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Production of Callus Mediated Gynogenic Haploids in Winter Squash (*Cucurbita maxima* Duch.) and Pumpkin (*Cucurbita moschata* Duch.)

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Abstract

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Although haploids were successfully produced via irradiated pollen technique and anther culture in *Cucurbita maxima* and *Cucurbita moschata*, the haploidization efficiency is still low due to genotype dependence. Thus, as an alternative technique, the efficacy of the ovule culture was investigated. Ovules were extracted at different flowering time and then cultured on a solid MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP), thidiazuron (TDZ), and naphthaleneacetic acid (NAA) to induce callogenesis and plant regeneration. The gynogenic response was influenced by the combination of plant growth regulators, genotype and culture time. The medium containing of 4.0 mg/l BAP + 0.05 mg/l NAA + 0.1 mg/l TDZ provided the highest response at anthesis time. Plantlets were rooted and elongated on a solid MS medium supplemented with 0.01 mg/l indole-3-acetic acid (IAA) + 1.0 mg/l BAP. The ploidy observations of 122 plants revealed that 70 plants were haploid, 46 plants were diploid and the others were mixoploid.

Keywords: Cucurbits; dihaploidization; growth regulators; ovule culture

Although most of the cultivars are open pollinated in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.), limited numbers of F_1 cultivars are currently released. Since winter squash and pumpkin are open-pollinated species, improved an F_1 cultivars are highly labour and time-consuming (Gémes-Juhász *et al.* 2002; Datta 2005).

Doubled haploid (DH) technology is a tool for the recovery of genetically uniform lines for the commercial production of F_1 hybrid varieties (Kurtar & Balkaya 2010). These unique uniform lines are provided in a short time by anther-microspore culture, ovule-ovary culture, and pollen irradiation.

The first successful production of haploid plants was achieved by irradiated pollen technique in pumpkin (Kurtar *et al.* 2009) and in winter squash (Kurtar & Balkaya 2010) and by anther culture in winter squash and pumpkin (Kurtar *et al.* 2016). The frequency of dihaploidization by these techniques mainly depends on the genotypic response, whereby their practical use in breeding programs is still limited.

Gynogenesis is one of the approaches used to generate haploid plants in Cucurbits, but only few reports have been published, e.g. in melon (Ficcadenti *et al.* 1999; Malik *et al.* 2011), cucumber (Gémes-Juhász *et al.* 2002; Diao *et al.* 2009; Tantasawat *et al.* 2015) and summer squash (Metwally *et al.* 1998; Shalaby 2007). The first gynogenic response was reported by Kwack and Fujieda (1988) in pumpkin (*Cucurbita moschata*), but regenerated plants were found to be diploid and tetraploid. Sun *et al.* (2009) obtained only gynogenic embryos in pumpkin ovaries.

There are no successful reports on obtaining gynogenic haploid plants in winter squash and pumpkin

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via ovule culture, and the aim of this work was to optimize the medium composition to increase the frequency of gynogenic haploid plants in breeding lines of winter squash and pumpkin. Therefore, the effects of benzylaminopurine (BAP), thidiazuron (TDZ), naphthaleneacetic acid (NAA) and ovary collection time were evaluated.

MATERIAL AND METHODS

The study was carried out with four winter squash and two pumpkin lines. Female flowers were collected one day before anthesis (BA) and at anthesis (A) time (Figure 1A). Female flowers were isolated one day before anthesis to avoid pollen contamination. Briefly, all parts except the ovary were removed gently and ovaries were rinsed for 15–20 min, and they were then immersed in ethanol (70%) for 2 min, followed by sterilization in 10% commercial bleach solution + 0.1% Tween-20 solution for 15 min. Ovaries were rinsed three times with sterile double distilled water for 5 min each time.

For induction of callogenesis and regeneration of plantlets, the ovules were cultured onto a solid MS medium (pH 5.8) supplemented with different combinations and concentrations of PGRs (Table 1). The ovules were excised and transferred to Magenta boxes according to Kurtar *et al.* (2016) (Figure 1B, C). Magenta boxes were maintained in the dark at 35 ± 1°C for 3 days, followed by transfer to a growth chamber at 26 ± 1 °C with 22 µmol/m^2/s light intensity under 12/12 h for a week and then 16/8 h for 3 to 4 weeks. When calli enlarged and reached a convenient size (Figure 1D), they were subcultured onto callus maturation media (M) for 3 or 4 weeks.

