Australian Journal of Crop Science AJCS
AJCS 6(1):23-30 (2012) ISSN:1835-2707

Evaluation of regenerated strains from six *Cucurbita* interspecific hybrids obtained through anther and ovule *in vitro* cultures

M.T. Rakha, E.I. Metwally, S.A. Moustafa, A.A. Etman, Y.H. Dewir*

Horticulture Department, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh 33516, Egypt

*** Corresponding author: ydewir@hotmail.com**

Abstract

Homozygous plants can be obtained in a single generation using doubled haploid technology, which can tremendously facilitate breeding programs. The present study reports on *in vitro* regeneration and production of double haploid plants through anther and ovule cultures resulting from the hybridization of cultivated species of *Cucurbita pepo* L. with three other Cucurbita species i.e. *C. moschata* L., *C. ficifolia* and *C. martinezii* L. Among six sucrose-2,4-Dichlorophenoxyacetic acid (2,4-D) combinations tested, the addition of 90 g l^{-1} sucrose and 1 mg l^{-1} 2,4-D into the medium was found to be more suitable for callus regeneration from anthers. Anthers from the hybrid *C. pepo* L. (Queen F_1) × *C. moschata* L. regenerated the highest percentage (40%) of plantlets from callus and highest average number of plantlets per callus (7.5) when the culture medium was supplemented with the aforementioned concentrations of sucrose and 2,4-D. Ovules collected from the hybrid *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. produced the highest percentage of regenerated plantlets (25.33%), the highest percentage of responded ovules (18.67%) and the highest average number of plantlets per ovule (3.37). Cold pretreatment had negative effects on gynogenesis with the exception of the hybrid *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. Chromosome counting in the root tips of androgenic plantlets revealed 50% of haploid and ($n = x = 20$) and 50% of dihaploid plantlets ($2n = 2x = 40$). The assessment of root tips from gynogenic plantlets resulted in 60% haploid ($2n = x = 20$) and 40% diploid ($2n = 2x = 40$) plants. The androgenic strains were evaluated for their vegetative and reproductive traits compared with open local cultivar *C. pepo* L. (Eskandarani cv.). While the majority of regenerated strains were intermediate between the cultivated species of *C. pepo* L. and other Cucurbita species, two regenerated strains proved superior to the local cultivar 'Eskandarani' in number of fruits (early and total yield/plot) as well as in total fruit yield per plot.

Keywords: Anther culture, Cucurbitaceae, Genotype, Haploid, Ovule culture.
Abbreviations: 2,4-D - 2,4-Dichlorophenoxyacetic acid; MS - Murashige and Skoog's medium; NAA - 1-Naphthalene acetic acid; PGR - plant growth regulators; PPF- photosynthetic photon flux .

Introduction

Doubled haploidy is the fastest route to homozygosity in plants. Resulting doubled haploids are useful in fixing traits rapidly in a line/variety and facilitate hybrid breeding. Haploid plants can be generated from microspores through direct embryogenesis or via a callus phase in both anther and isolated microspore culture (Wang et al., 2000). Chromosome doubling, either spontaneous or induced, converts haploids into doubled haploid plants. In a further step, homozygous plants can be obtained in a single generation, facilitating breeding programs tremendously (Forster and Thomas, 2005). In addition to the short period of time required to obtain inbred lines for F1-hybrid, haploid technology facilitates selection for recessive and polygenic traits and revealed to be of benefit in genetic transformation and mutation studies as well (Ferrie et al., 2008).

Pure lines are considered as the first step in genetic improvement of vegetable crops as the lines may be the source of superior hybrids new cultivars in self pollinated crops, and high yielding hybrids (Veilluex, 1994; Metwally et al., 1998a). The production of pure lines in vegetable crops especially from open pollinated plants such as Cucurbita requires several years of conventional plant breeding program (Bajaj, 1990; Metwally et al., 1998a). On the other hand, the morphogenesis of the gametophytic cells is remarkably propice for the rapid production of homozygous lines and the detection of mutation. Thus, i*n vitro* anther and/or ovule culture may be used to generate homozygous lines through the production of haploid plants. Although anther culture has been reported to be more efficient for haploid induction compared to ovary/ovule culture (Bajaj, 1990; Ferrie et al., 1995), one of its drawbacks consist of the female plants and male sterile plants that may result from (Doctrinal et al., 1989). Squash (*Cucurbita pepo* L.) is the most widely grown and polymorphic among the *Cucurbita* species. The production of haploids in squash has been reported through anther culture (Metwally et al., 1998a), unpollinated ovules (Metwally et al., 1998b) and irradiated pollen technique (Kurtar et al., 2002). Recently, Shalaby (2007) studied some factors affecting *in vitro* gynogenic embryo yield. The objectives of the present study were to evaluate the potential for *in vitro* regeneration of interspecific Cucurbita hybrids through anther and ovule cultures, to produce double haploid plants and to assess the vegetative and reproductive traits of the regenerated genotypes in the field.

