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Comparison of different androgenesis protocols for doubled haploid plant production in ornamental pepper (*Capsicum annuum* L.)

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Abstract: Doubled haploid (DH) plant production plays an important role in pepper (*Capsicum annuum* L.) breeding for development of new varieties. However, information is lacking for DH plant production in pepper genotypes carrying ornamental value. The major goal of this study was to produce DH ornamental pepper lines having the mentioned value. Androgenic responses of 48 genotypes at the F_2 or F_3 generation were compared in each of three androgenesis protocols to determine the most effective method. Of the three protocols tested, anthers were placed on two different semisolid culture media and on a double-layer medium also called shed-microspore culture medium. The results revealed that the shed-microspore culture protocol was superior to both semisolid anther culture protocols. The average numbers of total and normal-looking embryos per bud of the most responsive genotype were 102.90 and 34.11, respectively. We report here, a total of 122 regenerated ornamental pepper plants with ornamental value; 63 DH, 52 haploid, and 7 mixoploid plants were produced in the present study. The spontaneous diploidization rate was 51.6% based on flow cytometry analyses. The results showed that the shed-microspore culture protocol could be used effectively for the development of DH lines in ornamental pepper breeding programs.

Key words: Anther culture, breeding, *Capsicum annuum* L., doubled haploid, microspore embryogenesis, shed-microspore culture, ornamental pepper, ornamental plant

1. Introduction

Aside from the main purpose of pepper (*Capsicum annuum* L.) breeding programs conducted mainly for vegetable and spice consumption, many varieties or genotypes showing ornamental potential have also been produced. Choices of different fruit and leaf colors and shapes and their compact forms offer different opportunities (Stommel and Bosland, 2006) for use as pot plants, seasonal garden plants, or cut flowers in the ornamental plants sector.

According to Stommel and Griesbach (2005), when peppers were introduced to Europe in the 15th century, they were held in higher esteem as ornamental plants than as a food source. Ornamental peppers were recognized as Christmas peppers for a long time (Hammer, 1980). Because they bear brightly colored fruits, they were the most popular Christmas gift plants until the 1960s. Ornamental peppers are still popular today in Europe as pot or bedding plants. Because of easy seed propagation, relatively short cropping time, and heat and drought tolerance, they have also begun to gain their old popularity again in the United States lately (Armitage and Hamilton, 1987; Bosland, 1999; Stommel and Bosland, 2006). In addition, ornamental pepper growing for landscape aims is a new trend in the Brazilian consumer market (Silva et al., 2015). This renewed interest has been gradually increasing worldwide and has encouraged studies on the breeding of ornamental type peppers. According to the literature, most of these breeding studies were executed with classical methods, and lately marker-assisted selection and molecular-based biotechnological methods have been used. However, there are no efficient reports on doubled haploid (DH) plant production in ornamental peppers with ornamental value as far as we know.

Homozygosity can be attained promptly and stable homozygous DH lines can be selected for desired features thanks to different haploidy methods. In vitro androgenesis techniques have been used effectively in plant breeding programs of many species, including pepper. Therefore, studies conducted on vegetable or spice peppers can give ideas for which methods could be used for ornamental-type peppers.

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The first studies in anther culture of pepper were reported in 1973 using semisolid media by Wang et al. (1973), Kuo et al. (1973), and George and Narayanaswamy (1973). Pepper is accepted to be the third solanaceous crop that could be defined as recalcitrant in regard to the response to androgenesis induction (Segui-Simarro et al., 2011). Many studies have been conducted to overcome this bottleneck and elaborate the critical factors involved in anther culture response of peppers, such as media, culture system, and genotype, since 1973. The two-step anther culture system, first introduced by Sibi et al. (1979) and then optimized by Dumas de Vaulx et al. (1981), was used in various bell pepper breeding programs (Abak et al., 1982; Hendy et al., 1985; Daubeze et al., 1990; Caranta et al., 1996). However, the two-step procedure did not produce positive results for many accessions in different pepper germplasms. Later, the double-layer anther culture system was developed by Dolcet-Sanjuan et al. (1997). This technique, also called shed-microspore culture, was successfully refined by Supena et al. (2006a, 2006b) and Supena and Custers (2011) for Indonesian hot peppers.

