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Research Article

EVALUATION OF FACTORS AFFECTING EMBRYO-LIKE STRUCTURE AND CALLUS FORMATION IN UNPOLLINATED OVARY CULTURE OF WATERMELON (*Citrullus lanatus*)

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ABSTRACT. An unfertilized ovary culture is an effective approach for inducing haploid plants that can significantly reduce breeding time. The present study aimed to investigate the effect of plant growth regulators (PGRs), floral stages, and heat pre-treatments on the formation of embryo-like structures (ELS) and calli in watermelon using the unfertilized ovary culture method. This method has already been used effectively for breeding across many species, but observations of it being used for watermelon breeding are limited. Three different watermelon varieties ('Ersin', 'Silva', 'Üstün') were used as plant materials. Ovaries were collected from plant materials at three floral stages (12 h before anthesis, at anthesis, and 12 h after anthesis). Different concentrations and combinations of 2,4-D (2,4 Dichlorophenoxy acetic acid), BAP (6-Benzylaminopurine), and NAA (Naphthaleneacetic acid) PGRs were used to induce callus and ELS. Ovaries were kept on induction media in the dark at either 25 °C or 35° C for 3 days followed by 25°C for 30 days, and then transferred to three different differentiation media. Different levels of callus and ELS formations were observed at different rates among varieties. Based on the results 'Üstün' variety had the highest rate of callus formation and ELS development among all varieties. I3 combination of plant growth regulation (3 mg/l 2,4D+ 2mg/l 6-BA+0.5mg/l NAA) had the highest rate of callus formation with a rate of 97%, and M3 had the highest rate of ELS at a rate of 6.94%. Anthesis time of ovaries also had the highest callus formation rate of all stages, at 85%, and 25°C was the best temperature for callus formation overall, at 79%.

Keywords: *Watermelon, Haploid, Unfertilized ovary, Ovary culture, Embryo-like structure*

INTRODUCTION

Cucurbitaceae or cucurbits are a plant family contained in 965 species in around 95 genera which are the most important to humans [1]. This family has worldwide distribution but is mostly tropical. More than 300 plant species are used by humans and only 150 plant species are cultivated expensively and 30 of these are essential for global food production. Cucurbits are a great and significant group of vegetables that are economically important and the most common vegetables are watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita moschata*), melon (*Cucumis melo*), summer squash (*Cucurbita pepo*) [2].

Watermelon is a flowering plant species of the Cucurbitaceae family that originated in Africa and have been cultivated since antiquity. It is widely grown in warmer climates around the world. However, they are now one of the most economically important crops in the world, as well as the most valued and refreshing fruit during the hot summer months [3].

Watermelon is botanically classified into two species: *C. lanatus* (Thunb.) Mastum and Nakai are the best-known sweet-type and seed-type watermelons in the genus *Citrullus* cultivated in Western Africa. *C. lanatus* var. citrodes (L. H. Bailey) citrones and tsamma genus are widely used in Eastern and Southern Africa as edible, seed production, and animal feed [4].

According to the data for 2020, around 101.6 million tons of watermelons are produced in the world and Türkiye is in among the most important producers in terms of world watermelon production and was ranked the second world producer with 3.49 million tones after China with 60.08 million tones along with India with 2.79 million tonnes and Iran with 2.74 million tones production in 2020 b [30].

The traditional method of breeding vegetables is used to achieve homozygosity, but this method is time-consuming and cannot achieve 100% homozygosity; it takes 10–12 years for open-pollinated plants and 6-7 years for self-pollinated plants. Vegetables, especially those in the Cucurbitaceae family, have used the haploid plant production method because it shortens the breeding cycle, achieves 100% homozygosity, and increases breeding efficiency. There are several ways to produce haploid plants in the Cucurbitaceae plant family, including ovary culture, ovule culture, anther culture, microspore culture, and pollination with irradiated pollen the latter of which is the most effective method. The main two determinants of haploidy success are genotype and radiation doses [5].

By rescuing parthenogenetic embryos produced by in situ pollination with an anther or an isolated microspore (androgenesis), an ovule (gynogenesis), or irradiated pollen (parthenogenesis) in an *in vitro* culture, haploid plants can be produced [6]. Gynogenesis is the process of cultivating female gametophytes, ovules, or unfertilized ovaries [7]. On the other hand, the androgenic method entails the in vitro placement of anthers from buds that contain immature pollen in artificial nutrient media [8].

Haploid plants are primarily formed from meiotically reduced tissue that goes through embryogenesis without fertilization. It can also refer to plants with a gametic number of chromosomes in sporophytes. Dorothy Bergner's observation of *Datura stamonium* L. sparked interest in haploids in 1921. Primary haploid plants in the Cucurbitaceae family were achieved in the 1950s. in 1958 Aalders isolated haploid embryos from normal cucumber seeds, whereas Singh and Swaminathan [8] observed a haploid shoot of watermelon on a diploid plant grown from X-ray-treated seeds [9].

Ovary culture has been used to generate homozygous lines for commercial production of F1 hybrid varieties and genetic research. In ovule and ovary cultures, unfertilized ovaries are used to produce haploid plants. A small number of cucurbit species respond positively to the ovary and ovule culture[12]. Metwally et al. [10] and Gemes et al. [11] reported *in vitro* gynogenesis induction in squash and cucumber, respectively.

Ovule-ovary in haploid production performs on watermelon genotypes, the haploid establishing potential of their cultures; it has been informed to be plagued by many conditions, including genotype, media, female gametophyte developmental stages, temperature shock applications growth regulators, and other components in the environment and culture conditions [11], [13].

Swaminathan and Singh [8] observed a haploid shoot of watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] on a diploid plant grown from an X-ray-treated seed. Sarı et al, [14] obtained parthenogenetic haploid embryos of watermelon after pollination with g-irradiated (200 or 300 Gy) pollen.

Gürsoy et al, [15] collected the unfertilized ovaries of the Halep karasi watermelon genotype during the anthesis period, 1 day before and 1 day after anthesis. Ovules were isolated from the ovaries for ovule culture under sterile conditions and ovaries were sliced for ovarian culture. Ovarian explants and ovules were cultured by adding 1 mg/l TDZ, 1 mg/l SPM (spermin) and 1 mg/l TDZ + 1 mg/l SPM to two different media (MS and CBM). After plant regeneration from ovaries and ovules, they were transferred to MS and CBM media without PGRs.

Zou et al. [13] accomplished an experiment to induce haploid plantlets in watermelon by evaluating the effects of some significant factors on unfertilized ovary cultures of watermelon,

including genotype, the duration of induction, medium, and the development stage of the ovaries.

