, followed by crushing in a 2% acetocarmine solution between slides. Subsequently, microspores were spread onto slides and subjected to examination under a light microscope to identify the appropriate size indicative of the late-uninucleate phase (Figure 1). The size of the flower buds was measured and correlated with the developmental stage of microspores in the late-uninucleate phase. Only anthers exhibiting the late-uninucleate microspore stage were selected for the subsequent *in vitro* culture. The flower buds underwent a 20-minute wash under tap water. Following the removal of sepals, the buds were cleansed using antibacterial soap. Under sterile conditions, the buds were subjected to a 1-minute sterilization in 70% ethanol. Subsequent rinsing was carried out with sterile distilled water (SDW), with at least two to three cycles, followed by immersion in 30% sodium hypochlorite (NaOCl, 4.5% v/v) solution (commercial domestos) along with two drops of tween 20 for 20 minutes. This was succeeded by additional SDW rinsing rounds until the foam on the explants was no longer present. Anther removal from the flower buds was meticulously performed using sterile forceps and a scalpel within a sterile bench environment, ensuring no damage, after which they were cultured on a B5 nutrient medium (Gamborg, 1968) (Table 1). The culture media were composed of 90 gL^{-1} sucrose and 3 gL^{-1} gelrite (Duchefa) (Table 1), along with varying concentrations of hormones, namely NAA (0.1, 1, 2 mgL^{-1}) or 2,4-D (0.1, 1, 2 mgL^{-1}) serving as auxins, and kinetin (0, 1 mgL^{-1}) as a cytokinin (Table 2). To attain a pH of 5.8, media were adjusted prior to autoclaving. The prepared media were poured into sterile petri dishes (measuring 90 × 15 mm) within a sterile bench setup. Subsequently, the petri dishes were sealed and covered with cling film, then placed in a dark environment at a temperature of 4°C within a refrigerator for a duration of 4 days.
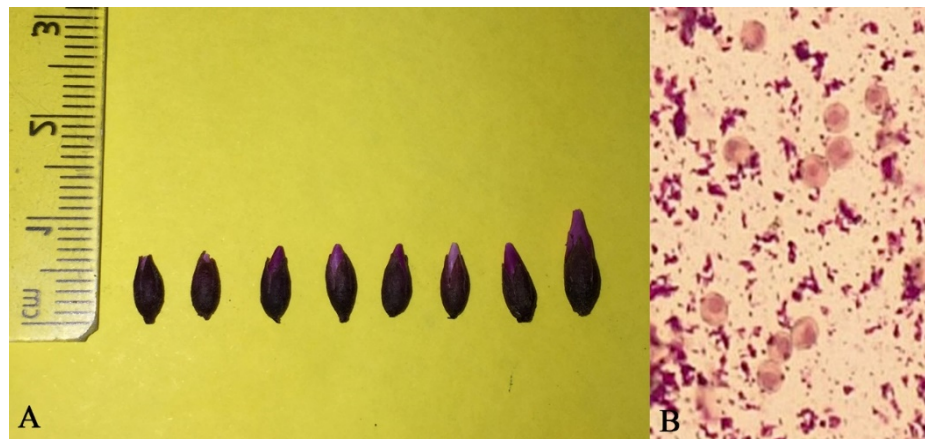

Figure 1. Determination of late-uninucleate stage of microspore in *Cyclamen co*um, A. Measuring of flower buds, B. Observation of microspore stages under the microscope.

Table 1. B5 Nutrient media and content used in anther culture experiments

Macro Elements (B5)	mgL^{-1}	Other Compounds (B5)	mgL^{-1}
NaH$_2$PO$_4$	130	FeSO$_4$.7H2O	27.8
(NH$_4$)$_2$SO$_4$	134	Na$_2$EDTA.2H$_2$O	37.3
CaCl$_2$.2H$_2$O	150	Myo-inositol	100
MgSO$_4$ x 7H$_2$O	250	Nicotinic acid	0.1
KNO$_3$	2500	Pyridoxine HCl	0.5
Micro Elements (B5)	mgL^{-1}	Thiamine HCl	0.5
CuSO$_4$.5H$_2$O	0.025	Carbohydrates	gL^{-1}
CoCl$_2$.6H$_2$O	0.025	Solidifier and regulators	gL^{-1}
Na$_2$MoO$_4$.2H$_2$O	0.25	Sucrose	90
Kl	0.75	Gelrite	3
ZnSO$_4$.7H$_2$O	2		
H$_3$BO$_3$	3		
MnSO$_4$.H$_2$O	10		

