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Research Article

The Effect of Shaker Use on Embryo Yield in Shed-Microspore Cultures of Ornamental Peppers**

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Anther, *Capsicum annuum* L., doubled haploid, microspore embryogenesis, culture shaking

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Abstract. Because of their morphological diversity and various rich content, ornamental peppers (*Capsicum annuum* L.) has become a favored plant in ornamental plant, food, cosmetics and medicinal sectors in recent years. This interest increased the ornamental pepper breeding studies. However, there are limited numbers of study based on haploid technology in ornamental peppers. In this study, we aimed to determine the effect of using shaker on embryo yield in the shed-microspore cultures of ornamental peppers. We used a total of 29 genotypes and compared the shaken and stationary cultures for each genotype. The shaken cultures gave more successful androgenic performance in terms of total and globular embryo formations. The most successful embryogenic performance was obtained from Genotype 754 in both stationary and shaken cultures. However, the shaken cultures of Genotype 754 formed 4.5 times more embryos when compared to its stationary control group. As the result, the use of shaker positively affected embryo yield in shed-microspore cultures of ornamental pepper depending on genotype.

Çalkalayıcı Kullanımının Süs Biberi Shed-Mikrospor Kültürlerinde Embriyo Verimine Etkisi

Anahtar kelimeler:
Anter, *Capsicum annuum* L., doubled haploid, mikrospor embriyogenesis, çalkalama

Özet. Morfolojik çeşitlilikleri ve çeşitli zengin içerikleri nedeniyle süs biberleri (*Capsicum annuum* L.) son yıllarda süs bitkisi, gıda, kozmetik ve ilaç sektörlerinde tercih edilen bitkiler haline gelmiştir. Süs biberine olan bu ilgi süs biberinde yapılan ıslah çalışmalarını son yıllarda arttırmıştır. Ancak süs biberlerinde haploid teknolojisine dayalı sınırlı sayıda ıslah çalışması bulunmaktadır. Bu çalışmada, süs biberi shed-mikrospor kültürlerinde çalkalayıcı kullanımının embriyo verimi üzerine etkisi belirlenmeye çalışılmıştır. Toplam 29 süs biberi genotipi kullanılmış ve her genotipin çalkalanan ve durağan kültürleri karşılaştırılmıştır. Çalkalanan kültürler, toplam ve globular embriyo oluşturma açısından daha başarılı androjenik performans göstermiştir. Çalışmada en başarılı embriyojenik performans, hem durağan hem çalkalanan kültürlerde Genotip 754'ten elde edilmiştir. Genotip 754'ün çalkalanan kültürleri, durağan kontrol grubuna kıyasla 4.5 kat daha fazla embriyo oluşturmuştur. Sonuç olarak, çalkalayıcı kullanımı, genotipe bağlı olarak süs biberi shed-mikrospor kültürlerinde embriyo verimini olumlu yönde etkilemiştir.

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INTRODUCTION

Capsicum annuum L. (Solanaceae) is one of the most cultivated and economical species in *Capsicum* genus in the world today. It is consumed freshly or dried in the kitchen and is utilized in the food industry as canned food, sauce, spice and pickle. Additionally, in the *C. annuum* species, the ones that have ornamental value have become favored for in landscaping due to their easy and fast growing traits, warm and drought tolerance and high fruit retention ratios (Stommel and Bosland, 2007). Thus, it is noteworthy that for all these reasons, suitable breeding studies for various pepper types have been emphasized all over the world and biotechnology, especially haploidy is widely used. In *C. annuum*, microspore embryogenesis based androgenic techniques are mostly used for the production of doubled haploids. However, the androgenesis studies in *C. annuum* are generally focused on edible varieties and studies for ornamental peppers are very few in the literature (Barraso *et al*., 2015; Arı *et al*., 2016a, 2016b).

