Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2019) 43: 123-137
© TÜBİTAK
doi:10.3906/tar-1802-35

Effects of season, genotype, and nutrient medium on pepper anther culture and microspore development

Atilla ATA[1], Davut KELEŞ[1], Hatıra TAŞKIN[2,*], Saadet BÜYÜKALACA[2]

[1]Alata Horticultural Research Institute, Mersin, Turkey

[2]Department of Horticulture, Faculty of Agriculture, Cukurova University, Adana, Turkey

Received: 08.02.2018 • **Accepted/Published Online:** 20.09.2018 • **Final Version:** 01.04.2019

Abstract: Pure lines of 3 pepper genotypes, including one tolerant (Alata 421) and one intolerant (Alata 195) to lower temperatures and one intolerant (Alata 277A) to higher temperatures, together with one variety (Inan3363) tolerant to higher temperatures, were selected from the pepper collection of Alata Horticultural Research Institute (Mersin, Turkey). Two different culture media (Medium 1 - Murashige and Skoog [MS] + 4 mg L^{-1} naphthalene acetic acid [NAA] + 0.1 mg L^{-1} 6-benzylaminopurine [BAP] + 0.25% activated charcoal + 8 g L^{-1} agar + 30 g L^{-1} sucrose + 15 mg L^{-1} silver nitrate [$AgNO_3$]; Medium 2 - MS + 4 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 0.25% activated charcoal + 8 g L^{-1} agar + 30 g L^{-1} sucrose + 15 mg L^{-1} $AgNO_3$) were used in this study. The anthers were cultured during different periods (12 months) in order to determine the highest embryo formation and haploid plant regeneration. In addition, all anthers were taken before culturing and on the 1st, 2nd, 3rd, 4th, 8th, and 14th days of different months and were fixed with Carnoy's solution. Development of microspores was observed cytologically using 4'-6-diamidino-2-phenylindole-2HCl (DAPI) and with acetocarmine stain. At the end of the study, it was determined that both embryo formation and haploid plant development varied depending on genotype and cultivation period. No significant differences in the number of embryos obtained were observed between the nutrient media. The highest percentage of haploid embryos was obtained from the Inan3363 variety, known to be tolerant to high temperature. In addition, the androgenic capacity of the Inan3363 variety increased to 65% during some anther culture cultivation periods. The anthers cultured in September and July–August produced the highest yielding results compared with the other periods in terms of number of haploid embryos. Obtaining healthy and developed plants from the embryos was more successful in April, August, September, March, and May than in the other months. Studies on the microspores revealed that the high percentage of uninucleate phase at the beginning of the culture decreased rapidly in the following days.

Key words: Androgenesis, *Capsicum annuum* L., genotype effect, microspore development, plant growth regulators

1. Introduction

Studies on pepper anther culture in Turkey were initiated by Abak (1983a, 1983b, 1986). The effects of growth regulators such as kinetin, 2,4-dichlorophenoxyacetic acid (2,4-D), sucrose, and iron components added to the nutrient medium for anther culture of local pepper materials were studied, together with determination of the proper flower bud size, in these studies. $AgNO_3$'s effect was tested in Şanlıurfa and Kahramanmaraş pepper populations by the same researchers. Effects of two different incubation conditions (keeping them at 35 °C for 8 days, then transferring to 29 °C with a 16/8-h light/dark photoperiod, followed by incubation at 29 °C in continuous light) on haploid embryo regeneration in the local Kahramanmaraş pepper population were investigated by Terzioğlu et al. (2000). In a study conducted by Ercan et al. (2001), 11 different nutrient media containing BA, NAA, 2,4-D, and activated charcoal were tested for 5 different pepper varieties in terms of embryo regeneration. Özkum Çiner and Tıpırdamaz (2002) tested effects of cold pretreatments (keeping buds at 4 °C for 48 and 96 h) and adding 0.25% activated charcoal to the nutrient medium. The researchers also observed the microspore development stage using paraffin and acetocarmine crushing methods. The effects of different doses of NAA, 2,4-D, BAP, kinetin, 0.25% activated charcoal, and 10 mg L^{-1} $AgNO_3$ supplemented to MS medium and pretreatments at 4 °C, 29 °C, and 35 °C in the dark on pepper anther cultures were studied by Çağlar et al. (2004). The influences of the growing conditions of donor plants (greenhouse and open field) and $AgNO_3$ (5, 10, 15, 20 mg L^{-1}) on anther culture of pepper were determined by Buyukalaca et al. (2004). In a study carried out by Sayılır and Özzambak (2005), 6 culture media containing different combinations of 4 mg L^{-1} NAA,

* Correspondence: hatirataskin1@gmail.com

This work is licensed under a Creative Commons Attribution 4.0 International License.

0.1 mg L^{-1} BA, activated charcoal, and carrot extract, as well as 3 flower bud sizes, were examined in 6 pepper varieties. Effects of growing season, age of donor plants, and genotypes on embryo regeneration were examined by Ercan et al. (2006) and Ercan and Ayar Şensoy (2011). Using 5 different pepper genotypes, effects of 4 different nutrient media, different culture periods, and abscisic acid (ABA) were investigated by Taşkin et al. (2011). A review was published recently about pepper anther culture studies in Turkey (Çömlekçioğlu and Ellialtıoğlu, 2018).

