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ORIGINAL PAPER

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THE EFFECTS OF ANTI-MITOTIC AGENTS ON DIHAPLOIDIZATION AND FERTILITY IN WINTER SQUASH (*Cucurbita maxima* Duch.) AND PUMPKIN (*Cucurbita moschata* Duch.) ANDROGENIC HAPLOIDS

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ABSTRACT

Double-haploidization (DH) is one of the favorable techniques to obtain 100% pure double haploid plants (DH's) for generating the new F_1 cultivars in a short time. The fecundity of this technique depends on the high quantity of haploids and also fertile DH's. However, there are no comprehensive reports on the chromosome doubling and fertility (fruit and seed-set) of winter squash and pumpkin haploids, currently. Thus, to obtain high frequency and fertile DH's, the efficiency of different anti-mitotic agents (colchicine, amiprophos methyl, trifluralin and oryzalin) was tested at various concentrations and exposure times for both *in vitro* and *in vivo* conditions. Haploid plantlets recovered from anther cultures of winter squash and pumpkin lines were used for DH program. The haploid plants were wholly immersed in aqueous solutions of anti-mitotic agents *in vitro* and apical parts of haploid plantlets were treated with anti-mitotic agents three times *in vivo*. Since some plants remained haploid, and the highest DH efficiency was obtained from multiple treatments of colchicine to shoot tips of haploid plants in the rate of 93.3%. *In vivo* multiple treatments of 1% colchicine for an hour was found to be the best doubling procedure for the recovery of high-frequency fertile DH's in our winter squash and pumpkin breeding program.

Key words: dihaploidization, anti-mitotic agents, fertility, pumpkin, winter squash

INTRODUCTION

Achieving the pure lines is the preliminary stage for the production of superior F_1 hybrid cultivars. Traditional breeding methods are highly labor and time-consuming, and lines still are not 100% homozygous [Germana 2006]. Currently, doubled haploid (DH) technique is used for the recovery of 100% pure dihaploid lines (DH's) in lots of plant species [Kurtar and Balkaya 2010, Galazka and Niemirowicz-Szczytt 2013]. *In vitro* androgenesis, gynogenesis and parthenogenesis are the unique methods for the recovery of fertile homozygous DH's. In Cucurbitaceous, irradiated pollen technique is the efficient method to generate the genetically uniform DH's in melon [Sari et al. 2010] and cucumber [Lotfi et al. 1999], but this technique has not yet been fully optimized for production of high quantity haploids in squash [Kurtar et al. 2002], pumpkin [Kurtar et al. 2009] and winter squash [Kurtar and Balkaya 2010]. Thus, the alternative method, we conducted to anther culture with the aim of developing high quantity DH's in winter squash and pumpkin [Kurtar et al. 2016].

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Nevertheless the importance of the DH's, this unique technology has some limitations in large-scale breeding efforts, such as genotype dependency, low haploid frequency, requirement for labour-intensive works (detection and extraction of embryos, acclimatization etc.) and production of fertile plants (lack of efficient doubling methods) [Kurtar and Balkaya 2010, Kurtar et al. 2016]. Therefore, to produce an efficient dihaploidization method with high-level of fertile DH's, sterile haploids should be subjected to some anti-mitotic agents with convenient dose and durations, because the fertility (fruit-set and seed-set) is a key factor for maintaining the dihaploid lines in a DH breeding effort.

Colchicine is the mainly used anti-mitotic agent [Lim and Earle 2009, Galazka and Niemirowicz-Szczytt 2013, Aslam et al. 2017] along with chromosome duplication methods categorized as immersion of whole plantlets or nodal explants [Sari and Abak 1996, Caglar and Abak 1997], treatment on apical meristems [Nikolova and Niemirowicz-Szczytt 1996], shoot tips [Yetisir and Sari 2003, Solmaz et al. 2011] and lateral shoots in Cucurbits. Trifluralin, amiprophos methyl, and oryzalin have been reported as alternative anti-mitotic agents for colchicines due to less phytotoxicity, high efficiency at low concentration, and insignificantly morphological abnormalities [Grzebelus and Adamus 2004, Alan et al. 2007, Chen et al. 2016].

To our knowledge, there are no reports about chromosome doubling procedure and relationship between anti-mitotic agents and fertility in winter squash and pumpkin. Hence, the aim of the present study was to determine the efficacy of four anti-mitotic agents (colchicine, amiprophos methyl, trifluralin and oryzalin) at various doses and durations for both *in vitro* and *in vivo* conditions to increase the frequencies of fertile double-haploid lines in winter squash and pumpkin. Fertility of double-haploids was also evaluated by pollen viability test, fruit-set, and seed-set.

MATERIAL AND METHODS

Plant material and preparation of anti-mitotic agents. The ploidy levels of all plantlets of winter squash and pumpkin breeding lines derived from anther culture were checked by root tip chromosome counting [Kurtar et al. 2016]. Nodal explants of haploid plantlets, having a leaf and a node, were transferred to modified solid E20A medium with the addition of 0.1 mg l^{-1} IAA and 0.1 mg l^{-1} IBA for micropropagation. Micro-propagated haploid plants were used as plant material and they were treated with anti-mitotic agents at various concentrations and durations.

A stock solution of colchicine – CL (Sigma), amiprophos methyl – APM (Fluca), trifluralin – TF (Duchefa) and oryzalin – OR (Duchefa) were dissolved in dimethyl sulfoxide DMSO (Sigma), diluted in distilled water and stored in deep freeze at –18°C untill use.

***In vitro* chromosome doubling.** After 4–5 weeks of micropropagation, fully developed haploid plantlets were treated with filter sterilized doubling solutions of CL (0.5 and 1.0 g l^{-1}), APM, TF and OR (0.1 and 0.15 g l^{-1}) for 12 and 24 h, before acclimatization process. Plantlets were wholly immersed in Magenta boxes containing 100 ml sterile anti-mitotic solution in the sterile cabinet. Magenta boxes were closed and wrapped with stretch film in the sterile cabinet and placed on a rotary shaker (120 rpm) at 18±1°C under 16/8 (day/night) photoperiod in the growth cabinet. Then plantlets were rinsed three times with sterile distilled water for 5 min each time before culture in sterile conditions. Plantlets were blotted on sterile filter paper to eliminate excessive surface water. Nodal explants of plantlets were cultured and micro-propagated in Magenta boxes containing 25 ml solid E20A elongation medium modified as described by Lim and Earle [2009]. Plantlets were observed in two-day intervals, vitrified and browning explants were eliminated and survival plants were noted, regularly. When regenerated shoots reached 10–12 cm height and 5–6 nodes, the acclimatization process was set up as described by Kurtar and Balkaya [2010]. Acclimatized plants were planted in vials (28 cells) filled with a mixture of peat moss: perlite (2:1, v/v) and well-developed plants were then transferred to the field conditions.

