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Androgenic responses of 64 ornamental pepper (*Capsicum annuum* L.) genotypes to shed-microspore culture in the autumn season

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Abstract: The recent gradual increase in interest and demand for multipurpose ornamental peppers has encouraged intensive breeding studies, especially for use in landscape. However, information in the literature about haploidy is limited. Thus, first, we intend to identify proper morphological markers for quick selection of suitable flower buds containing convenient microspores in two sample ornamental pepper genotypes with and without anthocyanin in external appearance. Secondly, the androgenic responses of 64 genotypes are determined in the autumn season through shed-microspore culture. Based on DAPI-assisted results, the calyx/bud ratio can be used as an effective morphological marker for proper bud selection in both full anthocyanin (70%–80%) and green-looking (70%–85%) genotypes. The indicator of anthocyanin pigmentation in anthers, as used in peppers, is a convenient marker for green-looking genotypes (10%–40%), although it is not suitable for genotypes with full anthocyanin (70%–90%). Of a total of 64 genotypes, 48 responded to the shed-microspore culture and produced embryos in varying ratios. The highest average yields of total and normal-looking embryos per flower bud were found to be 12.7 and 1.48, respectively. The promising embryo formation results obtained in autumn verified that the shed-microspore culture technique can be used successfully in different ornamental pepper genotypes with landscape value for double haploid plant production.

Key words: Anther, bud morphology, *Capsicum annuum*, haploid, microspore embryogenesis, ornamental pepper, ornamental plant, shed-microspore culture

1. Introduction

Ornamental peppers (*Capsicum annuum* L.) have the advantage of multipurpose use in fresh, spice, and pickle consumption, besides being pot and garden plants and cut flower arrangement species. Lately, interest and demand for ornamental peppers has increased. According to Stommel and Bosland (2006), ornamental peppers have begun to regain their previous popularity due to easy seed propagation, relatively short cropping time, and heat/drought tolerance. The varied foliage color and colorful fruits characterizing ornamental peppers ensure a vivid summer garden display that rivals the chrysanthemum (*Dendramthema grandiflora* Tzvelev) for brilliant fall color (Stommel and Griesbach, 2005).

Steadily growing interest has encouraged intensive breeding studies of ornamental peppers. When evaluated in terms of method, it was seen that the majority of these studies (Stommel and Griesbach, 1993, 2004, 2005, 2008a, 2008b; Taychasinpitak and Taywiya, 2003; Gajanayake et al., 2011; Barroso et al., 2012; Santos et al., 2014) were carried out with classical breeding methods. However, biotechnological methods have also begun to be included in marker development for marker-assisted selection (Lee et al., 2008) and in molecular-level research for anthocyanin biosynthesis (Lightbourn et al., 2007; Stommel et al., 2009).

Among the biotechnological breeding methods, the in vitro double haploid (DH) technique can provide prompt homozygosity and stable 100% homozygous lines for desired features only in one generation. Even though the DH method has been used effectively in breeding programs of different pepper types, information is lacking for haploidy reports on ornamental peppers for landscape aims.

Anther or microspore cultures have been used to produce DH plants in pepper since 1973. In spite of their simplicity, pepper anther cultures show certain problems, such as low efficiency, uncontrolled tapetum secretion, and the possibility of somatic regenerants (Segui-Simarro et al., 2011). Thus, the number of relevant research studies has increased in order to improve an efficient microspore culture protocol in pepper. Kim et

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al. (2008) obtained fairly successful results in isolated microspore culture. Their improved method (Kim et al., 2013), a two-step culture system, provided the best results thus far in mechanically isolated microspore culture of pepper. However, certain disadvantages, such as increased labor and sensitivity, complex lab equipment, and higher contamination risk, are important limitations of its easy application in the laboratory. Therefore, several research studies have been conducted to develop a simpler method to isolate the microspores and produce DH plants in an easier manner. Among these, the liquid anther cultures in tobacco (Sunderland and Roberts, 1979), colza (Lichter, 1981), *Anemone* sp. and *Clematis* (Johansson et al., 1982), potato (Uhrig, 1985), barley (Ziauddin et al., 1990), and winter triticale (Ponitka and Ślusarkiewicz-Jarzina, 2007) are remarkable. As a type of submethod between anther and microspore culture, the shed-microspore technique has lately drawn attention. The merits of this technique are based on Dolcet-Sanjuan et al.'s (1997) double-layer anther culture system. Additionally, its name originates from Ziauddin et al.'s (1990) study. Supena et al. (2006a, 2006b) modified this method and Supena and Custers (2011) refined the technique for Indonesian hot peppers. Due to its practical application and high efficiency, shed-microspore culture has advanced among pepper androgenesis techniques.

There are many factors influencing the success of in vitro androgenesis, including genotype, microspore developmental stage, culture medium ingredients, and plant growth conditions including culture season. Among them, the effect of genotype on haploid embryogenesis in pepper has been shown to be the most important factor in many studies (Kristiansen and Andersen, 1993; Qin and Rotino, 1993; Mitykó et al., 1995; Rodeva et al., 2004; Gémes Juhász et al., 2009; Nowaczyk et al., 2009).

Among the factors affecting microspore embryogenesis, proper microspore stage is generally the second critical factor after genotypes. It is known that late unicellular, vacuolated microspores, and, in some cases, young bicellular pollen are suitable for haploid induction (Segui-Simarro, 2010). Indirect indicators have been used due to the difficulties in cytologically determining the presence of the optimal microspores, described above, every time. The most commonly used indirect morphologic indicators in pepper are the size of the flower buds and the relation of the calyx length to the bud (calyx/bud ratio) (Sibi et al., 1979; Nowaczyk and Kisiała, 2006).