Studies on anther culture of the genus *Capsicum* in other parts of the world were initiated by Wang et al. (1973), George and Narayanaswamy (1973), Novak (1974), Harn et al. (1975), and Dumas de Vaulx et al. (1981). Since then, many researchers have focused on this subject. Effects of activated charcoal and FeEDTA supplemented to MS medium on pepper anther culture were investigated by Vagera and Havranek (1985). Genotype effect on anther culture was evaluated by Morison et al. (1986), Mityko et al. (1995), Rodeva et al. (2004), Irikova et al. (2011), and Niklas-Nowak et al. (2012). Kristiansen and Andersen (1993) determined the effects on anther culture of the age of donor plants, temperature (22 °C, 26 °C, and 30 °C), and photoperiod during incubation. In a study conducted by Matsubara et al. (1998), different culture periods for pepper anthers were studied. Different durations of colchicine application (culturing in nutrient medium containing 0.04% colchicine for 2, 4, and 6 days) were tested by Gemesne Juhasz et al. (2001). Different nutrient media and incubation conditions were studied by Koleva-Gudeva et al. (2007). Influences of different carbohydrate sources (sucrose and maltose) and growth regulators (2,4-D, NAA, zeatin, kinetin, and 6-BA) on pepper anther culture were investigated by Zhao et al. (2010). Olszewska et al. (2014) determined effects of genotype, 2 different kinetin doses (0.1 and 0.3 mg L^{-1}) added to CP nutrient medium, and 3 different incubation durations (culturing 12, 14, and 16 days in CP medium) on anther culture.

Anther culture technique has been commonly used in breeding studies of pepper, and thereby new varieties have been developed. However, embryo and plant regeneration are influenced by different factors such as genotype, growing conditions of donor plants, nutrient medium, and the culture period; therefore, a high percentage of embryos cannot be guaranteed in some genotypes. The aims of this study can be summarized as follows: (i) to determine embryo and plant regeneration percentage of pepper genotypes requiring different climate conditions (both sensitive and tolerant to low and high temperatures) for every month during a 1-year period in two different nutrient media; (ii) to reveal the existence of a relationship between different climatic conditions and embryo-plant regeneration; and (iii) to examine microspore development.

2. Materials and methods

2.1. Anther culture studies

In this study, genotypes Alata 421, 195, and 277A and the Inan3363 variety were used as plant material (Figure 1). Previous studies showed that genotypes 421 and 195 were tolerant and sensitive to low temperatures, respectively.

Figure 1. The fruits of the pepper genotypes and variety used in this study: a) genotype 277A, b) genotype 195, c) genotype 421, d) Inan3363 variety.

Genotype 277A and variety Inan3363 were identified as sensitive and tolerant to high temperatures, respectively. All the plant materials were obtained from the pepper breeding projects of Alata Horticultural Research Institute (Mersin, Turkey). In a study carried out by Buyukalaca et al. (2004), embryo stimulation of donor plants cultured in a greenhouse was found to be higher than in those cultured in an open field. Therefore, the pepper plants used in this study were cultured in a greenhouse. Seeds were planted into plugs containing peat : perlite (2 w/1 w) to obtain seedlings, and the seedlings were then transferred to 50 × 100 cm spaces in the greenhouse. While pepper seedlings planted at the beginning of September were used during the anther culture period from October to April, new seedlings planted at the beginning of April were used for the other months (May, June, July, August, and September). The pepper seedlings planted at the beginning of September and April were respectively used during the anther culture period from October to April and during other months. Normal horticultural cultivation practices were implemented throughout the growing period. The late uninucleate or early binucleate phase (the beginning of the first mitotic division) is the best anther stage for culturing. According to Buyukalaca et al. (2004), the length of the corolla should be equal to that of the calyx or slightly longer, and almost half of the anthers have anthocyanin at this phase. Therefore, the same morphological markers were used in this study. However, proper phase was controlled by staining with acetocarmine at the beginning of the study. Flower buds were sterilized using 15% sodium hypochlorite solution including 1 or 2 drops of Tween 20 for 15 min and then rinsed 5 times using sterile distilled water in the sterile bench. After sterilization, the flower buds were dissected, the filaments were removed, and the anthers were placed on nutrient medium with sterile forceps and scalpels. The cultured anthers were incubated at 35 °C in the dark for the first 2 days, and then they were transferred to the growth chamber at 25 °C with an 8-h dark and 16-h light photoperiod. Two different MS nutrient media combinations containing 2 different BAP doses were tested in this study:

Medium 1: MS + 4 mg L^{-1} NAA + 0.1 mg L^{-1} BAP + 0.25% activated charcoal + 8 g L^{-1} agar + 30 g L^{-1} sucrose + 15 mg L^{-1} $AgNO_3$;

Medium 2: MS + 4 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 0.25% activated charcoal + 8 g L^{-1} agar + 30 g L^{-1} sucrose + 15 mg L^{-1} $AgNO_3$.

Hormone-free MS medium was used for regeneration of plants from embryos (Figure 2). Five anthers taken from each flower bud were placed into the same glass petri dish. This study was designed with 5 replicates, with 25 anthers in each replicate. During the experiments, totals for anthers cultured, embryos obtained, embryos per 100 anthers, anthers producing embryos, anthers producing embryos per 100 anthers, embryos producing plants, and plants were recorded. Ploidy level of plants was detected using a flow cytometer (Partec GmBH) as described by Keleş et al. (2015).

2.2. Microspore development studies

Anthers were fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) before the culture and on the 1st, 2nd, 3rd, 4th, 8th, and 14th days of different months to determine the microspore development of cultured anthers. The nucleus of the pepper cell is invisible due to the fact that pepper anthers have structures that prevent the transmittance of light. In order to break this structure, 40% ferric chloride distilled with water was prepared, and 300 µL of this mixture was added to 10 µL of Carnoy fixation solution. The anthers were immersed in this solution for 30 min; 80 µL of 40% ferric chloride solution was again added, and the anthers were incubated for 1 day (Kim and Jang, 2000). After the application, the anthers were maintained in 70% ethanol until stained with DAPI. One microliter of DAPI stock solution was diluted with 1 mL of water. The fixed pepper anthers were crushed on glass slides with forceps and a scalpel, and DAPI solution was added to the anthers. The anthers were then closed with coverslips to determine the development stages of the microspores. Microspore developmental stages were examined with a fluorescence microscope. Considering the time requirements of this process, microspore developmental stage was also determined by light microscope using the acetocarmine staining method. For this purpose, a mixture containing 45% glacial acetic acid, 55% distilled water, and 2 g of carmine red was prepared and heated. Following the filtration of the solution 2 times, an acetocarmine solution was obtained. The prepared acetocarmine was dropped onto the pepper anthers placed on glass slides. The anthers were crushed and closed with the coverslips. After 3 min, the anthers were examined by light microscope. Four anthers were used for each application and 5 observations were performed per anther. Microspore development was calculated by the ratio of microspore numbers in the uninucleate, binucleate, and multinucleate stages with total microspore numbers in the same anther.