***In vivo* chromosome doubling and ploidy analyses.** Well developed and subsequently acclimatized haploid plantlets regenerated from anther culture were treated with CL (5.0 and 10 g l^{-1}), APM, TF and OR (0.5 and 1.0 g l^{-1}) for 1 and 2 h. *In vivo* treatments of anti-mitotic agents were based on modified multiple methods of Nikolova and Niemirowicz-Szczytt [1996]. A piece of cotton was imbibed into doubling solutions and placed on apical parts of seedlings at 4–5 true-leaf stages. The cotton piece was covered with aluminum foil and stretch film to prevent light influx and evaporation.

Doubling agents were applied for three times and once a day, and treated seedlings were kept at 25 ±2°C day and 18 ±2°C night under daily photoperiod conditions. The apical parts of plants were rinsed three times with distilled water for 5 min each time. The plants were shaded with the net material (green colored and 40% shaded) to protect sensitive apical parts from destructive effects of excessive sunlight and heat. The plants were grown in controlled greenhouse then were transferred to the open field conditions. Ploidy status of treated plants was identified *via* chromosome counting in root tips as described by Kurtar et al. [2016]. A plantlet for each Magenta box and ten Magenta boxes for three replicates (totally 30 plantlets) were cultured for each treatment.

Pollen viability, fruit-set, and seed-set. In order to evaluate the reproductive potential of the DH's, pollen viability test and fruit- and seed-set observations were carried out. For pollen viability tests, pollens of four flowers of each well developed double-haploid plant were mixed and placed in three areas on glass slides and stained with a drop of 1% TTC solution. Stained pollens were incubated in a growth chamber at 25 ±1°C in dark condition. After 4–6 h, approximately 300 pollen grains (100 pollen grains at three different slide areas) were scored by direct microscopy. Pollen grains stained with dark red or red were evaluated viable, and pollen viability was classified as highly viable – HV (>80%), viable – V (50–80%) and low viable – LV (<50%).

One day before anthesis, female and male flowers were isolated with cloth bags. Following day in the morning, female flowers were self-pollinated and isolated again to avoid undesired pollen contamination. Cloth bags were removed on the 2nd or 3rd day of pollination. Four female flowers were pollinated for each double-haploid plant. Fruit-set and seed-set were evaluated as a number of the plant with fruit (PF) and the number of average full seed (FS).

Data collection and statistical analysis. A factorial experiment based on a Completely Randomised Design with three replications was applied. Ten plantlets for each replication (thirty plantlets in total) were used for each doubling solution, concentration and exposure time. The data were subjected to an analysis of variance by SPSS statistical program and the mean values were separated based on Duncan's multiple range test (DMRT).

RESULTS

***In vitro* chromosome doubling.** *In vitro* doubling experiments were based on whole plants strategy. In the first week of culture, some phototoxic effects (browning, vitrification and growth abnormalities) of anti-mitotic agents were observed at high doses and longer durations. Thus, significant reductions were observed in survival rate (SR), in comparison to the control series. The SR ranged from 60.0% to 80.8% in winter squash and from 52.5% to 76.7% in pumpkin, on average. A significant correlation was also observed between doses and durations, and higher doses and longer durations induced a decrease in SR. It is quite obvious that CL was found to be the highest phytotoxic among the anti-mitotic agents that were used. Moreover, 36.7% and 33.3% of plantlets were only survived due to 1.0 g l^{-1} CL for 24 h *in vitro* condition. On the other hand, APM, TF and OR had weaker phytotoxic effect than CL (Tab. 1).

Table 1. The effects of anti-mitotic agents, and their doses (D) and durations (DR) on ploidy status of winter squash and pumpkin *in vitro*

