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Full Length Research Paper

Induction of microspore-derived embryos by anther culture in selected pepper genotypes

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Five pepper genotypes (A71, A269, A313, A109 and A74) and four different culture media were tested in this study carried out at the University of Çukurova, Turkey. The anthers were cultured at different periods in order to optimize the frequency of embryo production. Moreover, the embryos that were unable to complete their growth in the culture medium studied were placed in a medium containing 0.5 mgL^{-1} abscisic acid for 10 days. At the end of the study, it was determined that embryo development varied with genotype, anther cultivation period and culture medium. The highest yield of embryos was obtained from A269, one of the genotypes tolerant to low temperature. The anthers cultured from April to May, gave the highest yields of embryos compared to anthers from the other periods. Most of the embryos were obtained from Medium III (MS medium containing 4 mg/L NAA, 1 mg/L BAP, 0.25% activated charcoal, 15 mg/L $AgNO_3$, 30 g/L sucrose) and the Medium IV (modified MS medium containing 0.25% activated charcoal, 15 mg/L $AgNO_3$, 4 mg/L NAA, 0.1mg/L BAP and with 0.5 mg/L ABA). There was no positive effect of abscisic acid on the mature embryos.

Key words: Pepper (*Capsicum annuum)*, genotype, anther culture, embryo quality.

INTRODUCTION

The technique of haploid induction has great value in vegetable breeding as it has the ability to shorten the breeding period. The most significant application of haploid plant production is the rapid production of homozygous doubled haploid lines even with self-incompatible species. Homozygous lines can also be obtained via conventional methods, although this process takes 10 to 12 years in open pollinated plants, and 6 to 7 years in self pollinated plants. However, this period can be shortened for 1 to 2 years with haploidy techniques. Haploid plants and homozygous dihaploid plants can be obtained in a few months with the anther culture method. These homozygous plants are very important to obtain homozygous lines that are necessary to develop hybrid varieties. Dihaploid plants undergo normal meiotic segregation and do not lose the desirable features as a consequence of segregation (Reinert and Bajaj, 1977). Since dihaploid lines are absolute homozygous, they can be used to determine and isolate recessive mutants. Any dominant effect disappears and additional gene effects double. Another advantage of dihaploid plants is that multi disease resistance tests could be applicable.

The technique of anther culture is one of the current techniques to obtain haploid plants (Dunwell, 2010). The advantage of this method is that there exist thousands of microspores in each anther and numerous haploid plants can be obtained from a single anther. The main principle of anther culture is the prevention of the pollen cells development that forms male gamete, and instead the induction of immature pollen cells to directly form embryos similar to somatic cells.

Turkey is the third biggest pepper producing country following China and Mexico with 1.8 million tons of pepper production (Anonymous, 2009). Pepper production in Turkey can be observed in several regions in both open fields and greenhouses. Problems such as

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Abbreviations: MS, Murashige and skoog; **NAA,** naphthalene acetic acid; **BAP,** 6-Benzylaminopurine; **ABA,** Abscisic acid.

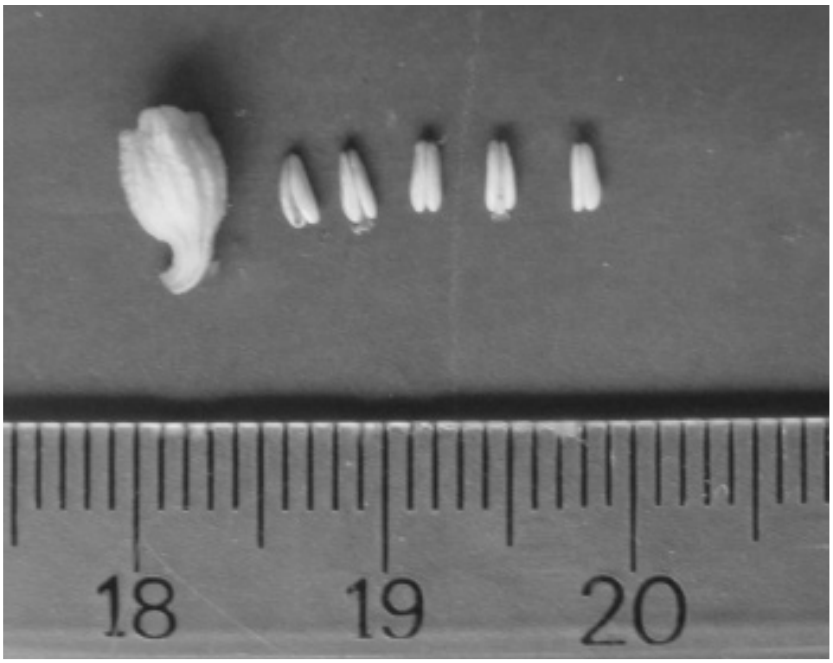

Figure 1. Suitable bud and anther size for the culture.