In a review study, Segui-Simarro *et al*. (2011) stated that the most efficient results in microspore embryogenesis studies for pepper were gained from the protocols: (1) anther culture method introduced from Dumas de Vaulx *et al*. (1981), (2) two-phased method developed by Dolcet-Sanjuan *et al*. (1997), (3) two-phased stationary shed-microspore culture consisting of a solid medium layer with activated charcoal and a liquid medium layer improved by Supena *et al*. (2006b) and (4) isolated microspore culture method. However, due to genotype factor and embryo development difficulties, there is not still a routine used androgenesis protocol for pepper. Among these protocols, the stationary shed-microspore culture technique developed by Supena *et al*. (2006b) has become prominent with its practical application and high yield of embryos (Supena *et al*., 2006a, 2006b; Supena and Custers, 2011; Arı *et al*., 2016a, 2016b). Besides these advantages, in the solid phase of shed-microspores culture, activated charcoal has some critical roles in embryo development. The pore structures of activated charcoal adsorb the toxic and phenolic components released from anther tissues and microspores in the liquid media. Also activated charcoal stores these substances inside their large internal volume. Thus, the adsorption of phenolics prevents the damages in culture material and provides a better output (Thomas, 2008). Supena *et al*. (2006b) pointed out that they got the higher numbers of total and normal looking healthy embryos from the activated carbon added stationary shed-microspore cultures of Indonesian hot peppers.

The shed-microspore culture can be regarded as a kind of anther culture in the liquid medium in terms of the development of microspores released from anther walls in the liquid medium of biphasic media. A better contact of explant with liquid media results in faster growth than with gel media. By providing agitation to liquid media, the explants use more efficiently the nutrients and growth regulators in the medium and accumulation of toxic metabolites near the explants efficaciously spread out (George, 1993). Kim *et al*. (2013) interpreted the reason of high yield of embryo development in liquid medium as the microspores in the liquid medium could reach the necessary nutrients more easily thanks to their freedom of movement. Another advantage of liquid medium to gels is its aeration opportunity (Smith and Spomer, 1995). Takahashi *et al*. (1992) demonstrated that agar-based medium suppressed the production of lily-bulblets. Conversely, the best propagule quality was obtained from the aeration optimized liquid tank cultures in their study.

The growing habits of some cultures are better in liquid media than on solid media. A gentle agitating with a rotator or a shaker may help to aeration of medium and so prevents the explants from submerging (Kyte and Kleyn, 1996). The advantages of shaken liquid cultures are noticed in various micropropagation studies with their high carbohydrate reserves, easy acclimatization abilities and better root developments. Among these, the results of shaken liquid cultures of *Hosta tokudoma* (Newberry Gold) and *Hosta x hybrid* Tratt. (Blue Cadet) (Adelberg *et al*., 2000; Adelberg, 2005), *Colocasia esculenta* L. Schott Fontanesii and *Alocasia macrorrhizos* G. Don (Adelberg and Toler, 2004), and *Ophiopogon planiscapus* 'Nigrescens' (Black Mondo) (Arı *et al*., 2015) are considerable.

In regard of haploidy studies, only Yang *et al*. (2013) examined the effect of shaker use. They compared the embryo formations and plant regeneration capacities of stationary *Brassica rapa* L. ssp *chiensis* microspore cultures and cultures on shakers with different frequencies (40 rpm, 50 rpm, 80 rpm, 100 rpm). According to the results, embryos with higher quality and more regeneration capacities were obtained from the cultures on shakers with 50 rpm frequency than the embryos obtained from stationary cultures.

There are a few haploidy studies (Barraso *et al*., 2015; Arı *et al*., 2016a, 2016b) in ornamental peppers. The increasing their embryo yields would help to improve the efficiency of ornamental pepper breeding programs. The aim of this study is to detect the effect of shaker use on embryo yield of 29 ornamental pepper genotypes' shed-microspore cultures.

MATERIAL AND METHOD

Plant Material

The material for present study consisted of 29 ornamental pepper (*Capsicum annuum* L.) genotypes. From those, 25 genotypes were originated from cultivars encoded with (C) letter and 4 genotypes were originated from local cultivars encoded with (L) letter. The seeds of the plant material were procured from Pey-Art Ltd. located in Antalya, Turkey. The seeds of 29 ornamental pepper genotypes were planted in viols containing 10% vermiculite, 25% perlite and 65% peat mixture, then the developed seedlings were planted with a distance of 30 cm as row distance and 80 cm between two seedlings in a high plastic greenhouse in Antalya in spring. The plants fertilized with NPK (15-15-18 % w/w) by drip irrigation in every two weeks. Despite not being recommended; an insecticide has been applied only once due to intensive pest attack.