In ornamental peppers, several commercial varieties were improved from diverse origins such as *C. baccatum*, *C. chinense*, and *C. frutescens*, as well as *C. annuum* via interspecific crosses (Bosland, 1992; Stommel and Bosland, 2006). Therefore, there are very different growth habits among ornamental peppers, such as short, intermediate, or tall habitus. In addition, several have dense anthocyanin pigmentation not only in their anthers but also in their stems, leaves, calyxes, or bud leaves, as opposed to the green-looking genotypes showing anthocyanin pigmentation only in their aging anthers. Their bud sizes are also different, with some bigger or smaller than each other. However, it is certain that the general habitus and subsequently the flower bud sizes of ornamental peppers are mostly smaller than those of their vegetable relatives. Therefore, calyx/bud ratios or other morphologic markers, such as anthocyanin pigmentation in the anthers used for vegetable peppers, might not be good morphological markers for ornamental peppers due to the large variation in their different flower bud morphology. Consequently, it may be useful to separately identify the suitable morphological markers for the selection of the desired flower buds containing appropriate microspore developmental stages in both anthocyanin-pigmented and green-looking genotypes as external plant appearance.

The purposes of this study are to identify the proper morphological markers for the selection of convenient flower buds in two ornamental pepper genotypes with and without anthocyanin, and to determine the androgenic responses of 64 ornamental pepper genotypes with landscape value through shed-microspore culture in the autumn season.

2. Materials and methods

2.1. Plant material

Sixty-four ornamental pepper (*Capsicum annuum* L.) genotypes, originating from both commercial cultivars and local accessions represented with the letters "C" and "L", respectively, were used. Fifty-one segregated genotypes originating from 21 commercial cultivars and 13 segregated genotypes derived from 10 local accessions were tested for their androgenic response through shed-microspore culture. Eight seedlings were grown for each genotype from the seeds of the segregated population in F_3 (C) or F_4 (L) generations provided by Pey-Art Ltd., Antalya, Turkey. Donor plants were grown in an unheated plastic greenhouse in Antalya during the 2013 autumn season. They were fertilized with 0.50 g L^{-1} per plant of 15-15-18 (15% nitrogen-15% phosphorus-18% potassium) water soluble fertilizer twice a week via drip irrigation and were subjected to an insecticide application only once out of necessity.

Desired flower buds containing more than 50% late unicellular microspores, as well as midunicellular ones and young bicellular pollens, as suggested by Supena et al. (2006b), were collected early in the morning in November and December and exposed to cold pretreatment at 4 °C for 24 h. Afterwards, they were sterilized in 15% commercial bleach with Tween 20 added for 10 min, followed by rinsing three times with sterile dH_2O.

2.2. Determination of morphological markers for selection of proper flower buds

Among 64 ornamental pepper genotypes, genotypes 1 and 117 (Figures 1A and 1B) were chosen randomly as samples to represent genotypes with and without anthocyanin in their external plant appearance. Their flower buds were used to determine the proper morphological markers for the selection of convenient flower buds containing appropriate microspore developmental stages. The flower buds of each genotype were separated into six groups based on their size (Figure 1C) on 28 October 2013. Morphological and cytological observations were then performed in each group with three replications. Bud and calyx length were measured with a digital caliper. Calyx length was divided by bud length for the determination of calyx/bud ratio. The ratio of anthocyanin pigmentation in an anther was estimated visually. The presence of proper microspore developmental stages in the buds was determined with 4',6-diamidino-2-phenylindole (DAPI) DNA-specific fluorochrome dye (Sigma-Aldrich, St. Louis, MO, USA) according to Custers (2003).

2.3. Shed-microspore culture

The combined protocols of Supena et al. (2006b) and Supena and Custers (2011) were followed for shed-microspore culture with a small modification. A double-layer medium was used for the culture, the underlayer of which consisted of Nitsch and Nitsch medium (NN; Nitsch and Nitsch, 1969) with 1% activated charcoal, 2% maltose, and 0.6% plant agar. The liquid upper layer was composed of NN medium with 2% maltose. After the anthers (5–7) of one flower bud were placed on a double-layer medium in each petri dish (60 mm in diameter; each layer containing 4 mL), the cultures were incubated at 9 °C for 1 week and then transferred to 28 °C for 3 weeks. Later, the cultures were moved to 21 °C for an additional 3–5 weeks in the dark. According to Supena and Custers (2011), a combination of filter-sterilized 2.5 µM zeatin and 5 µM indole-3-acetic acid should be added to the upper layer of the cultures after 3 weeks of incubation. To prevent contamination risk, it was added to the upper layer during the initial culture in the present study as a small modification. The pH of the media was adjusted to 5.8 and media were autoclaved at 121 °C for 20 min and poured into petri dishes. Three replications of each 5–10 petri dishes were cultured per genotype. Duchefa chemicals (Duchefa Biochemie, Haarlem, the Netherlands) were used in the entire study.

2.4. Plant regeneration

During the incubations, the formation of embryos was checked frequently under a stereomicroscope. Normal-looking embryos with two cotyledons were placed onto half-strength MS medium (Murashige and Skoog, 1962) with 0.1 µM benzyl adenine, 2% sucrose, and 0.6% plant agar in Magenta B-cap (Sigma-Aldrich) for germination. The vessels were incubated at 25 °C under 16 h of light (50 µmol m^{-2} s^{-1}). After 4–5 weeks, healthy seedlings with a few real leaves and enough root systems were subjected to acclimatization treatments. Following acclimatization and ploidy analysis, the surviving seedlings, including haploid and spontaneous DH, were transferred to the greenhouse for further growth, colchicine treatment, or selfing procedures.