high temperatures, low temperatures, diseases and pests affect the yield and quality of pepper production. The development of species resistant to such environmental factors is a significant way to improve the yield. Although, a lot of research has been conducted in the development of new hybrid types with conventional breeding methods, this process takes quite a lot of time. Not only to shorten the breeding process with a conventional breeding method but also to provide breeding lines, various *in vitro* methods have been used. Nowadays, amongst other techniques, one of the most widely used and the most successful method is anther culture. The first haploid plant via anther culture was obtained by Guha and Maheswari (1964). After that anther culture for haploid plant production was studied by a lot of researchers in many species (Kaloo, 1986). Androgenetic haploid plants of barley, colza, eggplant (Veilleux, 1994), watermelon and cabbage (Cao et al., 1995) were commonly used to develop hybrids. The first haploid pepper plant via anther culture was obtained by Wang et al. (1973). Subsequently, researchers as George and Narayanswamy (1973), Saccardo and Devreux (1974), Novak (1974), Harn et al. (1975) and Abak (1983) conducted such studies in pepper. Although, anther culture is an important method in homozygous line production, the yield of haploid plants is low in most species (Kristiansen and Andersen, 1993). Culture medium is one of the main factors affecting androgenesis. In addition, androgenetic embryo ratio can change depending on genotype (Çömlekçioğlu et al., 2001).

The purpose of this research was to obtain in high ratio quality haploid embryos from cold tolerant genotypes via anther culture. With this objective, the best medium previously reported was modified, different media and additional reasons to determine the most suitable one were examined. There were also some experiments to improve embryo quality and number with different media.

MATERIALS AND METHODS

Five pepper genotypes (A71, A269, A313 tolerant, A109 moderately tolerant and A74 susceptible to low temperatures) derived from a joint study between Horticulture Department of Cukurova University of Turkey and Alata Horticultural Research Institute, Mersin, Turkey, were examined in the present study. Seeds were planted in plugs containing soil mixture (2 volumes peat: 1 volume perlite). Throughout the growing period, normal horticultural cultivation practices were implemented, while during the flowering period of the plants, every month, anthers were cultured in different media by means of removing flower buds which were in the proper phase of anther development. Collected buds (Figure 1) were exposed to surface sterilization in 15% sodium hypochlorite solution including 1 to 2 drops Tween 20 for 15 min, then followed by rinsing in sterile distilled water for 3 to 4 times.

After sterilization the buds were dissected, the anther filaments removed and the anthers placed on nutrient medium in Petri dishes. Five anthers belonging to a flower bud were placed in a Petri dish in 5 cm diameter containing 7.5 ml medium. The experiment was designed in a completely randomized experimental design with three replications and five Petri dishes included per replication. Four culture media were examined in this study; Medium I: MS medium containing 4 mg/L NAA, 0.1 mg/L BAP, 0.25% activated charcoal, 15 mg/L $AgNO_3$, 30 g/L sucrose; Medium II: MS medium containing 4 mg/L NAA, 0.5 mg/L BAP, 0.25% activated charcoal, 15 mg/L $AgNO_3$, 30 g/l sucrose; Medium III: MS medium containing 4 mg/L NAA, 1 mg/L BAP, 0.25% activated charcoal, 15 mg/L $AgNO_3$, 30 g/l sucrose; and Medium IV: a modification of MS