	TR	D (g l^{-1})	DR (h)	TE	SR (no. (%))	Ploidy status (no. (%))			
						n	2n	n + 2n	4n
Winter squash	C	–	–	30	30 (100.0) ^{A}	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
	CL	0.5	12	30	23 (76.7) ^{AB}	11 (47.8) ^{C}	5 (21.7) ^{D}	7 (30.4) ^{C}	0
			24	30	19 (63.3) ^{B}	6 (31.6) ^{D}	8 (42.1) ^{B}	5 (26.3) ^{CD}	0
		1.0	12	30	19 (63.3) ^{B}	3 (15.8) ^{E}	11 (68.8) ^{A}	4 (21.1) ^{CD}	1 (5.3)
			24	30	11 (36.7) ^{C}	0 ^{F}	6 (54.5) ^{AB}	3 (27.3) ^{CD}	2 (18.2)
		Total/mean		120	72 (60.0) ^{B}	20 (27.8) ^{B}	30 (41.7) ^{A}	19 (26.4) ^{B}	3 (4.2)
	APM	0.1	12	30	26 (86.7) ^{A}	12 (46.2) ^{C}	0 ^{F}	14 (53.8) ^{A}	0
			24	30	23 (76.7) ^{AB}	9 (39.1) ^{C}	3 (13.0) ^{DE}	11 (47.8) ^{B}	0
		0.15	12	30	25 (83.3) ^{AB}	9 (15.6) ^{E}	5 (20.0) ^{D}	11 (44.0) ^{B}	0
			24	30	21 (70.0) ^{B}	7 (33.3) ^{D}	7 (33.3) ^{C}	6 (28.6) ^{CD}	1 (4.8)
		Total/mean		120	95 (79.2) ^{A}	37 (38.9) ^{A}	15 (15.8) ^{B}	42 (44.2) ^{A}	1 (1.1)
	TF	0.1	12	30	26 (86.7) ^{A}	9 (34.6) ^{C}	0 ^{F}	17 (65.4) ^{A}	0
			24	30	25 (83.3) ^{AB}	11 (44.0) ^{C}	4 (16.0) ^{DE}	10 (40.0) ^{B}	0
		0.15	12	30	24 (80.0) ^{AB}	8 (33.3) ^{D}	6 (25.0) ^{CD}	8 (33.3) ^{C}	2 (8.3)
			24	30	22 (73.3) ^{A}	7 (31.8) ^{D}	6 (27.3) ^{CD}	6 (27.3) ^{CD}	3 (13.6)
		Total/mean		120	97 (80.8) ^{A}	35 (36.1) ^{A}	16 (16.5) ^{B}	41 (42.3) ^{A}	5 (5.2)
	OR	0.1	12	30	24 (80.0) ^{AB}	15 (62.5) ^{B}	0 ^{F}	9 (37.5) ^{C}	0
			24	30	22 (73.3) ^{AB}	11 (47.8) ^{C}	3 (13.6) ^{DE}	8 (36.4) ^{C}	0
		0.15	12	30	21 (70.0) ^{B}	9 (42.9) ^{C}	4 (19.0) ^{D}	6 (28.6) ^{CD}	2 (9.5)
			24	30	19 (63.3) ^{AB}	8 (42.1) ^{C}	5 (26.3) ^{CD}	3 (15.8) ^{D}	3 (15.8)
		Total/mean		120	86 (71.7) ^{A}	43 (50.0) ^{A}	12 (14.0) ^{B}	26 (30.2) ^{B}	5 (5.8)
		General		480	350 (72.9)	135 (38.6)	73 (20.9)	128 (36.6)	14 (4.0)
Pumpkin	C	–	–	30	30 (100.0)	30 (100.0)	0 ^{F}	0	0
	CL	0.5	12	30	20 (66.7) ^{AB}	9 (45.0) ^{C}	4 (20.0) ^{D}	7 (35.0) ^{BC}	0
			24	30	16 (53.3) ^{B}	8 (50.0) ^{BC}	6 (37.5) ^{B}	2 (12.5) ^{CD}	0
		1.0	12	30	17 (56.6) ^{B}	3 (17.6) ^{E}	13 (68.4) ^{A}	1 (5.9) ^{D}	0
			24	30	10 (33.3) ^{C}	0 ^{F}	4 (40.0) ^{B}	4 (40.0) ^{B}	2 (20.0)
		Total/mean		120	63 (52.5) ^{B}	20 (31.7) ^{B}	27 (42.9) ^{A}	14 (22.2) ^{B}	2 (3.2)
	APM	0.1	12	30	24 (80.0) ^{AB}	13 (54.2) ^{BC}	0 ^{F}	11 (45.8) ^{A}	0
			24	30	22 (73.3) ^{AB}	14 (63.6) ^{B}	4 (18.2) ^{D}	4 (18.2)	0
		0.15	12	30	25 (83.3) ^{AB}	9 (36.0) ^{CD}	6 (24.0) ^{CD}	8 (32.0) ^{C}	2 (8.0)
			24	30	21 (70.0) ^{AB}	9 (42.9) ^{C}	8 (38.1) ^{B}	2 (9.5)	2 (9.5)
		Total/mean		120	92 (76.7) ^{A}	44 (47.8) ^{A}	18 (19.6) ^{B}	24 (26.1) ^{AB}	4 (4.3)
	TF	0.1	12	30	24 (80.0) ^{AB}	15 (62.5) ^{B}	0 ^{F}	9 (37.5) ^{BC}	0
			24	30	22 (73.3) ^{AB}	12 (54.5) ^{BC}	5 (22.7) ^{D}	5 (22.7) ^{C}	0
		0.15	12	30	23 (76.7) ^{AB}	8 (34.8) ^{CD}	6 (26.1) ^{CD}	8 (34.8) ^{BC}	1 (4.3)
			24	30	21 (70.0) ^{AB}	6 (28.6) ^{DE}	7 (33.3) ^{C}	6 (28.6) ^{C}	2 (9.5)
		Total/mean		120	90 (75.0) ^{A}	41 (45.6) ^{A}	18 (20.0) ^{B}	28 (31.1) ^{AB}	3 (3.3)
	OR	0.1	12	30	25 (83.3) ^{AB}	17 (68.0) ^{B}	0	8 (32.0) ^{C}	0
			24	30	23 (76.7) ^{AB}	13 (56.5) ^{BC}	3 (13.0) ^{DE}	7 (30.4) ^{C}	0
		0.15	12	30	20 (66.7) ^{AB}	9 (45.0) ^{C}	4 (20.0) ^{D}	6 (30.0) ^{C}	1 (5.0)
			24	30	18 (60.0) ^{B}	8 (44.4) ^{C}	4 (22.2) ^{D}	5 (27.7) ^{C}	1 (5.6)
		Total/mean		120	86 (71.7) ^{A}	47 (54.7) ^{A}	11 (12.8) ^{BC}	26 (30.2) ^{AB}	2 (2.3)
		General		480	331 (70.0)	153 (46.2)	74 (22.4)	93 (28.1) ^{B}	11 (3.3)

Each value followed by different letters indicates a significant difference ($P < 0.05$) to (DMRT)

C: control; TR: treatments; TE: number of treated explants; SR: survival rate; CL: colchicine; APM: amiprophos methyl; TF: trifluralin; OR: oryzalin

Table 2. The effects of anti-mitotic agents, and their doses (D) and durations (DR) on ploidy status of winter squash and pumpkin *in vivo*