2.5. Ploidy analysis

The young leaf pieces of the regenerants were processed using the CyStain UV Precise P Kit for extraction and staining, and were then analyzed in CyFlow Ploidy Analyzer (Partec GmbH, Münster, Germany).

2.6. Statistical analysis

After 8 weeks of culture, the data of the average number of total embryos produced and the average number of normal-looking embryos were recorded for each genotype. The total and normal-looking embryo yields of the genotypes were presented as per bud, and not per 100 anthers, as in the protocol of Supena et al. (2006b). One-way analysis of variance (ANOVA) was performed to determine the differences between genotypes. In order to meet the assumptions of ANOVA, data were normalized prior to analysis by Sqrt (x + 0.5), where x represents the number of embryos per bud. The general linear model procedure of MINITAB 15 (MINITAB, State College, PA,

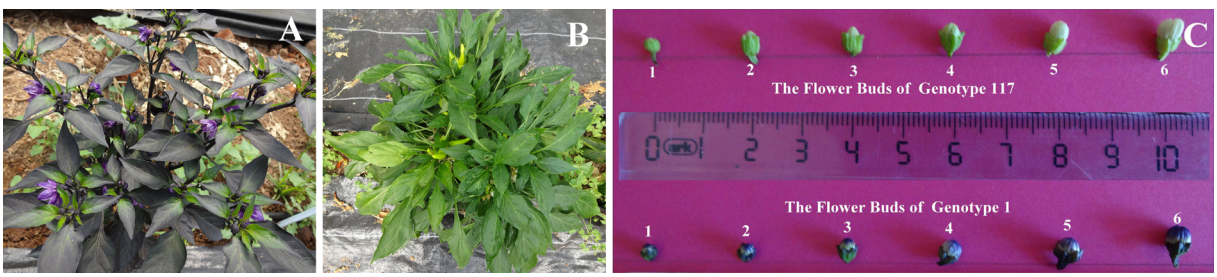


Figure 1. Two ornamental pepper genotypes and their flower buds used for the determination of proper morphological markers for the selection of convenient flower buds. (A) Genotype 1 with anthocyanin; (B) genotype 117 without anthocyanin in external plant appearance; (C) flower buds of Genotype 117 (above) and genotype 1 (below).

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 HSD post hoc test at an alpha 0.05 level.

3. Results

3.1. Determination of morphological markers for selection of proper flower buds

The measured and observed morphological and cytological characteristics of the flower buds belonging to genotypes 1 and 117 are presented in Table 1.

Based on the DAPI staining results, the proper microspore stages were determined in the first and second flower bud groups in genotype 1, with full anthocyanin, and in the second and third flower bud groups in genotype 117, without anthocyanin in the external appearance of the plant. The calyx/bud ratios of the proper flower buds were 70%–80% and 70%–85% in genotypes 1 and 117, respectively. The ratios of anthocyanin pigmentation in anthers of the suitable buds belonging to genotypes 1 and 117 were estimated visually as 70%–90% and 10%–40%, respectively. Several developmental stages of the microspores in the flower buds of genotypes 1 and 117, determined by DAPI staining, are presented in Figures 2A–2H.

As seen from Figure 2, the desired microspore stages for haploid induction are the late unicellular, vacuolated microspores (Figures 2B and 2F) and young bicellular pollens (Figures 2C and 2G). The flower buds containing more than 50% of late unicellular microspores as well as midunicellular and young bicellular pollens were in bud groups 1 and 2 in genotype 1 and in bud groups 2 and 3 in genotype 117.

3.2. Shed-microspore culture

ANOVA results showed very significant differences in the mean number of both total and normal-looking embryos produced per bud among the genotypes (both at $P \leq 0.001$). Of the tested 64 genotypes, 48 were responsive to shed-microspore culture (Table 2).

As presented in Table 2, the genotypes originating from commercial cultivars were outstandingly more responsive and generated more embryos per bud compared to the genotypes derived from local accessions. Genotype 75 (12.68 ± 4.03), genotype 9 (11.74 ± 3.43), and genotype 73 (10.50 ± 9.03) were the three most successful genotypes, producing the highest average number of embryos. In

Table 1. Morphological and cytological characteristics of different-sized flower buds belonging to two ornamental pepper genotypes, genotypes 1 and 117.

No. of bud	Bud length (mm)	Calyx length (mm)	Ratio of calyx/ bud (%)	Ratio of anthocyanin pigmentation in anther (%)*	Presence of proper microspore stage** (+: present) (-: absent)
Genotype 1 with anthocyanin					
1	2.85 ± 0.22	2.23 ± 0.12	75–80	70–80	+
2	3.38 ± 0.24	2.47 ± 0.06	70–75	80–90	+
3	4.04 ± 0.17	2.67 ± 0.32	60–70	90–100	-
4	5.16 ± 0.31	2.74 ± 0.35	50–60	100	-
5	6.29 ± 0.36	2.83 ± 0.50	40–50	100	-
6	7.60 ± 0.64	2.97 ±1.03	30–40	100	-
Genotype 117 without anthocyanin					
1	3.00 ± 0.14	2.73 ± 0.12	85–90	0	-
2	4.37 ± 0.55	3.61 ± 0.38	80–85	10–20	+
3	5.28 ± 0.20	3.87 ± 0.33	70–80	20–40	+
4	6.06 ± 0.33	3.93 ± 0.11	60–70	40–60	-
5	7.22 ± 0.33	4.05 ± 0.41	50–60	60–95	-
6	8.66 ± 0.54	4.07 ± 0.32	40–50	100	-

*These ratios were estimated visually.