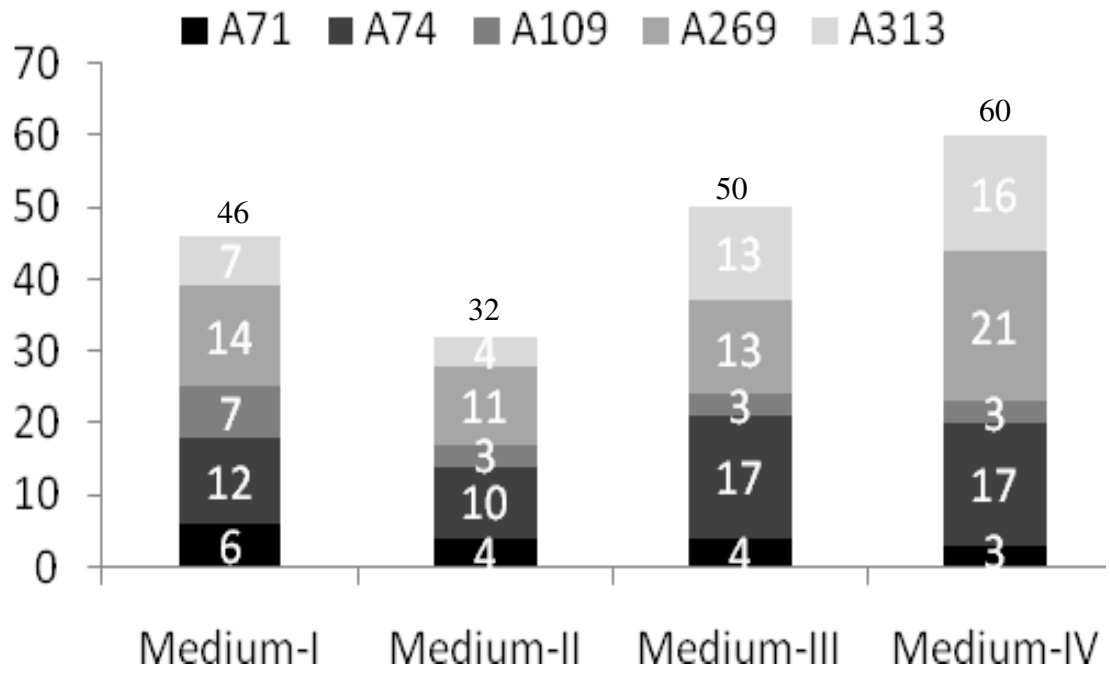

Figure 2. Numbers of embryogenic anthers.

(nitrate: ammonium ratio was changed from 40:20 to 55:5) (Büyükalaca, 1993) and containing 0.25% activated charcoal,15 mg/L $AgNO_3$, 4 mg/L NAA, 0.1mg/L BAP. Globular embryos were placed on MS medium including 0.5 mg/L ABA for maturation. Furthermore, cultured anthers were incubated at 35°C during the first two days in the dark and were then transferred to the growth chamber at 28°C and 8 hours dark and 16 h light photoperiod conditions.

RESULTS AND DISCUSSION

Data related to the embryogenic anther numbers showed that the best available medium was Medium-IV (Figure 2). A total of 60 embryogenic anthers out of 2700 anthers were observed in this medium. Medium-II was found to be the least effective for embryogenesis. Fifty anthers were found to be embryogenic in Medium-III and 46 anthers in Medium-I. The A269 pepper genotype gave the highest frequency of embryogenic anthers (21 embryogenic anthers) in Medium-IV. A269 and A313 gave 17 and 16 embryogenic anthers respectively. The genotypes A71 and A109 were found to be recalcitrant genotypes to form embryos in the best medium of the present study. The two genotypes gave the lowest embryogenic anther number values for all media. The overall numbers of embryogenic anthers according to the genotypes were 59 anthers for A269 and 56 anthers for A74 genotypes. A313, A71 and A109 followed by 40, 17 and 16 anthers respectively.

Furthermore, nutrient medium and growing condition (Koleva-Gudeva, 2003; Koleva-Gudeva et al., 2007) has an important effect on embryogenic capacity of the anthers. Different media and pre-thermal treatments of anthers give different embryogenic responses. These researchers obtained embryos only from CP medium and heat pretreatment application (+35°C), while the other medium and cold treatments induced callus formation. The present study showed that embryogenic response of the anthers was highly influenced by the growing season of the donor plants. Anthers of almost all genotypes were found to be embryogenic in April, May and June, but in the other months pepper genotypes produced very low number of embryogenic anthers (Figures 3 and 4). A total of 188 anthers produced embryos in this study. Moreover, no embryogenic anthers were shown in July and only one anther was observed in August. The higher values were observed in April, May and June, with 58, 56 and 45 embryogenic anthers, respectively. According to the genotypes, A269 gave the higher number of embryogenic anthers in April and June with 30 and 22 anthers, respectively. 26 and 24 anthers were scored as embryogenic in A74 and A313 genotypes respectively in May.