Table 2. The hormone types and concentrations

Kinetin (mgL⁻¹)	NAA (mgL⁻¹)	Kinetin + NAA	Media number
	0.1	(0 + 0.1)	1
0	1	(0 + 1)	2
	2	(0 + 2)	3
	0.1	(1 + 0.1)	4
1	1	(1 + 1)	5
	2	(1 + 2)	6
Kinetin (mgL⁻¹)	2,4-D (mgL⁻¹)	Kinetin + 2,4 D	
	0.1	(0 + 0.1)	7
0	1	(0 + 1)	8
	2	(0 + 2)	9
	0.1	(1 + 0.1)	10
1	1	(1 + 1)	11
	2	(1 + 2)	12

The petri dishes containing both the media and anthers were shifted to an incubator under dark conditions, maintaining a temperature of 25±1°C. The incubation continued until the development of embryo/embryoid structures became evident. Upon emergence of embryos and callus from the anthers under dark conditions, the anthers were transitioned to a growth chamber setup. The growth chamber environment comprised a photoperiodicity of 16 hours of light (75 μmolm⁻²s⁻¹) followed by 8 hours of darkness, and a constant temperature of 25±1 °C. Within this chamber, the anthers were cultured in a B5 nutrient medium devoid of hormones to facilitate maturation and germination processes.

2.2. Experimental Design and Statistical Analysis

A fully randomized factorial design was employed for conducting the experiments. Each application consisted of 15 replicates, with 5 anthers placed within each replication. Analysis of variance was conducted to distinguish means, and the least significant difference (LSD) test was executed to assess significant variations in callus and somatic embryo formation across distinct culture media. Prior to statistical analysis, percentage data underwent an arcsine transformation. The analyses were performed using SPSS Statistics version 21.0.

3. Results

This study focused on investigating the morphological dimensions of anther buds housing microspores in the late-uninucleate stage. Flower buds of varying sizes were gathered, sorted according to their length, and subsequently subjected to observation under a light microscope following microspore cell staining using acetocarmine (Figure 1). It was ascertained that microspore cells in the late uninucleate stages were acquired from flower buds measuring 7.64-8.23 mm in length.