**The presence of proper microspore stages containing more than 50% late unicellular microspores besides midunicellular microspores and young bicellular pollens in the convenient flower buds was determined by DAPI staining.

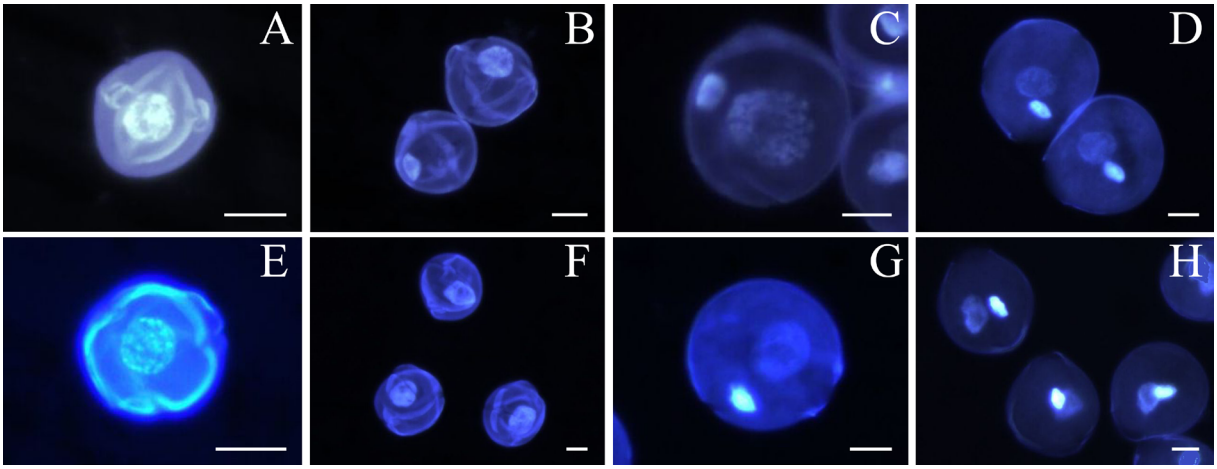


Figure 2. Different developmental stages of the microspores determined by DAPI staining in the flower buds of genotype 1 (A–D) and genotype 117 (E–H) Bars in A–H: 10 µm. (A) Early and (B) late unicellular, vacuolated microspore stage in the first bud; (C) young bicellular pollen stage in the second bud; (D) late pollen stage in the fourth bud; (E) early unicellular microspore stage in the first bud; (F) late unicellular, vacuolated microspore stage in the second bud; (G) young and middle bicellular pollen stage in the third bud; (H) late pollen stage in the fifth bud.

terms of normal-looking embryo formation, genotype 9 (1.48 ± 0.68) was the most responsive. The average values for the mean number of total and normal-looking embryos produced per bud of the 64 genotypes were 1.14 ± 0.51 and 0.15 ± 0.08, respectively. The numbers of produced total and normal-looking embryos, normal-looking embryos transferred to germination medium, and germinated embryos are given in Table 3.

Of a total of 2406 embryos produced, 297 transformed to normal-looking embryos occurring from radicle, hypocotyl, and two cotyledons with an average conversion ratio of 12.34% (Table 3). Genotypes 75, 73, and 9 were the most prolific genotypes in terms of the total number of embryos with 482, 399, and 317 embryos, respectively. However, the highest conversion ratio (47.83%) of total embryos to normal-looking embryos was found in genotype 17 (from 69 to 33 embryos), followed by genotype 200 (22.1%) (from 181 to 40 embryos).

The indicated total and normal-looking embryo yields would have been much higher if the numerous proembryos or globular embryos (Figure 3A) or the produced embryos had continued their normal growth. Most of the proembryos and globular embryos ceased their development and did not reach the next stage, whereas the others continued to grow (Figure 3B). In terms of abnormal embryos, the vast majority lacked either radicle or cotyledon, and several others possessed a single cotyledon (Figure 3C).

A total of 297 normal-looking embryos (Figures 3C and 3D) were obtained from 33 genotypes (Table 3). Of these, 263 embryos were transferred to a germination medium. Among those, a total of 132 embryos germinated by initially forming a few real leaves and roots. However, further growth of the germinated embryos and consequently the ratio of their transformation to whole plants was not realized as expected. Most stopped growing and then slowly turned brown in color. Only a few reached the acclimatization stage (Figures 3E and 3F) (data not presented), and then their ploidy levels were determined based on flow cytometer analysis (Figures 3G and 3H).

4. Discussion

Of a total of 64 ornamental pepper genotypes, 48 responded to shed-microspore culture and produced embryos in varying ratios. The genotype had a very significant effect ($P \leq 0.001$) on the androgenic responses of the genotypes towards both total and normal-looking embryo formation. This expected result is compatible with previously conducted studies on vegetable, spice, or culinary peppers (Dumas de Vaulx et al., 1981; Kristiansen and Andersen, 1993; Mitykó et al., 1995; Dolcet-Sanjuan et al., 1997; Rodeva et al., 2004; Gémes Juhász et al., 2009; Nowaczyk et al., 2009; Luitel and Kang, 2013).