Results and discussion

Effect of sucrose and 2,4-Dichlorophenoxyacetic acid (2,4-D) concentrations and hybrids on anther culture efficiency

Sucrose and 2,4-D concentrations had a significant impact on androgenesis of Cucurbita hybrids. The combination of 90 g l^{-1} sucrose and 1 mg l^{-1} 2,4-D was optimal for callus formation and plantlets regeneration for each hybrid. There was a trend towards a decrease in callus formation and plantlets regeneration with increasing either sucrose or 2,4-D concentrations. Callus regeneration was null when the medium was amended with 150 g l^{-1} sucrose and 1 or 5 mg l^{-1} 2,4-D with the exception for *C. pepo* L. (Eskandarani cv.) × *C. moschata* L. Significant differences were observed between the various interspecific hybrids in terms of percentage of callus giving plantlets and number of plantlets per callus (Table 1). Twenty one treatments out of 36 formed neither callus nor plantlets. The anthers resulting from *C. pepo* L. (Queen F_1) × *C. moschata* L. gave the highest percentage (40%) of fertile callus and an average of 7.5 plantlets per callus when the culture medium was supplemented with 90 g l^{-1} sucrose and 2,4-D at 1 mg l^{-1} . No plantlets were regenerated from the anthers of *C. pepo* L. (MHTC77 F_1) × *C. martinezii* L. Genotypes and medium composition are key factors influencing embryo/callus induction and subsequent plant regeneration. Androgenic response in *Cucurbita pepo* L. (Shail and Robinson, 1987) and haploid induction in carrot (Gorecka et al., 2005) have been reported to vary according to the genotype. In addition of being a source of carbon and energy, sugars are known to act as an osmotic regulator in the induction medium. The presence of sucrose in the induction medium has been found to influence androgenesis and embryogenesis in many species (Ferrie et al. 1995) However, its concentration in the medium seems to have a differential effect on different species. For example, concentrations of 30, 60, 100 and 150 g l^{-1} sucrose were found to be optimal for embryogenesis in *Quercus suber* (Bueno et al., 1997), *Oryza sativa* (Afza et al., 2000), *Avena sativa* and *A. sterilis* (Kiviharju and Pehu, 1998) and *Cucurbita pepo* (Metwally et al., 1998a), respectively. The negative effect of high sucrose concentration on plantlets regeneration may be due the rapid hydrolysis of sucrose into fructose and glucose which in turn increases the medium osmosis as reported previously in wheat (Navarro-Alvarez et al., 1994).

