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Obtention of Haploid Plant in *Citrullus lanatus* var. *lanatus* and *Citrullus lanatus* var. *citroides* Species by Anther Culture Method

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ABSTRACT

This research was conducted with *Citrullus lanatus* var. *lanatus* and *Citrullus lanatus* var. *citroides* species of watermelon in order to obtain haploid plants by anther culture method. In the experiment, 1 open-pollinated species (Halep Karası) and 1 hybrid cultivar (Crimson Tide) belonging to *Citrullus lanatus* var. *lanatus* subspecies and 2 genotypes of the *Citrullus lanatus* var. *citroides* subspecies (PI 296341 and PI 189225) were used. In anther culture applications, 2 mg/l 2.4-D + 90 g/l sucrose supplemented MS medium was used and 6- Benzylaminopurine (BAP) was put in different doses (1 and 1.5 mg/l). Additionally, spermidine (SPD) and putrescine (PUT) were added seperately and together to the media contents at different doses (500 and 1000 µM/l) to investigate the effect of polyamines. Fourteen different media combinations were evaluated in 2 different experiments. In the first experiment, flower buds were collected 1 day before anthesis and the anthers were stored at 32 °C for 2 days in dark condition and then cultured. In the second experiment, the buds were collected 2 days before anthesis and cultured without being subjected to pre-heat treatment. According to the results, anther development and callus formation were observed at varying rates on the basis of genotypes used in different medium. However, the influence of pre-heat treatment and ratio of callus formation were higher in *Citrullus lanatus* var. *lanatus* species. Even so, no transformation into plants was observed. The effect of polyamines (SPD and PUT) on anther culture in watermelon has not been clearly observed.

Keywords: Watermelon (*Citrullus lanatus* L.), haploid, spermidine, putrescine

INTRODUCTION

Watermelon is an important vegetable species in terms of production amount and cultivation area. Our country has 4 031 174 tons of watermelon production on 863 610 da (TUIK, 2018). Watermelon production is 118 413465 tons on 3 477 285 ha around the world (FAO, 2017). Turkey comes after China in the world ranking of watermelon production. It is observed that watermelon production is intense in the Mediterranean region (1 738 699 tons) on a regional base and especially in Adana (TUIK, 2018). Varieties of foreign origin are preferred in watermelon cultivation nowadays. The development of domestic hybrid varieties and their usage in production is important for the national economy (Solmaz and Sarı, 2011).

Citrullus genus belongs to the *Cucurbitaceae* family and contains 4 species. *C. lanatus* (Thunb.) Matsum and Nakai belong to the genus *Citrullus* and it is grown in tropical and subtropical regions of the world. It not only contains the most diverse species but also the high commercial value of culture form *C. lanatus* var. *lanatus* and *C. lanatus* var. *citroides* (L. H. Bailey Mansf.) (Tulukoglu, 2014; Solmaz et al., 2016). *Citrullus lanatus* var. *citroides* (L.H. Bailey Mansf.) has different names in the literature such as “citron”, “citron melon” and “canned melon”. The first form of “Citron” fruit is found in South Africa and is used in pickle, canning and jam making (Solmaz et al., 2016).

Haploids contain only the chromosome set found after meiosis in male or female gametes. This chromosome set ‘n’ corresponds to only half of the chromosome set found in the zygote and other

somatic cells. The leaves and flowers of haploid plants are small and short in size when compared to diploid plants (Solmaz et al., 2014; Tulukoğlu, 2014). Haploid plants are not capable of producing. Its chromosome numbers should be doubled with some chemicals (most commonly used colchicine) in order to make haploid plants usable in breeding studies. Haploid plants become diploid in this method it is known as dihaploidization (Sarı, 1994). In today's plant breeding, homozygous lines are required since hybrids are preferred most in plant cultivation (Kosmrlj et al., 2013). Haploidy is one of the most powerful methods of plant breeding, because of its possibility to obtain completely homozygous plants in a short time (Bobkov, 2014).

Haploid plants are formed by gynogenesis, androgenesis, semigami, polyembryony and chromosomal elimination, although the frequency is low (Gürsöz and Sarı, 1990). Androgenesis and gynogenesis are two commonly used methods in haploid plant production. Androgenesis is the production of haploid plants by culturing anthers in vitro. In the gynogenesis method, haploid plants are obtained by culturing unfertilized egg cells under in vitro conditions (Ellialtıoğlu et al., 2001).