After genotypes, the proper microspore stage is probably the second most decisive factor affecting the success of microspore embryogenesis. The suggested proper developmental stages for haploid induction in pepper are mostly late unicellular, vacuolated microspores and, in some cases, young bicellular pollens (Segui-Simarro, 2010). In order to culture the proper microspores in a speedy manner, the size of flower buds and the calyx/bud ratio are generally used as indirect morphological indicators in pepper androgenesis studies (Sibi et al., 1979; Nowaczyk and Kisiała, 2006). However, these indicators are

Table 2. Comparison of the androgenic responses of 64 ornamental pepper genotypes originating from commercial and local sources in shed-microspore culture in terms of average yield of total and normal-looking embryos produced per flower bud.

Origin *	Genotype	Average yield of embryos per flower bud				Origin	Genotype	Average yield of embryos per flower bud			
		No. of total embryos produced		No. of normal-looking embryos				No. of total embryos produced		No. of normal-looking embryos	
		M ± SE**		M ± SE				M ± SE		M ± SE	
C1	1	0.00 ± 0.00	a	0.00 ± 0.00	a	C10	69	0.58 ± 0.24	acdj	0.13 ± 0.13	ay
	2	0.05 ± 0.05	a	0.00 ± 0.00	a		92	0.69 ± 0.27	amdfj	0.19 ± 0.09	az
	4	0.00 ± 0.00	af	0.00 ± 0.00	acf	C11	70	0.39 ± 0.21	acdj	0.04 ± 0.04	ay
C2	7	0.05 ± 0.03	a	0.00 ± 0.00	a		72	1.16 ± 0.35	acdhj	0.21 ± 0.11	ay
	8	0.10 ± 0.05	a	0.02 ± 0.02	acf	C12	73	10.50 ± 9.03	d	0.13 ± 0.09	ay
C3	9	11.74 ± 3.43	be	1.48 ± 0.68	bghjkm		75	12.68 ± 4.03	e	0.90 ± 0.62	cgnsuyz
	11	0.40 ± 0.18	ac	0.05 ± 0.03	ano		77	3.51 ± 3.02	ak	0.19 ± 0.09	az
C4	15	0.06 ± 0.06	a	0.00 ± 0.00	ap		78	0.00 ± 0.04	a	0.00 ± 0.00	a
	21	0.00 ± 0.00	a	0.00 ± 0.00	ay		100	1.03 ± 0.26	amdfghj	0.31 ± 0.13	az
	22	0.05 ± 0.04	a	0.05 ± 0.04	ay	C13	80	0.17 ± 0.12	af	0.00 ± 0.00	az
	24	0.00 ± 0.00	a	0.00 ± 0.00	a	C15	83	1.35 ± 1.22	amdj	0.03 ± 0.03	az
	27	0.00 ± 0.00	a	0.00 ± 0.00	ay		84	0.08 ± 0.05	a	0.03 ± 0.03	az
	99	0.67 ± 0.29	amdfghj	0.13 ± 0.13	az	C16	85	0.03 ± 0.03	a	0.00 ± 0.00	a
	204	0.07 ± 0.05	ak	0.00 ± 0.00	az	C17	88	0.82 ± 0.36	amdj	0.03 ± 0.03	az
C5	31	0.11 ± 0.11	a	0.05 ± 0.05	ay	C18	91	0.10 ± 0.08	af	0.00 ± 0.00	az
C6	33	1.00 ± 0.58	acdj	0.46 ± 0.25	ay	C19	93	0.33 ± 0.19	amdfj	0.11 ± 0.08	az
	35	0.74 ± 0.27	acdj	0.21 ± 0.12	ay	C20	200	4.89 ± 1.68	gmnde	1.08 ± 0.37	djru
C7	37	0.00 ± 0.00	a	0.00 ± 0.00	a	C21	B5	4.28 ± 1.02	hmnd	0.55 ± 0.20	efkoptvyz
	38	0.06 ± 0.04	a	0.03 ± 0.03	ay	C22	B6	3.41 ± 0.88	jmnd	0.46 ± 0.16	amz
C8	39	0.05 ± 0.05	a	0.02 ± 0.02	ay	L2	117	0.00 ± 0.00	a	0.00 ± 0.00	az
	43	0.03 ± 0.03	a	0.03 ± 0.03	ay	L3	124	0.00 ± 0.00	a	0.00 ± 0.00	az
	96	0.00 ± 0.00	a	0.00 ± 0.00	a	L6	139	0.04 ± 0.04	af	0.00 ± 0.00	az
	97	0.00 ± 0.00	a	0.00 ± 0.00	a	L7	143	0.25 ± 0.14	amdf	0.00 ± 0.00	az
C9	45	0.00 ± 0.00	a	0.00 ± 0.00	a	L11	171	0.09 ± 0.07	a	0.00 ± 0.00	az
	49	0.00 ± 0.00	a	0.00 ± 0.00	a	L12	180	0.77 ± 0.35	amfj	0.13 ± 0.08	az
C10	55	0.18 ± 0.09	ac	0.05 ± 0.03	ay	L13	185	0.40 ± 0.26	adf	0.10 ± 0.10	az
	56	0.05 ± 0.05	a	0.00 ± 0.00	a	L15	18	1.57 ± 0.33	acdkn	0.23 ± 0.10	ast
	57	0.00 ± 0.00	a	0.00 ± 0.00	ay		19	0.00 ± 0.00	a	0.00 ± 0.00	a
	60	0.00 ± 0.00	a	0.00 ± 0.00	a		20	0.34 ± 0.17	acd	0.16 ± 0.08	auv
	62	0.00 ± 0.00	a	0.00 ± 0.00	a	L16	17	2.88 ± 0.60	cdekm	1.38 ± 0.94	br
	66	0.08 ± 0.06	a	0.00 ± 0.00	a		192	4.79 ± 2.26	fcndek	0.46 ± 0.22	ahzu
	68	0.18 ± 0.10	ac	0.00 ± 0.00	a	L17	194	0.06 ± 0.04	am	0.00 ± 0.00	az

Mean average yield of total and of normal-looking embryos per flower bud: 1.14 ± 0.51 and 0.15 ± 0.08.