In shed-microspore cultures, anthers float on the liquid layer for a while, and then they open and release their microspores into the medium as the microspores grow and undergo dehiscence (Supena et al., 2006b). Microspores are isolated in a nonmechanical way in this system (Segui-Simarro et al., 2011) and grow individually in the medium. Thus, the regenerants obtained from shed-microspore culture systems are generally accepted to have microspore origin.

Among the many factors influencing successful DH plant production in pepper, the genotype effect on haploid embryogenesis has been demonstrated to be the most critical one in many studies (Kristiansen and Andersen, 1993; Qin and Rotino, 1993; Mityko et al., 1995; Rodeva et al., 2004, 2006; Gemes Juhasz et al., 2009; Nowaczyk et al., 2009).

The main purpose of this study was to produce DH ornamental pepper breeding lines with ornamental value. In order to determine the most effective method for this purpose, androgenic responses of 48 ornamental pepper genotypes at the F_2 or F_3 generation were compared in 3 androgenesis protocols, two of which had different semisolid media and one of which had shed-microspore culture consisting of a double-layer system.

2. Materials and methods

2.1. Plant material

A total of 48 ornamental pepper genotypes with ornamental value were used. They originated from both commercial cultivars and local accessions, denoted with the letters C and L, respectively. The ornamental value and quality of the genotypes that originated from commercial cultivars were higher than that of the genotypes derived from local accessions. Thirty segregated genotypes originating from 15 commercial cultivars and 18 segregated genotypes derived from 18 local accessions were tested for their androgenic responses using 3 protocols. Eight seedlings per genotype were grown from seeds, segregating the population at F_2 (C) and F_3 (L) generations, provided by PEYART Ltd., Antalya, Turkey. Donor plants were grown in a plastic greenhouse during the spring of 2013 in Antalya. They were fertilized twice a week with 0.50 g L^{-1} $plant^{-1}$ of 15-15-18 (15% nitrogen-15% phosphorus-18% potassium) water-soluble fertilizer via a drip irrigation system.

Flower buds of the desired size containing mostly late uninucleated as well as early binucleated microspores were determined by DAPI staining (Coleman and Goff, 1985). Buds were collected randomly from 8 plants of each genotype early in the morning and exposed to cold pretreatment at 4 °C for 1 day in all culture protocols studied. After cold pretreatment, they were sterilized for 10 min in 15% household bleach with Tween 20 added, and then rinsed three times with sterile dH_2O. The sterilized buds were chosen again randomly to open. All the anthers (5–7) in a bud were cultured in each petri dish (60 mm in diameter) for all of the protocols tested.

2.2. Culture protocols

Three different androgenesis protocols were tested: 1) Anthers were cultured on semisolid Murashige and Skoog (1962) (MS) medium with 1 mg/L 6-benzylaminopurine (BAP), 4 mg/L naphthalene acetic acid (NAA), 15 mg/L silver nitrate, 0.25% activated charcoal (AC), 3% sucrose, and 0.8% plant agar according to Taskin et al. (2013). 2) The same components as in the first protocol except B5 medium (Gamborg et al., 1968) were used. For the first two protocols, petri dishes containing the anthers were exposed to 35 °C heat shock for 2 days in dark and then transferred to 25 °C with a 16/8-h photoperiod. 3) A double-layer medium (shed-microspore culture) was used according to Dolcet-Sanjuan et al. (1997), Supena et al. (2006a, 2006b), and Supena and Custers (2011), in which the underlayer was composed of Nitsch and Nitsch (1969) (NN) medium with 2% maltose, 1% AC, and 0.6% plant agar. The liquid upper layer was NN medium with 2% maltose. Once anthers were placed on double-layer medium in petri dishes (each layer 4 mL), cultures were incubated at 9 °C for 1 week, then transferred to 28 °C for 3 weeks. Later, a combination of filter-sterilized 2.5 µM zeatin and 5 µM indole acetic acid was added to the upper layer of the cultures and then moved to 21 °C for an additional 3–5 weeks in darkness. In all protocols, the pH was adjusted to 5.8 and media were autoclaved at 121 °C for 20 min. For each genotype, three replications of each 10 petri dishes and four replications of each 20–30

petri dishes were used for semisolid medium protocols and the shed-microspore culture protocol, respectively. Unless indicated otherwise, Duchefa chemicals (Duchefa Biochemie, Haarlem, the Netherlands) were used in all of the protocols described in the study.