Maturated calli were cultured onto differentiation media under 37 µmol/m^2/s light intensity for plantlet initiation (Figure 1E, F). The media were refreshed 4 times at 10 days intervals. The plantlets that reached 4–5 mm in height were cultured in MS medium supplemented with 0.01 mg/l IAA + 1.0 mg/l BAP for root and shoot elongation. Then,

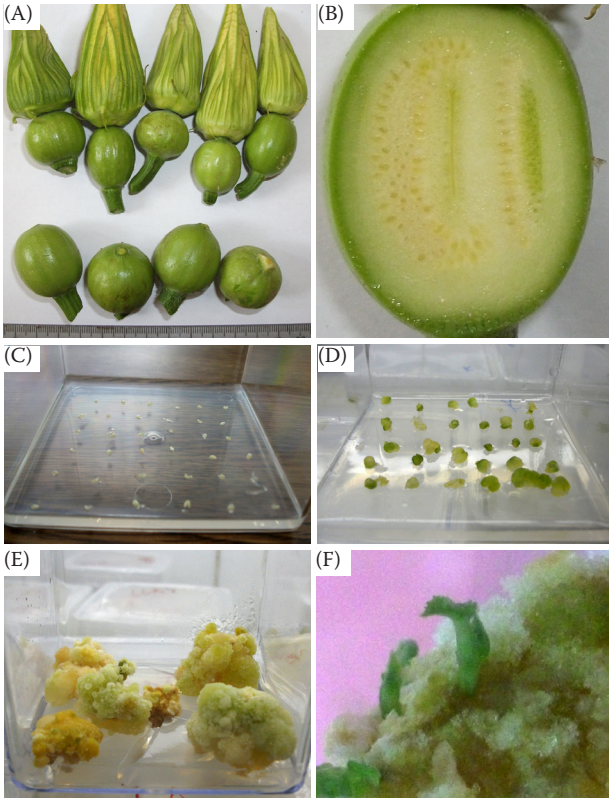

Figure 1. The stages of ovule cultures in winter squash and pumpkin: female flowers (A), ovules in unpollinated female flowers (B), ovules on culture medium (C), callus induction at 3rd–4th weeks of culture (D), callus maturation at 6th–8th weeks of culture (E), plantlets initiation on the callus at 12th–15th weeks of culture (F)

Table 1. The composition of MS medium for callus induction and plant regeneration in winter squash and pumpkin

		Agar	Sucrose	2,4-D	BAP	NAA	TDZ	IAA	Media
		(g/l)		(mg/l)					
Callus	induction	7	120	2.0	–	–	–	–	C
	maturation	7	120	2.0	0.5	–	–	–	M
Plant initiation		8	30	–	4	0.05	–	–	I1
							0.05	–	I2
							0.1	–	I3
							0.5	–	I4
Regeneration		7	30	–	1	–	–	0.01	R

2,4-D – 2,4-dichlorophenoxyacetic acid; BAP – benzylaminopurine; NAA – naphthaleneacetic acid; TDZ – thidiazuron; IAA – indole-3-acetic acid

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plants were micropropagated on R medium (Table 1; Figure 2A, B). The plantlets were acclimatized as described by Kurtar and Balkaya (2010) (Figure 2D).

Ploidy determination and dihaploidization. The ploidy level of all plants was determined by chromosome counting in root tips and the haploid plantlets were treated with 1% colchicine for chromosome doubling as described in our previous work (Kurtar *et al.* 2016).

Data collection and statistical analysis. A factorial experiment based on a completely randomized design with four replications was used in this study. Four Magentas for each replication were cultured for each medium, line and flower collection time. The data were analysed by SPSS (Ver. 18.0, 2009) and mean values were separated based on Duncan's multiple range test.