As previously mentioned, there have been a lot of studies using this method in cucurbitaceous plants that have been published; these reports have been a great source of inspiration for this study. According to these reports, auxin (such as 2,4-D and NAA) and cytokinin (such as BAP and KT) are frequently used for the culture of unfertilized ovules and ova. PGRs are also important for the induction of in vitro gynogenesis [16].

Consequently, we carried out this experiment to investigate the effects of genotype, media, temperature, and the floral stages on the embryo-like structure and callus formation from unpollinated ovary culture in watermelon. The objective of this study was to choose species that have higher response and induction rates as well as the best environmental conditions and ovarian development stages for unfertilized ovary culture on watermelon.

MATERIAL METHODS

Plant material

Three different cultivated watermelon varieties (‘Ersin’, ‘Silva’, ‘Üstün’) were used as plant materials. The seedlings were obtained from a private company in Antalya, Türkiye. Plants were grown in the open field at Erciyes University, Agriculture Faculty, Kayseri, Türkiye. A drip irrigation system was used in irrigation, fertilization, and spraying processes were applied for plant growth. Watermelon plants that were 32-37 days old were used as ovary donor material and the ovaries were harvested after the second female flower appeared and the remaining works were conducted in The plant biotechnology laboratory at Genome and Stem Research Centre of Erciyes University, Kayseri, Türkiye.

Ovary culture procedure

Ovaries were collected 12 hours before anthesis, during anthesis and 12 hours after anthesis (Fig. 1.) To avoid unwanted cross-pollination, the flowers were closed or bagged the day before anthesis or 12 hours after anthesis. After taking the flowers were washed in a flow of water for 15 minutes before being surface sterilized in 75% ethanol for 5 minutes and 20% sodium hypochlorite with 4 drops of tween 20 after that rinsed four times with distilled sterile water. The sterilized ovaries were cut into 2-3 mm ovarian slices, which were then dried on sterile filter paper before being cultured in sterile Petri dishes containing an induction medium supplemented with NAA, 2,4 D, and BAP in combination. As a pre-treatment, the Petri dishes were kept in a dark incubator at 25 °C and 35 °C for three days until being transferred to a normal tissue culture growing toom with 16/8 hours light and dark photoperiod conditions at 25±2°C. The induced callus and ELS were transferred to an embryo maturation medium supplemented with BAP and NAA after 5 weeks. Each medium was supplemented with 3% (w/v) sucrose and solidified with 0.75 (w/v) agar. Before autoclaving at 121 °C for 21 min. the pH was adjusted to 5.7±0.1.

Fig. 1. *Flower Stages; A) 12 hours before anthesis, B) at anthesis, C) 12 hours after anthesis*

Combinations of PGRs concentration

In this study, the experiment was carried out in 4 treatments (Table 1.). All treatments contained basal MS medium (Murashige and Skoog, 1962) with 2,4 D, 6-BA, NAA, 7 g/L agar, and 30 g/L sucrose. The pH was adjusted to 5.7 ± 1 and autoclaved at 121 °C for 21 min.

Table 1. *Combination of PGRs concentrations*

Induction Medium	MS + 30 g/l sucrose + 8 g/l agar + pH 5.7 was standart		
Medium No	2, 4-D (mg/l)	6- BAP (mg/l)	NAA (mg/l)
I1	3	1	0.5
I2	4	1	0.5
I3	3	2	0.5
I4	4	2	0.5
Maturation Medium	MS + 30 g/l sucrose + 8 g/l agar + pH 5.7 was standart		
Medium No	BAP (mg/l)	NAA (mg/l)	
M1	0.8	0.2	
M2	0.8	0.4	
M3	1	0.25	
M4	1	0.5	

Data Analysis

Ovarian Development (%): This was calculated by dividing the total number of ovaries cultured by the number of ovules that developed and swelled on each ovary as a result of the culture period. Callus Formation in Ovarian Culture (%): The number of ovaries forming callus was calculated by dividing the total number of ovaries in culture by the number of ovaries forming callus. All data were expressed as means, and an analysis of variance was performed. Means detected the statistically different were separated by least significant difference test (LSD). JMP® software (SAS Institute, Cary, NC) ver. 8.00 was used for statistical analysis.

RESULTS AND DISCUSSION

The results of the four different PGRs, three different floral stages, and two different temperatures of ovary cultures varied, but some data, including the effects of PGRs, the effects of PGRs with stages, and the effects of PGRs with temperatures and stages, were shown as tables as well as the results of ELS formation shown as tables. The remaining data was displayed on graphs, including the impact of temperatures, stages, temperature and stages, and PGRs and temperatures on callus formation.

'Ersin' watermelon callus formation

'Ersin' watermelon ovary culture was established on May 24, 2021. Female flower buds from the 'Ersin' watermelon were collected for the experiment at three different times: 12 hours before anthesis, at anthesis, and 12 hours after anthesis. The buds were then cultured in four different MS media with various hormone combinations. Two groups of cultures were created. One group was heat shocked at 35 °C for three days in the dark before going into climate chambers, while the other group went right into growing chambers at 25 °C. The ovary produced callus and showed ELS as a result of 'Ersin' watermelon Fig.4.

Effects of PGRs, PGRs with stages, PGRs with temperature, and stages on callus formation

The outcomes of the PGRs' effects are displayed in Table 4.1. The 'Ersin' variety's ovary culture I3 Medium showed the highest callus development at a rate of 84 percent, while the I2 medium had the lowest callus development, at a 64 % rate. The table also shows the relationship between hormone level and stage, and it demonstrates that the flowers harvested at 12 hours after anthesis in the I3 medium at a rate of 92 % had the best hormone level and stage for callus formation. While those harvested 12 hours before anthesis in the I4 medium had the lowest callus development rate at 42%. The interaction of PGRs with temperature and stage reveals that the flowers harvested at 12 hours after anthesis and cultured in an I3 medium shocked at 35 °C at a rate of 96% had the best hormone with stage and temperature for callus development. The flowers harvested at 12 hours before anthesis and cultured in the I4 medium containing at 25 °C had the lowest callus development rate, with a rate of 32%, under the interaction of hormone with temperature and stage.