	TR	D (g l^{-1})	DR (h)	TE	Ploidy status (no. (%))			
					n	2n	n + 2n	4n
Winter squash	C	–	–	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
	CL	5.0	1	30	6 (20.0) ^{C}	15 (50.0) ^{C}	9 (30.0) ^{BC}	0
			2	30	7 (23.3) ^{C}	21 (70.0) ^{B}	2 (6.7) ^{E}	0
		10.0	1	30	0 ^{D}	28 (93.3) ^{A}	1 (3.3) ^{E}	1 (3.3) ^{B}
			2	30	0 ^{D}	23 (76.7) ^{B}	2 (2.67) ^{E}	5 (16.7) ^{A}
		Total/mean		120	13 (10.8) ^{B}	87 (72.5) ^{A}	14 (11.7) ^{B}	6 (5.0)
	APM	0.5	1	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
			2	30	25 (83.3) ^{AB}	0	5 (16.7) ^{C}	0
		1.0	1	30	15 (50.0) ^{B}	5 (16.7) ^{DE}	10 (33.3) ^{B}	0
			2	30	15 (50.0) ^{C}	7 (23.3) ^{DE}	8 (26.7) ^{B}	1 (3.3) ^{B}
		Total/mean		120	85 (63.3) ^{A}	12 (10.0) ^{B}	23 (19.2) ^{B}	1 (0.8)
	TF	0.5	1	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
			2	30	26 (86.7) ^{A}	0 ^{F}	4 (13.3) ^{D}	0
		1.0	1	30	19 (63.3) ^{C}	6 (20.0) ^{DE}	5 (16.7) ^{D}	0
			2	30	14 (46.7) ^{C}	10 (33.3) ^{D}	3 (10.0) ^{DE}	3 (10.0) ^{B}
		Total/mean		120	89 (74.2) ^{A}	16 (13.3) ^{B}	12 (10.0) ^{B}	3 (2.5)
	OR	0.5	1	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
			2	30	28 (93.3) ^{A}	0 ^{F}	2 (6.7) ^{E}	0
		1.0	1	30	12 (40.0) ^{C}	0 ^{F}	18 (60.0) ^{A}	0
			2	30	15 (50.0) ^{B}	4 (13.3) ^{E}	11 (36.7) ^{B}	0
		Total/mean		120	85 (70.8) ^{A}	4 (3.3) ^{C}	31 (25.8) ^{A}	0
		General		480	272 (56.7)	119 (24.8)	80 (16.7)	10 (2.1)
Pumpkin	C	–	–	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
	CL	5.0	1	30	9 (30.0) ^{C}	14 (46.7) ^{C}	7 (23.3) ^{C}	0
			2	30	7 (23.3) ^{CD}	19 (63.3) ^{B}	4 (13.3) ^{E}	0
		10.0	1	30	0 ^{D}	24 (80.0) ^{A}	6 (20.0) ^{D}	0
			2	30	0 ^{D}	21 (70.0) ^{AB}	5 (16.7) ^{DE}	4 (13.3)
		Total/mean		120	16 (13.3) ^{B}	78 (65.0) ^{A}	22 (18.3) ^{B}	4 (3.3)
	APM	0.5	1	30	24 (80.0) ^{AB}	0 ^{F}	6 (20.0) ^{D}	0
			2	30	27 (90.0) ^{A}	0 ^{F}	3 (10.0) ^{E}	0
		1.0	1	30	16 (53.3) ^{BC}	6 (20.0) ^{DE}	8 (26.7) ^{C}	0
			2	30	13 (43.3) ^{C}	11 (36.7) ^{CD}	4 (13.3) ^{E}	2 (6.7)
		Total/mean		120	80 (66.7) ^{A}	17 (14.2) ^{B}	21 (17.5) ^{B}	2 (1.7)
	TF	0.5	1	30	29 (96.7) ^{A}	0 ^{F}	1 (3.3) ^{EF}	0
			2	30	26 (86.7) ^{AB}	0 ^{F}	4 (13.3) ^{E}	0
		1.0	1	30	13 (43.3) ^{C}	6 (20.0) ^{DE}	11 (36.7) ^{BC}	0
			2	30	15 (50.0) ^{BC}	9 (30.0) ^{D}	4 (13.3) ^{E}	2 (6.7)
		Total/mean		120	83 (69.2) ^{A}	15 (12.5) ^{B}	20 (16.7) ^{B}	2 (1.7)
	OR	0.5	1	30	30 (100.0) ^{A}	0 ^{F}	0 ^{F}	0
			2	30	22 (73.3) ^{AB}	0 ^{F}	8 (26.7) ^{C}	0
		1.0	1	30	13 (43.3) ^{C}	0 ^{F}	17 (56.7) ^{A}	0
			2	30	10 (33.3) ^{C}	5 (16.7) ^{DE}	15 (50.0) ^{A}	0
		Total/mean		120	75 (62.5) ^{A}	5 (4.2) ^{C}	40 (33.3) ^{AB}	0
		General		480	254 (52.9) ^{A}	115 (24.0)	103 (21.5) ^{B}	8 (1.7)

Each value followed by different letters indicates a significant difference ($P < 0.05$) to (DMRT)
C: control; TR: treatments; TE: treated explants

Chromosome counting in root tips indicated that anti-mitotic agents provided different ploidy status in regenerants such as diploid (2n), mixoploid (n + 2n) and tetraploid (4n). Spontaneous duplications were not observed and all plants remained at a haploid level in control series. When haploid plants were treated with 1.0 g l^{-1} CL for 12 h, DH frequency was highest at the rate of 68.4% for pumpkin and 68.8% for winter squash. Despite the fact that APM, TF and OR resulted in impressive survival rate, they produced small quantities of DH's *in vitro*. While the average duplication rate was 41.7% and 42.9% in CL treatments, it was 15.8% and 19.6% in APM, 16.5% and 20.0% in TF, and 14.0% and 12.8% in OR for winter squash and pumpkin, respectively. Thus APM, TF and OR were not found to be comparable with CL. The DH frequency was approximately 3-fold that of APM, TF and OR treatments by *in vitro* CL application. Interestingly, haploid plants were not detected at the treatment of 1.0 g l^{-1} CL for 24 h and plantlets were only of diploid, mixoploid and tetraploid nature.

***In vivo* chromosome doubling.** Multiple treatments of anti-mitotic agents on apical parts of acclimatized haploid plantlets were experienced *in vivo* doubling experiments in winter squash and pumpkin lines. It has been well documented that the ploidy level of plantlets was highly dependent on anti-mitotic agents, application doses and durations. Anti-mitotic substances had significant effects on ploidy levels and duplication rates, whereas control plants sustained their haploid level. DH's were obtained from all CL treatments in all lines more or less.

Chromosome doubling was only achieved by 1.0 g l^{-1} APM and TF for 1 or 2 h, and 1.0 g l^{-1} OR for 2 h. The lower dose (0.5 g l^{-1}) of APM, TF and OR was not successful on duplication. Besides, treatments of 0.5 g l^{-1} + 1 h of APM, TF and OR were found to be similar to non-treated series and all plants remained their haploid level. Likewise, the treatment of 10.0 g l^{-1} CL for 1 h produced the best duplication rates from 80.0% to 93.3% in pumpkin and winter squash, respectively. Subsequently, 10.0 g l^{-1} CL for 2 h produced notable duplication rate. When the haploid plantlets were treated with 5.0 g l^{-1} CL for 1 h, DH frequency was the lowest at a rate of 46.7% in pumpkin *in vivo* (Tab. 2). Moreover, haploids were not detected at 1 or 2 h treatment with 10.0 g l^{-1} CL for all lines. Although higher doses and longer durations of CL generated low-level phytotoxicity at some apical leaves, these negligible abnormalities were recovered at the growth processing and then the plants were growing normally.