2.3. Germination of embryos

All petri dishes were investigated under a stereo microscope and the healthy normal-looking individual embryos with two cotyledons and noncontiguous anthers were chosen to be transferred to the germination medium. The embryos obtained from the first two protocols (semisolid anther cultures) were placed onto MS medium with 3% sucrose and 0.8% plant agar. In the shed-microspore culture protocol, half-strength MS with 0.1 µM BA, 2% sucrose, and 0.6% plant agar in petri dishes (90 mm in diameter) were used for embryo germination. Once germination occurred, the embryos were transferred to baby food jars containing the same germination medium in each protocol for further development. After 4–5 weeks, seedlings with a few true leaves were placed into glass jars of 175 mL containing a 1-cm-thick layer of vermiculite moistened with liquid half-strength MS medium with 1% sucrose. All of the vessels were incubated at 25 °C under 16 h of light (50 µmol m^{-2} s^{-1}). Later, seedlings forming 5–7 leaves were transferred to pots containing a peat moss, perlite, and vermiculite mixture (3:1:1 v/v/v) for acclimatization.

2.4. Ploidy analysis and chromosome doubling

Young leaf pieces sampled from regenerant plants were processed using the CyStain UV Precise P Kit for nuclear extraction and staining, then analyzed in the CyFlow Ploidy Analyzer for determination of the ploidy levels of the regenerants (Partec GmbH, Münster, Germany).

Haploid plants were subjected to in vivo colchicine treatment in which 0.05% colchicine solution was applied with a piece of cotton for 2 h in the dark to axillary buds, then rinsed with tap water. Later, spontaneous DH plants and colchicine-applied haploid plants were selfed.

2.5. Statistical analyses

As in the protocol of Supena et al. (2006b), average numbers of total and normal-looking embryo yields of the genotypes were presented in our study, and they were calculated per bud, not per 100 anthers. Total and normal-looking embryos produced from each genotype were recorded after 4–5 weeks for the semisolid medium protocols and 8–10 weeks for the shed-microspore culture protocol. One-way analysis of variance (ANOVA) was carried out to determine the differences between genotypes in only the shed-microspore culture protocol due to the inadequate embryo yields obtained from the MS and B5 protocols. The data were normalized by Sqrt (x + 0.5), where x represents the number of embryos per bud prior to analysis, to meet the assumptions of ANOVA. The GLM procedure of Minitab 15 statistical software (Minitab, State College, PA, USA) was used for data analyses. All main effects were considered as fixed effects. Multiple comparisons of genotypes within the shed-microspore culture protocol were made using Tukey's HSD post hoc test at an alpha 0.05 level.

3. Results

3.1. Comparison of the protocols

Microspore embryogenesis inductions of the genotypes cultured in the first and second protocol media based on semisolid anther culture medium were far below expectations compared to the third one on shed-microspore culture medium. The numbers of average total embryos per bud produced from 3 protocols were 0.13, 0.70, and 6.50 for MS, B5, and shed-microspore culture medium, respectively (data not presented).

Only 7 and 13 out of 48 genotypes were responsive to the semisolid MS and B5 protocols, respectively, compared to 46 genotypes with the shed-microspore culture protocol (Table 1). The numbers of total embryos produced on MS, B5, and shed-microspore culture medium were 180, 1011, and 18,578, respectively. Only a few embryos forming on semisolid media developed into normal-looking embryos, of which four transformed into whole plants belonging to genotypes 55, 89, and 92 from B5 and genotype 83 from MS medium. Therefore, the conversion ratios of total embryos to normal embryos were given only for the shed-microspore culture protocol (Table 1).

As shown in Table 1, the genotypes that originated from local accessions were remarkably more responsive to induce microspore embryogenesis compared to the genotypes derived from commercial cultivars in all protocols. The total embryo numbers produced from a total of 18 genotypes coming from local accessions were 158, 590, and 15,361 in MS, B5, and shed-microspore culture protocols, respectively. However, 22, 421, and 3217 embryos were recorded from a total of 30 genotypes that originated from commercial cultivars in the same protocols.

The shed-microspore culture protocol was superior to the other two protocols in producing total and normal-looking embryos. Of the 18,578 embryos, 5587 transformed to normal-looking embryos with an average of 39%. Depending on genotype, conversion percentages ranged between 0% and 100% in the shed-microspore culture protocol.