Table 2. *'Ersin' watermelon callus formation and the effects of PGRs, PGRs with stages, PGRs with temperature, and stages*

Application			Percentage of callus formation		
Hormone	Flower Stages	Temperature (°C)	H*Temp*Stage	H*S	H
I1	12 h B/A	25	84 (71.00)	80abc (67.50)	74b (62)
		35	76 (63.60)		
	At anthesis	25	60 (48.00)	64cd (51.90)	
		35	68 (55.80)		
	12 h A/A	25	80 (72.00)	80abc (67.50)	
I2	12 h B/A	25	48 (40.80)	56de (47.10)	64c (53)
		35	64 (53.40)		
	At anthesis	25	72 (61.20)	68bcd (57.30)	
		35	64 (53.40)		
	12 h A/A	25	76 (60.60)	68bcd (55.80)	
		35	60 (51.00)		
I3	12 h B/A	25	72 (61.20)	82ab (70.20)	84a (72)
		35	92 (79.20)		
	At anthesis	25	68 (55.80)	78abc (64.80)	
		35	88 (73.80)		
	12 h A/A	25	88 (79.80)	92a (82,20)	
		35	96 (84.60)		
I4	12 h B/A	25	32 (34.20)	42e (40.20)	66bc (58)
		35	52 (46.20)		
	At anthesis	25	76 (76.80)	74bc (65.40)	
		35	72 (64.20)		
	12 h A/A	25	88 (76.80)	82ab (68.70)	
		35	76 (60.60)		

LSD $_{Hormone}$: 8, 49, LSD $_{Hormone*Stage}$: 14, 71, LSD $_{Hormone*Stage*Temperature}$: N.S., LSD $_{Temperature}$: N.S., LSD $_{Stage}$: 7.36, LSD $_{hormone*temperature}$: N.S, LSD $_{Stage*Temperature}$: N.S.

Effect of temperature and stages on 'Ersin' watermelon callus formation.

Fig.2a. compares the callus formation on 'Ersin' watermelon under 35 °C and 25 °C temperatures. For the 'Ersin' watermelon variety, 35 °C performed only marginally better at a rate of 74% than 25 °C at its rate of 70%. Fig.3b illustrates the results of our study on the callus formation in 'Ersin' watermelon under the influence of three floral stages: 12 hours before anthesis, at anthesis, and 12 hours after anthesis. Our research showed that the third stage (12 hours after anthesis) had the highest callus formation rate at 80.5 percent, while the first stage (12 hours before anthesis) had the lowest callus formation rate at 65 percent.

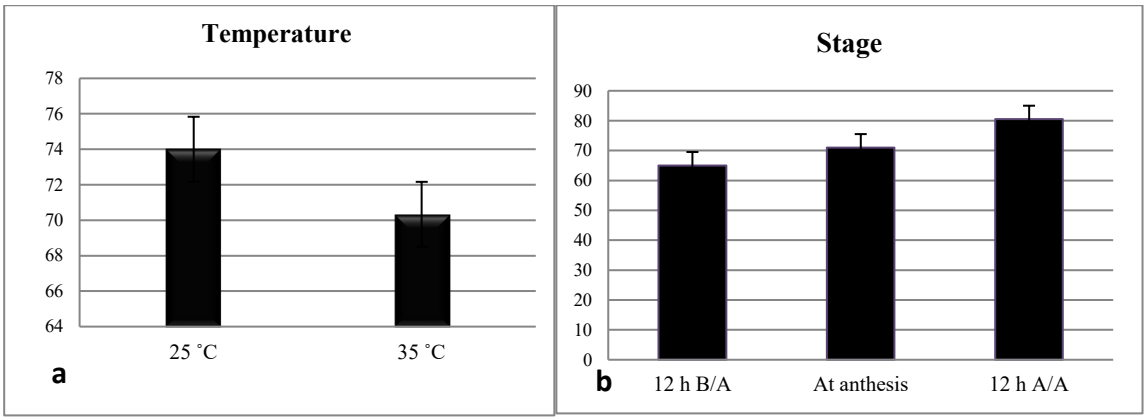

Fig. 2*a. Effect of temperature on 'Ersin' watermelon callus formation, Fig.2b. Effect of stages on 'Ersin' watermelon callus formation*

Effect of Temperature with hormone and temperature with stages on 'Ersin' watermelon callus formation

As depicted in Fig.3a. we investigated the effects of four different PGRs—I1, I2, I3, and I4 at two different temperatures (35 °C and 25 °C) on 'Ersin' watermelon callus formation. Our research revealed that the flowers cultured in the I1 medium and shocked at 35 °C had the lowest callus formation rate, with a rate of 60 percent, while the flowers cultured in the I3 medium and directly placed at 25°C growing chambers had the highest callus in formation rate at 84 percent. We also looked at how the callus formation of 'Ersin' watermelon affected two different temperatures (35 °C and 25 °C) and three floral stages. According to our research, flowers placed at 25 °C collected at 12 hours before anthesis had the lowest callus formation rate, at 59%, while flowers placed at 25 °C collected at 12 hours after anthesis, had the highest callus formation rate, at 83%. (Fig 3b.)

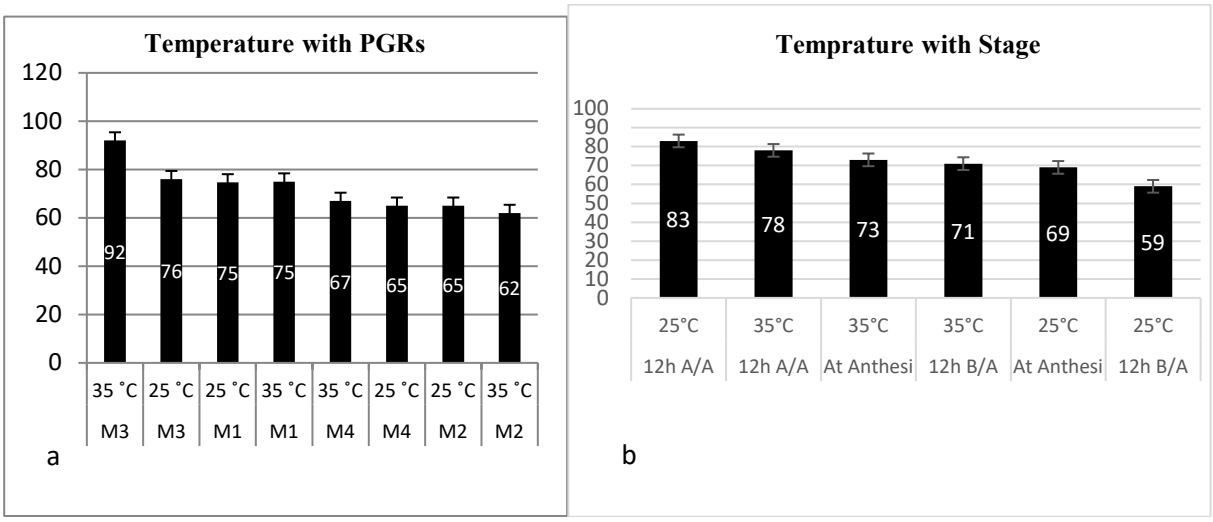

Fig. 3*a. Effect of Temperature with hormone on 'Ersin' watermelon callus formation, Fig.3b Effect of Temperature with stage on 'Ersin' watermelon callus formation*

'Ersin' Watermelon ELS Formation

Table 3. displays the effects of four different PGRs on the ELS formation of 'Ersin' watermelon: M1, M2, M3, and M4. The 'Ersin' variety's ovary culture showed that the medium with the highest ELS development, M3 developed ELS at a 6.94% rate, while the remaining three media M1, M2, and M4 had zero ELS development.