In average, duplication rate was obtained to be 10.0% and 14.2% (by APM) and 13.3% and 12.5% (by TF) in winter squash and pumpkin, sequentially. The OR was an inefficient anti-mitotic agent for *in vivo* duplication process, and it only produced about 4.0% DH's for both lines. Likewise, CL was the most efficient anti-mitotic agents and frequencies of DH's were approximately five- or 20-fold those of the other chemicals for *in vitro* duplications.

The effects of anti-mitotic agents on pollen viability. As provided in Table 3 and 4, the results of pollen viability (PV) tests implied that the fertility of DH's was significantly influenced by doses and durations of anti-mitotic agents for both *in vitro* and *in vivo*. In relation to increasing the dose and duration, all anti-mitotic agents showed similar trend on PV. The PV rates of DH's were also affected by *in vitro* and *in vivo* treatments; besides, PV was found to be highest *in vivo*. The highest HV (highly viable pollen) was obtained from non-treated plantlets varying between 93.3% and 100.0% and the genotypic effect was not detected on PV.

Accordingly, along with the increasing dose and duration of anti-mitotic agents, the rate of HV continuously decreased and viable (V) and low-viable pollen (LV) increased for *in vitro* DH's. In respect to anti-mitotic agents, the treatment with 1.0 g l^{-1} CL for 24 h produced the lowest HV, while it was ranged from 40% to 75.0% with treatment of APM, TF and OR. When comparing the anti-mitotic agents, CL had significantly lower HV and higher LV, averagely. Besides, HV values were found to be similar to the treatment of the APM, TF and OR ranging from 53.3% to 66.7%. Moreover, LV was only noted at 0.15 g l^{-1} dose and 24 h duration of APM, TF and OR.

Table 3. The effects of anti-mitotic agents, and their doses (D) and durations (DR) on pollen viability, number of plants with fruit (PF), and number of full seeds (FS) of *in vitro* DH's

	TR	D (g l^{-1})	DR (h)	APN*	Pollen viability (no. (%))			PF	FS
					HV (>80)	V (50–80)	LV (<50)	(no. (%))	(no. $fruit^{-1}$)
Winter squash	C	–	–	15	14 (93.3)^{A}	1 (6.7)^{EF}	0^{F}	15 (100.0)^{A}	334^{A}
	CL	0.5	12	4	3 (75.0)^{B}	1 (25.0)^{D}	0^{F}	3 (75.0)^{B}	312^{A}
			24	8	4 (50.0)^{C}	3 (37.5)^{D}	1 (12.5)^{E}	6 (75.0)^{B}	297^{AB}
		1.0	12	9	3 (33.3)^{D}	3 (33.3)^{D}	3 (33.3)^{D}	5 (55.6)^{BC}	252^{B}
			24	5	1 (20.0)^{DE}	2 (40.0)^{CD}	2 (40.0)^{CD}	2 (40.0)^{D}	205^{C}
		Total/mean		26	11 (42.3)^{CD}	9 (34.6)^{C}	6 (23.1)^{D}	16 (61.5)^{C}	267^{C}
	APM	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	3	2 (66.7)^{BC}	1 (33.3)^{D}	0^{F}	3 (100.0)^{A}	329^{A}
		0.15	12	4	3 (75.0)^{B}	1 (25.0)^{D}	0	3 (75.0)^{B}	318^{A}
			24	5	3 (60.0)^{CD}	1 (20.0)^{DE}	1 (20.0)^{DE}	3 (60.0)^{BC}	284^{AB}
		Total/mean		12	8 (66.7)^{BC}	3 (25.0)^{C}	1 (8.3)^{E}	9 (75.0)^{B}	310^{B}
	TF	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	3	2 (66.7)^{BC}	1 (33.3)^{D}	0^{F}	3 (100.0)^{A}	314^{A}
		0.15	12	6	3 (50.0)^{C}	3 (50.0)^{D}	0^{F}	5 (83.3)^{AB}	327^{A}
			24	4	2 (50.0)^{C}	1 (25.0)^{D}	1 (25.0)^{D}	2 (50.0)^{C}	271^{AB}
		Total/mean		13	7 (53.8)^{BC}	5 (38.5)^{C}	1 (7.7)^{EF}	10 (76.9)^{B}	304^{B}
	OR	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	3	2 (66.7)^{BC}	1 (33.3)^{D}	0^{F}	3 (100.0)^{A}	326^{A}
		0.15	12	3	2 (66.7)^{BC}	1 (25.0)^{D}	0^{F}	2 (75.0)^{B}	315^{A}
			24	4	2 (50.0)^{C}	1 (25.0)^{D}	1 (25.0)^{D}	2 (50.0)^{BC}	281^{AB}
		Total/mean		10	6 (60.0)^{BC}	3 (30.0)^{C}	1 (10.0)^{E}	8 (80.0)^{B}	307^{B}
		General		61	32 (52.5)	20 (32.8)	9 (14.8)	43 (70.5)	297^{B}
Pumpkin	C	–	–	15	15 (100.0)^{A}	0^{F}	0^{F}	15 (100.0)^{A}	221^{E}
	CL	0.5	12	4	2 (50.0)^{C}	2 (50.0)^{C}	0^{C}	3 (75.0)^{B}	178^{CD}
			24	5	3 (60.0)^{C}	2 (40.0)^{CD}	0^{F}	3 (60.0)^{C}	161^{D}
		1.0	12	12	5 (41.7)^{CD}	5 (41.7)^{CD}	2 (16.7)^{E}	7 (58.3)^{C}	152^{D}
			24	3	1 (33.3)^{D}	1 (33.3)^{C}	1 (33.3)^{D}	1 (33.3)^{D}	104^{E}
		Total/mean		24	11 (40.7)^{CD}	10 (41.7)^{C}	3 (12.5)^{E}	14 (58.3)^{C}	149^{F}
	APM	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	3	2 (75.0)^{B}	1 (25.0)^{D}	0^{F}	3 (100.0)^{A}	189^{CD}
		0.15	12	5	3 (60.0)^{BC}	2 (40.0)^{CD}	0^{F}	4 (80.0)^{B}	178^{CD}
			24	6	3 (50.0)^{C}	2 (33.3)^{D}	1 (16.7)^{E}	3 (50.0)^{BC}	143^{D}
		Total/mean		14	8 (57.1)^{BC}	5 (35.7)^{C}	1 (7.1)^{EF}	10 (71.4)^{B}	170^{E}
	TF	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	5	3 (60.0)^{BC}	2 (40.0)^{CD}	0^{F}	5 (100.0)^{A}	191^{CD}
		0.15	12	5	3 (60.0)^{BC}	2 (40.0)^{CD}	0^{F}	4 (80.0)^{BC}	181^{CD}
			24	5	2 (40.0)^{CD}	2 (40.0)^{CD}	1 (20.0)^{DE}	3 (57.1)^{C}	154^{D}
		Total/mean		15	8 (53.3)^{BC}	6 (40.0)^{C}	1 (6.7)^{EF}	12 (80.0)^{B}	175^{E}
	OR	0.1	12	0	0^{F}	0^{F}	0^{F}	0^{E}	0^{E}
			24	3	2 (66.7)^{BC}	1 (33.3)^{D}	0^{F}	3 (100.0)^{A}	187^{CD}
		0.15	12	4	2 (50.0)^{C}	2 (50.0)^{C}	0^{F}	3 (75.0)^{B}	185^{CD}
			24	3	2 (66.7)^{BC}	0	1 (33.3)^{D}	2 (66.7)^{C}	161^{D}
		Total/mean		10	6 (60.0)^{BC}	3 (30.0)^{C}	1 (10.0)^{E}	8 (80.0)^{B}	178^{E}
		General		63	33 (47.6)	24 (38.1)	6 (9.5)	45 (66.2)	168^{D}