3. Results

In this study, the effects of two nutrient media and different seasons on pepper anther culture of three pepper genotypes and one commercial variety were investigated. For this aim, the experiments were repeated with two nutrient media and four types of pepper every month over the course of 1 year. Development of microspores was

Figure 2. Different in vitro steps in the anther culture of pepper from culturing anthers in petri dishes to obtaining plants: a) plant formation from the pepper embryo in the petri dish, b) in vitro pepper plants developed from embryos, c) acclimatization of in vitro pepper plants in the plug, d) acclimatized in vitro pepper plants.

also observed using the method of fixation of anthers in Carnoy solution.

The responses of genotypes to anther culture in two different nutrient media and different seasons are given in Table 1 in detail. The main outcome of the study is discussed here in terms of the success of genotypes, seasons, and nutrient media. Considering the effect of the seasons, the highest results were obtained in September, with 21.88% embryos per 100 anthers, followed by July and August, both of which had the same value (17.60%). The lowest results were recorded in February, June, and November at 0.66%, 3.15%, and 4.85%, respectively. The data obtained from the other months were as follows: 12.39% in October, 5.44% in December, 6.44% in January, 7.17% in March, 5.33% in April, and 14.52% in May.

In terms of nutrient media, there are no considerable differences between the results. While the percentage of embryos per 100 anthers was found to be 10.05% in Medium 1, it was recorded as 9.03% in Medium 2.

When the results are discussed in terms of genotypes, the most positive response was obtained from the Inan3363 variety, with 22.14% embryos per 100 anthers. This was followed by genotype 277A with 10.01%. The lowest response was observed in genotype 421, with 1.40%

embryos per 100 anthers. The response of genotype 195 was between those of genotypes 277A and 421, with a percentage of 4.29%.

When the acclimatized plants are considered, the greatest number was obtained in April with 41 acclimatized plants (from 61 total plants obtained). This was followed by August (25 out of 56), September (23 out of 33), March (19 out of 38), and May (19 out of 36). When the genotypes were considered, the best results in terms of acclimatized plants were obtained from the Inan3363 variety (97 out of 172 plants) and 277A (39 out of 71 plants). Although similar results were recorded for both nutrient media tested (84 and 82 plants acclimatized from 158 and 137 of the plants obtained, and 10.05% and 9.03% embryos per 100 anthers for Medium 1 and Medium 2, respectively), both were found to be more successful in some cases. For example, while the embryo induction ratio per 100 anthers of the Inan3363 variety was 66.36% using Medium 2 in August, it decreased to 27.59% with Medium 1. Contrary to August, in September, these percentages for 277A were 56.67% and 3.33% using Medium 1 and Medium 2, respectively (Table 1).

The late uninucleate or early binucleate phase is the best stage for anther culture. Therefore, in this study, microspore development stage was observed both in different months and in the nutrient media before culturing and at the 1st, 2nd, 3rd, 4th, 8th, and 14th days of culture (Figure 3). In genotype 277A, while the percentage in the uninucleate stage varied between 98.97% and 100% before culturing in February, May, August, and October, it decreased in the following days with both nutrient media used (Table 2). The experiments were performed in May, August, and October for the Inan3363 variety (Table 3) and similar results were obtained. In this variety, the decrease in the percentage observed in the first and following days was slower in August and October. The uninucleate percentage of genotype 421 was found to be 93.59%–99.11% before culture in February, May, August, and October, and the percentage decreased rapidly in the following days (Table 4). In May, August, and October, the percentage of genotype 195 was 98.42%–99.55%, with a slower decrease (Table 5).

4. Discussion

The genotype effect on anther culture has already been described in other works (Morison et al., 1986; Mityko et al., 1995; Rodeva et al., 2004; Ercan et al., 2006; Ercan and Ayar Şensoy, 2011; Irikova et al., 2011; Taşkin et al., 2011; Niklas-Nowak et al., 2012; Olszewska et al., 2014; Arı et al., 2016; Ozsan and Onus, 2017). This study also revealed the genotype effect clearly. As stated in Section 3, the most positive responses were obtained from the Inan3363 variety. This variety was reported to be tolerant of high temperatures as a result of a breeding study carried out by the Alata Horticultural Institute (Erdemli, Mersin Province, Turkey). Therefore, obtaining successful results from this variety can be explained with two hypotheses: the first one is establishment of the experiments in Erdemli, which has a Mediterranean climate (high temperature and humidity). Obtaining positive results from the Inan3363 variety in August and September, which are known to be the hottest months in this province, may be proof for this hypothesis. The second one is the high androgenetic capacity of this variety. This result for the Inan3363 variety, which is tolerant of high temperatures, is of high importance for future breeding studies.

The Inan3363 variety was followed by genotype 277A, which has been identified as sensitive to high temperatures. The lowest results were obtained from genotypes 421 and 195, which are identified as tolerant and sensitive to low temperatures, respectively. However, genotype 195, which originated in India, had better performance in the summer season. This can be explained by the stimulation of embryo formation as a consequence of heat shock protein accumulation. Jan et al. (1992) reported that temperature stress alone is responsible for the induction of embryogenic development and causes an altered pattern of cell division; there may be direct involvement of Hsp 70 (heat shock proteins) in this process. Better results being obtained in December from genotype 421, which is tolerant to low temperatures, is another important finding.