Table 3. *'Ersin' watermelon ELS formation*

Hormone	Percentage of ELS formation
0.8 mg/l BAP+0.2mg/l NAA	0 (0.00)
0.8 mg/l BAP+0.4mg/l NAA	0 (0.00)
1 mg/l BAP+0.25mg/l NAA	6.94 (7.50)
1 mg/l BAP+0.5mg/l NAA	0 (0.00)

$LSD_{Hormone}$: N.S

Fig. 4. *a) Enlarging the ovaries after culture b) Callus formation c) ELS development*

'Silva' watermelon

On May 24, 2021, a 'Silva' watermelon ovary culture was established. As a previous variety, female flower buds from the 'Silva' watermelon were taken for the experiment three times: 12 hours before anthesis, at anthesis, and 12 hours after anthesis. Thereafter, the buds were grown in four different MS media using different hormone combinations. There were

two distinct cultures created. Before entering the growing chambers, one group was heat shocked at 35 °C for three days in the dark, while the other group entered the chambers at 25 °C right away. The ovary produced callus as well as ELS 'Silva' watermelon Fig.8.

Effects of PGRs, PGRs with stages, PGRs with temperature, and stages

Table 4. displays the effects of four different PGRs on the callus formation of 'Silva' watermelon: I1, I2, I3, and I4. Effects of these four PGRs at two different temperatures (35 °C and 25 °C), as well as at three different stages (12 hours before anthesis, at anthesis, and 12 hours after anthesis), on the development of calluses on 'Silva' watermelons. The 'Silva' variety's ovary culture revealed that the I3 medium promoted the most callus development produced calluses at an 82% rate, whereas the I4 and I2 media promoted the least callus and development at a 62% rate. The relationship between hormone level and stage shows that the best hormone level and stage for callus formation were found in flowers harvested 12 hours after anthesis in the I3 medium at a rate of 100%. The rate of callus development was 40% at anthesis in the I4 medium. The interaction of PGRs with temperature and stage shows that the best hormone with stage and temperature for callus development was observed in flowers harvested at 12 hours after anthesis and cultured in an I3 medium shocked at 35 °C and directly placed on 25 °C at a rate of 100%. According to the interaction of hormones with temperature and stage, the flowers harvested at anthesis and cultured in the I4 medium and then shocked at 35 °C had the lowest callus development rate, with a rate of 32%.

Table 4. *'Silva' watermelon callus formation and the effects of PGRs, PGRs with stages, PGRs with temperature, and stages*

Application			**Percentage of callus formation**		
Hormone	**Flower Stages**	**Temperature (°C)**	**H*Temp*Stage**	**H*S**	**Hormones**
I1	12 h B/A	25	56 (48.68)	50ef (45.00)	65b (56.23)
		35	44 (41.31)		
	At anthesis	25	88 (74.06)	78bcd (64.95)	
		35	68 (55.83)		
	12 h A/A	25	68 (61.62)	68cde (58.73)	
		35	68 (55.83)		
I2	12 h B/A	25	48 (43.62)	50ef (44.89)	62b (53.69)
		35	52 (46.25)		
	At anthesis	25	60 (50.99)	58def (49.73)	
		35	56 (33.69)		
	12 h A/A	25	76 (63.44)	78bcd (66.45)	
		35	80 (69.22)		
I3	12 h B/A	25	92 (82.15)	90ab (78.10)	82a (73.77)
		35	88 (74.06)		
	At anthesis	25	60 (57.00)	56ef (53.19)	
		35	52 (49.30)		
	12 h A/A	25	100 (90.00)	100a (90.00)	
		35	100 (90.00)		
I4	12 h B/A	25	92 (82.15)	86abc (72.79)	62b (54.69)
		35	80 (63.44)		
	At anthesis	25	44 (38.53)	40f (36.11)	
		35	36 (33.69)		
	12 h A/A	25	56(58.61)	62de (55.15)	
		35	68 (61.62)		

$LSD_{Hormone}$: 12.59, $LSD_{Hormone*stage:}$ 18.61, $LSD_{Hormone*Stage*Temperature}$: N.S., LSD_{Stage}: 9.30 $LSD_{Temperature}$: N.S, $LSD_{hormone*temperature}$: N.S, $LSD_{Stage*Temperature}$: N.S.

Effect of temperature and stages on 'Silva' watermelon callus formation

'Silva' watermelon variety was subjected to temperatures of 35 °C and 25 °C to compare the callus formation. When it came to the 'Silva' watermelon variety, 25 °C's performance at a rate of 70% and 35 °C's rate of 66% only slightly outperformed each other. (Fig.5a). Based on the findings of our investigation into how three floral stages affect the callus formation in 'Silva' watermelon the second stage (during anthesis) had the lowest callus formation rate, with a 58% rate, while the third stage (12 hours after anthesis) had the highest callus formation rate, at 77% (Fig. 5b.)