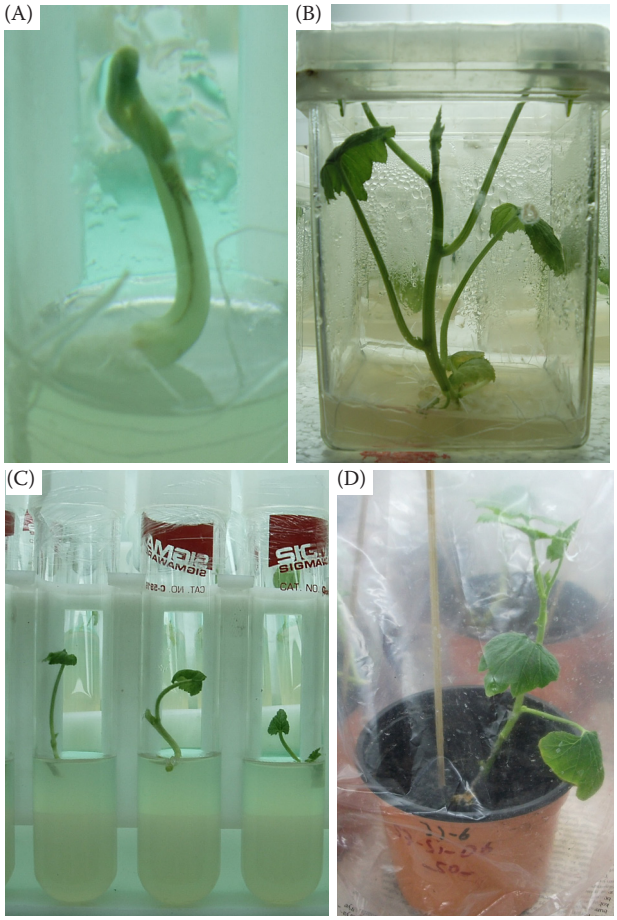

Figure 2. The regeneration processes of plantlets: a plantlet in the root and shoot development medium at 15th to 16th weeks of culture (A), a regenerated plant in a culture box at 17th–18th weeks of culture (B), microcuttings on regeneration medium at 19th–20th weeks of culture (C), an acclimatized plant in the growth chamber at 22th to 24th weeks of culture (D)

RESULTS

The ovules produced green and yellow calli and considerably enlarged within 6 or 8 weeks of culture. While genotype and ovary collection time were found to be effective for callus induction, callus maturation was influenced only by genotype. Nevertheless, flower collection time and media were not found to have a statistically significant effect on callus maturation (Table 2). The percentage of callus induction ranged from 61.3% to 86.4% at BA and from 70.4% to 93.3% at A for 14BO01 and 57SI21, respectively. On the other hand, the mean percentage of callus induction irrespective of genotypes was highest at A (50.9% for winter squash and 42.9% for pumpkin), while it was 43.7% for winter squash and 36.1% for pumpkin at BA. The greenish formation was maintained by consecutively subculturing onto callus maturation media. Most yellowish calli enlarged and turned firmly greenish within 4 or 5 weeks. Lines and species showed a different frequency of green callus formation and average values ranged from 32.9% (in 14BO01 at BA) to 58.2% (in 57SI21 at A). Likewise, the mean percentage was determined to be 45.1% and 48.1% in winter squashes and 37.9% and 40.1% in pumpkins at BA and A, respectively. 57SI21, 55CA15 and G9 were found to be fecund lines for both callus induction and callus maturation process.

The primary plantlets initiated growth from some calli within 12 or 15 weeks of culture. Genotype, medium, callus formation and ovary collection time were found to statistically affect the mean number of plantlets formed per callus (PPC) (Table 3). Control lots were capable of callus induction, but they did not produce any PPC for all lines and media. Eventually, all lines produced the highest PPC on I3 media. Although I2 medium appeared promising, the general frequency of PPC was lower at the highest TDZ level (I4 media).

Greenish calli were found to be fecund with regard to PPC in all lines. Yellowish calli produced PPC only on I3 media at BA and on I1, I2 and I3 media at A. Otherwise, PPC were obtained from I1, I2 and I3 media from yellowish calli at A. The highest PPC yield was from 57SI21 (1.89), followed by 55CA15 (1.66) and 55CA06 (1.58) with greenish callus form for A in winter squash. On the other hand, the mean PPC values varied from 0.02 to 0.27 in yellowish calli and from 0.39 to 1.54 in greenish calli.