Anther containing immature pollen is used in anther culture method. Anthers are separated from their buds and placed into the medium *in vitro* and haploid embryos are obtained from immature pollen. In this method, pollen grain, which will be transformed into a binuclear structure, is developed in a somatic direction rather than in the gametophytic direction. This change in growth direction is called 'microspore androgenesis' or 'androgenesis' only (Ellialtıoğlu, 2012).

The anther culture method was first tested on *Datura innoxia* which is a species belonging to the *Solanaceae* family (Guha and Maheshwari, 1964). Besides, there are anther culture studies on pepper (Olszewska et al., 2014), eggplant (Başay and Ellialtıoğlu, 2013), peas (Bobkov, 2014), asparagus (Feng and Wolyn, 1991) and canola (Lichter, 1981) as well as species belonging to the *Cucurbitaceae* family (Mohamed and Refaei 2004; Song et al., 2007; Suprunova and Shmykova, 2008).

Polyamines (PA) are low molecular weight polycation materials present in all living organisms. Diamine putrescine (PUT), triamine spermidine (SPD) and tetramine spermine (SPM) in plant cells are the main polyamines. Polyamines and their biosynthetic enzymes have been shown to be involved in a wide range of metabolic events ranging from cell division to organogenesis (Kaur-Sawhney et al., 2003). In addition, researchers think that the main role of polyamines is to be the direct protective component of the plant in disease, biotic and abiotic stress conditions (Alcázar et al., 2010; Carone et al., 2010; Hussain et al., 2011; Pál et al., 2015). Besides, it has been observed by researchers that polyamine use has a positive effect on ovule and ovarian culture (Gürsoy et al., 2012).

The effect of plant growth regulators on anther culture was investigated in many species. The basic nutrient medium is sufficient for induction in several species of the *Solanaceae* family. The presence of growth regulators (auxin, cytokinin or combinations) plays a crucial role in the production of microspore-derived embryos in important plant species, particularly those that respond negatively to anther culture, although no additional auxin is required in these species.

It was reported (Abdollahi et al., 2015) that cold or hot temperature pre-treatment has a positive effect on anther culture in some *Cucurbitaceae* species; squash (Metwally et al., 1998) and cucumber (Ashok Kumar et al., 2003; Song et al., 2007).

Watermelon cultivation has great importance in our country. Breeding studies on this plant are becoming widespread. However, the number of studies on anther culture, which is one of the methods of obtaining haploid plants in watermelon, is limited (Xue, 1983; Tulukoğlu, 2014; Abdollahi, 2015).

The purpose of this study is to produce haploid plants in different *Citrullus* species by using anther culture method.

MATERIAL AND METHOD

In this study a total of 4 genotypes were used; 2 genotypes and 1 variety were selected from watermelon genetic resources of Cukurova University, Department of Horticulture and 1 variety was obtained from commercial company.

Table 1. Watermelon genotypes used in this study.

Cultivars belong to ***C. lanatus* var. *lanatus***	Halep Karası (Open-pollinated)
	Crimson Tide (Hybrid)
Genotypes belong to ***C. lanatus* var. *citroides***	Kar 234 (PI 296341)
	Kar 375 (PI 189225)

Cultivation of plants

The seeds of genotypes belonging to 2 different species of *Citrullus* genus used in the research were sown in 2: 1 mixture of peat and perlite in order to obtain 40 seedlings from each genotype in Cukurova University Faculty of Agriculture Department of Horticulture. Seedlings were ready for planting in 2-3 true leaf stage and were planted in double rows in plastic greenhouses on 01/04/2018 with distances of (100-50) x 50 cm. The plants were grown in a single-stem. The drip-irrigation system was used in irrigation and cultural, maintenance works, disease and pest control were applied as required.

Content and preparation of the embryo-stimulation medium

The main nutrient medium used in the experiment was the MS medium, which was previously reported having positive results in anther culture studies (Metwally et al., 1998 a and b). 2 µM 2.4-D (Abdollahi et al., 2015) and 8 g / l agar were placed in standard MS medium and adjusted to pH 5.7. 90 g / l sucrose (Kumar et al., 2004; Rakha et al., 2012) was used as a carbon source in all mediums. BAP (1 and 1.5 µM doses) (Abdollahi et al., 2015) from the cytokinin hormone group, Spermidin (SPD) and Putresin (PUT) (500 and 1000 µM doses) were added to the nutrient media (Table 2).