When the genotype effect was evaluated in terms of months, Inan3363 was determined to be the most embryogenic genotype in October, November, March, May, June, August, and September, while 277A was the best in February and April, 421 in December, and 195 in July. Genotype 277A and variety Inan3363 produced similar results in January. Similar results were also reported in a study carried out by Ercan et al. (2006), in which the Kekova variety produced the highest results in summer, while the Sera Demre 8 variety was found to be more productive in winter. Five pepper genotypes having different responses to low temperatures were tested for their androgenesis capacity in different culturing periods (per month over 1 year) by Taşkin et al. (2011). The most promising results were obtained from the anthers cultured in April, May, and June. Grozeva et al. (2013) investigated the effects of temperature and growth period of donor plants on anther culture of 9 pepper hybrids and varieties. September was found to be the most successful period in their study, and 17.5–22.2 °C was recorded as the optimal temperature range for embryo induction. These findings clearly show that the response of genotypes to anther culture can vary depending on culture periods. It is known

Table 1. Pepper anther culture efficiency depends on different genotypes, periods, and nutrient media tested.

G	M	TAN	EN	EN/100A	EPAN	EPAN/100A	PPEN	APN
October								
277A	1	125	13	10.40	7	5.60	2	1
	2	170	37	21.76	14	8.23	1	1
I3363	1	138	47	34.06	24	17.39	5	4
	2	125	40	32.00	23	18.40	3	2
421	1	125	0	0.00	0	0.00	0	0
	2	125	1	0.80	1	0.80	0	0
195	1	180	0	0.00	0	0.00	0	0
	2	126	0	0.00	0	0.00	0	0
November								
277A	1	170	16	9.41	10	5.88	6	4
	2	153	4	2.61	4	2.61	1	1
I3363	1	150	17	11.33	13	8.67	0	0
	2	148	29	19.59	12	8.11	0	0
421	1	204	0	0.00	0	0.00	0	0
	2	225	0	0.00	0	0.00	0	0
195	1	150	0	0.00	0	0.00	0	0
	2	160	0	0.00	0	0.00	0	0
December								
277A	1	201	21	10.44	14	6.97	5	2
	2	131	0	0.00	0	0.00	0	0
I3363	1	126	5	3.97	4	3.17	3	0
	2	127	6	4.72	5	3.94	0	0
421	1	200	22	11.00	17	8.50	0	0
	2	155	11	7.10	10	6.45	0	0
195	1	130	0	0.00	0	0.00	0	0
	2	124	0	0.00	0	0.00	0	0
January								
277A	1	130	32	24.62	17	13.08	14	8
	2	125	3	2.40	3	2.40	3	2
I3363	1	130	15	11.54	14	10.77	7	3
	2	165	20	12.12	13	7.88	0	0
421	1	156	0	0.00	0	0.00	0	0
	2	131	0	0.00	0	0.00	0	0
195	1	125	0	0.00	0	0.00	0	0
	2	125	0	0.00	0	0.00	0	0

Table 1. (Continued).

February								
277A	1	125	3	2.40	3	2.40	3	0
	2	125	3	2.40	2	1.60	0	0
I3363	1	135	0	0.00	0	0.00	0	0
	2	152	1	0.67	1	0.67	0	0
421	1	112	0	0.00	0	0.00	0	0
	2	134	0	0.00	0	0.00	0	0
195	1	130	0	0.00	0	0.00	0	0
	2	142	0	0.00	0	0.00	0	0
March								
277A	1	125	9	7.20	3	2.40	4	2
	2	130	0	0.00	0	0.00	0	0
I3363	1	136	21	15.44	8	5.88	13	9
	2	135	35	25.93	14	10.37	15	5
421	1	140	0	0.00	0	0.00	0	0
	2	135	3	2.22	1	0.74	0	0
195	1	135	5	3.70	3	2.22	3	2
	2	124	3	2.42	2	1.61	3	1
April								
277A	1	125	26	20.80	18	14.40	20	13
	2	142	13	9.15	10	7.04	4	2
I3363	1	158	10	6.33	8	5.06	10	8
	2	149	0	0.00	0	0.00	7	6
421	1	135	0	0.00	13	9.63	4	3
	2	130	4	3.08	3	2.31	7	5
195	1	155	7	4.52	8	5.16	5	2
	2	132	0	0.00	7	5.30	4	2
May								
277A	1	150	0	0.00	0	0.00	0	0
	2	149	9	6.04	5	3.36	2	1
I3363	1	175	79	45.14	35	20.00	18	8
	2	125	79	63.20	38	30.40	16	10
421	1	125	0	0.00	0	0.00	0	0
	2	125	0	0.00	0	0.00	0	0
195	1	150	0	0.00	0	0.00	0	0
	2	151	0	0.00	0	0.00	0	0

Table 1. (Continued).

June								
277A	1	150	10	6.67	8	5.33	3	0
	2	125	1	0.80	1	0.80	0	0
I3363	1	175	9	5.14	6	3.43	0	0
	2	125	11	8.80	7	5.60	2	0
421	1	150	0	0.00	0	0.00	0	0
	2	175	0	0.00	0	0.00	0	0
195	1	125	4	3.20	3	2.40	0	0
	2	150	2	1.33	0	0.00	2	1
July								
277A	1	150	32	21.33	9	0.00	0	0
	2	160	27	16.88	10	0.00	0	0
I3363	1	136	19	13.74	16	11.65	0	0
	2	150	16	10.87	14	9.49	3	3
421	1	125	0	0.00	0	0.00	0	0
	2	125	0	0.00	0	0.00	0	0
195	1	150	29	19.33	9	6.00	3	3
	2	135	46	34.67	15	10.93	5	4
August								
277A	1	160	4	2.48	4	2.48	1	1
	2	125	6	5.00	4	3.40	2	1
I3363	1	195	52	27.59	40	20.95	16	6
	2	182	119	66.36	73	40.79	27	10
421	1	125	1	0.83	1	0.83	0	0
	2	125	0	0.00	0	0.00	0	0
195	1	145	13	8.67	6	4.00	1	0
	2	125	13	10.40	7	5.60	9	7
September								
277A	1	120	68	56.67	31	25.83	0	0
	2	150	5	3.33	4	2.67	0	0
I3363	1	155	89	57.42	38	24.52	6	5
	2	140	63	45.00	24	17.14	21	18
421	1	151	6	3.97	4	2.65	6	0
	2	155	1	0.65	1	0.65	0	0
195	1	145	20	13.79	8	5.52	0	0
	2	145	2	1.38	3	2.07	0	0

G: Genotype, M: medium, TAN: total number of anthers cultured, EN: number of embryos obtained, EN/100 A: number of embryos/100 anthers (%), EPAN: number of anthers producing embryos, EPAN/100 A: number of anthers producing embryos/100 anthers (%), PPEN: number of embryos producing plants, PN: number of acclimatized plants.