TDZ had an important role on PPC. It is more likely that when greenish calli were cultured on induction

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media combined with 0.5 mg/l of TDZ, the results of PPC were below average. However, PPC from greenish and yellowish callus were not observed in any medium without TDZ. It is noteworthy that low doses (0.05 and 0.1 mg/l) of TDZ combined with BAP and NAA stimulated plantlet initiation in winter squash and pumpkin lines. Winter squashes were found to be more productive than pumpkins. Among the lines 57SI21, 55CA15, 55CA06 and G9 gave favourable results, while 14BO01 and 55BA03 were non-responsive in terms of dihaploidization via gynogenesis.

As a result of the ovule culture, a total of 122 plants (94 of winter squash and 28 of pumpkin) were regenerated and micropropagated. Chromosome counting in root tips revealed that 70 plants were haploid (n = 20), 46 plants were diploid ($2n$ = 40) and 6 plants were mixoploid (Figure 3). The genotypes produced different frequency of haploidy, and the

Table 2. The effects of ovary collection time on callus induction and greenish calli formation in winter squash and pumpkin

Species	Lines	Media	BA	A	Avr.
Callus induction (%)					
Winter squash	57SI21	C0	8.3 (± 2.2)^{EF}	16.1 (± 3.1)^{E}	12.2 (± 4.8)^{D}
		C	86.4 (± 11.9)^{AB}	93.3 (± 9.4)^{A}	89.9 (± 10.2)^{A}
	55BA03	C0	5.7 (± 1.7)^{F}	10.9 (± 3.0)^{EF}	8.3 (± 2.9)^{D}
		C	70.8 (± 8.4)^{CD}	81.2 (± 9.1)^{B}	76.0 (± 11.3)^{B}
	55CA06	C0	7.2 (± 2.5)^{F}	13.1 (± 3.3)^{E}	10.2 (± 4.2)^{D}
		C	79.6 (± 8.3)^{BC}	86.5 (± 13.1)^{AB}	83.1 (± 9.9)^{AB}
	55CA15	C0	8.1 (± 2.2)^{EF}	15.6 (± 4.7)^{E}	11.9 (± 3.8)^{D}
		C	82.9 (± 9.9)^{B}	90.5 (± 9.2)^{A}	86.7 (± 12.4)^{A}
	Mean		43.7 (±7.4)^{AB}	50.9 (± 8.8)^{A}	
Pumpkin	14BO01	C0	3.7 (± 0.9)^{F}	6.1 (± 2.3)^{F}	4.9 (± 1.4)^{DE}
		C	61.3 (± 8.7)^{D}	70.4 (± 11.5)^{CD}	65.9 (± 10.8)^{C}
	G9	C0	4.9 (± 1.6)^{F}	11.7 (± 3.4)^{EF}	8.3 (± 5.1)^{D}
		C	74.4 (± 10.1)^{C}	83.3 (± 9.3)^{B}	78.9 (± 10.9)^{B}
	Mean		36.1 (±6.6)^{B}	42.9 (± 8.2)^{AB}	
Greenish calli formation (%)					
Winter squash	57SI21	M0	21.6 (± 4.3)^{G}	29.8 (± 4.9)^{F}	25.7 (± 5.3)^{D}
		M	84.3 (± 10.2)^{A}	86.6 (± 8.1)^{A}	85.5 (± 9.1)^{A}
	55BA03	M0	12.7 (± 3.2)^{H}	14.7 (± 3.6)^{GH}	13.7 (± 4.2)^{E}
		M	61.2 (± 9.3)^{D}	59.5 (± 7.4)^{D}	60.4 (± 6.7)^{C}
	55CA06	M0	19.8 (± 3.7)^{GH}	22.2 (± 4.5)^{G}	21.0 (± 4.9)^{D}
		M	67.6 (± 7.4)^{C}	68.8 (± 8.1)^{C}	68.2 (± 8.4)^{B}
	55CA15	M0	17.1 (± 2.9)^{GH}	23.6 (± 5.3)^{G}	20.4 (± 4.8)^{D}
		M	76.2 (± 7.4)^{AB}	79.1 (± 8.0)^{AB}	77.7 (± 7.7)^{AB}
	Mean		45.1 (±7.6)^{A}	48.1 (± 9.7)^{A}	
Pumpkin	14BO01	M	9.9 (± 2.7)^{H}	12.7 (± 4.1)^{H}	11.3 (± 5.2)^{E}
		M	55.8 (± 4.4)^{D}	56.2 (± 4.9)^{D}	56.0(± 4.1)^{C}
	G9	M0	14.6 (± 5.8)^{H}	17.9 (± 4.2)^{GH}	16.3 (± 5.5)^{DE}
		M	70.9 (± 8.2)^{BC}	73.2 (± 6.7)^{BC}	72.1(± 7.9)^{B}
	Mean		37.9 (±5.7)^{B}	40.1(± 4.7)^{B}	

