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RESEARCH PAPER

In vitro androgenesis in pepper and the affecting factors on success: I. Carbon source and concentrations

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Abstract

In vitro androgenesis methods are significant in rapid production of completely homozygous lines. Factors such as nutrient medium composition, types and concentrations of carbohydrate sources are effective on androgenetic success. Different sugar types were tested at different doses in anther culture of pepper to elucidate their influence on the yield of androgenesis. In this study, maltose was used as a source of sugar at 1.5, 3 and 6% doses. 3% sucrose was used as the control treatment. Murashige & Skoog (1962-MS) medium and 0.25% activated charcoal, 15 mg L^{-1} silver nitrate ($AgNO_3$), 4 mg L^{-1} naphthaleneacetic acid (NAA), and 2 different amounts of 6-benzylaminopurine (0.5, 1 mg L^{-1} BAP) with three breeding lines of pepper were used to know the genotypic effects on haploid embryo formation. When comparing 1.5, 3 and 6% maltose and 3% sucrose with each other in the medium, embryo response increased with maltose concentration. Maltose, which was generally compare to sucrose as carbohydrate source for anther culture, the embryo response had significantly affected at maltose concentrations. The combination with the best effect among the trial parameters was 30 g L^{-1} maltose and 0.5 mg L^{-1} BAP for obtaining embryos from anther culture. Also, it was found that genotype is one of the main factors affecting success in pepper anther and microspore culture.

Introduction

Pepper which belongs to the *Capsicum* genus and Solanaceae family is a domesticated species originated from the tropical and sub-tropical central regions of South America known as Bolivia (Olmstead et al., 2008). All pepper populations are diploid and have the same chromosome number (*2n=2x= 24*). *Capsicum annuum* L. is widely grown and produced all over the world. It was domesticated from wild bird pepper or 'Chiltepin' in Mexico (Perry et al., 2007; Yildiz et al., 2020). However, it is the most broadly distributed and economically crop of the world. Red pepper cv. Capia (*C. annuum*) type is an essential and widely used among other vegetables all over the world. Turkey, due to its geographical location and soil fertility has played an important role in the distribution of different plant species. It is one of the most important country having great cultivation of peppers. Annual production and harvested area of dry peppers and green peppers in Turkey were reported to be 16,355 tones and 6,749 ha and 2,608,172 tones and 94,444 ha, respectively (FAOSTAT, 2017).

One of the most important and original aims of the breeding programs in capia type pepper is development of new marketable cultivars. The most commonly used method by pepper breeders is pure line and pedigree selection. Biotechnological methods such as doubled haploid (DH) plant production, are intended for reducing the breeding process of these new homogenous and totally pure varieties (Lantos et al., 2012; Thomas et al., 2003). Obtaining high quality homozygous pure lines by classical methods is very time consuming (nearly 6-7

years) and labor intensive. Time can be shortened up to 1 year using androgenesis to obtain haploid plants via tissue culture. Microspore embryogenesis is an important and useful culture technique to obtain full homozygous lines from only the male gametes in pepper breeding (Comlekcioglu & Ellialtioglu, 2018). The first successful haploid embryo development from *C. annuum* anthers was happened by Wang et al. (1973) in China, George, and Narayanaswamy (1973) in India. In Turkey, the first *in vitro* androgenesis studies on domestic pepper genotypes were started by Abak (1983). Afterwards, many scientists in different parts of the world have investigated the factors affecting anther culture and published their valuable experiences. Some of the essential factors such as growing condition of donor plants, collection of donor buds in the optimal stage (Ari et al., 2016 a,b; Kim et al., 2004; Supena et al., 2006), pre-treatments of buds or anthers (Dumas de Vaulx et al., 1981), nutrient media (Comlekcioglu et. al., 2001; Dumas de Vaulx et al., 1981; Dumas de Vaulx, 1990; Irikova et al., 2011; Ozkum-Ciner & Tipirdamaz, 2002; Qin & Rotino, 1993; Supena et al., 2006), temperature treatment (Dolcet-Sanjuan et al., 1997; Koleva-Gudeva et al., 2009), culture season (Buyukalaca et al., 2004; Ercan et al., 2006; Gonzalez-Garcia, 2002; Rodeva & Cholakov, 2006; Taskin et al., 2011), incubation conditions (Ellialtioglu et al., 2001), plant growth regulators and genotype of the donor plant (Ari et al., 2016a,b; Buyukalaca et al., 2004; Comlekcioglu et. al., 2001; Wang & Zhang, 2001) which significantly influence the usefulness of haploidy could affect androgenetic responses.