Effect of cold treatment and 2,4-D on ovule culture efficiency

The type of hybrid significantly affected plantlet and callus regeneration from ovules and the number of plantlets obtained per ovule and responded ovule rates as well (Table 2). Overall, 75% (27) of the treatment combinations did not generate plantlets. Ovules from the hybrid *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. produced the highest percentage of regenerated plantlets (25.33%), the highest percentage of responded ovules (18.67%), and the highest average number of plantlets per ovule (3.37) in opposite to *C. pepo* L. (Queen F_1) × *C. ficifolia* L. and *C. pepo* L. (Jedida F_1) × *C. moschata* L.. Among treatments that produced calli, *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. generated the highest percent 9% versus 5 and 3% from *C. pepo* L. (Queen F_1) × *C. ficifolia* and *C. pepo* L. (Queen F_1) × *C. moschata L.* (Table 2). Ovules from the interspecific hybrid *C. pepo* L. (MHTC77 F_1) × *C. martinezii* L. did not respond to *in vitro* culture (data not shown). These results are consistent with previous repports on Cucurbita (Dumas de Vaulx and Chambonnet, 1986; Kwack and Fujieda, 1988; Shail and Robinson, 1987). Arafeh (2006) reported that the genotype proved to be one of the most important factors affecting in gynogenesis in summer squash. In sugar beet, the yield of haploid plants depended on the genotype, and the effect of genotype was significant only for certain media (Lux et al., 1990) as shown in the present study. These differences indicated that genotype specific results can be obtained depending on the genetic constitution of the individual used hybrids. In the present study, cold pretreatment had deleterious effects on gynogenesis except for the interspecific hybrid *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. Temperature shocks are believed to improve gynogenesis by diverting normal gametophytic development into a saprophytic pathway leading to the formation of haploid embryos. Lux et al. (1990) reported that cold pretreatment of sugar beet flower buds at 4 ^{o}C for 4 – 5 days led to increased embryo yield in consistence with other reports on *Cucurbita moschata* L (Kwack and Fujieda, 1988), onion (Hanna, 1994) and *Cucurbita pepo* (Shalaby, 2007), On the other hand, in consistence with our results, a deleterious effect of cold pre-treatment was reported by Metwally et al. (1998b) on squash and by Arefeh (2006) on flower buds. The combination of 2,4-D with cold pretreatment had a significant effect on gynogenesis in Cucurbita interspecific hybrids. The highest percentage of regenerated plantlets (25.33%) and highest number per ovule (3.37) were obtained when the ovules of *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. were cultured on media supplemented with 5 mg l^{-1} and cold pretreated for 7 and 14 days, respectively. Kwack and Fujieda (1988) indicated that plant growth regulators (PGRs) are important for embryogenesis of *Cucurbita moschata*. Bridgen (1994) found that somatic embryo induction was inhibited by high concentrations of exogenous auxin in the medium and stimulated by low concentrations. According to Evans et al. (1981), the primary medium for induction of somatic embryogenesis includes 2,4-D for the majority of crop species. Also, Campion et al. (1992) found that 2,4-D gave good results in both ovary and flower culture of onion.

Plantlets hardening and mitotic studies of androgenic and gynogenic genotypes

Androgenic and gynogenic plantlets were separated and cultured individually into MS medium without PGRs. Supplementation of MS medium with NaCl was beneficial for establishing a good root system. Plantlets cultured on medium without NaCl developed thin, friable roots and could not survive in greenhouse. Plantlets grown a medium amended with 1 mg l^{-1} NaCl developed 2 - 3 thick roots with 1- 2 cm in length (Fig. 1A). The plantlets were hardened in a growth chamber for 2 weeks followed by one week in greenhouse prior to their transplanting in the field (Fig. 1B, C and D). Chromosome count (Fig. 2 D, E) in root tips of the androgenic plantlets revealed that 50% of plants were haploid ($n = x = 20$) and the other 50% were dihaploid ($2n = 2x = 40$). The analysis of root tips of gynogenic plantlets revealed 60 % of haploid ($2n = x = 20$) and 40% of diploid plants ($2n = 2x = 40$). In summer squash, plants regenerated from anther and ovule culture where 50% and 25% where haploid, respectively, (Metwally *et al* 1998 b; Shalaby 1996) whereas plants derived from ovule cultures of Rosina F_1, Gabbla cv., and Eskandarani cv. were 64, 58, and 41% haploid and 36, 42, and 59% dihaploid (Arafeh, 2006) To the contrary, all

species from the Cucurbita genus were found to be dihaploid ($2n = 2x = 40$) (Robinson and Walters, 1997).

Field evaluation of vegetative traits in androgenic regenerated strains

Among 200 dihaploid regenerated plantlets, only 10 androgenic strains produced seeds while all other plants were either weak or abnormal. These 10 strains as well as a local open cultivar (Eskandarani) were evaluated for their traits. Table 3 indicates that the differences among genotypes were highly significant for all the vegetative traits. Strain 5 had the highest stem length, leaf area and fresh and dry weights per plant while strain 7 had the highest number of leaves per plant. Strain 8 had the smallest values of all the vegetative traits whereas Eskandarani showed intermediate values regardless of the trait. These evaluated strains were obtained from anther culture of interspecific hybrids between the cultivated species of *C. pepo* L. and other cucurbits species viz., *C. moschata* L., *C. ficifolia* and *C. martinezii* L. This indicates that the majority of the obtained strains had intermediate vegetative traits between the cultivated species of *C. pepo* L. and other Cucurbita species. It has been found that variance of stem length of the short species *C. pepo* L. was less than the variance of the long species *C. moschata* L. (Hassan et al. 1984). In summer squash, Eskandarani plants produced the largest values for all the vegetative traits evaluated, compared with its 16 dihaploid lines (Metwally et al.,1998c).