BA – before anthesis; A – anthesis; different letters indicate a significant difference (P < 0.05); C0 – control; $SD_{(0.05)}$ lines = 8.4; $SD_{(0.05)}$ media = 10.1; $SD_{(0.05)}$ mean = 8.9

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Table 3. The effects of media, ovary collection time and greenish (G) and yellowish (Y) calli formation on the mean number of plantlets per callus (PPC) in winter squash and pumpkin

Species	Lines	Media	BA		A		Avr.	
			G	Y	G	Y	G	Y
Winter Squash	57SI21	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.84 (±0.11) ^{F}	0.00 (±0.00) ^{H}	2.11 (±0.42) ^{CD}	0.16 (±0.03) ^{G}	1.48 (±0.39) ^{C}	0.08 (±0.03) ^{F}
		I2	1.11 (±0.16) ^{EF}	0.00 (±0.00) ^{H}	2.69 (±0.39) ^{BC}	0.38 (±0.07) ^{FG}	1.90 (±0.27) ^{B}	0.19 (±0.06) ^{F}
		I3	1.59 (±0.23) ^{DE}	0.23 (±0.08) ^{G}	3.72 (±0.51) ^{A}	0.63 (±0.12) ^{F}	2.66 (±0.44) ^{A}	0.43 (±0.19) ^{E}
		I4	0.32 (±0.09) ^{G}	0.00 (±0.00) ^{H}	0.91 (±0.17) ^{F}	0.00 (±0.00) ^{H}	0.62 (±0.24) ^{E}	0.00 (±0.00) ^{G}
		Avr.	0.77 (±0.21) ^{BC}	0.05 (±0.01) ^{D}	1.89 (±0.24) ^{A}	0.23 (±0.08) ^{CD}		
	55BA03	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.49 (±0.16) ^{FG}	0.00 (±0.00) ^{H}	1.12 (±0.19) ^{E}	0.00 (±0.00) ^{H}	0.81 (±0.27) ^{DE}	0.00 (±0.00) ^{G}
		I2	0.58 (±0.24) ^{F}	0.00 (±0.00) ^{H}	1.64 (±0.13) ^{DE}	0.11 (±0.09) ^{G}	1.11 (±0.29) ^{D}	0.06 (±0.01) ^{F}
		I3	0.82 (±0.15) ^{F}	0.03 (±0.01) ^{GH}	2.01 (±0.27) ^{D}	0.27 (±0.13) ^{G}	1.42 (±0.25)^{C}	0.15 (±0.03) ^{F}
		I4	0.07 (±0.02) ^{G}	0.00 (±0.00) ^{H}	0.34 (±0.06) ^{G}	0.00 (±0.00) ^{H}	0.21 (±0.05) ^{F}	0.00 (±0.00) ^{G}
		Avr.	0.39 (±0.11) ^{CD}	0.01 (±0.00) ^{D}	1.02 (±0.18) ^{B}	0.08 (±0.02) ^{D}		
	55CA06	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.72 (±0.20) ^{F}	0.00 (±0.00) ^{H}	1.84 (±0.31) ^{D}	0.12 (±0.02) ^{G}	1.28 (±0.16)^{CD}	0.06 (±0.01) ^{F}
		I2	0.97 (±0.24) ^{EF}	0.00 (±0.00) ^{H}	2.08 (±0.37) ^{CD}	0.27 (±0.05) ^{G}	1.53 (±0.19)^{C}	0.14 (±0.02) ^{F}
		I3	1.24 (±0.29) ^{E}	0.04 (±0.01) ^{GH}	3.19 (±0.29) ^{AB}	0.34 (±0.07) ^{G}	2.22 (±0.24)^{AB}	0.19 (±0.04) ^{F}