In addition, a total of 575 embryos were obtained from embryogenic anthers of all genotypes (Table 1). Among them, 270 (47%) embryos were globular and 305 (53%) embryos were mature. The highest value of embryo formation was obtained from medium-III with 190 embryos, followed by medium-IV and medium-I with 161 and 131 embryos respectively. Medium-II gave the lowest number of embryos with 93 embryos. In terms of embryo quality, Medium-IV was found to be the best medium. Mature embryo formation ratio was found to be 63% in

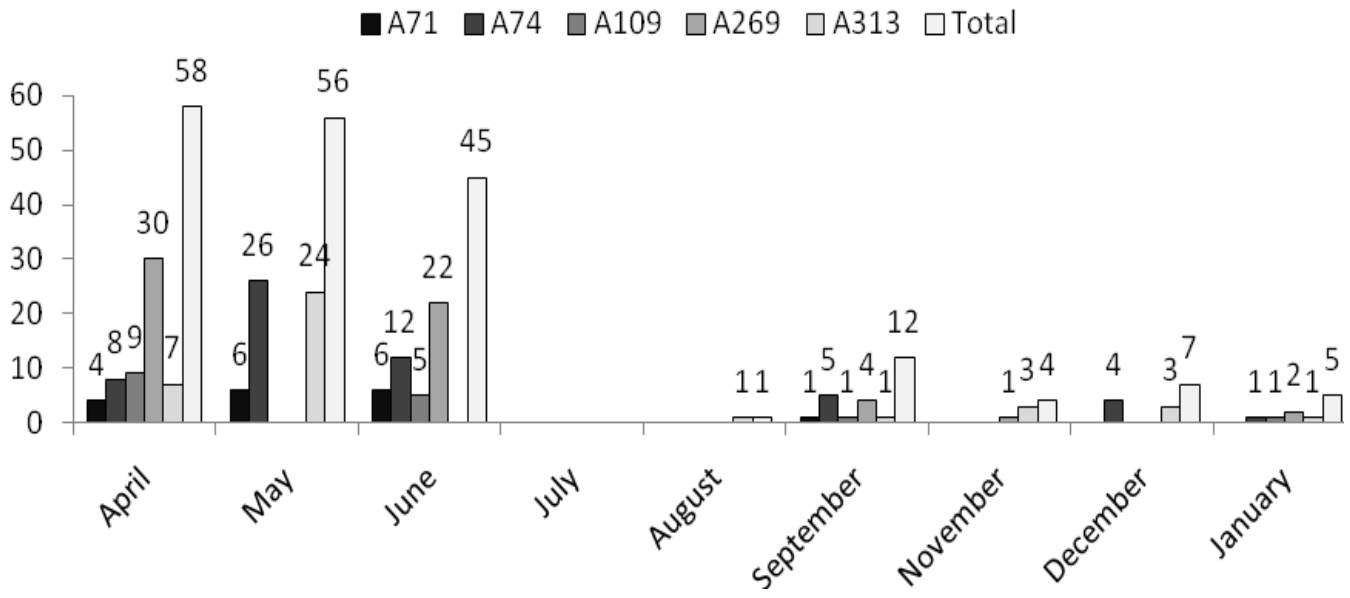

Figure 3. Numbers of embryo obtained in different months.