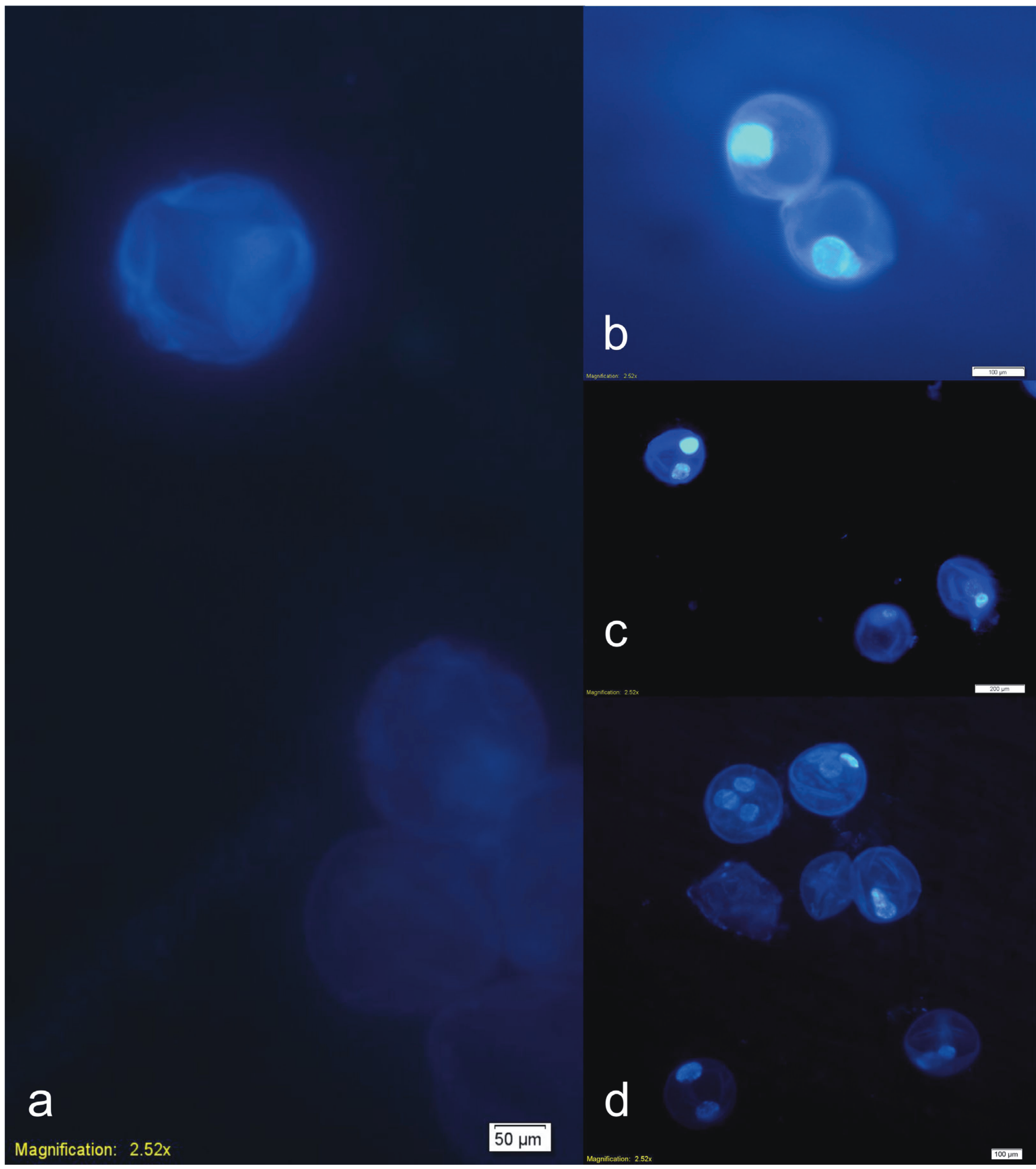

Figure 3. Microspore development stage of pepper observed: a) nucleus degeneration, b) uninucleate phase, c) binucleate phase, d) multinucleate phase.

Table 2. The percentage of microspore development of pepper genotype 277A in different nutrient media throughout the study period.

		Medium					
		Medium 1			Medium 2		
Month	Day	Empty	Uni	Bi	Empty	Uni	Bi
Feb	0	0.49	98.97	0.00	0.49	98.97	0.00
	1	55.33	44.67	3.52	85.72	14.28	3.63
	2	84.89	15.11	2.61	72.09	27.91	7.23
	3	92.52	7.48	0.72	93.62	6.38	0.86
	4	92.64	7.36	2.55	97.02	2.98	0.42
	8	96.04	3.96	0.40	96.19	3.81	1.06
	14	96.12	3.48	2.04	97.75	2.25	0.00
May	0	0.00	100	0.00	0.00	100	0.00
	1	38.14	58.27	3.59	26.38	65.04	8.58
	2	60.28	27.97	11.75	59.10	33.95	6.95
	3	72.24	20.79	6.97	80.08	18.64	1.28
	4	88.21	9.63	2.16	84.26	12.72	2.58
	8	96.41	3.59	0.00	98.65	1.35	0.00
	14	98.93	0.86	0.20	97.19	1.77	1.04
Aug	0	0.00	100	0.00	0.00	100	0.00
	1	55.21	38.82	5.96	5.94	26.26	60.05
	2	71.74	25.30	2.96	0.00	0.00	0.00
	3	70.56	23.58	5.87	71.75	23.90	4.35
	4	82.32	15.95	1.72	92.03	7.37	0.60
	8	97.21	2.79	0.00	95.38	4.25	0.38
	14	97.38	2.01	0.61	98.22	1.57	0.21
Oct	0	0.00	100	0.00	0.00	100	0.00
	1	64.72	32.60	2.69	62.52	33.52	3.96
	2	75.40	21.16	3.44	56.72	33.19	10.09
	3	73.69	17.65	8.66	74.75	22.23	3.02
	4	86.48	12.16	1.36	92.46	6.66	0.88
	8	94.46	5.54	0.00	96.76	3.24	0.00
	14	0.00	0.00	0.00	95.60	4.09	0.31