The proper ornamental pepper buds having appropriate staged microspores as described by Arı *et al*. (2016b) were used as material. We detected the stages of microspores by using 4′,6-diamidino-2-phenylindole (DAPI) staining technique (Sigma-Aldrich, St. Louis, MO, USA) as Coleman and Goff (1985) and Kim and Jang (2000) described. The candidate buds were collected in falcon tubes from the greenhouse between 8 am and 9 am in the morning and were kept cold until arrival at the laboratory. Subsequently, the buds were treated at 4^0C for 24 h and then sterilized in 15 % commercial bleach added with Tween 20 for 10 min, lastly rinsed with sterile dH_2O for three times. As culture material, the anthers of the genotypes were isolated from the buds under stereomicroscope in sterile laminar flow.

Media and Culture Conditions

The anthers (5-7) of each disinfected bud were sown in the two phased shed-microspore culture media which was the combination of Supena *et al*. (2006b) and Supena and Custers (2011) with minor amendments. The culture medium, in which the solid and liquid phases coexist, consists of the Nitsch and Nitsch (NN) (1969) components both in the liquid medium at the upper layer and solid medium at the under layer. The solid under layer was consisted of NN components with the addition of 2% maltose, 1% activated charcoal and 0.6% plant agar. The upper liquid layer was composed of NN liquid medium with 2% maltose, filter-sterilized 2.5 µM zeatin and 5 µM IAA (indole-3-acetic acid). Although Supena and Custers (2011) added zeatin and IAA in liquid medium 3 weeks after the incubation in their protocol, in our study, zeatin and IAA was added to the liquid medium before incubation to avoid contamination risk as used by Arı *et al*. (2016a, 2016b). The pH of the both medium was adjusted to 5.8 and autoclaved at 121 ^{o}C for 20 min., and then poured on petri dishes (60 mm in diameter; with solid layer containing 4 mL and liquid layer containing 5-6 mL).

As culture conditions, anthers from each bud were placed in one petri dish and incubated at 9 ^{0}C for one week. Afterwards, from the 10 petri dishes prepared for each genotype, 5 petri dishes were left stationary state for control and 5 were placed on an orbital shaker at 50 rpm per min for 3 weeks at 28^0C in continuous darkness. Lastly, the stationary and shaken cultures were transferred to 21 ^{o}C for 3-5 weeks in the dark.

Statistical Analysis

The experiment was repeated 3 times for stationary and shaken groups. The whole data were collected after 8 weeks of culture. The information of average numbers of total and normal-looking embryos for each genotype of stationary and shaken cultures were recorded. The averages of total and normal-looking embryo as per bud were evaluated as Supena *et al*. (2006a, 2006b) and Ari *et al*. (2016a, 2016b) described in their studies. One-way analysis of variance (ANOVA) was applied to define the differences in applications and genotypes. The data were normalized prior to analysis by Sqrt (x + 0.5), where x represents the number of embryos per bud in order to encounter the assumptions of ANOVA. The general linear model procedure of SPSS (Statistics 20) software (IBM Corp., Armonk, NY, USA) was used for data analyses. All main effects were considered as fixed effects. Multiple comparisons of the genotypes were performed by using Tukey's multiple range post hoc test at an alpha 0.05 level.

RESULTS

Shed-microspore Culture of Stationary Cultures

According to ANOVA results it was found that genotypes differ from each other statistically ($p < 0.001$) in the mean number of both total and normal looking embryos per bud. However, there was no significant difference between stationary and shaken cultures.

As given in Table 1, of the 29 genotypes, 13 were responsive to shed-microspore culture in stationary cultures. Between 0.14 and 4.69 embryos per bud were obtained from these 13 genotypes (Table 1). Statistically, the commercial originated genotype 754 had the highest performance (4.69 embryos per bud) and it was determined that this genotype was statistically different from the other genotypes. This genotype was followed by genotypes 735 and 283 with an average of 2.17 and 2.00 embryos per bud, respectively. In terms of average number of normal-looking embryos per bud, the highest formation of normal-looking embryos were obtained from local genotype 735 with a value of 0.67 embryos per bud.

Table 1. Comparison of the androgenic responses of stationary and shaken shed-microspore cultures of 29 ornamental pepper genotypes.
Çizelge 1. 29 süs biber genotipinin durağan ve çalkalanan kültürlerindeki androjenik tepkilerinin karşılaştırılması.