3.1. First Experimental Year of Anther Culture in *Cyclamen coum*

In the initial experimental year of anther culture within *C. coum*, varying rates of embryo and callus formation were observed in certain anther explants cultured on B5 medium supplemented with NAA, 2,4-D, and kinetin (Table 3, Figure 2). Statistical analysis for the first experimental year indicated no significant differences in callus and embryo formation rates among the different media (Table 3). A higher percentage of embryos was obtained from medium 5 containing NAA (1 mgL⁻¹) and Kinetin (1 mgL⁻¹) (8.57% of embryos), while medium 12, comprising 2,4-D (2 mgL⁻¹) and Kinetin (1 mgL⁻¹), yielded a lower embryo count (5.71% of embryos). Callus formation was exclusively observed in medium 12, containing 2,4-D (2 mgL⁻¹) and Kinetin (1 mgL⁻¹) (5.71% of calli), while the other media did not produce any calli. Following 24 weeks of culture in the dark, embryoid induction was evident in anther explants. Embryos formed from anthers cultured in B5 medium containing NAA (1 mgL⁻¹), Kinetin (1 mgL⁻¹) (8.57% embryos), and 2,4-D (2 mgL⁻¹), Kinetin (1 mgL⁻¹) (5.71% embryos). Callus production was exclusive to the medium with 2,4-D (2 mgL⁻¹) and Kinetin (1 mgL⁻¹) (5.71% calli). Conversely, anthers cultured in a B5 medium supplemented with varying concentrations of NAA and Kinetin or 2,4-D and Kinetin did not yield embryo development. Calli and embryos formed over approximately 24 weeks were transferred to B5 medium without hormones in a growth chamber under light conditions at 25±1ºC for maturation and germination. Embryo development and plantlet regeneration were evident in the first year within media 5 and 12 [NAA (1 mgL⁻¹) + Kinetin (1 mgL⁻¹) and 2,4-D (2 mgL⁻¹) + Kinetin (1 mgL⁻¹)] (Figure 2). Despite testing different plant growth regulator concentrations, development was not observed in certain explants. Anther explants cultured for 48 weeks exhibited no growth in media 1, 2, 3, 4, and 7 [NAA (0.1, 1, 2 mgL⁻¹), Kinetin (0, 1 mgL⁻¹), and 2,4-D (0.1 mgL⁻¹), Kinetin (0 mgL⁻¹)]. The absence of development at the end of the first year is attributed to cultural conditions and potential genotype influence.
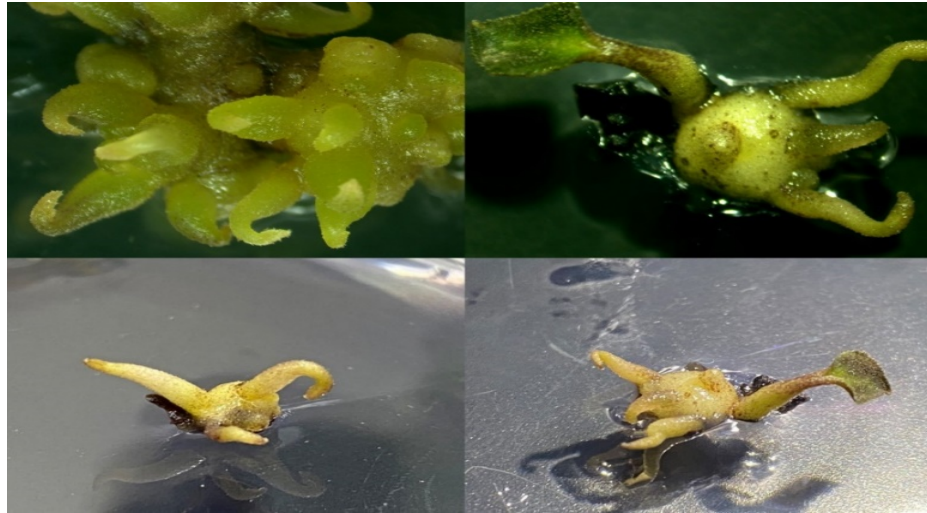
Figure 2. Transformation of embryos and calli to plantlets in *C. coum*.

Table 3. Callus and embryo formation rates for the first experimental year

Medium number	(%) Callus	(%) Embryo
1	0	0
2	0	0
3	0	0
4	0	0
5	0	8.57
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	5.71	5.71

3.2. Second Experimental Year of Anther Culture in *Cyclamen coum*

In the second-year experiment, the examination of appropriate bud size yielded similar outcomes, aligning with the microspore's developmental stage. Within the second-year trials, seven successful media (5, 6, 8, 9, 10, 11, and 12) from the prior year were utilized for anther culture in *C. coum*. As a result of the second-year experiments, the inclusion of NAA (1, 2 mgL^{-1}), and Kinetin (1 mgL^{-1}) in B5 medium produced callus (10 and 14.5%) and embryos (1.32 and 4%). Additionally, B5 medium supplemented with 2,4-D (0.1, 1, 2 mgL^{-1}) and Kinetin (0, 1 mgL^{-1}) yielded callus (2.1, 2.2, 1.25, 2.5, and 5%) and embryos (1.1, 1.1, 2.5, and 1.25%) (Table 4, Figure 3). The statistical analysis for the second experimental year showed significant differences among the different media for the number of developed calli (Table 4). The most substantial callus formation occurred in medium 6 (14.5% of callus), while the lowest was observed in medium 10 (1.25% of callus). Media 5, 6, and 12 shared the same statistical grouping, as did media 5, 8, 9, 10, 11, and 12. In terms of embryo count, statistical analysis indicated insignificant distinctions among the media. However, the highest embryo count (4%) originated from medium 6, whereas the lowest (1.1%) was evident in media 8 and 9, with no embryos detected in medium 10. Unlike the initial anther culture findings in *C. coum*, the second-year anther culture experiments revealed that embryo and callus formation occurred during the initial 9 weeks of culture in media 5, 6, and 12, encompassing NAA (1, 2 mgL^{-1}), Kinetin (1 mgL^{-1}), and 2,4-D (2 mgL^{-1}), Kinetin (1 mgL^{-1}) (Figure 3). The transformation of anthers into embryos and callus was observable in the B5 medium containing NAA, 2,4-D, and kinetin. Anther explants were cultured in various hormone concentrations within a B5 medium for 18 weeks under dark conditions. Nonetheless, some explants did not progress to embryo development during the second experimental year in *C. coum*. Calli and embryos formed over approximately 12 weeks of culture were subsequently transferred to a hormone-free B5 medium within a growth chamber under light conditions at 25±1 °C for maturation and germination.