Each value followed by different letters indicates a significant difference ($P < 0.05$) to (DMRT)
C: control; TR: treatments; APN: acclimatized plant number; HV: highly viable; V: viable; LV: low viable

Table 4. The effects of anti-mitotic agents, and their doses (D) and durations (DR) on pollen viability, number of plants with fruit (PF), and number of full seeds (FS) of *in vivo* DH's

	TR	D (g l^{-1})	DR (h)	OPN	Pollen viability (no. (%))			PF	FS
					HV (>80)	V (50–80)	LV (<50)	(no. (%))	(no. $fruit^{-1}$)
Winter squash	C	–	–	15	14 (93.3) ^{A}	1 (6.7) ^{B}	0 ^{F}	15 (100.0) ^{A}	334 ^{A}
	CL	5.0	1	15	13 (86.6) ^{AB}	2 (13.3) ^{E}	0 ^{F}	14 (93.3) ^{A}	328 ^{A}
			2	21	15 (71.4) ^{B}	6 (28.6) ^{D}	0 ^{F}	18 (85.7) ^{AB}	317 ^{A}
		10.0	1	28	18 (64.3) ^{BC}	7 (25.0) ^{D}	3 (10.7) ^{E}	22 (78.6) ^{B}	314 ^{A}
			2	23	12 (52.7) ^{C}	6 (26.1) ^{D}	5 (30.4) ^{D}	14 (60.9) ^{BC}	277 ^{AB}
		Total/mean		87	58 (66.7) ^{BC}	21 (24.1) ^{D}	8 (9.2) ^{E}	68 (78.2) ^{B}	309 ^{AB}
	APM	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	5	5 (100.0) ^{A}	0 ^{F}	0 ^{F}	5 (100.0) ^{A}	321 ^{A}
			2	7	6 (85.7) ^{B}	1 (14.3) ^{E}	0 ^{F}	6 (85.7) ^{AB}	328 ^{A}
		Total/mean		12	11 (91.2) ^{A}	1 (8.8) ^{E}	0 ^{F}	11 (91.2) ^{A}	325 ^{A}
	TF	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	6	6 (100.0) ^{A}	0 ^{F}	0 ^{F}	6 (100.0) ^{A}	327 ^{A}
			2	10	9 (90.0) ^{A}	1 (10.0) ^{CD}	0 ^{F}	8 (80.0) ^{B}	316 ^{A}
		Total/mean		16	15 (93.8) ^{A}	1 (6.2) ^{E}	0 ^{F}	14 (87.5) ^{AB}	322 ^{A}
	OR	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	4	3 (75.0) ^{B}	1 (25.0) ^{D}	0 ^{F}	3 (75.0) ^{B}	326 ^{A}
		Total/mean		4	3 (75.0) ^{B}	1 (25.0) ^{D}	0 ^{F}	3 (75.0) ^{B}	326 ^{A}
		General		119	87 (73.1)	24 (20.2)	8 (6.7)	95 (79.8)	322 ^{A}
Pumpkin	C	–	–	15	14 (93.3) ^{A}	1 (6.7) ^{EF}	0 ^{F}	15 (100.0) ^{A}	221 ^{C}
	CL	5.0	1	14	12 (85.7) ^{AB}	2 (14.3) ^{E}	0 ^{F}	12 (85.7) ^{AB}	205 ^{C}
			2	19	15 (78.9) ^{B}	4 (21.1) ^{E}	0 ^{F}	16 (84.2) ^{AB}	198 ^{C}
		10.0	1	24	16 (66.7) ^{BC}	5 (20.8) ^{DE}	3 (12.5) ^{E}	20 (83.3) ^{AB}	201 ^{C}
			2	21	9 (42.9) ^{CD}	5 (23.8) ^{D}	7 (33.3) ^{D}	14 (66.7) ^{BC}	144 ^{D}
		Total/mean		78	52 (66.7) ^{BC}	16 (20.5) ^{DE}	10 (12.8) ^{E}	62 (79.5) ^{B}	187 ^{E}
	APM	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	6	6 (100.0) ^{A}	0 ^{F}	0 ^{F}	6 (100.0) ^{A}	212 ^{C}
			2	11	9 (81.8) ^{B}	2 (18.2) ^{E}	0 ^{F}	9 (81.8) ^{B}	218 ^{C}
		Total/mean		17	15 (88.2) ^{AB}	2 (11.8) ^{E}	0 ^{F}	15 (88.2) ^{AB}	215 ^{D}
	TF	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	6	6 (100.0) ^{A}	0 ^{F}	0 ^{F}	6 (100.0) ^{A}	216 ^{C}
			2	9	7 (77.8) ^{BC}	2 (22.2) ^{D}	0 ^{F}	7 (77.8) ^{4B}	201 ^{C}
		Total/mean		15	13 (86.7) ^{AB}	2 (13.3) ^{E}	0 ^{F}	13 (86.7) ^{AB}	209 ^{D}
	OR	0.5	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
		1.0	1	0	0 ^{F}	0 ^{F}	0 ^{F}	0 ^{E}	0 ^{E}
			2	5	4 (80.0) ^{B}	1 (20.0) ^{DE}	0 ^{F}	4 (75.0) ^{B}	218 ^{C}
		Total/mean		5	4 (80.0) ^{AB}	1 (20.0) ^{DE}	0 ^{F}	4 (75.0) ^{B}	218 ^{D}
		General		115	84 (73.0)	21 (18.3)	10 (8.7)	94 (81.7)	207 ^{C}