Origin of the Genotype*	Genotype	Average yield of embryos per flower bud			
		No. of total embryos produced		No. of total normal-looking embryos produced	
		Stationary cultures	Shaken cultures	Stationary cultures	Shaken cultures
		M±SE**	M±SE	M±SE	M±SE
C1	3	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	17	0.14±0.14 ^{bc}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C2	34	1.00±0.54 ^{bc}	0.00±0.00 ^{b}	0.27±0.15 ^{abc}	0.00±0.00 ^{b}
C3	47	0.69±0.40 ^{bc}	0.00±0.00 ^{b}	0.08±0.08 ^{abc}	0.00±0.00 ^{b}
C4	54	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	96	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C5	107	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C6	108	0.57±0.40 ^{bc}	0.00±0.00 ^{b}	0.07±0.07 ^{bc}	0.00±0.00 ^{b}
C8	127	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	132	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C9	419	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	143	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	146	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	155	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	166	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
	174	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C10	230	0.47±0.27 ^{bc}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C11	283	2.00±0.79 ^{ab}	1.23±0.90 ^{b}	0.20±0.14 ^{abc}	0.23±0.17 ^{b}
	292	0.27±0.21 ^{bc}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C12	313	1.27±0.40 ^{abc}	0.57±0.39 ^{b}	0.27±0.12 ^{abc}	0.00±0.00 ^{b}
C13	324	0.07±0.07 ^{bc}	0.00±0.00 ^{b}	0.07±0.07 ^{bc}	0.00±0.00 ^{b}
	330	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C14	337	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
C18	383	0.27±0.19 ^{bc}	0.38±0.38 ^{b}	0.00±0.00 ^{c}	0.08±0.08 ^{b}
C20	754	4.69±2.33 ^{a}	20.4±9.76 ^{a}	0.46±0.22 ^{ab}	1.27±0.44 ^{a}
L1	441	0.00±0.00 ^{c}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
L5	564	0.00±0.00 ^{c}	0.13±0.13 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
L12	707	0.31±0.31 ^{bc}	0.00±0.00 ^{b}	0.00±0.00 ^{c}	0.00±0.00 ^{b}
L16	735	2.17±2.17 ^{abc}	3.15±3.15 ^{b}	0.67±0.67 ^{a}	0.08±0.08 ^{b}

*Letter C and L symbolizes commercial and local cultivars, respectively.
**Values are mean and standard error (SE). Means were separated by using Tukey's multiple range post hoc test. Different letters near the means represent significant difference at P≤0.05.

The total number of embryos obtained from genotypes 754, 283 and 735, which were recorded to have the best performance, were determined to be 61, 30 and 13, respectively (Table 2). The normal looking embryos were developed again mostly from genotype 754. Among the total embryos, those that could not transform into

normal looking embryos with two cotyledons either remained in globular shape or showed abnormal growth such as embryos with only one cotyledon.

When the embryo yield of stationary cultures was evaluated in general, a total of 177 embryos obtained from 13 genotypes, of which 63 were in the globular stage, 90 embryos with one cotyledon and 24 normal-looking embryos with two cotyledons (Table 2).

Table 2. The number of total, globular, abnormal (with one cotyledon) and normal-looking (with two cotyledons) embryos from the stationary and shaken shed-microspore cultures of 29 ornamental pepper genotypes.
Çizelge 2. 29 süs biberi genotipinin durağan ve çalkalanan kültürlerindeki toplam, globular, anormal (tek kotiledonlu) ve normal görünümlü embriyo sayıları.