Table 4. Callus and embryo formation rates for the second experimental year anther culture findings of in *Cyclamen coum*

Medium number	(%) Callus	(%) Embryo
5	10 [ab]	1.32
6	14.5 [a]	4
8	2.1 [b]	1.1
9	2.2 [b]	1.1
10	1.25 [b]	0
11	2.5 [b]	2.5
12	5 [ab]	1.25

a,b= different letters in same colon shows the statistical difference (P<0.05).
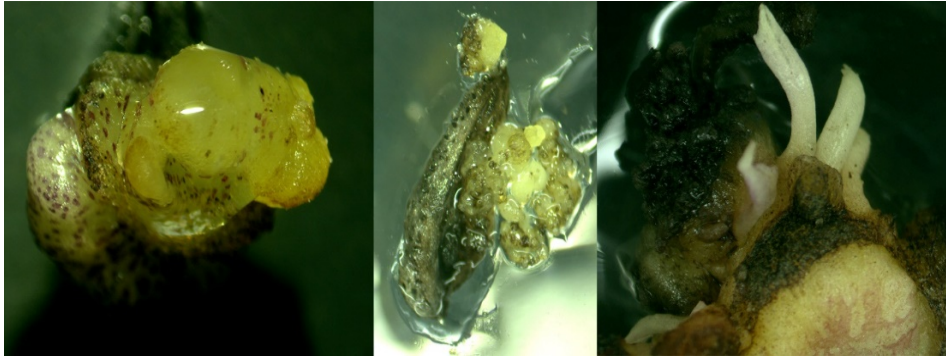
Figure 3. Embryo and callus formation in anther culture during the second experimental year.

4. Discussion

In the present study, successful regeneration was accomplished using a B5 medium supplemented with NAA, 2,4-D, and kinetin. A study by Ishizaka and Uematsu (1993) yielded comparable results, reporting effective embryo development rates of 9% in *C. persicum* species through anther culture experiments with B5 and N6 media. Their findings suggested that subjecting *C. persicum* species to a cold treatment at +4 °C for 4 days positively stimulated embryo formation. Başar (2018) studied anther culture in *C. persicum* and *C. persicum* Melody F1, achieving embryo formation in *C. persicum* culture medium containing 1 μM NAA (35.68% embryos) and callus in *C. persicum* Melody F1 culture medium supplemented with 1 μM NAA and $AgNO_3$ (38.27% callus). However, no embryo or callus formation was observed in *C. coum* cultured in B5 medium with varying concentrations. In our study, embryos were successfully obtained from the medium supplemented with NAA (1 mgL^{-1}) and kinetin (1 mgL^{-1}) (8.57% embryos) during the initial experimental year. Similarly, embryo and callus induction was achieved from the medium containing NAA and 2,4-D (1 mgL^{-1}) (1.32% embryos and 10% calli) in the second experimental year. Ishizaka and Uematsu (1993) reported embryos from a medium containing NAA (2 mgL^{-1}) and BA (0 mgL^{-1}) (2.3% embryos) in *C. persicum* Mill. In our study, embryos were successfully produced from the medium containing NAA and kinetin (1 mgL^{-1}) (8.57% embryos) in the first experimental year. Furthermore, embryo and callus formation were observed in the medium containing NAA (2 mgL^{-1}) and kinetin (1 mgL^{-1}) (4% embryos and 14.5% calli) during the second experimental year. Ishizaka (1998), examined anther culture in *C. persicum* and *C. purpurascens*, noting embryos from media containing NAA (0.1 and 1 mgL^{-1}) (2.4 and 2.6% embryos) and 2.4-D (0.1 and 1 mgL^{-1}) (0 and 2.6% embryos). De Vaulx and Chambonnet (1982) found the highest number of embryos in medium supplemented with 2,4-D (2 mgL^{-1}) and kinetin (1 mgL^{-1}) (4.91% embryos) in eggplant. In our study, embryos and calli were obtained from the medium containing 2,4-D (2 mgL^{-1}) and kinetin (1 mgL^{-1}) at the same concentration during the first experimental year (5.71% embryos and 5.71% calli). Similarly, in the second experimental year, embryo and callus formation occurred from the medium