Among the various factors used to increase the rate of haploid plants and cross-species hybrids of *C. annuum*, carbohydrate sources as nutrient components play a very important role. Many researchers have reached substantial results by changing source and concentration of carbohydrates (sucrose, maltose, and glucose) in the nutrient medium, in order to increase the effectiveness of microspore embryogenesis. Sucrose at different concentrations is commonly used as a carbohydrate source for *in vitro* cultivation of pepper anthers by Binzel et al. (1996), Morrison et al. (1986), and Supena et al. (2006). Maltose also used by Dolcet-Sanjuan et al. (1997), Gémesné et al. (2000), and Supena et al. (2006). There are very few research about using honey as a carbohydrate source instead of sucrose. Gebologlu et al. (2017) stated that the use of honey contributes positively to embryo formation, but this effect varies depending on genotypes. Consequently, different experiments are carrying out to create new protocols for effective embryo production in pepper.

In order to be used as parents in F1 hybrid variety breeding, 3 genotypes of sweet capia type pepper were studied to developed pure lines. The main propose of this work was to investigate the effect of different concentrations of maltose and sucrose as a carbohydrate sources in anther culture.

Materials and Methods

This research was carried out in the Tissue culture Laboratory owned by United Genetics Seed. A.Ş Company in Bursa, Turkey.

Plant material

In the current study, three different breeding lines (G-23, G-24, and G-26) of capia type peppers were used as a plant material. Pepper seeds were planted in styrofoam containing peat: perlite with the ratio of 2:1 in the greenhouse conditions, of the United Genetics Turkey Vegetable Seeds Company at Mustafakemalpaşa/Bursa location. Seedlings (42 day-old) were transferred into a controlled greenhouse to be grown as donor plants (Figure 1a). Due to the importance of healthy growth and development conditions of the donor plant, they were subjected to growth free from any stress like insects, weeds, diseases, water and nutrient deficiencies (Figure 1b).

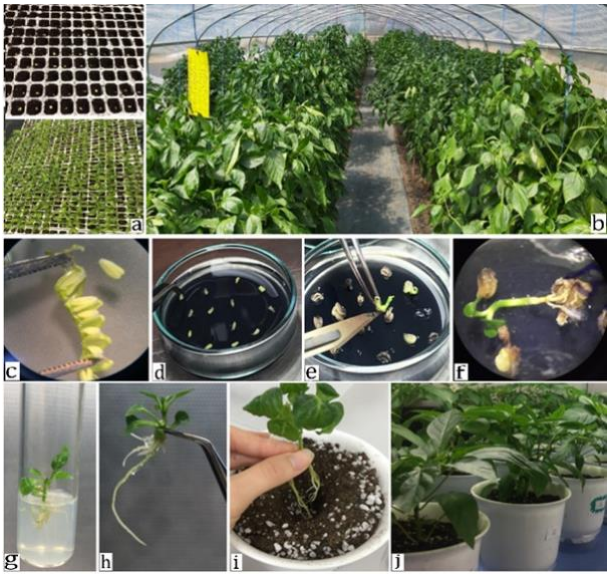

Figure 1. (a) Planting donor plants seeds and growing the seedlings, **(b)** donor plants in the controlled greenhouse, **(c)** isolation of anthers from buds under aseptic conditions, **(d)** putting the anthers into the petri dishes, **(e)** transferring the embryos to hormone-free MS medium, **(f)** haploid plantlet under stereomicroscope, **(g)** growing of embryos *in vitro*, **(h)** removing advanced embryos from the test-tube, **(i)** acclimatization of pepper plantlets, **(j)** healthy androgenic pepper plants.