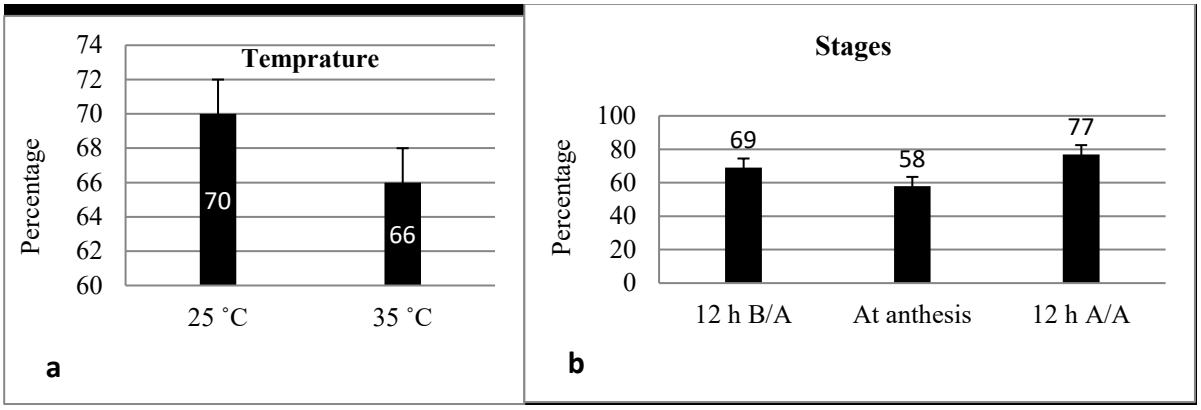

Fig. 5*a. Effect of temperature on 'Silva' watermelon callus formation, Fig.5b. Effect of stages on 'Ersin' watermelon callus formation*

Effect of temperature with stage on 'Silva' watermelon callus formation

As shown in Fig. 6, we looked at how the callus formation of 'Silva' watermelon affected two different temperatures (35 °C and 25 °C) and three floral stages (12 hours before anthesis, at anthesis, and 12 hours after anthesis). According to our research, flowers shocked at 35 °C during anthesis had the lowest callus formation rate, at 53%, while flowers shocked at 35 °C 12 hours after anthesis, had the highest callus formation rate, at 79%.

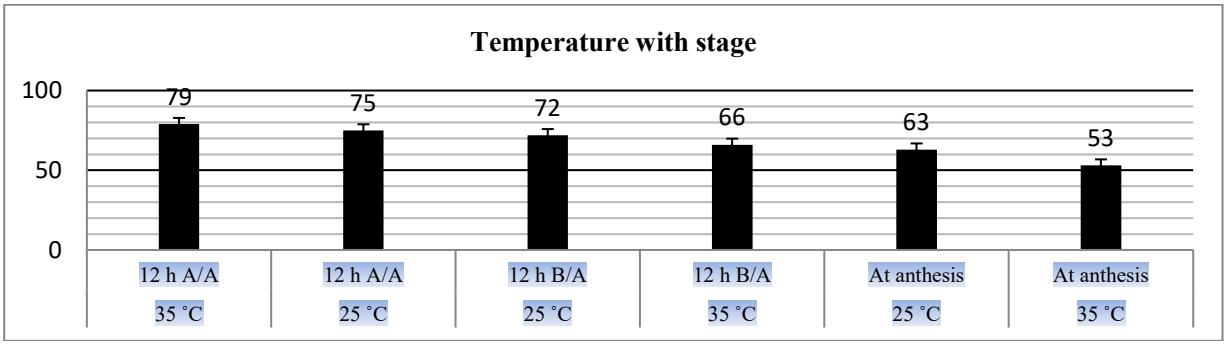

Fig. 6*. Effect of temperature with stage on 'Silva' watermelon callus formation*

Effect of temperature with hormone on 'Silva' watermelon callus formation

Fig.7. depicts the effects of four different PGRs—I1, I2, I3, and I4 with two different temperatures (35 °C and 25 °C). Our research found that flowers cultured in I1 and shocked at 35 °C had the lowest callus formation rate, at 60%, while flowers cultured in I3 and directly placed at 25 °C growing chambers had the highest callus formation rate, at 84%.

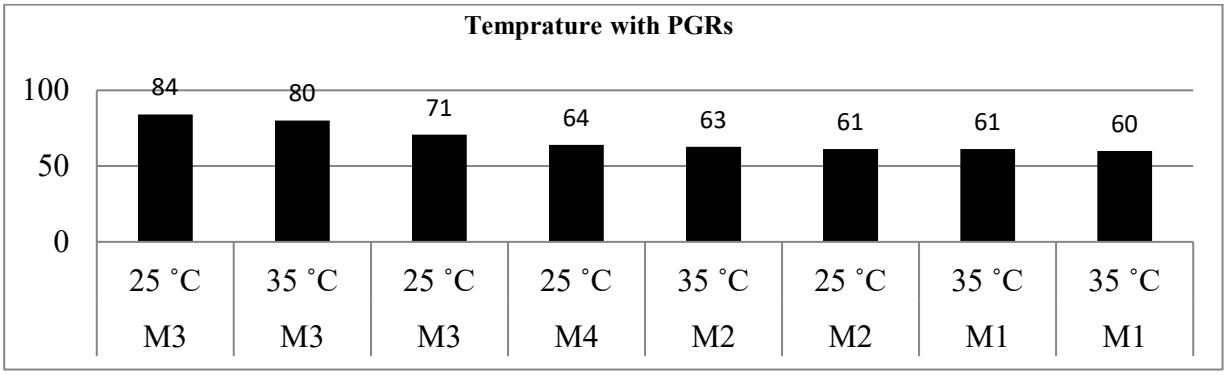

***Fig.** 7 Effect of temperature with hormone on 'Silva' watermelon callus formation*

'Silva' Watermelon ELS Formation

Table 3.3 displays the effects of four different PGRs on the ELS formation of 'Silva' watermelon: M1, M2, M3, and M4. The 'Silva' variety's ovary culture showed that the medium with the highest ELS development, M3, developed ELS at a 2.63 % rate, while the remaining three media M1, M2, and M4 had zero ELS development.

Table 5. *'Silva' watermelon ELS formation*

Hormone	Percentage of ELS formation
1 mg/l BAP+0.25mg/l NAA	2.63 (3.16)
1 mg/l BAP+0.5mg/l NAA	0 (0.00)
0.8 mg/l BAP+0.2mg/l NAA	-0 (-0.00)
0.8 mg/l BAP+0.25mg/l NAA	-0 (-0.00)

$LSD_{Hormone}$: N.S

***Fig. 8.** Enlarging the ovaries after culture b) Callus formation c) ELS development*

'Üstun' watermelon

An 'Üstun' watermelon ovary culture was established on May 24, 2021. Female flower buds from the 'Üstun' watermelon were collected for the experiment three times: 12 hours before anthesis, at anthesis, and 12 hours after anthesis. The buds were then grown in four different MS media with different hormone combinations. Two distinct cultures were created. One group was heat shocked at 35 °C for three days in the dark before entering the growing chambers, while the other group entered the chambers at 25 °C right away. The ovary produced callus and ELS as a result of 'Üstun' watermelon Fig. 10.