Uni: Uninucleate phase (%) Bi: binucleate phase (%).

that air temperature in the period when donor plants are grown affects the performance of the anther culture. Therefore, androgenesis capacity of a genotype cannot be determined alone by culturing it in a specified growing time.

One of the important findings of this study was obtaining a high percentage of haploid embryos (65%) with the combination of August–Medium 2–Inan3363 variety. Such a high rate has not been obtained previously in our studies on anther culturing of pepper (Çömlekçioğlu et al.,

Table 3. The percentage of microspore development of pepper variety Inan3363 in different nutrient media throughout the study period.

		Medium					
		Medium 1			Medium 2		
Month	Day	Empty	Uni	Bi	Empty	Uni	Bi
May	0	0.51	99.49	0.00	0.51	99.49	0.00
	1	55.34	39.98	4.68	50.5	39.75	9.75
	2	54.94	31.86	13.21	71.93	23.19	4.88
	3	69.56	25.93	4.51	82.29	14.53	3.18
	4	79.96	14.10	5.95	68.72	23.85	7.44
	8	94.87	4.01	1.12	92.43	5.41	2.16
	14	93.85	6.15	0.00	0.00	0.00	0.00
Aug	0	8.76	89.54	1.71	8.76	89.54	1.71
	1	36.68	59.94	3.38	16.08	81.05	2.87
	2	64.77	26.62	8.61	64.39	30.26	5.36
	3	69.84	16.79	13.38	91.70	7.66	0.64
	4	62.69	22.64	14.07	61.56	28.57	9.87
	8	0.00	0.00	0.00	71.83	18.23	9.94
	14	49.32	37.86	12.81	77.47	18.63	3.45
Oct	0	3.53	96.47	0.00	3.53	96.47	0.00
	1	24.40	70.49	5.12	15.04	77.81	7.15
	2	54.95	30.33	14.72	57.38	33.41	9.21
	3	70.63	22.79	6.58	48.77	36.88	14.36
	4	42.21	39.62	18.17	60.13	31.47	8.40
	8	79.19	17.19	3.62	29.76	49.85	20.39
	14	57.68	33.11	9.21	93.84	4.69	1.47

2001; Buyukalaca et al., 2004; Taşkin et al., 2011; Al Remi et al., 2014). The results of various studies carried out by different researchers throughout the world are also not as high, according to our knowledge to date.

It was observed that microspores divided to form double their number and more nuclei, or died due to nucleation caused by culture conditions (Kim et al., 2004; Gonzalez and Jouve, 2005). It is known that the dead pollen cells increase and division begins from the first day of culture (Kim et al., 2004; Gonzalez and Jouve, 2005). From the fourth day of culture, microspores having 3 and 4 nuclei were observed even if they were less numerous. Symmetric and asymmetric divided multinucleate structures on the fourth day of the culture were reported by Kim et al. (2004). It is known that the embryo is formed with symmetrical (division of both vegetative and generative nuclei) and asymmetrical division of the microspores placed in nutrient media for anther or microspore culture. In this study, it was found that the asymmetric division ratio was greater than the symmetric division ratio. Embryo and microspore-derived callus formation by asymmetric division has been reported in maize (Pretova et al., 1993) and pepper (Kim et al., 2004). It was reported by Kim et al. (2004) that symmetrical division was observed in pepper; however, no embryos formed as a result of this division.

In conclusion, in this study, androgenesis efficiency of three pepper genotypes and one pepper variety that reacted differently to low and high temperatures was investigated in two different nutrient media and in different cultivation periods over the course of 1 year. At the end of the study,

Table 4. The percentage of microspore development of pepper genotype 421 in different nutrient media throughout the study period.

		Medium					
		Medium 1			Medium 2		
Month	Day	Empty	Uni	Bi	Empty	Uni	Bi
Feb	0	0.89	99.11	0.66	0.89	99.11	0.66
	1	65.95	34.05	0.00	0.00	0.00	0.00
	2	76.58	23.42	1.76	83.38	16.62	1.56
	3	88.39	11.61	0.76	89.47	10.53	1.15
	4	95.90	4.10	0.00	100	0.00	0.00
	8	100	0.00	0.00	100	0.00	0.00
	14	99.10	0.90	0.00	95.11	4.42	1.69
May	0	0.00	93.59	4.86	0.00	93.59	4.86
	1	67.85	31.74	0.41	80.92	18.20	0.88
	2	88.51	11.05	0.44	85.09	14.65	0.27
	3	83.63	16.37	0.00	89.99	9.69	0.32
	4	93.64	5.47	0.83	95.37	4.33	0.30
	8	93.99	5.30	0.72	98.95	1.05	0.00
	14	99.19	0.68	0.07	99.47	0.26	0.07
Aug	0	0.92	98.09	0.99	0.92	98.09	0.99
	1	71.09	27.47	1.44	60.64	37.22	2.14
	2	69.44	26.11	4.44	72.52	22.17	4.88
	3	60.22	34.35	5.43	82.03	16.40	1.57
	4	85.22	12.91	1.87	80.44	14.64	4.84
	8	84.69	10.24	4.96	97.19	2.48	0.00
	14	92.57	7.07	0.27	93.91	4.26	1.50
Oct	0	4.46	95.54	0.00	4.46	95.54	0.00
	1	45.57	47.68	6.75	67.61	32.39	0.00
	2	85.78	14.22	0.00	85.80	13.19	1.01
	3	0.00	0.00	0.00	91.66	8.34	0.00
	4	87.89	11.72	0.38	0.00	0.00	0.00
	8	86.10	11.46	2.44	0.00	0.00	0.00
	14	100	0.00	0.00	100	0.00	0.00