*Letters C and L denote commercial cultivars and local accessions, respectively.

**Values are mean (M) and standard error (SE). One-way ANOVA was used for the comparison of the means, which were separated by using Tukey's HSD multiple range test. Means within a column followed by different letters represent significant difference at $P \leq 0.05$.

Table 3. Numbers of total and normal-looking embryos produced from 64 ornamental pepper genotypes, and normal-looking embryos transferred and germinated.

Origin* and no. of genotype		No. of total embryos	No. of normal-looking embryos	No. of normal-lookingembryos transferred	No. of normal-looking embryos germinated	Origin and no. of genotype		No. of total embryos	No. of normal-looking embryos	No. of normal-looking embryos transferred	No. of normal-looking embryos germinated
C1	1	0	0	0	0	C10	69	23	5	5	3
	2	2	0	0	0		92	25	7	7	3
	4	0	0	0	0	C11	70	9	1	1	0
C2	7	2	0	0	0		72	44	8	8	5
	8	4	1	1	0	C12	73	399	5	5	2
C3	9	317	40	28	13		75	482	34	20	9
	11	16	2	2	0		77	130	7	7	5
C4	15	2	0	0	0		78	2	0	0	0
	21	0	0	0	0		100	37	11	11	6
	22	2	2	2	1	C13	80	5	0	0	0
	24	0	0	0	0	C15	83	50	1	1	1
	27	0	0	0	0		84	3	1	1	0
	99	10	2	2	0	C16	85	1	0	0	0
	204	2	0	0	0	C17	88	28	1	1	0
C5	31	5	2	2	1	C18	91	3	0	0	0
C6	33	39	18	14	9	C19	93	9	3	3	1
	35	29	8	8	5	C20	200	181	40	40	23
C7	37	0	0	0	0	C21	B5	124	16	16	9
	38	2	1	1	0	C22	B6	99	13	11	4
C8	39	2	1	1	1	L2	117	0	0	0	0
	43	1	1	1	1	L3	124	0	0	0	0
	96	0	0	0	0	L6	139	1	0	0	0
	97	0	0	0	0	L7	143	6	0	0	0
C9	45	0	0	0	0	L11	171	3	0	0	0
	49	0	0	0	0	L12	180	24	4	4	3
C10	55	7	2	2	1	L13	185	12	3	3	1
	56	2	0	0	0	L15	18	55	8	8	4
	57	0	0	0	0		19	0	0	0	0
	60	0	0	0	0		20	11	5	5	2
	62	0	0	0	0	L16	17	69	33	32	14
	66	3	0	0	0		192	115	11	10	6
	68	7	0	0	0	L17	194	2	0	0	0
Total		454	80	64	31	Total		1952	217	199	101
Overall		2.406	297	263	132						

*Letters C and L denote commercial cultivars and local accessions, respectively.