		I4	0.15 (±0.06) ^{G}	0.00 (±0.00) ^{H}	0.77 (±0.18) ^{F}	0.00 (±0.00) ^{H}	0.46 (±0.09) ^{E}	0.00 (±0.00) ^{G}
		Avr.	0.62 (±0.11) ^{C}	0.01 (±0.00) ^{D}	1.58 (±0.21) ^{A}	0.15 (±0.02) ^{D}		
	55CA15	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.79 (±0.14) ^{F}	0.00 (±0.00) ^{H}	2.03 (±0.31) ^{D}	0.22 (±0.04) ^{G}	1.41 (±0.19) ^{C}	0.11 (±0.06) ^{F}
		I2	1.09 (±0.23) ^{EF}	0.00 (±0.00) ^{H}	2.91 (±0.37) ^{B}	0.31 (±0.08) ^{G}	2.00 (±0.27) ^{B}	0.16 (±0.03) ^{F}
		I3	1.41 (±0.21) ^{E}	0.13 (±0.04) ^{G}	3.47 (±0.46) ^{A}	0.81 (±0.17) ^{F}	2.44 (±0.33) ^{A}	0.47 (±0.14) ^{E}
		I4	0.24 (±0.07) ^{G}	0.00 (±0.00) ^{H}	0.69 (±0.13) ^{F}	0.00 (±0.00) ^{H}	0.47 (±0.07) ^{E}	0.00 (±0.00) ^{G}
		Avr.	0.71 (±0.13) ^{C}	0.03 (±0.01) ^{D}	1.66 (±0.18) ^{A}	0.27 (±0.04) ^{CD}		
		Mean	0.62 (±0.11) ^{C}	0.03 (±0.01) ^{D}	1.54 (±0.17) ^{A}	0.18 (±0.03) ^{D}		
Pumpkin	14BO01	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.09 (±0.05) ^{GH}	0.00 (±0.00) ^{H}	0.71 (±0.18) ^{F}	0.00 (±0.00) ^{H}	0.40 (±0.08) ^{E}	0.00 (±0.00) ^{G}
		I2	0.31 (±0.07) ^{G}	0.00 (±0.00) ^{H}	0.95 (±0.21) ^{EF}	0.00 (±0.00) ^{H}	0.63 (±0.19) ^{E}	0.00 (±0.00) ^{G}
		I3	0.63 (±0.11) ^{F}	0.00 (±0.00) ^{H}	1.22 (±0.33) ^{E}	0.08 (±0.02) ^{GH}	0.93 (±0.14) ^{D}	0.04 (±0.01) ^{FG}
		I4	0.06 (±0.02) ^{G}	0.00 (±0.00) ^{H}	0.18 (±0.04) ^{G}	0.00 (±0.00) ^{H}	0.12 (±0.02) ^{F}	0.00 (±0.00) ^{G}
		Avr.	0.22 (±0.06) ^{CD}	0.00 (±0.00) ^{E}	0.61 (±0.13) ^{C}	0.02 (±0.01) ^{D}		
	G9	I0	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{H}	0.00 (±0.00) ^{G}	0.00 (±0.00) ^{G}
		I1	0.52 (±0.26) ^{F}	0.00 (±0.00) ^{H}	1.18 (±0.15) ^{E}	0.00 (±0.00) ^{H}	0.85 (±0.14) ^{DE}	0.00 (±0.00) ^{G}
		I2	0.98 (±0.19) ^{EF}	0.00 (±0.00) ^{H}	1.54 (±0.19) ^{DE}	0.11 (±0.03) ^{G}	1.26 (±0.31) ^{CD}	0.06 (±0.01) ^{F}
		I3	1.14 (±0.31) ^{E}	0.14 (±0.03) ^{G}	2.12 (±0.26) ^{CD}	0.22 (±0.05) ^{G}	1.63 (±0.32) ^{C}	0.18 (±0.03) ^{F}
		I4	0.15 (±0.04) ^{G}	0.00 (±0.00) ^{H}	0.36 (±0.07) ^{G}	0.00 (±0.00) ^{H}	0.26 (±0.04) ^{EF}	0.00 (±0.00) ^{G}
		Avr.	0.56 (±0.08) ^{C}	0.03 (±0.01) ^{D}	1.04 (±0.18) ^{B}	0.07 (±0.02) ^{D}		
		Mean	0.39 (±0.08) ^{CD}	0.02 (±0.01) ^{D}	0.83 (±0.11) ^{B}	0.05 (±0.01) ^{D}		