Table 2. Mediums and nutrient concentrations (MS + 2 µm 2.4-D + 90 g / l sucrose, pH 5.7 standard)

Medium	**Growth Regulators**		
	BAP (mg/l)	SPD (µM)	PUT (µM)
1	1	-	-
2	1	500	-
3	1	-	500
4	1	500	500
5	1	1000	-
6	1	-	1000
7	1	1000	1000
8	1.5	-	-
9	1.5	500	-
10	1.5	-	500
11	1.5	500	500
12	1.5	1000	-
13	1.5	-	1000
14	1.5	1000	1000

The medium was placed in an autoclave at 121 °C under 1.2 atm pressure for 15 minutes sterilization. The mediums were cooled to 50-60 °C at room temperature and were dispensed into sterile petri dishes under aseptic conditions.

Collection of flower buds

In the first study, the flowers were collected one day before the anthesis and in the second one, flowers 2 days before the anthesis were collected by cutting off the pedicle in the early hours (Fig. 1).

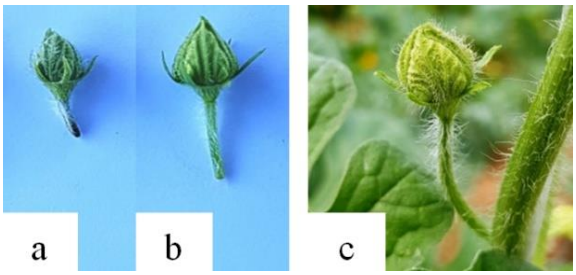

Fig. 1. Male flowers in different growth periods. (a) 2 days before the anthesis, (b-c) 1 day before the anthesis

In the first experiment, male flowers collected from the greenhouse on 22/05/2018 and were brought to the Tissue Culture Laboratory of Cukurova University Biotechnology Research and Application Center. In surface sterilization, the flowers were first kept under tap water for 30 minutes (Gürsoy et al., 2012). After this application, the anthers were taken to the sterile cabinet, kept in 70% ethyl alcohol solution for 2 minutes, and rinsed 3-4 times with sterile pure water (Tulukoğlu, 2014; Abdollahi et al., 2015). In the next step, the flower buds were incubated for 15 minutes in 15% sodium hypochlorite solution with 1-2 drops of Tween 20. Finally, the surface sterilization was completed after rinsing 3-4 times with sterile purified water.

In the second experiment, male flowers 2 days before the anthesis were collected in early hours from the greenhouse and were brought to the center on 07/06/2018. The same method was applied for surface sterilization.

Culturing of anther from embryo-stimulation medium

After the sterilization, the male flowers were dried on sterile filter paper, they were first separated from sepals and petals. Then the anther filaments were carefully removed, isolated anthers were separated and placed in petri dishes containing embryo-stimulation medium.

In this study, totally 1792 anthers were cultured (4 genotype x 2 anthesis stages x 14 different media combinations x 4 repetitions x 4 anthers in each petri dishes).

Pre-treatment

In the first experiment, cultured anthers were subjected to temperature pre-treatment in dark conditions for 2 days at 32 °C (Abdollahi et al., 2015). After the heat shock, the petri dishes containing the cultured anthers were taken into climatic chambers at a temperature of 25-26 °C, with a light intensity of 3000-4000 lux, having a light time of 16 hours and dark time of 8 hours and developments were observed.

Culturing callus developed from anther

Approximately 2-months-old embryonic calluses were sub-cultured with 1 anther in each petri dish containing 13.32 µM BAP and 0.54 µM NAA (Song et al., 2007) (Figure 2).

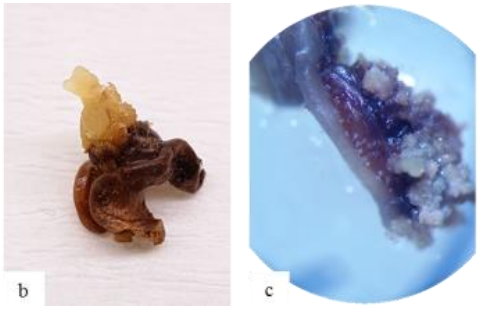

Figure 2. a, b) Development of embryonic calluses

Anther development was daily observed and callus formation (%) was calculated by the number of callus-forming anthers divided to the total number of cultured anthers.