Effects of PGRs, PGRs with stages, PGRs with temperature, and stages

Table 3.3 displays the effects of four different PGRs on the callus formation of 'Üstun' watermelon: I1, I2, I3, and I4 effects of these four PGRs at two different temperatures (35 °C and 25 °C), as well as at three different stages (12 hours before anthesis, at anthesis, and 12 hours after anthesis), on the development of calluses on 'Üstun' watermelons. The 'Üstun' variety's ovary culture revealed that the I3 medium promoted the most callus development and produced calluses at a 97 % rate, whereas the I2 and I4 media promoted the least callus development at a 53 % rate. The relationship between PGRs level and stage shows that the best PGRs level and stage for callus formation were found in flowers harvested at 12 hours after anthesis in the I3 medium at a rate of 100%. The rate of callus development was 46% at 12 hours after anthesis in the medium of I2. The interaction of PGRs with temperature and stage shows that the best PGRs with stage and temperature for callus development were observed in flowers harvested during anthesis and cultured in the I1 medium placed at 25°C as well as in flowers directly placed on 25 °C containing I3 collected 12 hours after anthesis also flowers collected 12 hours after anthesis cultured I3 shocked at 35 °C, flowers cultured I3 collected after anthesis placed at 25 °C and flowers cultured in I3 collected at anthesis in for 35°C and 25 °C at a rate of 100%. According to the interaction of PGRs with temperature and stage, the flowers harvested at 12 hours before anthesis and cultured in a medium containing I2 and then shocked at 35 °C had the lowest callus development rate, with a rate of 40%.

Table 6. *'Üstun' watermelon callus formation and the effects of PGRs, PGRs with stages, PGRs with temperature, and stages*

Application			Percentage of callus formation		
PGRs	Flower Stages	Temperature (°C)	H*Temp*Stage	H*Stage	PGRs
3 mg/l 2,4D+ 1mg/l 6-BA+0.5mg/l NAA	12 h B/A	25	52 (46.15)	58de (49.84)	77b (67.42)
		35	64 (53.53)		
	At anthesis	25	100 (90.00)	96a (86.04)	
		35	92 (82.15)		
	12 h A/A	25	84 (74.31)	78bc (66.34)	
		35	72 (58.37)		
4 mg/l 2,4D+ 1mg/l 6-BA+0.5mg/l NAA	12 h B/A	25	56 (48.46)	48e (43.73)	53c (46.57)
		35	40 (39.01)		
	At anthesis	25	60 (51.00)	66cd (56.18)	
		35	72 (61.38)		
	12 h A/A	25	48 (41.07)	46e (39.80)	
		35	44 (38.53)		
3 mg/l 2,4D+ 2mg/l 6-BA+0.5mg/l NAA	12 h B/A	25	96 (84.00)	90ab (79.60)	97a (86.53)
		35	84 (74.53)		
	At anthesis	25	100 (90.00)	100a (90.00)	
		35	100 (90.00)		
	12 h A/A	25	100 (90.00)	100a (90.00)	
		35	100 (90.00)		
4 mg/l 2,4D+ 2mg/l 6-BA+0.5mg/l NAA	12 h B/A	25	68 (55.84)	72cd (61.15)	82b (70.76)
		35	76 (66.46)		
	At anthesis	25	84 (68.75)	78bc (66.45)	
		35	72 (64.15)		
	12 h A/A	25	100 (90.00)	96a (84.68)	
		35	92 (79.37)		

LSD $_{PGRs}$: 8.29 LSD $_{PGRs*stage}$: 14.37, LSD $_{PGRs*Stage*Temperature}$: N.S., LSD $_{Stage}$: 7.18, LSD $_{Temperature}$: N.S, LSD $_{PGRs*temperature}$: N.S, LSD $_{Stage*Temperature}$: N.S.

Effect of temperature and stages on 'Üstün' watermelon callus formation

Temperatures of 35 °C and 25 °C were applied to the 'Üstun' watermelon variety to compare callus formation. When it came to the 'Üstun' watermelon variety, 25 °C performed at a rate of 79 percent, while 35 °C performed at a rate of 76 percent. (Fig.9a). As shown in (Fig.9b.) the results of our study into how three floral stages—12 hours before anthesis, at anthesis, and 12 hours after anthesis—influence callus formation in 'Üstun' watermelon. According to our findings, the first stage (12 hours before anthesis) had the lowest callus formation rate (67%), while the second stage (during anthesis) had the highest callus formation rate (85%).

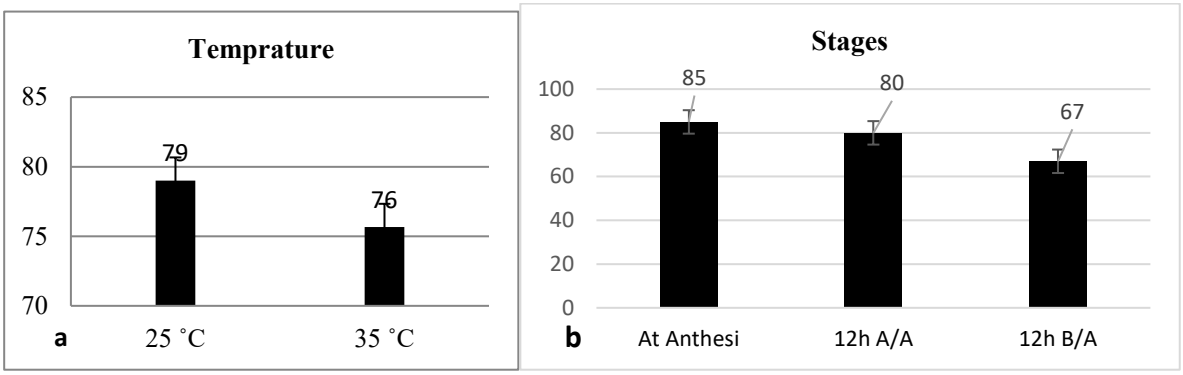

Fig. 9*a. Effect of temperature on 'Üstün' watermelon callus formation, Fig.9b. Effect of stages on 'Üstun' watermelon callus formation.*

Effect of Temperature with stage and temperature with PGRs on 'Üstun' watermelon callus formation

Fig. 10a. represents how two different temperatures (35 °C and 25 °C) and three floral stages affected the callus formation of 'Üstun' watermelon (12 hours before anthesis, at anthesis, and 12 hours after anthesis). According to our findings, flowers placed at 25 °C during the second stage (at anthesis) had the highest callus formation rate, at 86 percent, while flowers shocked at 35 °C collected at the first stage (12 hours before anthesis) had the lowest callus formation rate, at 66 percent. Fig.10b. describes the effects of four different PGRs—I1, I2, I3, and I4 with two different temperatures (35 °C and 25 °C) on 'Üstun' watermelon callus formation. Our research found that flowers cultured in I3 that were directly placed at 25 °C had the highest callus formation rate, at 99%, while flowers cultured in I2 heat shocked at 35 °C had the lowest callus formation rate, at 52%.