One-way ANOVA results of the shed-microspore culture protocol exhibited very significant differences in the number of total and normal-looking embryos produced per bud among the genotypes (both at $P \leq 0.001$). The average values for the number of total and normal-looking embryos produced per bud were 6.49 ± 1.13 and 1.89 ± 0.35, respectively (Table 2).

Table 1. The number of total and normal-looking* embryos produced from 48 ornamental pepper genotypes cultured in each of 3 androgenesis protocols.

Origin code of genotype**	Genotype no.	Androgenesis culture protocols				
		Semisolid MS	Semisolid B5	Shed-microspore culture		
		Total number of embryos		Total number of embryos	Total number of normal-looking embryos	%
C1	1	0	0	0	0	0
C2	3	4	132	393	188	48
	4	0	79	196	51	26
C3	5	0	0	235	78	33
	96	0	0	306	93	30
C5	7	0	0	88	25	28
C6	8	0	0	215	82	38
C7	10	0	0	3	1	33
C8	11	0	4	20	17	85
	12	0	0	3	3	100
C9	13	6	6	5	1	20
C10	15	0	0	70	25	36
	16	0	152	79	38	48
	17	0	0	96	50	52
	18	0	0	18	12	67
	19	2	0	11	4	36
	20	0	0	154	56	36
	21	0	0	275	122	44
	98	0	0	1	0	0
C11	22	0	0	484	218	45
C12	23	0	0	40	19	48
	24	0	14	224	89	40
C13	25	0	12	49	28	57
C15	27	0	0	15	3	20
	28	10	22	5	0	0
	29	0	0	5	2	40
	30	0	0	7	1	14
	31	0	0	5	0	0
C16	99	0	0	5	1	20
C17	32	0	0	210	102	49
Total of C-originated genotypes	30 genotypes	22	421	3217	1309	36.43
L1	34	0	0	0	0	0
L2	41	0	0	10	1	10
L3	45	0	0	3	2	67

Table 1. (Continued).

L4	49	0	0	5	5	100
L5	52	0	0	8	6	75
L6	55	0	22	219	62	28
L7	58	0	96	151	77	51
L8	63	0	0	6	4	67
L9	67	0	0	75	61	81
L10	70	0	0	11	6	55
L11	73	0	0	32	5	16
L12	79	0	0	81	24	30
L13	83	132	146	896	315	35
L14	86	0	0	1610	402	25
L15	89	14	230	6276	2081	33
L16	90	0	0	5589	1065	19
L17	91	0	0	62	30	48
L18	92	12	96	327	132	40
Total of L-originated genotypes	18 genotypes	158	590	15,361	4278	43.33
Total of overall genotypes	48 genotypes	180	1.011	18,578	5587	Average 39.02

*The conversion rates of total embryos to normal embryos were given only for shed-microspore culture protocol due to inadequate normal embryo yields obtained from semisolid MS and B5 protocols.
**Letters C and L denote commercial cultivars and local accessions, respectively.

Among all the genotypes, genotype 89 originating from a local accession (L15) was the most responsive one and its total and normal-looking embryos per bud were 102.90 ± 14.30 and 34.11 ± 4.27, which was followed by genotype 90 (98.10 ± 12.10, 18.68 ± 1.54) and genotype 86 (32.20 ± 6.18, 8.04 ± 1.24).

A total of 5587 normal-looking embryos were obtained from 43 genotypes in shed-microspore culture. Since an approximately equal number of normal-looking embryos could not be supplied from each genotype, an equal number of embryos could not be cultured in the germination media per genotype. Hence, the embryo germination results of the genotypes were not given here. However, many of the 5587 normal-looking embryos germinated and some of the in vitro-grown plantlets were exposed to acclimatization. A total of 204 in vitro-germinated plantlets were subjected to an acclimatization procedure, which resulted in 122 plants that originated from 16 genotypes with a 59.8% survival rate.

3.2. Ploidy analysis of the regenerants

The ploidy levels of 122 regenerated ornamental pepper plants were determined by flow cytometry. Sixty-three of 122 acclimatized plants were spontaneous DH plants at a rate of 51.6%, in addition to 52 haploid (42.6%), and 7 mixoploid (5.7%) plants (Table 3).