containing 2,4-D (2 mgL^{-1}) and kinetin (1 mgL^{-1}) at the same concentration (1.25% embryos and 5% calli). The success of anther culture is influenced by various factors, with donor plant selection being a pivotal determinant (Ellialtıoğlu et al., 2000). Studies on somatic embryogenesis in *C. persicum* genotypes have demonstrated distinct regeneration abilities across different genotypes under identical medium and incubation conditions. The formation of callus and embryos has been shown to differ based on genotypes and explant types (Kocak et al., 2014). In our study, the lack of high success may be attributed to the genotypic attributes of the source plants from which anthers were derived. Similar to investigations conducted on *C. persicum*, this study underscores the significant role of genotype in anther culture. Takamura et al. (2011) explored various carbon sources, identifying maltose at 394.4 mM as the most effective in *C. persicum* anther culture. Consistent with this, our study found sucrose to be a successful carbon source in *C. persicum* anther culture experiments. Conversely, Ishizaka and Umatsu (1993) suggested sucrose at 90 gL^{-1} as the optimal carbon source for *C. persicum* anther culture. Carbon source preferences have exhibited considerable variation across different species. The impact of carbon sources on anther culture has been noted, with high carbon concentrations favoring haploid development over diploid structures (Ellialtıoğlu et al., 2000). Sucrose concentration has been proposed to alter the water content of pollen grains, rendering them more susceptible to androgenesis (Canhoto et al., 1990). Sucrose has shown effectiveness as a carbon source in rice anther culture (Trejo et al., 2002). The choice of carbohydrate source significantly influences the osmotic and nutritional aspects of microspore embryogenesis (Powell, 1990). Sucrose, commonly utilized as a carbon source, also serves as an osmotic regulator and energy source in culture media (Reinert and Bajaj, 1977). Adequate carbon availability is pivotal for successful androgenesis in culture media (Nitsch, 1969). Carbon source has a profound influence on anther culture responses in the induction medium. Anther culture in cultivated cyclamen (*C. persicum*) has resulted in the production of microspore-derived plants, significantly influenced by factors such as high sucrose

concentrations, dark culture conditions, and basal medium selection Ishizaka and Umatsu (1993). Başar (2018) reported the highest number of plants in the B5 medium containing 5 μM NAA in *C. persicum*. Anthers were cultured by applying 4 °C cold applications for 2 days. Cultured anthers and 2-day cold pre-treatment of flowering buds proved to be optimal as it gave the best callus/embryo induction in *C. persicum* in B5 medium containing 1, 3, and 5 μM NAA. Keleş et al. (2015) studied anther culture to obtain haploid plants in some pepper varieties. They found variations among genotypes and types in embryo formation and the number of spontaneous haploid plants. Anther culture emerges as a critical technique in haploidization studies, offering advantages such as the generation of pure lines and expedited breeding processes, particularly for annual garden plants. In the development of embryoids from *C. persicum* pollen grains, the B5 medium demonstrated superiority over other media Ishizaka and Umatsu (1993). Similarly, B5 outperformed MS medium in *Lilium longiflorum* anther culture (Amaury, 1997). Thiamine HCl content sets B5 apart from other media, containing a significantly higher vitamin concentration of 10 mgL^{-1}. Thiamine serves as an enzymatic cofactor in carbohydrate energy extraction (Goyer, 2010). In contrast, light was found to inhibit embryo development in *C. persicum* anther culture by Ishizaka and Umatsu (1993). Our study revealed that darkness fostered androgenesis preceding pollen-callus production. Incubation of media under light conditions as opposed to darkness accelerates auxin oxidation, possibly explaining the improved response observed in anthers cultured under darkness. Additionally, light conditions have been identified as a pivotal element in anther culture for certain plant species. While *L. longiflorum* anther culture produced callus under darkness, no callus formation occurred under light conditions (Amaury, 1997). Beyond the genotype and microspore developmental stage, additional factors influencing androgenesis such as donor plant growth conditions, anther pretreatment, culture media, and incubation conditions (Seguí-Simarro and Nuez, 2008). Generally, anther culture facilitates the production of microspore-derived embryos, with genotype significantly influencing the percentage of microspores favoring embryogenesis and successfully transitioning into embryos (Seguí-Simarro and Nuez, 2008; Irikova et al., 2011).