Anther culture studies

Flower buds were collected in the morning. In the phase when the corolla and the calix are at the same height or when the the height of corolla is a little above the height of the calix and also when there is an anthocyanin production, mostly late uninucleate and mid uninucleate microspore stage were found alongside anthers that contain pollen in the young binucleate stage (Comlekcioglu & Ellialtioglu, 2018).

Surface sterilization and anther extraction is also critical in anther culture. In the laboratory this method

started with washing flower buds with sterile distilled water very quickly. Then 70% ethanol sprayed on buds and kept in 2.5% sodium hypochlorite and a drop of Tween-20 for 10 minutes and washed in sterile distilled water 3 times or more to remove the traces of sodium hypochlorite. And later all buds are carefully cut (Figure 1c) and examined with using a microscope inside laminar flow cabinet (anther should not be damaged during cutting) on a sterile paper and cultured horizontally on the pepper agar medium (Figure 1d).

Treatments and observations

Anthers of three capia (G-23, G-24, and G-26) pepper genotypes (breeding lines) were cultured on MS (Murashige & Skoog, 1962) media including vitamins and plant growth regulators. It was previously reported (Buyukalaca et al., 2004; Keles et al., 2015 and Taskin et al., 2011) that MS nutrient media supplemented with NAA, BAP, activated charcoal, sucrose and $AgNO_3$ were successful in the androgenesis of pepper. Actually, 8 different types of culture media were prepared supplemented with 4 mg L^{-1} NAA, 15 mg L^{-1} $AgNO_3$, 0.25% activated charcoal, and 8 g L^{-1} agar for all under the same condition. Variable factors were BAP with (0.5, 1 mg L^{-1}) doses and carbon source and concentrations as maltose with 15, 30 and 60 g L^{-1} and sucrose 30 g L^{-1} as a control treatment.

The cultures were incubated in the dark conditions for 2 days at 35°C, later at 25°C for 4 to 6 weeks to induce direct embryogenesis continuously. Embryo formation (Figure 1e and 1f), development, germination and transformation into a complete plantlet were regularly observed. After growing embryos were transferred to MS medium without any plant growth regulator (Figure 1g) and were kept in light with 16 hours photoperiod at 25°C. After few days the embryos were transferred into sterile coco peat pots (Figure 1h and 1i) for shoot and root elongation (Figure 1j).

Experiment design and statistical analyses

The experiment was carried out in a "completely randomized design" with four replications and 5 petri dishes per repetition. Five anthers obtained from a flower bud were planted in each petri dishes (20 Petri dishes and 100 anthers were used in each medium). Data were subjected to analysis of variance (ANOVA, Tarist Statistical Software) and means were separated by LSD test (A probability level $P < 0.01$ was used to test significance of differences between means).

Results and Discussion

In this study, for evaluating of embryo induction of anthers of three Capia pepper genotypes (G-23, G-24, and G-26) were cultured on MS medium including (0.5 and 1.0 mg L^{-1}) BAP with different concentrations of maltose (15, 30 and 60 g L^{-1}) and sucrose (30 g L^{-1}) as a carbon source.

The data presented in Table 1 show the effect of different carbon sources and BAP doses on number of embryos per 100 anther obtained and developed embryos obtained on pepper anther culture. According to data, G-26 genotype produced significantly higher embryos at each carbon and BAP level than the other two genotypes. This technology consists of stimulation and germination of haploid plants via anther or

Table 1. Number of embryos from different carbon sources, doses and two different doses of BAP in the culture medium