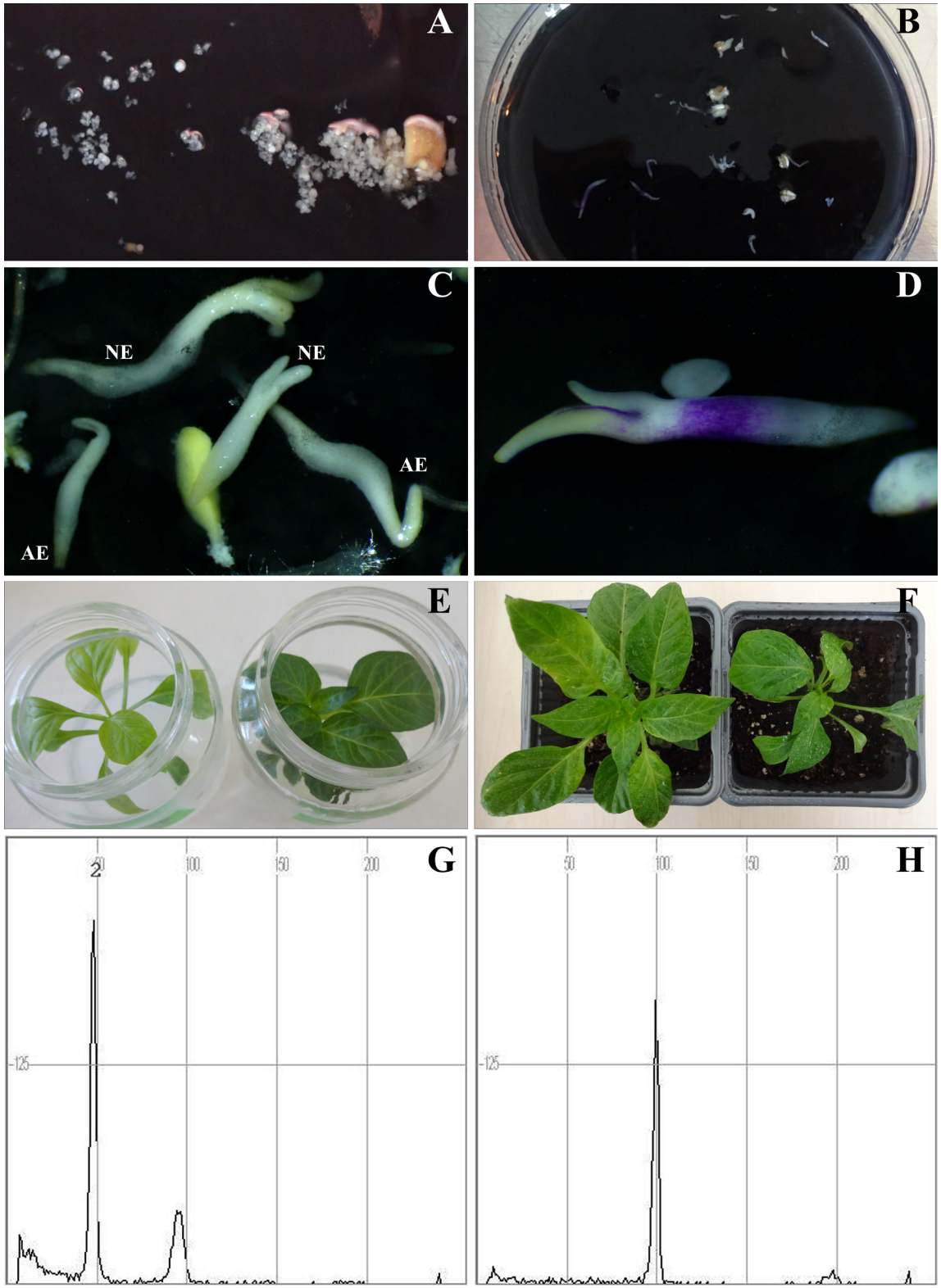


Figure 3. Double haploid plant regeneration in ornamental peppers through shed-microspore culture. (A) Proembryos and globular embryos; (B) embryo development in different stages; (C) normal-looking (NE) and abnormal embryos (AE); (D) a normal-looking embryo showing anthocyanin pigmentation; (E) healthy growth of in vitro ornamental pepper plants; (F) acclimatized ornamental pepper seedlings; (G) flow cytometer peaks of a haploid; and (H) spontaneous double haploid plant.

not applicable to all varieties due to significant differences in bud morphology among genotypes (Rodeva, 2001; Irikova, 2008; Irikova et al., 2011). In the 64 ornamental pepper genotypes used in the present study, there was large variation in terms of different growth habits, such as short, medium, or tall habitus. Different plant sizes also affected their flower bud sizes. In addition, almost half of the genotypes had anthocyanin pigmentation not only in their anthers but also in their fruits, stems, leaves, calyxes, or bud leaves. We tried to use flower buds containing more than 50% late unicellular microspores in addition to midunicellular and young bicellular pollens for each genotype, as suggested by Supena et al. (2006b). For a quick estimation of the mentioned microspore stages, we used the morphologic markers of calyx/bud ratio, anther pigmentation ratio, and bud length for each of the two sample ornamental pepper genotypes, genotypes 1 and 117, with or without anthocyanin, respectively. Several research groups suggested diverse calyx/bud ratios or described various flower bud morphologies based on their own plant materials. The markers of calyx/bud ratio and anther pigmentation ratio reported for vegetable peppers (Özkum Çiner and Tıpırdamaz, 2002; Nowaczyk and Kisiała, 2006; Supena et al., 2006b; Kim et al., 2008; Parra-Vega et al., 2013) were valid for our green-looking ornamental pepper genotypes carrying anthocyanin only in their anthers. Parra-Vega et al. (2013) proposed the combined use of calyx/bud ratio and anther pigmentation for anthocyanin-producing pepper cultivars. They reported a proper bud morphology, which mostly contained vacuolated microspores and young bicellular pollen grains with a sepal length of around 80% of the petal length and anthers with purple distal tips for Spanish sweet pepper F1 hybrids Herminio, Gacela, and Aguila. In our study, the calyx/bud ratio was 70%–80% in genotype 1 and 70%–85% in genotype 117. Hence, the marker of calyx/bud ratio can also be used for genotypes with full anthocyanin in addition to green-looking genotypes.

With regard to the ratio of anthocyanin pigmentation in anthers, this was 70%–90% in genotype 1 and 10%–40% in genotype 117 for proper buds containing more than 50% late unicellular microspores. The difference between the two ornamental pepper genotypes was rather significant. Dense anthocyanin pigmentation in certain genotypes influenced the amount of anthocyanin in anthers. However, this does not mean that the flower buds of genotype 1, containing 70%–90% anther pigmentation, cannot be used in androgenesis study, since DAPI staining of the microspores proved their compatibility. Therefore, the morphologic marker of anther pigmentation used for the vegetable pepper types cannot be used in ornamental pepper genotypes with full anthocyanin.

The convenient flower bud length differed in both genotypes due to the diverse bud sizes caused by the varied habitus of ornamental peppers. The proper bud length was determined to be ≈3 mm in genotype 1 and ≈4–5 mm in genotype 117. In vegetable peppers, bud lengths between 4 and 5 mm were reported to be an accurate marker by Parra-Vega et al. (2013). These results revealed that the use of the bud length criterion needs initial work supported by DAPI staining for different ornamental pepper types. As an important observation on bud size, working with overly small-sized buds in some genotypes brought about difficulty in the isolation of anthers and consequently risk of damage to anthers. Hence, the use of mechanically isolated microspore cultures might be an easier way for DH plant production in ornamental peppers, especially for genotypes with small buds.