It was found that 96.7% of the regenerated plants occurred in the shed-microspore culture protocol, in addition to 4 regenerants that originated from semisolid anther culture protocols. Normally, there is a risk for the regenerants that form especially in semisolid anther cultures in terms of androgenic origin due to the diploid characterized cells of the anthers like tapetum cells. We did not conduct molecular analysis to detect the origins of these plants. However, we observed homogeneity among the selfed progenies of diploid (DH) regenerants obtained from both shed-microspore culture and the semisolid anther culture protocols even though the donor F_2 or F_3 plants of the regenerants were heterogeneous. Thus, the uniform progenies may be a sign of the homozygosity of the diploid regenerants derived from microspores and the diploid regenerants might be of real androgenic origin.

The stages of androgenesis protocols followed in this study for DH plant production of different ornamental pepper genotypes are presented in Figures 1A–1O.

4. Discussion

Androgenic responses of 48 genotypes were tested in each of three different androgenesis protocols in order to determine the most effective androgenesis method for

Table 2. Comparison of the androgenic responses of 48 ornamental pepper genotypes that originated from commercial and local sources in shed-microspore culture protocol. Means within a column followed by different letters represent significant difference at P = 0.05.

Origin code*	Genotype no.	Average yield of embryos per flower bud			
		Number of embryos produced		Number of normal-looking embryos	
C1	1	0.00 ± 0.00	a	0.00 ± 0.00	a
C2	3	5.24 ± 0.66	ab	2.57 ± 0.42	cbf
	4	2.58 ± 0.54	abf	0.67 ± 0.17	ae
C3	5	3.41 ± 1.13	abgh	1.13 ± 0.46	ae
	96	6.24 ± 2.43	odfhjkm	1.90 ± 0.67	ce
C5	7	1.10 ± 0.25	ah	0.31 ± 0.08	ae
C6	8	2.65 ± 0.56	abj	1.01 ± 0.23	ae
C7	10	0.04 ± 0.04	a	0.01 ± 0.01	a
C8	11	0.24 ± 0.12	a	0.21 ± 0.11	a
	12	0.04 ± 0.03	a	0.04 ± 0.03	a
C9	13	0.06 ± 0.04	a	0.01 ± 0.01	a
C10	15	1.07 ± 0.39	ak	0.39 ± 0.22	ae
	16	0.94 ± 0.04	ak	0.45 ± 0.16	ae
	17	1.75 ± 0.91	ak	0.91 ± 0.53	ae
	18	0.29 ± 0.09	ak	0.19 ± 0.09	ae
	19	0.13 ± 0.07	a	0.05 ± 0.02	a
	20	2.27 ± 0.62	abm	0.82 ± 0.25	ae
	21	3.62 ± 1.18	abnm	1.61 ± 0.64	abe
	98	0.02 ± 0.02	a	0.00 ± 0.00	a
C11	22	8.80 ± 3.47	fgjmno	3.96 ± 1.47	cf
C12	23	0.77 ± 0.35	am	0.37 ± 0.15	ae
	24	2.64 ± 0.56	abmn	1.05 ± 0.37	ae
C13	25	0.60 ± 0.15	am	0.34 ± 0.11	ae
C15	27	0.18 ± 0.07	a	0.03 ± 0.02	a
	28	0.08 ± 0.05	am	0.00 ± 0.00	a
	29	0.06 ± 0.03	a	0.03 ± 0.02	a
	30	0.10 ± 0.05	a	0.01 ± 0.01	a
	31	0.07 ± 0.03	a	0.00 ± 0.00	a
C16	99	0.09 ± 0.06	a	0.02 ± 0.02	a
C17	32	2.92 ± 0.77	abmn	1.42 ± 0.39	abe
L1	34	3.33 ± 0.75	abmn	0.00 ± 0.00	a
L2	41	0.12 ± 0.05	a	0.01 ± 0.01	a
L3	45	0.04 ± 0.03	a	0.03 ± 0.03	a
L4	49	0.06 ± 0.04	a	0.06 ± 0.04	a
L5	52	0.10 ± 0.08	a	0.08 ± 0.06	a
L6	55	2.55 ± 0.67	abmn	0.72 ± 0.14	ae
L7	58	1.91 ± 0.37	abmn	0.98 ± 0.22	abe
L8	63	0.07 ± 0.04	a	0.05 ± 0.03	a
L9	67	1.04 ± 0.35	am	0.85 ± 0.27	ae
L10	70	0.17 ± 0.06	am	0.09 ± 0.04	a

Table 2. (Continued).