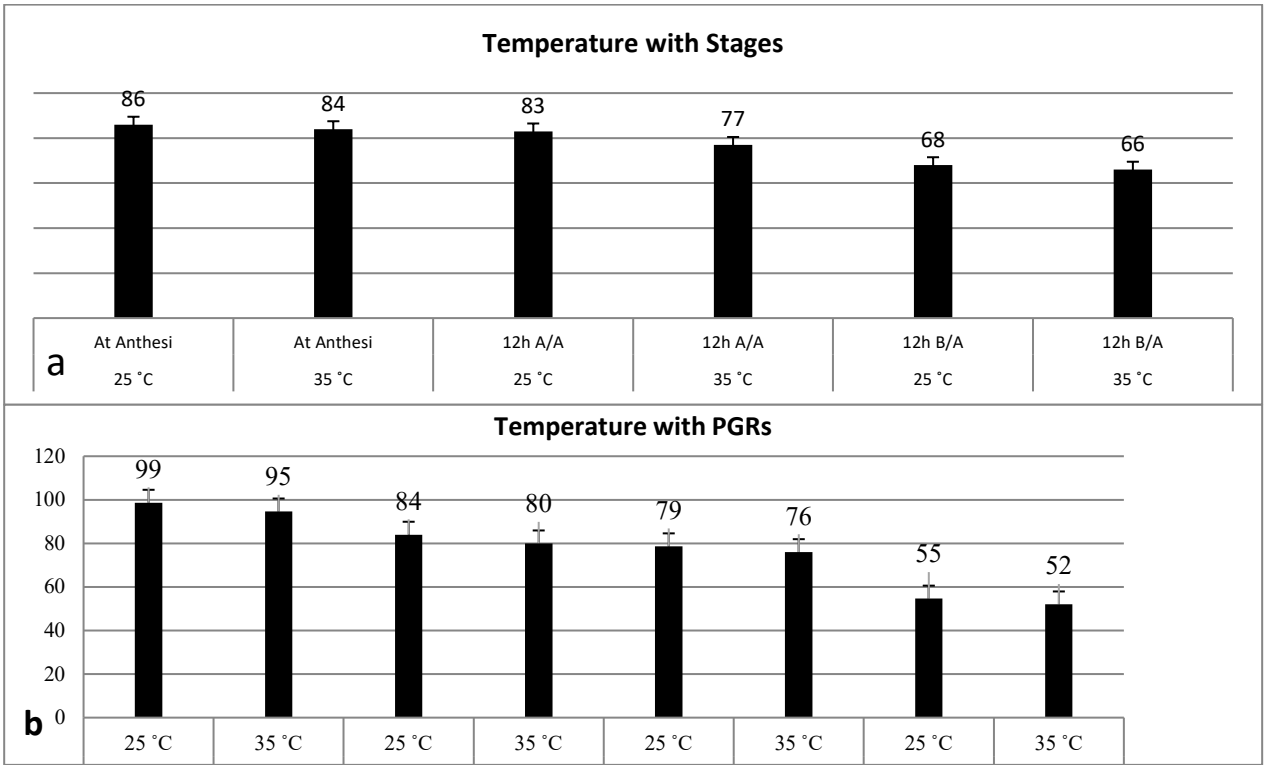

Fig. 10*a Effect of temperature with stage on 'Üstün' watermelon callus formation, Fig.10b. Effect of temperature with PGRs on 'Üstün' watermelon callus formation*

'*Silva' Watermelon ELS Formation*

Table 3.3 displays the effects of four different PGRs on the ELS formation of 'Üstun' watermelon: M1 (0.8 mg/l BAP+0.2mg/l NAA), M2 (0.8 mg/l BAP+0.4mg/l NAA), M3 (1 mg/l BAP+0.25mg/l NAA), and M4 (1 mg/l BAP+0.5mg/l NAA). The 'Silva' variety's ovary culture showed that the medium with the highest ELS development, M3 (1 mg/l BAP+0.25mg/l NAA), developed ELS at a 3.94 % rate, while the lowest media M1 (0.8 mg/l BAP+0.2mg/l NAA) had a zero rate for ELS development.

Table 7. *'Silva' watermelon ELS formation*

PGRs	Percentage of ELS formation
1 mg/l BAP+0.25mg/l NAA	3.94 (4.73)
1 mg/l BAP+0.5mg/l NAA	2.63 (3.16)
0.8 mg/l BAP+0.2mg/l NAA	1.32 (1.58)
0.8 mg/l BAP+0.25mg/l NAA	-0 (-0.00)

LSD_{PGRs}: N.S

Fig. 11. *Enlarging the ovaries after culture b) Callus formation c) ELS development*

Various methods included in the haploidization method are used to produce plants in species of the Cucurbit family. Many researchers favor gynogenesis as a method for growing plants; it is one of these techniques. Studies using the watermelon gynogenesis method are extremely rare. Studies on ovule-ovarian culture [15], and ovarian culture have been published in the literature [13]. It is believed that protocols must be created for the gynogenesis technique to be used successfully on watermelons, as it has been for other cucurbit species. It has been discovered that many factors, including the genotype of the donor plant, low/high-temperature pretreatments, the developmental stage of the female gametophyte, growth regulators or other media compositions, and culture conditions demonstrated in studies, all influence the success of ovary culture in producing haploid and doubled haploids [16].

The effects of PGRs combinations, $AgNO_3$, and temperature pretreatment on cucumber were investigated by Golabadi et al. [17]. In their study, which consisted of three trials, the NAA+2,4-D+KIN+BAP (0.5+0.7+1+1.8) mg/l PGRs combinations produced the highest embryogenesis rate and embryo formation in the first trial.

In vitro culture of unfertilized ovule-ovaries, auxin (2,4-D, NAA, IAA), and cytokinin (BAP, Kin) are the most frequently used plant growth regulators [16]. Therefore, we used our study 2,4-D, NAA as auxin, and BAP as cytokinin. According to our research, combining NAA and BAP was superior to BAP alone for callus and ELS induction. When BAP concentration was increased from 1 mg/l to 2 mg/l along with NAA, better results were obtained. Certain PGR combinations (BAP: NAA) may be crucial for callus differentiation. Additional studies should focus on the relationship between endogenous hormone levels and the supplied exogenous PGRs to confirm the critical values. This investigation supported the findings of Song et al's study [12].