Carbon Source	BAP content (mg L^{-1})	Donor genotype	Number of embryos obtained	Total obtained embryos		Number of developed plantlets	Total developed plantlets	
Sucrose 30 g L^{-1}	0.5	G-23	4	18	55	2	5	16
		G-24	0			-		
		G-26	14			3		
	1.0	G-23	3	37		1	11	
		G-24	4			-		
		G-26	30			10		
Maltose 15 g L^{-1}	0.5	G-23	2	43	69	-	8	12
		G-24	0			-		
		G-26	41			8		
	1.0	G-23	2	26		-	4	
		G-24	0			-		
		G-26	24			4		
Maltose 30 g L^{-1}	0.5	G-23	6	44	84	-	13	24
		G-24	3			-		
		G-26	35			13		
	1.0	G-23	0	40		-	11	
		G-24	19			6		
		G-26	21			5		
Maltose 60 g L^{-1}	0.5	G-23	1	36	46	-	11	17
		G-24	2			-		
		G-26	33			11		
	1.0	G-23	3	10		1	6	
		G-24	1			1		
		G-26	6			4		

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microspores culture. Anther culture is one of the most interested method because of its high yield performance and number of male gametes existing in each bud. The genotype has the main role and often restricting aspect in the pepper androgenesis (Buyukalaca et al., 2004; Koleva-Gudeva et al., 2007; Rodeva et al., 2004). According to Morrison et al. (1986), the donor's plant genotype is the most important factor influencing the embryo production from anther culture of pepper. In our experiment, G-26 was the most responsive genotype for anther culture.

As a result of variance analysis, it was determined that two-way and three-way interaction among all studied factors (carbon source, BAP dose, and genotypes; CS×BAP×G) statistically were significant. This significance was realized for both the number of embryos obtained and the number of embryos developed. ($P \leq 0.01$) (Table 2, Table 3).

The highest average percentage of embryo induction rate in MS medium was obtained from G-26 with 10.25% including 15 g L^{-1} Maltose + 0.5 mg L^{-1} BAP per anther and this was followed by the same genotype created higher embryos than other genotypes with 8.75% embryos in 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP, and 8.25% embryos in 60 g L^{-1} Maltose + 0.5 mg L^{-1} BAP (Table 2).

Table 2. Average of obtained embryos according to different carbon sources, BAP doses and genotypes

Carbon source	BAP (mg L^{-1})	Genotype			Carbon×BAP Mean	Carbon Mean
		G-23	G-24	G-26		
Sucrose	0.5	1.00	0.00	3.50	1.50	**2.29^{c}**
30 g L^{-1}	1.0	0.75	1.00	7.50	3.08	
Mean		**0.88^{a}**	**0.50^{b}**	**5.50^{c}**		
Maltose	0.5	0.50	0.00	10.25	3.58	**2.88^{b}**
15 g L^{-1}	1.0	0.50	0.00	6.00	2.17	
Mean		**0.50^{a}**	**0.00^{c}**	**8.13^{a}**		
Maltose	0.5	1.50	0.75	8.75	3.67	**3.50^{a}**
30 g L^{-1}	1.0	0.00	4.75	5.25	3.33	
Mean		**0.75^{a}**	**2.75^{a}**	**7.00^{b}**		
Maltose	0.5	0.25	0.50	8.25	3.00	**2.04^{d}**
60 g L^{-1}	1.0	0.75	0.25	2,25	0.86	
Mean		**0.50^{a}**	**0.38^{bc}**	**5.25^{c}**		
BAP	0.5	**0.81^{a}**	**0.31^{b}**	**7.69^{a}**		
Mean	1.0	**0.50^{a}**	**1.50^{a}**	**5.25^{b}**		
Genotype Mean		**0.66^{c}**	**0.91^{b}**	**6.47^{a}**		

Means were separated by LSD test at $P \leq 0.01$. Column having different letter(s) are statistically significant.

Factor A: Carbon source; Factor B: BAP dose; Factor C: Genotype.
LSD (%)
Factor A: 0.240, Factor B: 0.170. A×B: 0.339,
Factor C: 0.208,
A×C: 0.415, B×C: 0.294 , A×B×C: 0.587

It was followed by G-24 including 30 g L^{-1} Maltose + 1.0 mg L^{-1} BAP with the highest average percentage of 4.75 embryos per anther. The lowest average of 1.5 embryos per anther was observed in G-23 genotype including 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP (Table 2).