L11	73	0.56 ± 0.38	am	0.09 ± 0.05	a
L12	79	1.16 ± 0.32	am	0.34 ± 0.11	ae
L13	83	14.22 ± 3.35	d	5.00 ± 1.36	f
L14	86	32.20 ± 6.18	e	8.04 ± 1.24	g
L15	89	102.90 ± 14.30	p	34.11 ± 4.27	j
L16	90	98.10 ± 12.10	p	18.68 ± 1.54	h
L17	91	0.97 ± 0.33	am	0.47 ± 0.18	a
L18	92	4.30 ± 1.08	fhjkmo	1.74 ± 0.47	ce
Average	48 genotypes	6.49 ± 1.13		1.89 ± 0.35	

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

Table 3. Ploidy analysis results of the 122 regenerated ornamental pepper plants that originated from 16 genotypes.

Origin code of regenerants**	Genotype no. of regenerants	No. of plants analyzed	No. of regenerated plants *		
			Haploids	Diploids	Mixoploids
C2	3	2	1	1	
C4	27	1	1		
C10	16	3	3		
	17	2	2		
	18	1		1	
	20	1		1	
	21	1	1		
C17	32	2	1	1	
L2	41	1		1	
L6	55	1			1*
L7	58	1	1		
L13	83	5		4*	1
L14	86	7	2	5	
L15	89	73	35*	35	3
L16	90	12	2	8	2
L18	92	9	3*	6	
Total		122	52	63	7

*Except 4 regenerated plants obtained from genotypes 55, 89, and 92 from the semisolid B5 and genotype 83 from the semisolid MS protocol, all other regenerants were generated in the shed-microspore culture protocol.
**Letters C and L denote commercial cultivars and local accessions, respectively.

DH plant production in ornamental-type peppers. The shed-microspore culture protocol consisting of a double-layer system was confirmed to be the most effective one in comparison to two semisolid anther culture protocols. The haploid embryogenesis induction, total and normal-looking embryo formation, and conversion of the normal-looking embryos to whole plants in shed-microspore culture were superior to others. This protocol exhibited