RESULTS AND DISCUSSION

Results of first anther culture experiment

The highest callus formation was observed in the anthers belonging to the genotype of 'Halep Karası' with 81% rate from the control medium (1 µM BAP). When the embryo development is examined, development was seen only in medium 4 (1 µM BAP + 500 µM SPD + 500 µM PUT), 7 (1 µM BAP + 1000 µM SPD + 1000 µM PUT) and 12 (1.5 µM BAP + 1000 µM SPD). Embryo-like structures were observed but were not evaluated due to the lost of vitality.

When anther development data of 'Crimson Tide' were examined, 100% anther development was observed except medium 2 (1 µM BAP + 500 µM SPD). The highest rate of callus formation (31%) was obtained from the medium number 4 (1 BM BAP + 500 µM SPD + 500 µM PUT). Embryo development was not observed in this variety.

Anther growth rates showed differences according to the medium observations of 'Kar 234'. However, there have been no reports of both callus formation and embryo development.

According to the data of 'Kar 375', the highest rate (50%) of callus formation was determined in medium 8 (1.5 µM BAP)., however embryo development was not observed.

Results of second anther culture experiment

According to the results 'Halep Karası', the effect of media on callus formation was varied. While the highest callus formation was found in the medium 1 (control) with 93%, callus was not observed in medium 3 (1 µM BAP + 500 µM PUT). Although a large number of callus was formed, there was no conversion to the embryo-like structure.

The results showed that the highest values in callus formation were observed in medium 7 (1 µM BAP + 1000 µM SPD + 1000 µM PUT) and number 10 (1.5 µM BAP + 500 µM PUT) in 'Crimson Tide' variety. When embryo development was examined, only medium 8 (1.5 µM BAP) and 12 (1.5 µM BAP + 1000 µM SPD) were observed.

Anther growth was found to be highest in the 'Kar 234' genotype when compared with other genotypes. However, callus formation developed only in medium 9 (1.5 µM BAP + 500 µM SPD).

Anther development of the 'Kar 375' was found to be 90%. However, callus could not be obtained in the second anther culture experiment performed in this genotype.

Comparison of anther culture findings of first and second experiment

Two different experiments were established for anther culture. In the first study, the flowers one day before the anthesis and in the second one, flowers 2 days before the anthesis were collected. The effects of anthers, which were taken during different flowering periods, on embryogenesis were compared on the basis of genotype. As shown in Figure 3, the data of 'Halep Karası' showed that the callus formation was highest in medium 1 (1 µM BAP) for both experiments. In addition, callus formation in the medium number 3 (1 µM BAP + 500 µM PUT), 6 (1 µM BAP + 1000 µM PUT) and 13 (1.5 µM BAP + 1000 µM PUT) containing putrescine alone was decreased in second experiment.

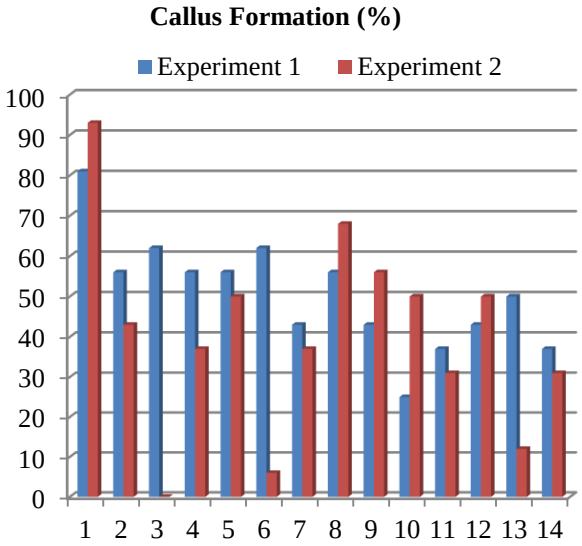

Fig. 3. Comparison of first and second anther culture experiment results of 'Halep Karası'

A high rate of callus formation was detected in Crimson Tide' in medium 4 (1 µM BAP + 500 µM SPD + 500 µM PUT), 7 (1 µM BAP + 1000 µM SPD + 1000 µM PUT) and 10 (1.5 µM BAP + 500 µM PUT) containing different doses of BAP, spermidine and putrescine (Figure 4).