Evaluation of flowering and yield traits in double haploid genotypes

The differences among genotypes were highly significant for all the flowering traits (Table 4). The number of days to the first male flower ranged between 38.6 days for Eskandarani and 55.3 days for strain 1 whereas the number of days to the first female flower ranged between 35.0 days for Eskandarani and 54.3 days for strain 2. Strain 10 and 3 and 5 produced the largest number of male and female flowers per plant respectively, and Eskandarani cv. only produced lower numbers of male and female flowers compared to the induced strains. The lowest and highest sex ratios was observed for strains 5 (0.9) and strain 7 (1.6) (Table 4). Eskandarani cv. produced nearly equal number of male and female flowers per plant and therefore, sex ratio was nearly equal to one. The above results indicate that female flowering occurred earlier than male flowering. It has been reported that female flowering occurs one or two days before male flowering (Metwally, 1985). In summer squash, the number of days from planting to the first male flower differed between dihaploid lines and ranged from 39.8 to 50.6 days (Metwally et al., 1998c). The same authors reported 43.3 day-period between planting to the first female flower and a sex ratio of 1.3 for Eskandarani cv. For fruit characteristics, the highest fruit weight (127 g), fruit length (15.6 cm), fruit diameter (3.5 cm) and early yield per plot (23.9 Kg) were obtained from Eskandarani cv. while strain 3 had the lowest values. The highest number of fruits in terms of early yield (308.4) and total yield (911.2 g) per plot were obtained from strain 6 (Fig. 3 A). Strain 5 recorded the highest total yield per plot (45.1 kg). The lowest values of early and total yield per plot were obtained from strain 1 and 2 (Fig. 3 B). It has been found that fruit shape of F_1 plants of the cross between *C. moschata* L. × *C. maxima* was intermediate (Robinson et al. 1978). In summer squash, fruit traits, i.e., average fruit weight, length and diameter, the differences within dihaploid lines and

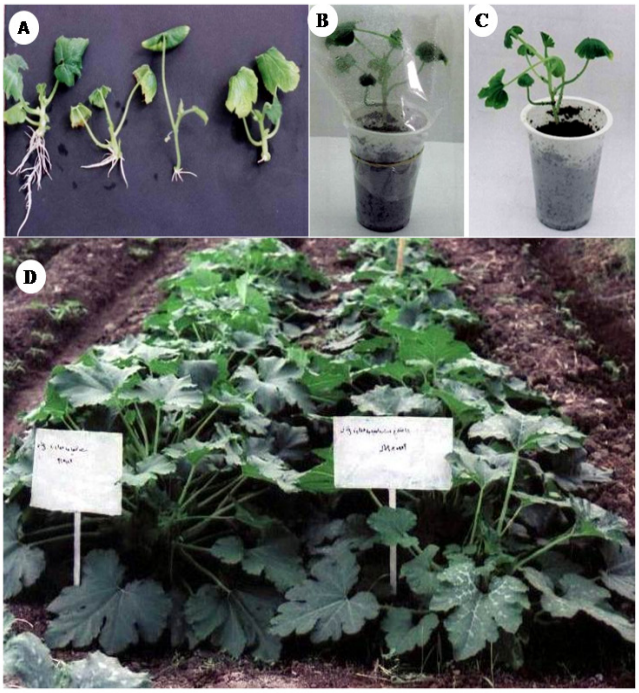

Fig 1. Acclimatization of the regenerated plantlets: (A) *in vitro* rooting; (B, C) acclimatization of plantlets in growth chamber; (D) evaluated strains grown in the field.

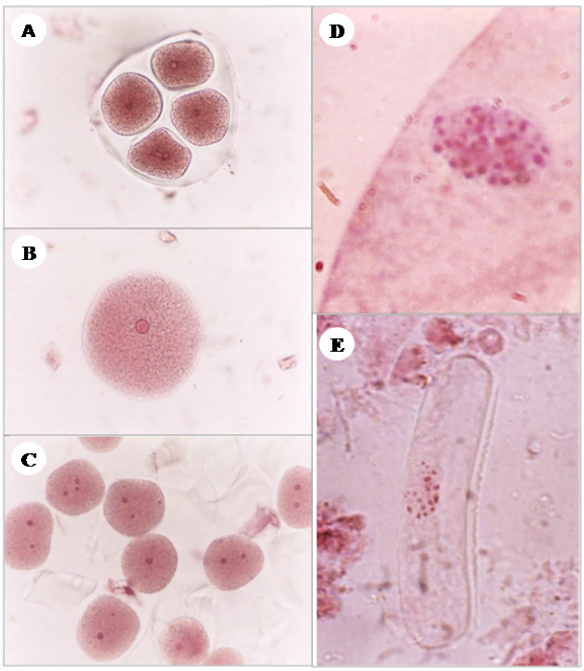

Fig 2. Cytological observations on anther's microspores (Mag. X 40): (A) tetrad after meiosis; (B) uninucleate stage; (C) binucleate stage; (D) chromosomes in diploid plantlets; (E) chromosomes in haploid plantlets.