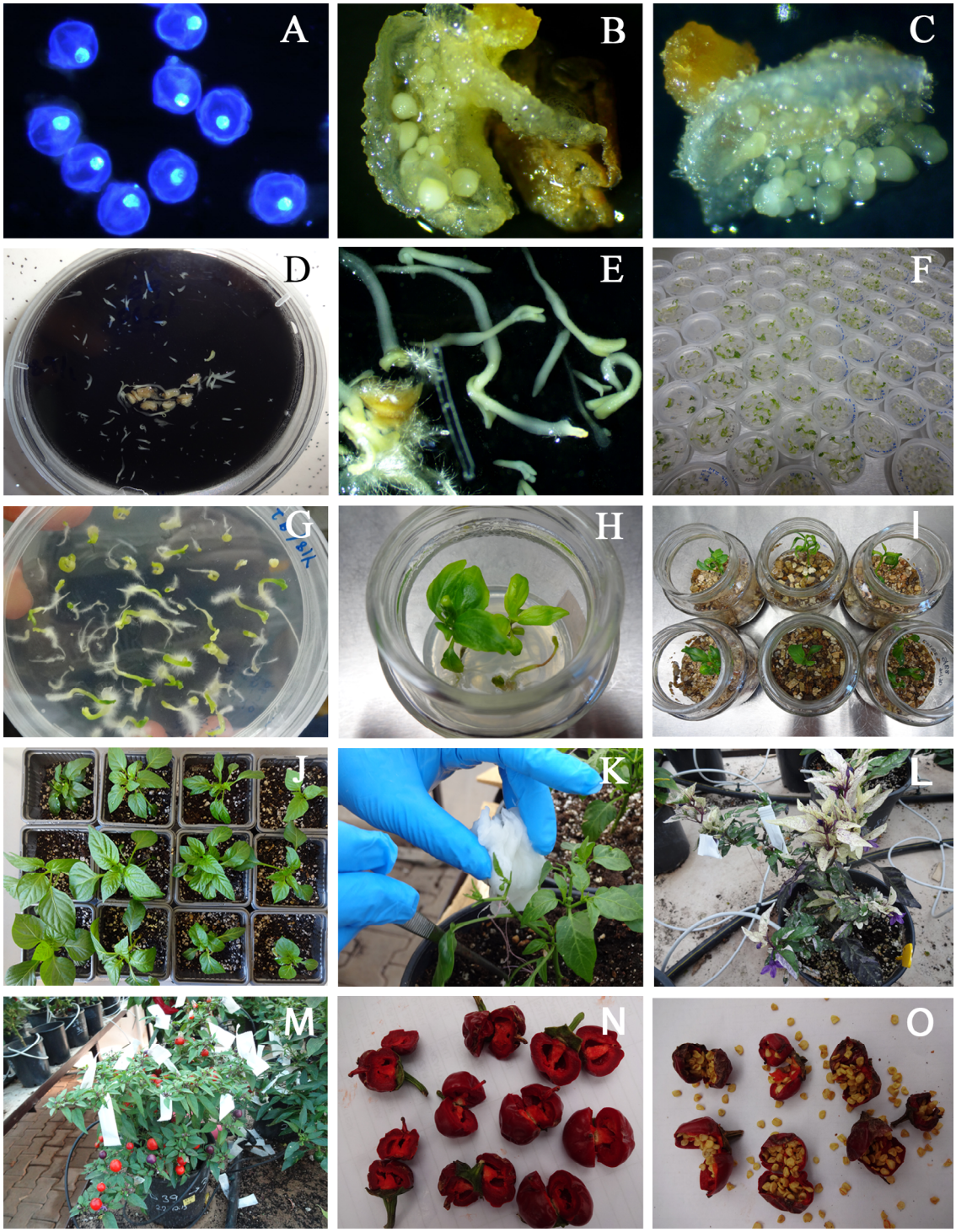


Figure 1. The stages of androgenesis protocols followed for DH plant production of different ornamental pepper genotypes: the appropriate microspore stage for the culture; late uninucleated microspore determined by DAPI (A); androgenic embryos of genotypes 89 and 83 obtained from semisolid B5 and MS media, respectively (B, C); prolific embryo production in a shed-microspore culture petri dish (D); close-up of the petri dish with normal embryos (E); germination of the embryos obtained from shed-microspore cultures (F, G), in vitro plant growth in semisolid media in baby food jar and liquid media with vermiculite (H, I); acclimatized ornamental pepper seedlings (J); colchicine application to a haploid plant (K); selfing study in a colchicine-applied haploid plant (L); selfing study in a spontaneous DH plant (M); fruits of a haploid plant without seeds prior to colchicine application (N); fruits and seeds of a spontaneous DH plant (O).

statistically significant differences in the number of total and normal-looking embryos produced per bud among the genotypes ($P \leq 0.001$).

The medium protocol used in anther or microspore cultures is generally one of the most critical factors affecting androgenetic induction. Besides the effectiveness of the double-layer system, the presence of maltose in the medium instead of sucrose, which was used in both semisolid media, could provide an explanation for our positive results in shed-microspore cultures. As an alternative carbohydrate source, maltose has been in use for haploidy studies of many plant species, especially cereals. Scott et al. (1995) attributed this effect to its slower metabolism and consequently the availability of sufficient oxygen in the medium, allowing cells to live longer. The employment of maltose in pepper androgenesis studies was first reported by Dolcet-Sanjuan et al. (1997), followed by Gyulai et al. (2000), Supena et al. (2006a, 2006b), Kim et al. (2008), Gemes Juhasz et al. (2009), and Parra-Vega et al. (2013). Except for Kim et al. (2008), all of these researchers reported positive results by using maltose in their studies. Moreover, Vizintin and Bohanec (2004) inferred that the beneficial effect of maltose on androgenesis was dependent on the genotype.

The effect of genotype on the success of anther or microspore cultures of vegetable peppers has been demonstrated as the main factor in previous studies (Kristiansen and Andersen, 1993; Qin and Rotino, 1993; Mityko et al., 1995; Rodeva et al., 2004, 2006; Gemes Juhasz et al., 2009; Nowaczyk et al., 2009). However, there is no efficient protocol on the androgenic responses of ornamental pepper genotypes carrying ornamental value in the literature as far as we know. In this haploidy study carried out with 48 ornamental pepper genotypes, we also found that androgenic induction is significantly related to the genotypes tested in shed-microspore culture protocol ($P \leq 0.001$). The average yields of total and normal-looking embryos per flower bud ranged from 0 to 102.9 ± 14.3 and 0 to 34.1 ± 4.3, respectively, among the genotypes. These numbers are very similar to the results reported by Supena and Custers (2011), who obtained such numbers as 106.4 and 35.3 for the Galaxy-DH vegetable pepper line. These findings imply that the usage of DH plants as donor plants in future haploidy studies would further improve the androgenic response in ornamental peppers.