Li et al. [18] cultured unfertilized ovules in Chinese long cucumber. They found that adding 0.05 mg dm3 NAA, 0.2 mg dm3 BA, and 5-10 mg dm^3 $AgNO_3$ to the CBM medium resulted in the most plant formation.

Additionally, we employed 2,4 D, which responded best when its concentration was decreased from 4 mg/l to 3 mg/l in combination with other PGR. Our results demonstrated the same as the research done by Zou et al. [13]. But Rekha et al. [19] conducted a study on anther culture in six interspecific Cucurbita hybrids, and the researcher demonstrated that an induction medium containing 1.0 mg/l 2, 4-D is superior to media containing other concentrations for callus regeneration. Several previous studies looked at the effects of only using plant growth regulators. [10], [20], [21], and [19].

Prior studies discovered that when Crimson sweet and Charleston gray watermelon cultivars were grown in MS medium supplemented with 0.44 mg/l 2,4-D and 0.372 mg/l KT or 0.338 mg/l BAP, 1.47 and 1.20 embryos were produced, respectively. Auxin-cytokinin combinations are one example of how plant growth regulators can be combined. The highest ME/EC was obtained in a culture medium containing 2M 2,4-D and 1.5M KIN, as well as 1 and 1.5M BAP, with embryos averaging 1.90 and 1.85, respectively [22].

Numerous species, including cucumber, summer squash, winter squash, and pumpkin, have demonstrated that genotype is a significant determinant of in vitro gynogenesis/androgenesis [23], [24], [25]. In this study, the induction rates of three varieties greatly varied, the ‘Üstun’ variety had the highest callus formation at a rate of 97% demonstrating that genotype strongly affects watermelon unfertilized ovary culture. According to Shalaby [24], the percentage of squash gynogenic ovules varies from 0 to 48.8% depending on genotype. To be more specific, 'Raad F1' had the highest percentage of responding ovules and number of plantlets per dish, at 48.8% and 15 plants, respectively, whereas 'Yellow Bik F1' cultured ovules remained undeveloped on the culture medium.

Investigation of the interaction between variety and temperature shock pretreatment on embryogenesis success showed that the highest proportion of embryogenesis was obtained in all cultivars when pretreated at 35 °C for 3 days, although the response of each cultivar to this treatment was not the same for all treatment durations. Gemes et al. [11] demonstrated that heat pretreatment at 32 °C during the embryo induction phase increased haploid embryo development from ovule cultures of cucumber. High temperatures applied to the ovary or during *in vitro* culture have a strong influence on embryo formation [26], [23]. Currently, Abdollahi et al [22] showed that 4 days of heat pre-treatments of the cultured anthers at 30 °C for 7 days and 32 °C for 2 days produced the highest percentage of embryonic callus compared to the control and other heat pre-treatments in Crimson Sweet watermelon cultivar.

Medium culture is a primary factor controlling the induction and development of intact plants, and the formulation of media culture also contributes to the advancement of gynogenic methods. The most frequently modified components are the organic nitrogen base, carbohydrates, and growth regulators [16].

In Cucurbitaceae haploids, via in vitro unfertilized ovule/ovary culture, most researchers have used MS medium as a basal medium such as [27], [11], [24], [21], [28]. Also [16] reported that only two researchers used N6 and MS medium as basal medium.

Abdollahi et al [29] conducted research in anther culture. They discovered cultivar differences in MS culture medium with altered macronutrients (half, full, 1.5 fold, and double). Isfahani anther cultures on full- and half-strength MS medium showed the highest percentage of callogenesis (100%) in a doubled strength of macronutrients, while the full strength of macronutrients showed the highest embryogenic callus (83.33 percent) and the highest number of gametic embryos (1.26 and 1.23 embryos/anther) distinguished from 'Isfahani' anther cultures on full- and half-strength.

Considering prior studies, we revealed that in this study, to evaluate factors affecting the embryo-like structure and callus formation in unpollinated ovary culture of watermelon we discovered that media containing different doses of auxin and Cytokinin were found separately and together. Three floral stages were used (12 hours before anthesis, at anthesis, and 12 hours after anthesis). When we analyzed the data from the ovary culture experiment, in the ‘Üstun’ variety I3 hormone (3 mg/l 2,4D+ 2mg/l 6-BA+0.5mg/l NAA) had the highest callus formation of all varieties with a rate of 97 percent. According to [13], the highest callus ratio was observed in 3 mg/l 2,4D+ 2 mg/l 6-BA+0.5 mg/l NAA using the "Zao-Jia" cultivar, which is a finding that is similar to the result of higher callus formation in this medium combination. The ‘Üstun’ variety had the highest data in this study, with an anthesis stage rate of 85%, when we evaluated the stages. According to a similar finding of higher callus induction based on stage, particularly at anthesis, made by [13], the "Hei-Mei-Niang" cultivar's highest callus ratio occurred at the anthesis stage.

CONCLUSION

Even though watermelon is a significant species that is widely cultivated in Turkey and around the world, it is the second-highest-producing vegetable after tomatoes. In traditional watermelon breeding, pure lines are produced through inbreeding and selection over several generations. The traditional method is time-consuming because it requires at least eight generations of inbreeding. Ovule-ovarian culture is a quick way to obtain homozygous materials [13].

The ovary culture technique, which is one of the haploidization methods, the small number of studies on watermelon in the literature, and the very low gynogenesis efficiency have all contributed to watermelon's limited success in comparison to other cucurbit species.

Considering the current findings, each new study will contribute to the biotechnological development and advancement of this technique.

Among all variety es, the 'Üstun' variety had the highest rate of callus formation, and the I3 hormone (—3 mg/l 2,4D+ 2 mg/l 6-BA + 0.5 mg/l NAA—) had the highest rate of callus formation. The second stage (at anthesis) also had the highest callus formation rate of all stages, and 25 °C was the best temperature for callus formation overall, with a rate of 79%.

There have been few studies on the ovary culture technique in watermelon. Due to the low success rate of gynogenesis in watermelon, it is thought that new studies should be conducted to develop a suitable protocol to make it a better alternative method for obtaining haploid plants. Other watermelon genotypes in the watermelon genetic resource collection should be tested as well to obtain haploid plants in gynogenesis. The findings received in the study will be a valuable source of information and a guide for future research on ovule-ovarian culture in watermelon.

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