Origin of the Genotype*	Genotype	No. of total embryos		No. of globular embryos		No. of abnormal embryos with one cotyledon		No. of normal-looking embryos with two cotyledons	
		Stationary cultures	Shaken cultures	Stationary cultures	Shaken cultures	Stationary cultures	Shaken cultures	Stationary cultures	Shaken cultures
C1	3	0	0	0	0	0	0	0	0
	17	2	0	2	0	0	0	0	0
C2	34	15	0	8	0	3	0	4	0
C3	47	10	0	1	0	8	0	1	0
C4	54	0	0	0	0	0	0	0	0
	96	0	0	0	0	0	0	0	0
C5	107	0	0	0	0	0	0	0	0
C6	108	8	0	3	0	4	0	1	0
C8	127	0	0	0	0	0	0	0	0
	132	0	0	0	0	0	0	0	0
C9	419	0	0	0	0	0	0	0	0
	143	0	0	0	0	0	0	0	0
	146	0	0	0	0	0	0	0	0
	155	0	0	0	0	0	0	0	0
	166	0	0	0	0	0	0	0	0
	174	0	0	0	0	0	0	0	0
C10	230	7	0	4	0	3	0	0	0
C11	283	30	16	13	6	14	7	3	3
	292	4	0	4	0	0	0	0	0
C12	313	19	8	5	8	10	0	4	0
C13	324	1	0	0	0	0	0	1	0
	330	0	0	0	0	0	0	0	0
C14	337	0	0	0	0	0	0	0	0
C18	383	3	5	1	0	2	4	0	1
C20	754	61	306	17	230	38	57	6	19
L1	441	0	0	0	0	0	0	0	0
L5	564	0	2	0	2	0	0	0	0
L12	707	4	0	0	0	4	0	0	0
L16	735	13	41	5	30	4	10	4	1
	Total	**177**	**378**	**63**	**276**	**90**	**78**	**24**	**24**

*Letter C and L symbolizes commercial and local cultivars, respectively.

Shed-microspore Culture of Shaken Cultures

As given in Table 1, of the 29 genotypes, 6 were responsive to shed-microspore culture in the shaken cultures. Between 0.13 and 20.4 embryos per bud were obtained from these 6 genotypes. The genotype 754 with commercial origin apparently produced the highest number of total (20.4 embryos per bud) and normal-looking embryos (1.27 embryos per bud) and distinguished from the other genotypes. This genotype was followed by genotypes with an average of 3.15 and 1.23 total embryos per bud, respectively, from local genotype 735 and genotype 283 with commercial origin. In terms of producing normal-looking embryos, the highest average per bud was obtained from 754 with a value of 1.27.

The total number of embryos obtained from genotypes 754, 735 and 283, which had the best performance, were determined to be 306, 41 and 16, respectively (Table 2). The highest transition to normal looking embryos was observed in genotype 754. As in the stationary cultures, the embryos that could not normally develop, stayed in globular stage or developed abnormally.

In terms of embryo production of shaken cultures, a total of 378 embryos obtained from 6 genotypes, of which 276 were in the globular stage, 78 embryos with one cotyledon and 24 normal-looking embryos with two cotyledons (Table 2). After one month of culture when the embryos appeared in the medium, in the shaken cultures, the normal-looking embryos were kept in the medium to complete their developments. However, these embryos turned into brownish color and died in a short span of time.

DISCUSSION

In androgenesis studies, the genotype is accepted as one of the most important factor for the androgenic response of microspores (Segui-Simarro, 2010). The tendency to microspore embryogenesis may vary in different plant varieties even they are in the same species. Some variants do not respond microspore embryogenesis at all, while some highly response (Ferrie *et al*., 1995; Touraev *et al*., 2001; Malik *et al*., 2008; Segui-Simarro, 2010). Also in this study, genotype had a very significant effect in terms of total embryo and normal-looking embryo formations ($p \leq 0.001$). The results agree with those obtained by Arı *et al*. (2016a, 2016b) and by Barraso *et al*. (2015) working on haploid embryogenesis in different ornamental pepper genotypes.

In regard to culture type, there was no significant difference between the stationary and shaken cultures in the study. The genotype 754 became prominent in terms of average yield of total and normal-looking embryo formations in both stationary and shaken culture which shows again the dominant effect of genotype. We detected about 4.5 times more average yield of total embryo formation in the shaken cultures of genotype 754 in comparison to its stationary group. The number of embryos per bud of genotype 754 increased from 4.69 to 20.4. This result agrees with the findings of Yang *et al*. (2013) who revealed the positive results of shaker use on microspore embryogenesis. In their study, of the 7 tested genotypes belongs to *Brassica rapa* L. ssp. *chinensis,* the microspore embryogenesis and embryo development performances were higher in shaken cultures when compared to their stationary control groups. In particular, shaking culture success of the varieties of Huaguan and YS07 significantly increased in comparison to the stationary culture. The number of embryos per bud increased from 1.87 to 3.13 in Huaguan while from 24.00 to 28.53 in YS07, the most responsive variety. Six of the 29 ornamental pepper genotypes tested in our study showed positive responsive to the shaken cultures. Thus, we deduced that shaking cultures could strongly enhance microspore embryogenesis in ornamental pepper. However, this influence had quite strong genotype-dependent effect.