Commercial ornamental pepper cultivars have not only been developed from crosses involving *C. annuum*, but interspecific hybridizations of different *Capsicum* species like *C. baccatum*, *C. chinense*, and *C. frutescens* have also been used in breeding programs. As reported in earlier studies (Rodeva et al., 2004; Nowaczyk et al., 2006; Irikova et al., 2011; Lantos et al., 2012; Olszewska et al., 2014), androgenic responses among different *Capsicum* species vary greatly. The plant materials employed in our study originated from segregating populations of 15 commercial F_1 hybrids and 18 local accessions. Even though we do not know the real origins of each cultivar or accession, it is clear that *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* also contributed to the variation in our initial plant materials due to the diversity in their different pod types, flower colors, and plant and fruit growth habits. Therefore, the large differences of haploidy response among our genotypes might be explained by the genotypic background and again the genotypic effect.

On the other hand, spontaneous diploidization is a desired feature for haploid breeding programs since it speeds up the production of pure lines by eliminating the need for chromosome doubling treatment as well as avoiding the use of harmful and expensive chemicals like colchicine. Thus, it is important to find the genotypes carrying high spontaneous DH ratios. In the literature on vegetable pepper androgenesis, different spontaneous DH formation rates were reported such as 65% (Dolcet-Sanjuan et al., 1997), 35.6% (Dumas de Vaulx et al., 1981), 32.6% (Gyulai et al., 2000), 14%–51% (Supena et al., 2006b), and 16.3 % (Kim et al., 2013). In our ploidy analysis, 51.6% of the acclimatized plants were found to be DH (63 out of 122). The most responsive genotypes, genotypes 89, 90, 92, 86, and 83, generated 48% (35/73), 67% (8/12), 67% (6/9), 71% (5/7), and 80% (4/5) spontaneous DH rates, respectively. In general, the reason for a high level of spontaneous DH is most likely the genetic structure of the genotype. However, spontaneous DH lines can derive from different types of cells and involve different processes (Foisset et al., 1997). Spontaneous duplication of a haploid genome is accepted to originate from mainly three mechanisms: endomitosis, endoreduplication, and nuclear fusion (Meyer, 1925; Hu and Kasha, 1999; Testillano et al., 2004; Shim et al., 2006; Segui-Simarro and Nuez, 2008; Wu et al., 2014). However, they result in either homozygous or heterozygous spontaneous DHs. The formation of homozygous DHs was caused by endomitosis/endoreduplication, while the heterozygous ones were caused by nuclear fusion (Perera et al., 2014). According to Perera et al. (2008), homozygous versus heterozygous lines can be determined by SSR marker analysis. In our study, we observed homogeneity among the selfed progeny of diploid (DH) regenerants. Thus, we did not conduct molecular analysis. However, the origins of our spontaneous DH regenerants may rely on endomitosis or endoreduplication.

Alongside the temperature pretreatments and in vitro early application of chemicals containing antimicrotubule agents, other culture conditions might also help for genesis of this phenomenon. Hence, the rate of spontaneous DH formation in ornamental peppers would be further improved by modifications in the protocol used.

Sharing one of our personal observations might be helpful here. The genotypes with a prostrate growth habit, such as genotypes 3, 83, 86, 89, and 92, that derived from different origins produced relatively higher androgenic responses compared to upright-growing genotypes. This can be an important morphological marker in future studies if it is supported with further findings based on molecular studies.

In conclusion, we report here 122 regenerated ornamental pepper plants developed in the present study through in vitro androgenesis, of which 63 plants were DH, 52 haploid, and 7 mixoploid. Spontaneous DH plants and colchicine-applied haploid plants were evaluated as breeding lines to use in F_1 hybrid improvement for ornamental aims. Among the three compared androgenesis protocols for determination of the most efficient method to produce in vitro androgenic regenerated plants, shed-microspore culture was superior and 118 of 122 regenerated plants were produced with this protocol. Therefore, we verified that the shed-microspore culture technique could be used effectively for the development of DH breeding lines. Further modifications in the protocol, e.g., colchicine application and improvement of acclimatization, might increase the efficiency of the protocol.

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