Each value followed by different letters indicates a significant difference ($P < 0.05$) to (DMRT)
C: control; TR: treatments; OPN: observed plant number; HV: highly viable; V: viable; LV: low viable

On the other hand, the HV of *in vivo* DH's was higher than *in vitro*, likewise, HV was interrupted with the application of 1.0 g l^{-1} CL for 24 h. The lowest HV values were noted to be 66.7% for both of winter squash and pumpkin. In average, HV values ranged from 75.0% to 93.8%. On the other hand, APM, TF and OR produced higher rate of HV, and the percentage of HV reached up to 100.0% due to application of 1.0 g l^{-1} APM and TF for 1 h. Interestingly, LV was only observed in 10.0 g l^{-1} dose + 1 or 2 h CL treatments in the range of 10.7–33.3%. In addition, V was only recorded in the 2 h treatments of 1.0 g l^{-1}APM, TF and OR for *in vivo* DH's.

Effects of anti-mitotic agents on fruit set and seed set. Based on the results of the fruit-set and seed-set observations it was inferred that number of plants with fruit (PF) and the number of average full seeds (FS) were significantly influenced by the anti-mitotic agents, application doses and durations (Tab. 3 and 4).

In relation to the increasing dose and duration of anti-mitotic agents, fruit-set (PF) was interrupted for both *in vitro* and *in vivo* DH's (Tab. 3 and 4). Similarly, CL has been determined as highly phytotoxic agents, so that a gradual decrease in fruit-set rate was detected *in vitro* DH's at the rate of 33.3% to 40.0% at higher concentration and longer duration of CL. On the other hand, mean PF was 58.3% and 61.5% with CL applications, and APM, TF and OR had significantly favorable PF values in the range of 71.4–80.0%. Moreover, all *in vivo* DH's had the fruit-set by the treatment of 0.1 g l^{-1} APM, TF and OR for 24 h. Although a clear depression was detected on mean PF by the treatment of CL for both *in vitro* and *in vivo* DH's, CL gave considerably PF results of *in vivo* DH's. Likewise, higher concentration and longer duration of CL yielded the lowest PF values about 60.0%, but other concentration and durations of CL reached to the favorable PF varying between 78.6% and 93.3%.

However, full seed-set (FS) was markedly influenced by CL treatments and lines for both *in vitro* and *in vivo* DH's. Furthermore, doses and durations of APM, TF and OR were not affected on FS and they produced similar FS results. The FS was the highest in control series and it was reached up to 334, whereas a wide range of seed-set frequency was noted between lines. The lowest FS values were recorded at the 1.0 g l^{-1} CL + 24 h for both species, and it was 104 and 144 in pumpkin, and 205 and 277 in winter squash for *in vitro* and *in vivo*, respectively. DH's reached significantly higher FS results *in vivo* than *in vitro*, therefore the mean FS varied from 149 to 310 for *in vitro*, while it varied from 187 to 326 for *in vivo*. Correlatively, genotypes also showed different FS response, and higher FS was obtained from winter squash for both conditions.

DISCUSSION

To our knowledge, this is the first comprehensive report on the efficacy of colchicine, amiprophos methyl, trifluralin and oryzalin on double-haploidization and fertility of winter squash and pumpkin androgenic haploids for both *in vitro* and *in vivo* conditions. Our results indicated that anti-mitotic agents, treatment doses and durations, and *in vitro* and *in vivo* conditions had significant effects on double-haploidization and fertility.

In order to perform the chromosome duplication, various concentrations and durations of CL, APM, TF and OR were applied to micro-propagated haploids of winter squash and pumpkin for both *in vitro* and *in vivo*. When plantlets were wholly immersed in anti-mitotic solutions and then cultured in modified E20A medium, subsequently, tissue browning, vitrification and growth abnormalities were seen in some regenerants as a result of the phytotoxic effects of anti-mitotic agents, especially at higher doses and longer durations for *in vitro* conditions during the first and the second week of culture. Although our acclimatization process has been well established and routinely used for *in vitro* for a long time by us, some DH's did not survive at the least. Thus, low SR was obtained from *in vitro* DH experiment both due to the phytotoxicity of anti-mitotic agents and acclimatization process. Our findings provided a wide range of SR varying from 33.3% to 86.7%, depending on the anti-mitotic agents, and their dose and duration. It is clearly reflected that among the anti-mitotic agents, CL was found to be the highly phytotoxic, and SR gradually decreased as the dose and duration of CL increased. On the other hand,

APM, TF and OR had only little phytotoxic effect by the treatments with 0.15 g l^{-1} dose for 24 h and they produced favorable *in vitro* SR for both lines. Mean SR was about 40% in CL treatments, while it was up to 60.0% in OR. The phytotoxicity and lower SR of *in vitro* CL treatments was also reported in melon [Koksal et al. 2002, Yashiro et al. 2002, Lotfi et al. 2003, Yetisir and Sari 2003, Lim and Earle 2008, 2009, Solmaz 2011, Nasertorabi et al. 2012], or in cucumber [Caglar and Abak 1997, Claveria et al. 2005, Galazka et al. 2015]. In accordance with our results, in melon, Solmaz et al. [2011] reported the SR in the range of 23% and 53%, and findings of Lim and Earle [2008] also introduced the 39–83% SR; besides, it varied from 23% to 81% in Iranian melons [Nasertorabi et al. 2012]. Correlatively, 20–60% of regenerants were only survived in cucumber lines [Claveria et al. 2005].