In terms of normal-looking embryo formation, the transition rates of embryos to normal-looking embryos in stationary and shaken cultures were 6.34 % and 13.56 % respectively. From this point, the positive results of shaker use were also compatible with Yang *et al*. (2013). On the other hand, the normal-looking embryo formations were generally in low ratios both in stationary and shaken cultures in our study. The normal healthy embryos with two cotyledons that were kept waiting for a better development at 21 ^{0}C in dark conditions soon became brown and lost their vitality especially in shaken cultures. This circumstance was seen also in the globular embryos of shaken cultures. One month after the culturing process, the globular embryos were apparent in the cultures of both applications. However most of the globular embryos in the shaken cultures could not complete their development, stopped their maturation.

In the literature, there are limited number of studies focusing on the effects of shaker use on haploidy performance but there are more in other plant tissue culture studies. For instance, Raghuvanshi and Srivastava [1995] introduced positive effects of shaker use on micropropagation of mango. They solved the problem of phenolic-derived browning in explants by pretreating them in liquid medium on an automated shaker and refreshing the medium at certain intervals. Their work showed that pretreating explants in liquid shaker culture

and medium renovation helped to remove the phenolic exudates in mango thereby provided a better micropropagation.

The importance of culture medium renovation on embryo formation and regeneration were also revealed in several haploidy studies. Dias and Correia (2002) reported the positive effect of culture medium renovation on tronchuda cabbage "Couve Algarvia" microspore culture embryogenesis. The responsiveness to microspore embryogenesis increased when the medium was renewed. Li and Devaux (2003) reported that embryo development and regeneration capacities of *Hordeum vulgare* L. microspores incubated in liquid medium for 3 weeks and then in solid medium for 3 weeks were higher than cultures incubated only in liquid medium for 6 weeks. They stated that renewing the culture media allows the embryos to ventilate better and to develop in a healthier direction. The benefit of medium renovation on embryo yield is also revealed by Wei *et al*. (2008) in a study of isolated microspore culture of ornamental kale (*Brassica oleracea* var. *acephala*). They observed clusters of brown/dead cells on microspore suspensions cultures of not renewed medium while cells were alive where medium was renovated. In addition, Kim *et al*. (2013) showed the positive effects of media change on haploid performance of a hot pepper genotype (*C. annuum* L. cv. Milyang-jare) for microspore embryogenesis. They stated that liquid media have positive effects on embryo nutrient transport, but at the same time toxic and growth inhibiting substances are easily transported from the media to the embryos. Thereby, they reported that refreshing culture medium helps to dilute the detrimental compounds and reduces the negative effects of these harmful materials on embryo development.

When all these studies were considered, one possible reason of low embryo yield in our study can be explained by the fact that the anthers and microspores dispersed from the anthers in liquid medium were cultured in the same medium for about 2 months from the beginning. Thus, medium renovation at some intervals might contribute embryo quality and development for the future liquid culture studies in ornamental peppers.

As another important factor affecting androgenesis response, the donor plant growth conditions have a crucial role to induce haploid embryogenesis. The importance of this factor was underlined by Supena *et al*. (2006b). They emphasized that to obtain healthy buds is only possible from the plants grown in fully controlled phytotrones. In the present study, the donor plants were grown in a greenhouse and an insecticide had to be applied only once because of necessity. According to Custers (2003), the vitality of microspores is negatively affected from pesticides whether it is applied as spray or from soil. As Ferrie and Caswell (2011) stated, the essential condition in obtaining a successful and consistent microspore culture is using healthy and pesticide free donor plants.

CONCLUSION

Demand and interest in ornamental peppers due to their economic potential also increased the breeding studies on these plants. However, there are very few breeding studies using haploidy technique. In this study, the effect of shaker use on embryo yield was determined by using shed-microspore culture method based on haploidy technique. The shaken cultures gave more successful androgenic performance in terms of total and globular embryo formations. The use of shaker in shed-microspore cultures of ornamental peppers has positive effect on embryo yield, but this effect is genotype-dependent. In future studies, embryo yield in shaken cultures might be improved by cultivation of donor plants under controlled conditions and renovating the liquid medium of cultures at certain intervals.

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