BA – before anthesis; A – anthesis; Different letters indicate a significant difference ($P < 0.05$); I0 – control; $SD_{(0.05)}$ lines = 0.56; $SD_{(0.05)}$ media = 0.48; $SD_{(0.05)}$ mean = 0.41

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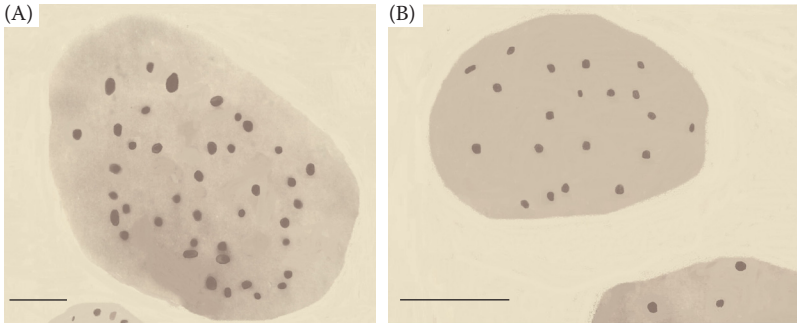

Figure 3. Chromosome numbers in root tips of winter squash regenerants: diploid ($2n$ = 40) (A), haploid (n = 20) (B); bars represent 5 μm

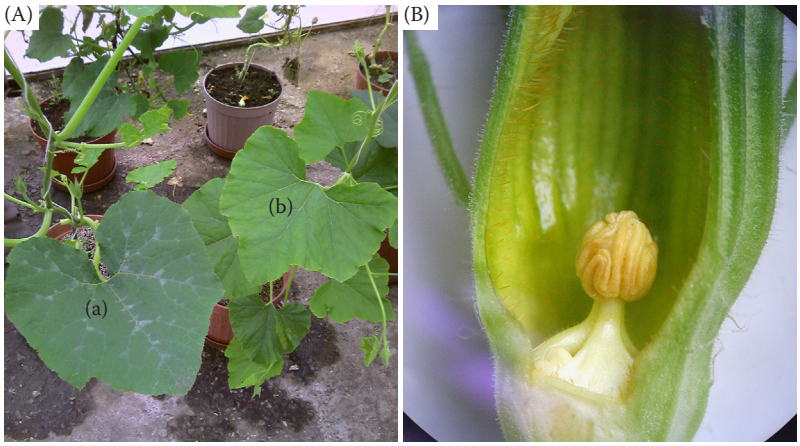

Figure 4. Diploid (a) and haploid (b) winter squash plants (A), anther of a haploid (sterile) pumpkin plant (B)

best responses were obtained from 57SI21 (72.7%), G9 (66.7%) and 55CA15 (62.1%) (Table 4). Dihaploid plants had larger leaves than haploid plants, and haploid plants did not produce pollen (Figure 4).

DISCUSSION

To our knowledge, this is the first successful report concerning the dihaploidization process via ovule culture in winter squash, while there are only two reports on pumpkin. Nevertheless, Kwack and Fujieda (1988) found diploid and tetraploid plants via ovule culture, and embryogenic structures were observed only in an ovary segment (Sun *et al.* 2009) from pumpkin.