5. Conclusion

The primary objective of this study was to employ the *in vitro* haploidization technique as the initial step toward developing pure lines within *Cyclamen* species. Haploid plants were successfully generated through the cultivation of anther explants *in vitro*, with the intention of subsequently collecting these pure lines for use as breeding material. The central focus of this research was to achieve haploid plants through *in vitro* cultivation of anther explants, and the outcomes of the study were systematically documented. A pivotal aspect in haploidization investigations revolves around the precise determination of bud sizes encompassing anthers containing microspore cells in the late-uninucleate phase. Within the study's framework, anther explants from varying bud sizes within *Cyclamen* species were subjected to acetocarmine staining, revealing that the late-uninucleate phase occurred within bud sizes ranging from 7.64 mm to 8.23 mm in *C. coum*. The acetocarmine staining method proved to be a straightforward means of identifying the uninucleate stage. Buds in the late-uninucleate phase underwent temperature treatment during anther culture experiment, being exposed to +4°C for a duration of 4 days. In future studies on *Cyclamen* species, it is advisable to explore cold or heat shock applications at different timings and temperatures tailored to the specific species. Anther culture studies were conducted using a B5 medium, incorporating varying concentrations of 2,4-D, NAA, and KIN. The outcomes of these experiments revealed that embryo and callus formation occurred in B5 medium containing 0.1 μM KIN, 0.1 and 1 μM NAA, 2 μM 2,4-D, and 1 μM KIN during the initial experimental year. In the second experimental year, embryo and callus formation were observed in B5 medium containing 1 μM KIN, 1 and 2 μM NAA, 0.1, 1, and 2 μM 2,4-D, and 0 and 1 μM KIN in *C. coum*. This underscores the significant role of genotype in the anther culture process within *Cyclamen* species. Consequently, it is advisable to conduct preliminary experiments aimed at determining the genetic susceptibility of selected genotypes, intended as starting material, prior to commencing haploidization studies in *Cyclamen* species. The thing to remember when culturing anthers in the anther culture process is that they should be cultured without damaging the anther walls and not contributing to the growth of diploid cells.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	M.A.K.	M.Y.	M.K.	M.T.
C	25	25	25	25
D	50	50		
S		100		
DCP	25	25	25	25
DAI	25	25	25	25
L	40	20	20	20
W	40	20	20	20
CR	40	20	20	20
SR	40	20	20	20
PM	40	20	20	20
FA	40	20	20	20

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

References

Amaury MAF. 1997. Production of doubled-haploid plants from Lilium longiflorum Thunb. anther culture. Plant Sci, 123: 179-187.

Amssa M, Buyser JD, Henry Y. 1980. Origin of the diploid plants obtained by in vitro anther culture of bread wheat (Triticum aestivum L.): effect of cold pretreatment and of in vitro culture on doubling, 290: 1095-1097.

Atalay İ. 1994. Vegetation geography of Turkey. Ege Üniversitesi Basımevi, İzmir, Türkiye, pp: 58.

Başar S. 2018. Obtaining haploid plant via anther and ovule culture techniques in cyclamen. PhD Thesis, Çukurova University, Institue of Natural and Applied Sciences, Adana, Türkiye, pp: 204.