Table 1. Effect of interspecific hybrids, sucrose and 2,4-D concentrations on plantlets regeneration in anther culture of *Cucurbita* interspecific hybrids

No.of plantlets/callus	Callus giving plantlets%	2, 4-D (mg l^{-1})	Sucrose (g l^{-1})	Interspecific hybrids
4.66 c	15 bc[z]	1	90	*C. pepo* L. (Eskandarani cv.) × *C. moschata* L.
3.00 d	5bc	5		
0.00 f	0 c	1	120	
0.00 f	0 c	5		
0.00 f	0 c	1	150	
2.33 de	15 bc	5		
7.50 a	40a	1	90	*C. pepo* L. (Queen F_1) × *C. moschata* L.
2.50 de	10bc	5		
0.00 f	0 c	1	120	
3.00 d	5bc	5		
0.00 f	0 c	1	150	
0.00 f	0 c	5		
2.80 de	25ab	1	90	*C. pepo* L. (Jedida F_1) × *C. moschata* L.
5.00 bc	10 bc	5		
6.50 ab	20abc	1	120	
5.00 bc	10 bc	5		
0.00 f	0 c	1	150	
0.00 f	0 c	5		
5.80 bc	25 ab	1	90	*C. pepo* L. (MHTC77 F_1) × *C. moschata* L.
6.00 b	15bc	5		
3.00 d	5bc	1	120	
4.25 c	20 abc	5		
0.00 f	0 c	1	150	
0.00 f	0 c	5		
0.00 f	0 c	1	90	*C. pepo* L. (Queen F_1) × *C. ficifolia* L.
1.25 e	20abc	5		
0.00 f	0 c	1	120	
0.00 f	0 c	5		
0.00 f	0 c	1	150	
0.00 f	0 c	5		
0.00 f	0 c	1	90	*C. pepo* L. (MHTC77 F_1) × *C. martinezii* L.
0.00 f	0 c	5		
0.00 f	0 c	1	120	
0.00 f	0 c	5		
0.00 f	0 c	1	150	
0.00 f	0 c	5		
		F-test[y]		
**	**	Interspecific hybrids		
**	**	Sucrose concentration		
NS	NS	2, 4-D concentration		
**	**	Interspecific hybrids × sucrose concentration		
*	*	Interspecific hybrids × 2, 4-D		
**	*	sucrose concentration × 2,4-D		
*	**	Interspecific hybrids × sucrose concentration × 2,4- D		

[Z] Mean separation within columns by Duncan's multiple range test at 5% level, [y] NS = not significant, * significant at 0.05 level, ** significant at 0.01 level.

Eskandarani cultivar were highly significant (Metwally et al. 1998c). In conclusion, the present study investigated androgenesis and gynogenesis of six Cucurbita interspecific hybrids. Genotypes and medium composition influenced the plantlets regeneration from anther and ovule cultures. There was a trend towards decreasing callus formation and plantlets regeneration from anthers with increasing either sucrose or 2,4-D concentrations. In most cases, cold pretreatment had negative effects on gynogenesis. Evaluation of the androgenic regenerated plants revealed that the majority of strains were intermediate in their traits between the cultivated species of *C. pepo* L. and other Cucurbita species. However, two regenerated strains proved superior to the local cultivar 'Eskandarani' in number of fruits (early and total yield/plot) as well as total fruit yield per plot.