Shed-microspore culture is not a commonly used androgenesis technique. However, Supena et al. (2006b) proved that it can be used as a highly effective and powerful method in pepper. The highest average yields of total and normal-looking embryos per flower bud were found to be 40.8 and 22.6 in an Indonesian large hot pepper type, Galaxy, in their first study. Later, these numbers were upgraded to 106.4 and 35.3 in a refined study (Supena and Custers, 2011) with the Galaxy-DH line. A 2.5-fold increase was achieved in total embryo production in 5 years. Therefore, the shed-microspore culture technique has strong potential for future pepper androgenesis studies. In our present shed-microspore culture study, the mentioned embryo yield criteria were 12.7 and 1.48. Here we verified the applicability of shed-microspore culture once again in the case of ornamental pepper.

Our total embryo formation results were quite promising for the initial work conducted on different ornamental pepper types grown in the autumn season. However, the normal-looking embryo formation and the germination and conversion of these embryos to whole plants were below our expectations. According to Supena et al. (2006b) and Parra-Vega et al. (2010), a common drawback in efficient DH plant production in pepper is the low germination success of the embryos. Efficiency is mostly lost during the transition of a proliferating yet undifferentiated globular embryo into a heart-shaped bilateral embryo (Parra-Vega et al., 2010). The presence of anatomical and functional abnormalities, ranging from mild to severe, is remarkably frequent in pepper androgenic embryos (Segui-Simarro et al., 2011) as well as in somatic embryos (Steinitz et al., 2003; Khan et al., 2006). Microspore-derived embryos display stunted or even absent cotyledons, indicating serious deficiencies in lateral expansion, which prevents further germination of embryos (Supena et al., 2006b; Kim et al., 2008; Parra-Vega et al., 2010; Segui-Simarro et al., 2011). We also frequently

encountered abnormal embryo formations without cotyledons or with a single cotyledon. Therefore, our low embryo germination results might be partially attributed to this frequently encountered embryo germination or growth problem in pepper. In addition, unlike Supena and Custers (2011), we added the combination of zeatin and indole-3-acetic acid to the upper layer of the double-layer media during the initial culture against contamination risk, instead of after 3 weeks of incubation. Hence, early hormone supplement might have influenced embryo quality regarding their transition towards maturation. Meanwhile, none of the embryos with anthocyanin were germinated in our germination media. Accordingly, the embryos with anthocyanin require a different medium and/or conditions. On the other hand, we had to apply an insecticide treatment to the donor plants only once out of necessity. It did not seem to have an impact on embryo induction, but it might have had an influence on the quality and ability of embryos for healthy germination.

Among the many factors affecting the success of haploid androgenesis, culture season is another important environmental factor. In the present study, the anthers were cultured in November and December, in other words in autumn and partly in winter. Our work is not a comparative study testing the influences of spring and autumn. However, Ellialtioğlu et al. (2001) reported that the donor plants of many species produced in their normal growing season generally have higher haploid performance compared to those grown outside the normal vegetation period. The effect of culture season has been reported previously in some pepper androgenesis studies. Ercan et al. (2006) compared the haploid responses of Kekova and Sera Demre 8, Turkish cultivars, in winter (from November to May) and in summer (from April to December) in Antalya. The genotypes showed diverse embryogenic responses to seasonal effects. Kekova and Sera Demre 8 cultivars had the highest embryogenic performance in summer and winter, respectively. Taskin et al. (2011) tested five Turkish pepper genotypes in different growing periods from April to January in Adana. Among them, April and May were determined as the best periods for the anthers to produce the highest yield of embryo. The number of clear sunny days and the mean monthly temperature during the growth period of the donor plants affected haploid induction in the pepper anther culture study of Rodeva and Cholakov (2006) (Irikova et al., 2011). According to Olszewska et al. (2014), unpredictable temperature fluctuation during donor plant growth can be a reason for the generally lower effectiveness of androgenesis in the *Capsicum* genus, native to tropical and subtropical climate zones. Kristiansen and Andersen (1993) tested different donor plant growth temperatures between 16 and 30 °C for three F_1 pepper hybrids under controlled conditions. They determined the optimum temperature to be 26.4 °C for embryo formation in pepper. The reported closest mean air temperature of Antalya was 25.4 °C in June as the actualized value in the long term (1950–2014). However, the same value for November and December was 14.9 and 11.4 °C, respectively (http://www.mgm.gov.tr/). Under greenhouse conditions, this term is earlier, as in our study the mean air temperature of the greenhouse was 19.3 and 16.2 °C in November and December 2013, respectively. The optimal pepper-growing period in Antalya is in early summer or late spring. Temperatures far from the optimum value in the vegetation period of donor plants can be a stress factor, reducing the androgenic response or embryo quality of the tested genotypes. Therefore, our results may have been affected by environmental conditions, mainly including temperature and photoperiod time in autumn.

In conclusion, the calyx/bud ratio can be used as an effective morphological marker for proper bud selection in ornamental pepper genotypes with both full anthocyanin (70%–80%) and green-looking appearance (70%–85%). The indicator of anthocyanin pigmentation in anthers as used in peppers is a convenient marker for green-looking ornamental pepper genotypes (10%–40%), while it is not suitable for genotypes with full anthocyanin (70%–90%). The use of bud length requires separate preliminary work assisted with DAPI staining for different ornamental pepper types. The promising embryo formation results of the present study revealed that the shed-microspore culture technique can be used successfully in different ornamental pepper genotypes for DH plant production. In future studies, the efficiency of this technique might be improved by culturing the anthers in the optimum growing season, in late spring or early summer, and the embryos in different germination media or conditions.

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