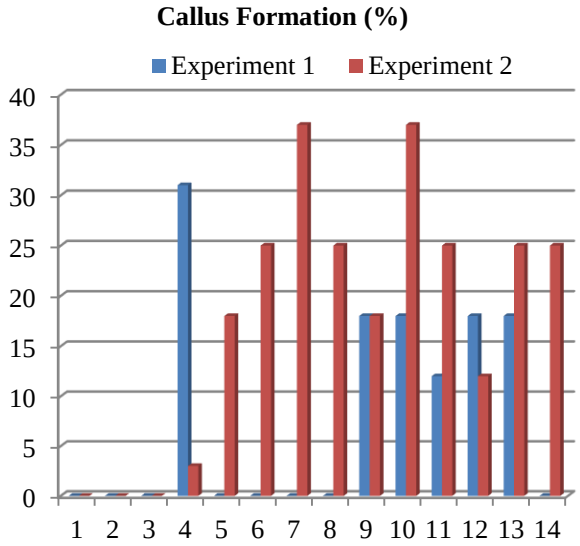

Fig. 4. Comparison of first and second anther culture experiment results of 'Crimson Tide'

According to the results of 'Kar 234', callus formation was observed only in the second experiment in the medium 9 (1.5 µM BAP + 500 µM SPD) (Figure 5). No positive response was obtained in both anther experiments performed on 'Kar 234'.

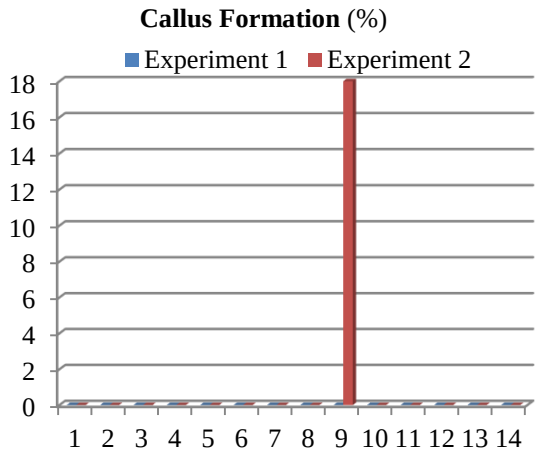

Fig. 5. Comparison of first and second anther culture experiment results of 'Kar 234'

In the data collected for the 'Kar 375', the highest rate of callus formation was found in medium 8 (1.5 µM BAP) of the first experiment (Figure 6). No results were obtained in the second study. The use of polyamine for this genotype did not have a positive effect.

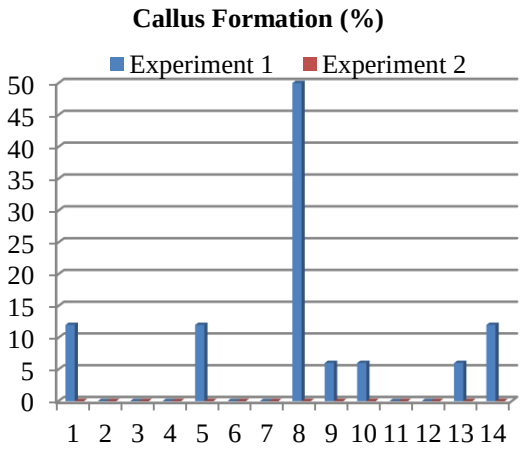

Fig. 6. Comparison of first and second anther culture experiment results of 'Kar 375'

Discussion

The nutrients used for haploid stimulation by anther culture method in watermelon and pre-temperature application were studied in parallel with each other. Therefore, the results obtained from the first and second experiments were evaluated together.

When the previous studies were examined, the number of anther culture methods in watermelon is limited (Xue et al., 1983; Zhang and Rhodes, 1992; Abdollahi et al., 2015). Due to this deficiency in the literature, the positive results of the recent research were tested in this study. In both experiments, 2.0 µM 2.4-D + 90 g / l sucrose + 8 g / l agar (Tulukoğlu, 2014) was added to MS medium as standard and 14 different media containing different doses of BAP, SPD and PUT were tested.

In the study of Kumar et al. (2003), it was reported that the usage of 2.0 µM 2.4-D + 1 µM BAP was the optimal concentration for embryogenic callus formation in cucumber. 2.0 µM 2.4-D was fixed in all mediums for our experiment.

It is reported that the usage of cytokine with auxin in some other species, except watermelon, increased callus formation (Bhaskaran and Smith, 1990; Peng and Wolyn, 1999; Zhao et al., 2006).