Considering to the carbon source showed that the highest amount of developed embryo number occurred in 30 g L^{-1} Maltose medium. Although, there are significant differences among BAP doses according to genotypes. The obtained average of 2.94 and 2.42 embryos were determined for 0.5 and 1.0 mg L^{-1} BAP in turn. Genotype averages were 0.66, 0.91, and 6.47 for G-23, G-24, and G-26, respectively (Table 2).

These results show that the genotype is the main limiting factor in androgenesis and that the success of androgenesis for the same genotype is also affected by growth regulators. Therefore, experimental determination of optimum culture conditions for each genotype is important.

In anther culture, the number of embryos obtained from different genotypes and their conversion rates to plant differs. There are problems in the development and transformation of some of the embryos formed into plants, or abnormal plant formation can be observed.

The highest rate of developed into plantlets according to the obtained embryos was determined from the G-26 genotype with 37.1%, in 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP contained medium. The highest total number of developed plantlets in all MS medium including maltose (15, 30, and 60 g L^{-1}) and sucrose (30 g L^{-1}) was obtained from G-26 (Table 1). The ratio of the total number of developed embryos into the plantlets obtained is proportional calculated 37.1%, 30.0%, and 33.3%, respectively.

In terms of the average percentage number of embryos per anther showed significant difference between genotypes, G-26, G- 24, and G-23 in 0.5 mg L^{-1} BAP medium respectively were determined 7.69, 0.31 and 0.81 embryos, and in 1 mg L^{-1} BAP medium it was 5.25, 1.50, and 0.50 embryos, respectively (Table 2).

According to carbon sources, the highest average of embryo at the genotypes is in the G- 26 genotype with 8.13, 7.00, and 5.50 embryos, including 15 g L^{-1} Maltose, 30 g L^{-1} Maltose and 30 g L^{-1} Sucrose medium and the lowest average of embryo with 5.25 related to 60 g L^{-1} Maltose media (Table 2).

The highest number of the developed plantlets at G-26 genotype in three-way interaction was determined from medium including 0.5 mg L^{-1} BAP and 15 g L^{-1} Maltose (10.25 embryo). Other combinations followed this medium respectively (30 g L^{-1} Maltose: 8.75 embryo, 60 g L^{-1} Maltose: 8.25 embryo, and 30 g L^{-1} Sucrose: 7.50 embryo) (Table 2).

Genotypes have shown different performance in terms of transformation of regenerated embryos from different MS media and BAP doses into complete plants. In G-26 genotype, 30 g L^{-1} Maltose + 0.5 mg L^{-1} BAP medium showed the highest number of plantlets with an average of 3.25 per anther. The highest number of average plantlets per anther was 0.93 for G-23 in 30 g L^{-1} Sucrose + 1.0 mg L^{-1} BAP contained medium and 1.5 for G-24 in 30 g L^{-1} Maltose + 1.0 mg L^{-1} BAP included medium (Table 3).

As an important result of this research, 30 g L^{-1} of maltose with both 0.5 and 1.0 mg L^{-1} BAP doses showed significantly higher "number of embryos obtained" and

"developed embryo into the plantlets" compared to other culture media and control. This was followed by maltose 15 g L^{-1}, sucrose 30 g L^{-1} and maltose 60 g L^{-1}. Thereby, the performance of genotypes differed significantly from each other, and also it varied according to different media culture. G-26 has been identified as the most successful genotype and the lowest performance occurred in G-23 genotype.