On the other hand, we observed a partial phytotoxicity in some plants (yellow spots and longitudinal growth at apical leaves) by *in vivo* treatments with CL at a higher dose and longer durations, but these slight abnormalities were subsequently recovered and all DH's were growing normally. In contrast, some permanent syndromes with CL application, such as yellowing on leaf surface and abnormal flowers [Koksal et al. 2002, Solmaz et al. 2011], and also clustering [Lim and Earle 2008] were reported for *in vivo* DH program in melon. This discrepancy may be associated with higher dose and/or longer durations of CL applications.

Ploidy status of treated haploids was directly identified by chromosome counting in root tips. As a result of chromosomal observations, in addition to DH's, haploid, mixoploid and low frequency of tetraploid were obtained. The spontaneous doubling was not identified, and genotypes showed similar trend in duplication process. Nevertheless, *in vitro* and *in vivo* experiments resulted in different duplication rate, and CL was found to be the most effective mitotic inhibitor and *in vivo* treatments produced the highest proportion of DH's. *In vitro* duplication rate was the highest at 1.0 g l^{-1} CL for 12 h about 68%, while it reached up to 93.3% with the multiple treatments of 10.0 g l^{-1} CL for 1 h *in vivo*. On the other hand, APM, TF and OR were ineffective agents in duplication and they had only 38.1% DH's *in vitro* treatment with 0.15 g l^{-1} APM for 24 h. Moreover, *in vitro*, the lower doses and shorter durations of APM, TF and OR did not produce DH's, and *in vitro* treatments of these agents were more productive than *in vivo*. Similarly, *in vivo* chromosome duplication of CL treatments was more successful than in vitro, and it was found to be three [Yetisir and Sari 2003] or four times [Solmaz et al. 2011] greater than *in vitro* treatments. Correspondingly, *in vivo* duplication treatments produced about 92% DH's in melon [Koksal et al. 2002]. Otherwise, the findings of Lotfi et al. [2003] and Lim and Earle [2008] were in conflict with those reports and our results, and they argued that *in vitro* CL treatment was more effective than *in vivo* for melon DH program.

Although the effects of antimitotic agents on dihaploidization and fertility were only extensively discussed in melon, there were not any reports on winter squash and pumpkin concerned with both DH efficiency and the effects on the fertility. It is quite obvious that the fecundity of double haploids is a key factor for a DH program, Because a pure-line, that has sufficient number of fruits with the high quantity of full seed, can only be utilized for a breeding program, we put the PV, PF and FS criteria to determine the fecundity of DH's for our breeding program.

Our results clearly reflected that mitotic inhibitors, their doses, and durations were effective on fecundity of DH's for both *in vitro* and *in vivo* DH's. The HV was determined in non-treated plantlets up to 100.0% and lines produced the similar HV, V and LV values. As a result of the higher dose and longer durations of anti-mitotic treatments, the pollens continuously lost their viability, in particular for *in vitro* conditions. Interestingly, all anti-mitotic agents, except from CL, did not provide LV, while the HV was highest *in vivo*. In relation to PV, PF was in similar trend and most of the fruits were obtained from DH's with higher HV. Likewise, along with increasing dose and durations of CL, PF continuously decreased and the mean PF was about 60% for *in vitro* DH's; however, it was similar to other agents for *in vivo* (about 79%). In contrast, even though HV and PF rate was reported with CL treatments for *in vitro* (about 60%), only four fruits were obtained from 103 plants in melon for *in vivo* treatments [Lim and

Earle 2008]. Additionally, Lim and Earle [2009] suggested that *in vitro* CL treatment with 0.5 g l^{-1} dose for 1 or 2 days gave favorable PV (41–57%) and FS (40–50%) in melon, interestingly. Besides, parthenogenetic melon plantlets produced no stained pollen *in vitro* [Lotfi et al. 2003]. Lines did not show statistical differences in FS; nevertheless, melon genotypes provided a wide range of fruit-set rate for both *in vivo* and *in vitro* conditions [Solmaz et al. 2011]. Lower fertility in some DH's was also reported in cucumber [Galazka et al. 2015].

In respect to SR, in relation to PV, *in vivo* DH's yielded the higher FS. Similarly, CL treatments had lower FS values among the mitotic inhibitors for both conditions. We also observed genotypic variation for FS and winter squash showed higher FS than pumpkin. Besides, seed number was changed from 29 to 421 *in vitro* parthenogenetic melon fruits, but some of seeds were germinated varying from 6–100% related to poor fruit quality and smaller seeds [Lim and Earle 2008].

To elucidate the effects of anti-mitotic agents in terms of partial fertility and infertility on DH's, some researchers concerned with microsporogenesis and pollen meiosis. Eventually, the meiotic (genetic) stability determines the pollen structure and physiology and unstable meiotic disturbance may cause significant changes in the pollen properties [Nepi and Pacini 1993]. When mitotic inhibitors were applied with a higher dose and longer durations, meiotic instability occurred with a high incidence of lower pollen viability or non-viable pollen grains, thus imbalanced in fecundity of progeny [Homer and Palmer 1995, Correa et al. 2005, Kumar and Dwivedi 2013]. On the other hand, the seed-set differences in ultimate dose and durations of anti-microtubules may be clarified by parthenocarpic response; because, in accordance with our previous work, winter squash and pumpkin have tendency to produce parthenocarpic fruits with empty or not fully developed seeds in a different manner, moreover some fruits were seedless [Kurtar 2009].

CONCLUSION

As a consequence of our comprehensive study, we highly recommend *in vivo* multiple colchicine treatments with 10.0 g l^{-1} dose for 1 h to apical parts of haploid plants for recovery high frequency and fertile doubled haploid lines in winter squash and pumpkin. Conversely, *in* vitro chromosome duplication and other mitotic inhibitors (amiprophos methyl, trifluralin, and oryzalin) were insufficient. Pollen viability, fruit-set, and seed-set criteria should be utilized to determine the fertility and stability of DH's for a forceful breeding program. Nevertheless, tetraploid regenerants were yielded at higher doses and longer durations of mitotic inhibitors, which may be a subject of our future interest.

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