September was determined to be the most successful month, followed by July and August, for the percentage of embryos per 100 anthers. Among genotypes, the Inan3363 variety gave the best results, while 421 was the weakest for the percentage of embryos per 100 anthers. In terms of acclimatized plants, similar results were obtained from different genotypes and seasons. However, the genotypes having different reactions to high and low temperatures produced variable results in different cultivation periods. In terms of nutrient media, there were no significant differences in the results. However, each was more successful in some periods and genotypes. This study

Table 5. The percentage of microspore development of pepper genotype 195 in different nutrient media throughout the study period.

		Medium					
		Medium 1			Medium 2		
Month	Day	Empty	Uni	Bi	Empty	Uni	Bi
May	0	0.45	99.55	0.00	0.45	99.55	0.00
	1	45.95	42.93	11.12	46.32	47.14	6.53
	2	73.80	19.86	6.34	54.82	29.86	15.32
	3	91.80	7.63	0.57	78.11	15.87	6.02
	4	90.35	8.23	1.42	79.27	16.57	4.16
	8	66.91	16.04	17.05	53.77	18.06	27.38
	14	77.22	7.99	13.27	73.76	7.98	15.04
Aug	0	1.24	98.76	0.00	1.24	98.76	0.00
	1	11.22	78.30	10.49	39.06	55.21	5.73
	2	30.38	56.85	12.76	44.84	39.94	15.22
	3	73.24	19.15	7.61	65.02	28.05	6.92
	4	49.50	36.00	14.15	36.88	47.45	15.67
	8	21.73	48.83	29.21	0.00	0.00	0.00
	14	64.12	25.52	10.36	20.79	50.35	27.36
Oct	0	1.58	98.42	0.00	1.58	98.42	0.00
	1	32.51	63.75	3.74	46.68	50.06	3.26
	2	52.52	35.50	11.98	68.55	30.15	1.30
	3	70.15	25.97	3.88	88.97	10.38	0.65
	4	86.39	13.61	0.00	88.00	12.00	0.00
	8	98.58	1.42	0.00	99.79	0.21	0.00
	14	89.34	10.05	0.61	100.00	0.00	0.00

has clearly showed the necessity for optimization of the method used for each genotype in anther culturing of pepper. In addition to this knowledge, determination of the anther culture performances of the breeding genotypes used in this study is very important for future studies aimed at development of varieties having tolerance to low or high temperatures.

Acknowledgments
The authors would like to thank the General Directorate of Agricultural Research and Policies of the Republic of Turkey Ministry of Food, Agriculture, and Livestock, and the Alata Horticultural Research Institute for supporting this research.

References

Abak K (1983a). Biberde (*Capsicum annuum* L.) anter kültürü yoluyla haploid bitki elde etme üzerinde araştırma. Ankara Üniversitesi Ziraat Fakültesi Yıllığı 33: 155-163 (in Turkish).

Abak K (1983b). Study on the anther culture in vitro of pepper (*Capsicum annuum*). Capsicum Newsletter 2: 72-73.

Abak K (1986). Biber ıslahında anter kültüründen yararlanma. In: TÜBİTAK Bitki Islahı Sempozyumu, 15–17 October 1986, İzmir, Turkey (in Turkish).

Al Remi F, Taşkin H, Sönmez K, Büyükalaca S, Ellialtioğlu Ş (2014). Effect of genotype and nutrient medium on anther culture of pepper (*Capsicum annuum* L). Turkish Journal of Agricultural and Natural Sciences 1: 108-116.

Arı E, Yıldırım T, Mutlu N, Büyükalaca S, Gökmen Ü, Akman E (2016). Comparison of different androgenesis protocols for doubled haploid plant production in ornamental pepper (*Capsicum annuum* L.). Turk J Biol 40: 944-954.

Buyukalaca S, Comlekcioglu N, Abak K, Ekbic E, Kilic N (2004). Effect of silver nitrate and donor plant growing conditions on production of pepper (*Capsicum annuum* L.) haploid embryos via anther culture. Eur J Hortic Sci 69: 206-209.

Çağlar Ç, Aras V, Bayram A (2004). Kurutmalık kırmızı biberlerde androgenesis yoluyla in vitro haploid embriyo uyartımı. Akdeniz Üniversitesi Ziraat Fakültesi Dergisi 17: 87-94 (in Turkish).

Çömlekçioğlu N, Büyükalaca S, Abak K (2001). Effect of silver nitrate on haploid embryo induction by anther culture in pepper (*Capsicum annuum*). In: Abak K, Büyükalaca S, Daşgan Y, editors. Proceedings of the 11th EUCARPIA Meeting on Genetics and Breeding of Capsicum & Eggplant, 9–13 April 2001, Antalya, Turkey. Adana, Turkey: EUCARPIA, pp. 133-135.

Çömlekçioğlu N, Ellialtıoğlu Ş (2018). Review on the research carried out on in vitro androgenesis of peppers (*Capsicum annuum* L.) in Turkey. Research Journal of Biotechnology 13: 75-84.

Dumas De Vaulx R, Chambonnet D, Sibi M (1981). Culture in vitro d'antheres de piment (*Capsicum annuum* L.): amelioration des taux d'obtention de plantes chez differents genotypes par de traitments at +35 °C. Agronomie 11: 859 (in French).

Ercan N, Ayar Şensoy F (2011). Androgenic responses of different pepper (*Capsicum annuum* L.) cultivars. Biyoloji Bilimleri Araştırma Dergisi 4: 59-61.