Table 3. Average of developed androgenetic plantlets number according to different carbon sources, BAP doses, and genotypes

Carbon source	BAP (mg L^{-1})	Genotype			Carbon×BAP Mean	Carbon Source (Mean)
		G-23	G-24	G-26		
Sucrose	0.5	0.50	0.00	0.75	0.42^{c}	**0.78^{ab}**
30 g L^{-1}	1.0	0.93	0.00	2.50	1.14^{a}	
Mean		**0.71^{a}**	**0.00^{b}**	**1.63^{b}**		
Maltose	0.5	0.00	0.00	2.00	0.67^{bc}	**0.50^{c}**
15 g L^{-1}	1.0	0.00	0.00	1.00	0.33^{b}	
Mean		**0.00^{b}**	**0.00^{b}**	**1.50^{b}**		
Maltose	0.5	0.00	0.00	3.25	1.08^{a}	**1.00^{a}**
30 g L^{-1}	1.0	0.00	1.50	1.25	0.92^{a}	
Mean		**0.00^{b}**	**0.75^{a}**	**2.25^{a}**		
Maltose	0.5	0.00	0.00	2.75	0.92^{ab}	**0.71^{bc}**
60 g L^{-1}	1.0	0.25	0.25	1.00	0.50^{b}	
Mean		**0.13^{b}**	**0.13^{b}**	**1.88^{ab}**		
BAP	0.5	**0.13^{a}**	**0.00^{b}**	**2.19^{a}**		
Mean	1.0	**0.29^{a}**	**0.44^{a}**	**1.44^{b}**		
Genotype Mean		**0.21^{b}**	**0.22^{b}**	**1.81^{a}**		

Means were separated by LSD test at $P \leq 0.01$. Column having different letter(s) are statistically significant.

Factor A: Carbon source; Factor B: BAP dose; Factor C: Genotype.
LSD (%)
Factor A: 0.259, Factor B: 0.183, A×B: 0.366
Factor C: 0.224,
A×C: 0.449, B×C: 0.317, A×B×C: 0.634

The addition of auxins, cytokines, or their combinations is very important for the production of microspore derived haploid embryos, especially in stubborn plant species (Germenà, 2011).

Some researchers reported successful results in combination with 4 mg NAA and 0.1 mg BAP (Alremi et al., 2014; Buyukalaca et al., 2004; Comlekcioglu et al., 2001). Some researchers reported that higher haploid embryos were obtained with the combination of 4 mg NAA and 1 mg BAP (Ozkum-Ciner & Tipirdamaz, 2011; Taskin et al., 2011). On the other hand, Keles et al. (2015) found that the combination of 4 mg NAA and 0.5 mg BAP successful.

The effects of many critical factors in anther culture studies have been determined to affect successful embryo formation. In this research, the genotype of the donor plants, the sugar composition of the nutrient medium, growth regulators microspore development stage, physiological condition and growing conditions of the donor plant, preliminary applications to the anthers and the incubation conditions of the cultures greatly affect the response to androgenesis. Dolcet-Sanjuan et al. (1997) used maltose, malt extract and sucrose in different concentrations in anther of pepper anther culture. They reported that sugar type and concentration had significant effects on the total number of embryos obtained and the best result was obtained from 40 g L^{-1} Maltose. These results are compatible with the literature information (Comlekcioglu & Ellialtioglu, 2018; Irikova et al., 2011; Koleva-Gudeva et al., 2007; Segui-Simarro et al., 2011).

Conclusion

The perfect potential of haploidy and doubled haploidy in breeding programs for the development of new standard and hybrid varieties with unique characteristics of homozygous genotypes is clearly evident. Plant regeneration from microspore derived embryos is one of the most critical steps in pepper microspore culture. Among the many factors affecting the success of haploid androgenesis, such as genotype of donor plants, carbon source and concentrations, growth regulator combinations are also important factors in anther culture. Comparing the effects of genotype and the content of nutrient medium on the direct embryogenesis, a higher influence of interaction was observed between two factors.

After the evaluation of our results, we have also found that the factors such as genotype and carbon source play an important role in direct embryogenesis in capia pepper.

Embryogenesis responses in pepper was found to be genotype-dependent. So, the different responses of genotypes to the applied nutrient media will enable the selection of the proper medium for each genotype.

In conclusion of this research, we showed that genotype is one of the main factors affecting success in pepper anther and microspore culture. Also, it was found that the use of maltose as a carbon source had significant effect on embryo formation frequency in pepper anther culture. Future studies on different concentrations and doses of carbon source in combination with BAP might shed more light on improving the androgenesis in peppers.

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