The success of dihaploidization via gynogenesis was found to be highly and statistically dependent on the genotype, medium composition, flower collection time and callus form. As a consequence of genotypic variation, winter squash lines were more productive than pumpkin lines for plantlet initiation. In this respect, genotypic differences were also observed for dihaploidization via irradiated pollen

Table 4. The frequency of haploid (H), diploid (D) and mixoploid (M) plants of winter squash and pumpkin

Species	Lines	Total	H		D		M	
			N	%	N	%	N	%
Winter Squash	57SI21	33	24	72.7	8	24.2	1	3.0
	55BA03	11	3	27.3	6	54.5	2	18.2
	55CA06	21	9	42.9	10	47.6	2	9.5
	55CA15	29	18	62.1	11	37.9	0	0.0
	Total/Avr.	94	54	57.4	35	37.2	5	5.3
Pumpkin	14BO01	7	2	28.6	4	57.1	1	14.3
	G9	21	14	66.7	7	33.3	0	0.0
	Total/Avr.	28	16	57.1	11	39.3	1	3.6
Mean		122	70	57.4	46	37.7	6	4.9

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in summer squash (Kurtar *et al.* 2002), pumpkin (Kurtar *et al.* 2009) and winter squash (Kurtar & Balkaya 2010), and also for androgenesis by anther culture in winter squash and pumpkin (Kurtar *et al.* 2016). A different gynogenetic response was reported in summer squash (Shalaby 2007). Conversely, in cucumber both a genotype-dependent reaction (Plapung *et al.* 2014; Tantasawat *et al.* 2015; Golabadi *et al.* 2017) and its absence (Diao *et al.* 2009) were reported.

We used MS medium with different doses of PGR and culture conditions also had to be optimized, because we did not succeed in producing plants by using the protocols of Kwack and Fujieda (1988) and Metwally *et al.* (1998), while only a few diploid regenerants were obtained with the protocol of Shalaby (2007). Plantlet initiation was highly influenced by the PGR concentrations. Species and lines produced various responses in different combinations of PGR. However, ovary collection time and genotype were effective on callus induction. The genotype was found to be a determining factor in the callus maturation procedure. 57SI21, 55CA15 and G9 were superior lines for both callus induction and callus maturation. In plantlet initiation, lower doses (0.05 and 0.1 mg/l) of TDZ with the addition of BAP and NAA had a stimulative effect on all lines. This is in agreement with findings of Kurtar *et al.* (2016), who did not observe plantlet initiation in MS medium without BAP. Accordingly, TDZ was found to be the most efficient PGR to produce a high quantity of plantlets in cucumber (Gémes-Juhász *et al.* 2002; Diao *et al.* 2009; Tantasawat *et al.* 2015). On the other hand, BAP (4 mg/l) and 2,4-D (1.5 mg/l) produced favourable outcomes in cucumber (Golabadi *et al.* 2017).

Firmly greenish calli were found to be highly productive on plantlet initiation. The gynogenic response was also influenced by ovary collection time. In agreement with this result, nearly mature or fully mature embryo sacs were the most responsive for plantlet initiation in cucumber (Gémes-Juhász *et al.* 2002). Conversely, Diao *et al.* (2009) and Golabadi *et al.* (2017) argued that plantlets were successfully procured in cucumber with ovaries which were harvested a day before anthesis.

The success of androgenesis and gynogenesis is highly dependent on the physiological condition of donors, and well-conditioned, healthy and young plants stimulate plantlet initiation and were hence profitable for the frequency of dihaploidization (Kurtar *et al.* 2016). Ploidy analyses revealed that the frequency of gynogenic haploids changed with genotypes, and diploid and mixoploid regenerants were also determined in chromosome counting in root tips (Table 4).

CONCLUSION

Gynogenesis (ovule culture) is highly recommended as an alternative method to irradiated pollen technique and anther culture in the dihaploidization process for winter squash and pumpkin. However, this technique needed improvements to realize the production of a high quantity of *in vitro* haploid regenerants. Thus, for the common use of this technique, further investigation should be planned on effective PGR combinations and concentrations (especially for BAP and TDZ), and also culture conditions (light intensity, media refreshment).

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