Kumar et al. (2004) found that a new type of plant growth regulator polyamines are considered as a key factor for organogenesis and embryogenesis. Erol (2018) was used spermidine and putrescine at 40-80-160-200 µM doses separately and together, and then examined the effect of these on ovule culture in cucumber plant. According to the results of study, it was observed that the development of ovules in polyamine-treated media was higher than the control. In our study, two of polyamines (spermidine and putrescine) was used. However, it was not found to have a positive effect on watermelon anther culture.

The usage of temperature pre-treatment and 2.4-D, BAP and kinetin (KIN) hormones in the base nutritional medium had a positive effect on embryogenesis. Besides, required concentrations for optimum development are depend on variety, pre-treatment, sterilization process and duration (Abdollahi et al., 2015). According to the results are obtained from this study, the positive effect of 2.4-D and BAP on callus formation was clearly seen in comparison with the literature.

Qin and Rotino (1993) reported that the combination of BAP and BAP with 2.4-D promotes embryogenesis in genotypes, especially that do not respond well to application, in anther culture applications. However, similar results were not found for both experiments conducted on the 'Kar 234' in the study.

According to the literature, it is known that the optimum concentration for androgenesis varies from species to species (Kasha et al., 1990). In this study, not only species but also genotype changes were observed.

Tulukoğlu (2014) examined the effect of buds collected in different flowering periods on anther culture from four different watermelon varieties According to the results, callus formation was higher in one-day-before anthesis flowers stage in ‘Halep Karası’, ‘Zeugma F1’ and ‘Starburst F1’ cultivars, whereas two-days-before anthesis stage was more successful in ‘Crimson Tide’. According to the data in our study, one-day-before anthesis stage was found successful for 'Halep Karası' and 'Kar 375' in callus formation and two-days-before anthesis stage was better for 'Crimson Tide' and 'Kar 234'. It is recommended that flowering period for anther culture is one-day-before anthesis in ‘Halep Karası’ and two-days-before anthesis in ‘Crimson Tide according to our experiments.

CONCLUSION

Watermelon is a species that great importance in breeding activities as well as production and consumption in our country and around the world. It is preferred by consumers because of its high nutritional value and its refreshing, aromatic structure.

Watermelon breeding has significant importance for commercial cultivation. Breeding methods are faster since pure-line is started to obtain. Pure lines are produced rapidly with the haploid plant obtained by using the androgenesis method, and hybrid breeding is made easier by haploidization, especially for institutions interested in this breeding. Insufficient studies on anther culture in watermelon makes the studies on this subject important.

In this study, the effect of buds collected at different flower development stages on anther culture was investigated. Anther culture method was used in two different *Citrullus lanatus* var. *lanatus* and 2 different *C. lanatus* var. *citroides* watermelon genotypes in order to obtain haploid plant.

In the first experiment callus formation rates were highest in the ‘Halep Karası’, which was collected 1 day before flowering, with 81% rate in the medium 1 (1 µM BAP) while no improvement was observed in the ‘Kar 234’ genotype.

Embryo-like structure development was observed in anthers belonging to the one-day-before anthesis period in medium 4 (1 µM BAP + 500 µM SPD + 500µM PUT), 7 (1 µM BAP + 1000µM SPD +

1000 µM PUT) and 12 (1.5 µM BAP + 1000µM SPD) on the genotype of 'Halep Karası'. When the one-day-before anthesis flowering period was examined, the embryo-like structure for 'Halep Karası' was developed in medium 8 (1.5 µM BAP) and in medium 8 (1.5 µM BAP) and 12 (1.5 µM BAP + 1000µM SPD) for the 'Crimson Tide'. However, these structures were lost their vitality 3-4 weeks after being sub-cultured and were not evaluated.

In this two-stage study, pre-heat treatment at 32 ° C for two days dark, combination of auxin-cytokinin hormone and polyamine usage were tested. However, pre-temperature treatment showed genotype-based effect. It was observed that 'Halep Karası' and 'Crimson Tide' were found to be high in rate of becoming dark, but not for the 'Kar 234' and 'Kar 375'.

High values in the one-day-before anthesis period were in the 'Kar 375', whereas the callus formation in the two-days-before anthesis period was better in the 'Crimson Tide'. The differences are thought to be caused by the genotype effect. Future researchers are advised to increase the number of different genotypes and then increase species diversity.

In literature, there is not enough information about anther culture in watermelon. However, it is recommended that positive results can be obtained if appropriate stress or developmental conditions are provided. When the results obtained from this and similar studies are evaluated, it is thought that testing different plant growth regulators and different stages of development about obtaining haploid plants in watermelon will become widespread.

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