Ercan N, Ayar Sensoy F, Sensoy AS (2006). Influence of growing season and donor plant age on anther culture response of some pepper cultivars (*Capsicum annuum* L.). Sci Hortic-Amsterdam 110: 16-20.

Ercan N, Boyacı F, Ayar F (2001). Biberde (*Capsicum annuum* L.) anter kültürü yoluyla haploid bitki eldesi üzerine farklı besin ortamlarının etkisi. In: GAP II. Tarım Kongresi, 24–26 October 2001, Şanlıurfa, Turkey. Şanlıurfa, Turkey: Harran University and GAP, pp. 121-128.

Gemesne Juhasz A, Petus M, Venzcel H, Sági ZS, Zatykó L (2001). Colchicine, an efficient genome doubling agent for anther derived haploid paprika (*Capsicum annuum* L.) plants. In: Abak K, Büyükalaca S, Daşgan Y, editors. Proceedings of the 11th EUCARPIA Meeting on Genetics and Breeding of Capsicum & Eggplant, 9–13 April 2001, Antalya, Turkey. Adana, Turkey: EUCARPIA, pp. 146-151.

George L, Narayanaswamy S (1973). Haploid *Capsicum* through experimental androgenesis. Protoplasma 78: 467-470.

González J, Jouve N (2005). Microspore development during in vitro androgenesis in triticale. Biol Plant 49: 23-28.

Grozeva S, Todorova V, Cholakov T, Rodeva V (2013). Effect of temperature and growth period of donor plants on pepper anther culture. In: 3rd International Conference: Research People and Actual Tasks on Multidisciplinary Sciences, Lozenec, Bulgaria, pp. 60-64.

Harn C, Kim MZ, Choi KT, Lee YI (1975). Production of haploid callus and embryoid from the cultured anther of *Capsicum annuum*. Sabrao J 7: 71-77.

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

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. HortScience 50: 1-6.

Kim M, Jang IC (2000). Rapid assessment of microspore development stage in pepper using DAPI and ferric chloride. J Plant Biotech 2: 129-134.

Kim M, Kim J, Yoon M, Choi DI, Lee KM (2004). Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). Plant Cell Tiss Org 77: 63-72.

Koleva-Gudeva LR, Spasenoski M, Trajkova F (2007). Somatic embryogenesis in pepper anther culture: The effect of incubation treatments and different media. Sci Hortic-Amsterdam 111: 114-119.

Kristiansen K, Andersen B (1993). Effect of donor plant temperature, photoperiod and age on anther culture response of *Capsicum annuum* L. Euphytica 67: 105-109.

Matsubara S, Yamamoto M, Jo MH, Murakami K (1998). Embryoid and callus formation from microspore by anther culture from July to November in pepper (*Capsicuum annuum* L.). Scientific Reports of the Faculty of Agriculture of Okoyama University 87: 117-122.

Mityko J, Andrasfalvy A, Csillery G, Fari M (1995). Anther culture response in different genotypes and F1 hybrids of pepper (*Capsicum annuum* L.). Plant Breeding 114: 78-80.

Morrison R, Koning RE, Evans DA (1986). Anther culture of an interspecific hybrid of *Capsicum*. J Plant Physiol 126: 1-9.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497.

Niklas-Nowak A, Olszewska D, Kisiala A, Nowaczyk P (2012). Study of individual plant responsiveness in anther cultures of selected pepper (*Capsicum* spp.) genotypes. Folia Horticulturae 24: 141-146.

Novak FJ (1974). Induction of a haploid callus in anther cultures of *Capsicum* sp. Z Pflanzenzenphysiol 68: 97-114.

Olszewska D, Kisiala A, Niklas-Nowak A, Nowaczyk P (2014). Study of in vitro anther culture in selected genotypes of genus *Capsicum*. Turk J Biol 38: 118-124.

Ozsan T, Onus N (2017). In vitro pepper (*Capsicum annuum* L.) anther culture: can be affected via vitamins B? Biotech J Int 20: 1-13.

Özkum Çiner D, Tipirdamaz R (2002). The effects of cold treatment and charcoal on the in vitro androgenesis of pepper (*Capsicum annuum* L.). Turk J Bot 26: 131-139.

Pretova A, Ruijter NCA, Van-Lammeren AAM, Schel JHN (1993). Structural observation during androgenic microspore culture of the 4c1 genotype of *Zea mays* L. Euphytica 65: 61-69.

Rodeva VN, Irıkova TP, Todorova VJ (2004). Anther culture of pepper (*Capsicum annuum* L.): Comparative study on effect of the genotype. Biotechnol Biotechnol Eq 18: 34-38.

Sayılır A, Özzambak E, (2005). Biber anter kültüründe uygun tomurcuk büyüklüğü ile besin ortamı içeriklerinin embriyo verimine etkileri üzerine bir araştırma. Ege Üniversitesi Ziraat Fakültesi Dergisi 42: 1-11 (in Turkish).

Taşkin H, Büyükalaca S, Keleş D, Ekbiç E (2011). Induction of microspore-derived embryos by anther culture in selected pepper genotypes. Afr J Biotechnol 10: 17116-17121.

Terzioğlu Ş, Ellialtioğlu, Ş, Abak K (2000). İnkübasyon koşullarının biber anter kültüründe embriyo oluşumu üzerine etkisi. In: III. Sebze Tarımı Sempozyumu, 11–13 September 2000, Isparta, Turkey, pp. 233-238 (in Turkish).

Vagera J, Havranek P (1985). In vitro induction of androgenesis in *Capsicum annuum* L. and its genetic aspects. Biol Plantarum 27: 10-21.

Wang YY, Sun CS, Wang CC, Chien NJ (1973). The induction of pollen plantlets of *Triticale* and *Capsicum annuum* from anther culture. Sci Sin 16: 147-151.

Zhao J, Zou X, Zhang Z, Yang B, Zhou S (2010). Influences of carbon sources and plant growth regulators on anther culture efficiency of pepper. Agricultural Science & Technology 11: 102-105.