Canhoto JM, Ludovina M, Guimaraes S, Cruz GS. 1990. In vitro induction of haploid, diploid and triploid plantlets by anther culture of Iochroma warscewiczii Regel. PCTOC, 21: 171-177.

Çürük P. 2013. Morphological and molecular characterization of cyclamen species growing naturally in Adana and its surroundings. PhD Thesis, Çukurova University, Institue of Natural and Applied Sciences, Adana, Türkiye, pp: 216.

Custódio L, Carneiro MF, Romano A. 2005. Microsporogenesis and anther culture in carob tree (Ceratonia siliqua L.). Sci Hortic, 104(1): 65-77.

de Vaulx RD, Chambonnet D. 1982. Culture in vitro d'anthères d'aubergine (Solanum melongena L.): stimulation de la production de plantes au moyen de traitements à+ 35° C associés à de faibles teneurs en substances de croissance. Agronomie, 2(10): 983-988.

Dole JM, Wilkins HF. 1999. Floriculture principles and species. Prentice Hall Inc., New York, US.

Ekim T, Koyuncu M, Güner A, Erik S, Yıldız B, Vural M. 1991. Taxonomic and ecologic researches on Econnomically important Geofits in Turkey. Publication Publicity, 669(65): 111.

Ellialtıoğlu Ş, Sarı N, Abak K. 2000. Haploid bitki üretimi. Bitki Biyoteknol, 1: 137-189.

Fehr WR. 1993. Principles of cultivar development: Theory and technique, 1.

Ferrie AMR, Caswell KL. 2011. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. PCTOC, 104: 301-309.

Finnie SJ, Powll W, Dyer AF. 1989. The effect of carbohydrate composition and concentration on anther culture response in barley (Hordeum vulgare L.). Plant Breed, 103(2): 110-118.

Folling L. 2002. Transformation of wheat (Triticum aesticum L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Rep, 20: 1098-1105.

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A. 2007. The resurgence of haploids in higher plants. Trends Plant Sci, 12(8): 368-375.

Gamborg OL. 1968. Nutrient requirements of suspension cultures of soybean root cultures. Exp Cell Res, 50: 148-158.

Germana MA. 2011. Anther culture for haploid and doubled haploid production. PCTOC, 104: 283-300.

Goyer A. 2010. Thiamine in plants: aspects of its metabolism and functions. Phytochem, 71(14-15): 1615-1624.

Grey-Wilson C. 1988. The genus Cyclamen (A Kew Magazine Monograph). The Royal Botanic Gardens, Kew in association with Christopher Helm and Timber Press. Chromosome association and fertility in interspecific hybrid of Cyclamen, pp: 371.

Heberle-Bors E. 1985. In vitro haploid formation from pollen: a critical review. Theor Appl Genet, 71: 361-374.

Heyne EG. 1987. Wheat and wheat improvement. American Society of Agronomy, Madison, US, pp: 322.

Irikova T, Grozeva S, Rodeva V. 2011. Anther culture in pepper (Capsicum annuum L.) in vitro. Acta Physiol Plant, 33: 1559-1570.

Ishizaka H, Uematsu J. 1993. Production of plants from pollen in Cyclamen persicum Mill. through anther culture. Jpn J Breed, 43(2): 207-218.

Ishizaka H. 1998. Production of microspore-derived plants by anther culture of an interspecific F1 hybrid between Cyclamen persicum and C. purpurascens. PTOC, 54(1): 21-28.

Jain SM, Sopory SK, Veilleux R. 1996. In vitro haploid production in higher plants. Springer Sci Busin Media, 2(24): 442.

Jalali N, Naderi R, Shahi-Gharahlar A, da Silva JAT. 2012. Tissue culture of Cyclamen spp. Sci Hortic, 137: 11-19.

Kasha KJ, Maluszynski M. 2003. Production of doubled haploids in crop plants. An introduction. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko (editors), Doubled Haploid Production in Crop Plants. Springer, Dordrecht, the Nederland, pp: 1-4.

Keleş D, Pınar H, Ata A, Taşkın H, Yıldız S, Büyükalaca S. 2015. Effect of pepper types on obtaining spontaneous doubled haploid plants via anther culture. Hort Sci, 50(11): 1671-1676.