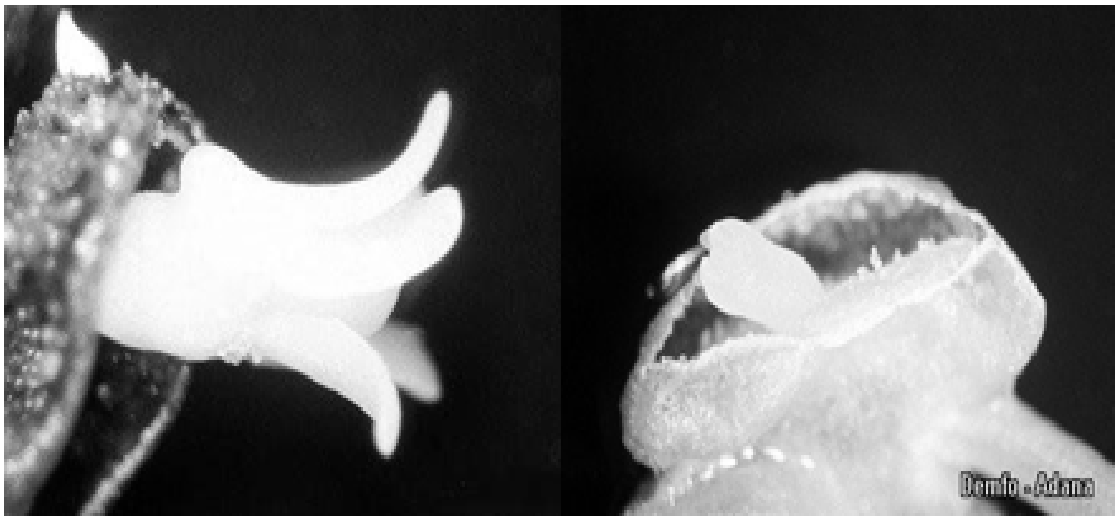

Figure 4. A view of embryos obtained in this study.

lower values of mature embryos. These results were supported by Çömlekçioğlu et al. (2001), Ercan et al. (2001), Çiner and Tıpırdamaz (2002), Büyükalaca et al. (2004) and Sayılır and Özzambak (2005). The genotype A269 was found to be the most productive genotype in terms of embryo numbers with 184 embryos, including 123 globular and 61 mature stage embryos, A74 and A313, followed with 153 and 151 total embryo numbers, respectively. The highest numbers of mature embryos were obtained from A313 and A74 genotypes with 98 and 87 mature embryos, respectively. While all genotypes were found to be most productive in April, May and June in general, the cold tolerant pepper genotype A313 also produced higher embryos than others in November and December.

The anthers cultured in April, May and June produced 85.4% of the embryos obtained in the study with 176, 176 and 139 embryos, respectively (Table 1). However, highest number of quality embryos was scored in May (122 mature embryos). A269 was found to be the highest embryo yielding genotype and it produced 101 embryos in April and 72 embryos in June. A109 was found to be the poorest genotype in this study for embryo production with total of 41 embryos. It was obvious from the study that the period of April, May and June was suitable for anther culture of the selected pepper genotypes. Hence,

Table 1. Numbers of embryos obtained from different media and during different months of the year.

Culture medium	Total	G*	Genotype	A71		A74		A109		A269		A313	
			M**	G	M	G	M	G	M	G	M	G	M
Medium 1	131	74	57	5	8	17	13	5	10	21	12	26	14
Medium 2	93	36	57	4	8	7	14	0	16	20	10	5	9
Medium 3	190	100	90	4	7	30	30	3	1	46	17	17	35
Medium 4	161	60	101	7	3	12	30	0	6	36	22	5	40
	575	**270**	**305**	20	26	66	87	8	33	123	61	53	98
Month													
April	176	94	82	6	3	11	9	4	9	65	36	8	25
May	176	54	122	4	13	33	55	0	0	0	0	17	54
June	139	80	59	9	10	16	11	0	21	55	17	0	0
July	0	0	0	0	0	0	0	0	0	0	0	0	0
August	4	4	0	0	0	0	0	0	0	0	0	4	0
September	30	9	21	1	0	0	10	3	3	2	6	3	2
November	23	19	4	0	0	0	0	0	0	1	0	18	4
December	21	9	12	0	0	6	1	0	0	0	0	3	11
January	6	1	5	0	0	0	1	1	0	0	2	0	2
	575	**270**	**305**	20	26	66	87	8	33	123	61	53	98

*Globular embryos; **Mature embryos

air temperature during the harvest of flower buds has an effect on the formation of haploid embryos. July and August are the warmest season of the Çukurova region and as seen in the study almost no embryos were obtained; it was assumed that embryo formation failed due to the high temperature during this time (Tiainen, 1992; Kristiansen ve Andersen, 1993). In addition, it was also assumed that plant age might have affected the androgenesis process since in July and August plants were 6 to 7 months old (Dunwell, 1976; Ercan et al. 2006). Moreover, outcome of the maturation experiment showed that ABA did not positively affect the maturation of globular embryos.

Conclusion

While the highest values of embryo formation was obtained from medium III with 190 embryos, the highest embryo quality was obtained in medium IV with 63% mature embryos. In terms of genotypes, while the most productive genotype was A269 with 184 embryos, the most mature embryos were obtained from A313 with 98 embryos. Also, when the effect of different periods was evaluated, it was observed that successful results were obtained from April, May and June with 176, 176 and 139 embryos, respectively. There was however no positive effect of abscisic acid on the maturation of globular embryos.

Acknowledgments

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