Kocak M, Izgu T, Sevindik B, Tutuncu M, Curuk P, Simsek O, Mendi YY. 2014. Somatic embryogenesis of Turkish Cyclamen persicum Mill. Sci Hortic, 172: 26-33.

Nitsch JP. 1969. Experimental andro-genesis in Nicotiana. Phytomorphology, 19: 389-404.

Olsen FL. 1992. Isolation and cultivation of embryogenic microspores from barley (Hordeum vulgare L.). Hereditas, 115(3): 255-266.

Pelletier G, Ilami M. 1972. Les facteurs de l'androgenese in vitro chez Nicotiana tabacum. Zeitschrift für Pflanzenphysiologie, 68(2): 97-114.

Powell W. 1990. Environmental and genetical aspects of pollen embryogenesis. Haploids Crop Improv, I: 45-65.

Pulido A, Bakos F, Castillo A, Vallés MP, Barnabas B, Olmedilla A. 2005. Cytological and ultrastructural changes induced in anther and isolated-microspore cultures in barley: Fe deposits in isolated-microspore cultures. J Struct Biol, 149(2): 170-181.

Reed SM. 2005. Haploid culture. In: Trigiano RN, Gray DJ, (editors), Plant Development and Biotechnology. CRC Press LLC, NW, US, pp: 225-234.

Reinert J, Bajaj YPS. 1977. Anther culture: haploid production and its significance. In: Reinert J, Bajaj YPS (editors). Applied and fundamental aspects of plant cell, tissue, and organ culture. Springer-Verlag, Berlin, Germany, pp: 251-267.

Schwartz-Tzachor R, Eisikowitch D, Dafni A. 2008. Flower characteristics and breeding system of two phenological

ecotypes of Cyclamen persicum Mill. (Myrsinaceae) in Israel. Pl Syst Evol, 274: 127-134.

Seguí-Simarro JM, Nuez F. 2008. How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant, 134(1): 1-12.

Seyring M, Ewald A, Mueller A, Haensch KT. 2009. Screening for propagation suitability in vitro of different Cyclamen species. Electron J Biotechnol, 12(4): 10-11.

Snape JW. 1989. Doubled haploid breeding: theoretical basis and practical applications. Rev Advan Plant Biotechnol, 2: 19-31.

Takamura T, Miyajima I. 1997. Micropropagation of Cyclamen persicum Mill. In: Bajaj YPS (editor). High-tech and micropropagation VI. biotechnology in agriculture and forestry, 40th ed. Springer, Berlin, Germany, pp: 96-112.

Takamura T, Sakamoto K, Horikawa M. 2011. Effect of carbon source on in vitro plant regeneration in anther culture of Cyclamen (Cyclamen persicum Mill.). In XXVIII International Horticultural Congress on science and horticulture for people (IHC2010): International Symposium on Micro, December 31, Lisbon, Portugal, pp: 129-134.

Testillano PS, Coronado MJ, Seguı JM, Domenech J, González-Melendi P, Raška I, Risueño, MC. 2000. Defined nuclear changes accompany the reprogramming of the microspore to embryogenesis. J Struct Biol, 129(2-3): 223-232.

Trejo-Tapia G, Maldonado Amaya U, Salcedo Morales G, De Jesús Sánchez A, Martínez Bonfil B, Rodríguez-Monroy M, Jiménez-Aparicio A. 2002. The effects of cold-pretreatment, auxins and carbon source on anther culture of rice. PTOC, 71: 41-46.

URL1: http://www.cyclamen.org/indexCS.html (accessed date: July 15, 2024).

Wehr WH. 1976. Plant production from cultured anthers of Hordeum vulgare L., Zeitschrift fuer Pflanzenzuechtung. 77: 198- 204.

Yesson C, Culham A. 2006. A phyloclimatic study of Cyclamen. BMC Evol Biol, 6:1-23.

Yilmaz R, Butt SJ, Korkut A. 2003. Plant diversity and effects of environmental problems in Turkey. J Environ Protect Ecol, 4: 924-930.

Zhang XQ, Yang JS, Zheng ZX. 2009. Study on induction of embryoid through in vitro anther of Cyclamen persicum. JAAS, 1: 57-58.