Materials and methods

Plant material

Six interspecific hybrids of Cucurbita were used to obtain male and female flower buds. The combination consisted of *C. pepo* L. (Eskandarani cv.) × *C. moschata* L. (Nigerian local cv.), *C. pepo* L. (Queen F_1) × *C. moschata* L. (Nigerian local cv.), *C. pepo* L. (Jedida F_1) × *C. moschata* L. (Nigerian local cv.), *C. pepo* L. (MHTC77 F_1) × *C. moschata* L. (Nigerian local cv.), *C. pepo* L. (Queen F_1) × *C. ficifolia* L. and *C. pepo* L. (MHTC77 F_1) × *C. martinezii* L. The hybrid *C. pepo* L. (MHTC77 F_1) × *C . martinezii* L. was produced through embryo culture technique and grown in the

Table 2. Effect of interspecific hybrids, cold treatment and 2,4-D concentrations on production of embryos in unfertilized ovule culture of *Cucurbita* interspecific hybrids

Callus formation %	No. of plantlets /ovule	responded ovules %	Regenerated plantlets %	2,4-D (mg l^{-1})	Cold treatment at 4 °C (days)	Interspecific hybrids
8.25 bc	1.99 b	6.67 bcd	13.33 b[z]	1	0	*C.pepo* L. (Eskandarani cv.) ×
8.00 bc	1.00 bc	2.67 cd	2.67 c	5		*C. moschata* L.
2.00 c	0.00 c	0.00 d	0.00 c	1	7	
1.00 c	0.00 c	0.00 d	0.00 c	5		
2.00 c	0.00 c	0.00 d	0.00 c	1	14	
0.00 c	0.00 c	0.00 d	0.00 c	5		
3.00 c	2.00 b	1.33 d	2.67 c	1	0	*C. pepo* L. (Queen F_1)
8.00 bc	1.14 bc	9.33 bc	10.67 bc	5		*C. moschata* L×
5.00 c	1.00 bc	2.67 cd	2.67 c	1	7	.
8.00 bc	0.00 c	0.00 d	0.00 c	5		
6.00 c	0.00 c	0.00 d	0.00 c	1	14	
5.00 c	0.00 c	0.00 d	0.00 c	5		
2.00 c	0.00 c	0.00 d	0.00 c	1	0	*C. pepo* L. (Jedida cv.)
7.00 bc	0.00 c	0.00 d	0.00 c	5		× *C. moschata* L.
2.00 c	0.00 c	0.00 d	0.00 c	1	7	
2.00 c	0.00 c	0.00 d	0.00 c	5		
0.00 c	0.00 c	0.00 d	0.00 c	1	14	
0.00 c	0.00 c	0.00 d	0.00 c	5		
9.00 bc	1.00 bc	18.67 a	18.67 ab	1	0	*C. pepo* L. (MHTC77F_1)
4.00 c	0.00 c	0.0 d	0.00 c	5		× *C. moschata* L.
11.00 b	0.00 c	0.00 d	0.00 c	1	7	
9.00 bc	3.37 a	2.67 cd	9.00 c	5		
7.00 bc	0.00 c	0.00	0.00 c	1	14	
17.00 a	1.90 bc	13.33 ab	25.33 a	5		
5.00 c	3.33 a	4.00 cd	13.33 b	1	0	*C. pepo* L. (Queen F_1)
0.00 c	0.00 c	0.00 d	0.00 c	5		× *C. ficifolia* L.
0.00 c	0.00 c	0.00 d	0.00 c	1	7	
2.00 c	0.00 c	0.00 d	0.00 c	5		
1.00 c	0.00 c	0.00 d	0.00 c	1	14	
0.00 c	0.00 c	0.00 d	0.00 c	5		
0.00 c	0.00 c	0.00 d	0.00 c	1	0	*C. pepo* L. (MHTC77F_1)
0.00 c	0.00 c	0.00 d	0.00 c	5		× *C. martinezii* L
0.00 c	0.00 c	0.00 d	0.00 c	1	7	.
0.00 c	0.00 c	0.00 d	0.00 c	5		
0.00 c	0.00 c	0.00 d	0.00 c	1	14	
0.00 c	0.00 c	0.00 d	0.00 c	5		
F-test[y]						
**	*	*	*	Interspecific hybrids		
NS	NS	**	**	Cold treatment		
NS	NS	NS	NS	2, 4-D concentration		
*	*	*	*	Interspecific hybrids × cold treatment		
*	*	*	*	Interspecific hybrids × 2, 4-D		
NS	*	*	**	Cold treatment × 2,4-D		
**	**	**	**	Interspecific hybrids × Cold treatment × 2,4- D		

[z] Mean separation within columns by Duncan's multiple range test at 5% level, [y] NS = not significant, * significant at 0.05 level, ** significant at 0.01 level.

experimental farm of Kafrelsheikh University using the same technique described by Metwally et al. (1998a).

Sterilization of male flower buds

Cytological observations on anther's microspores were conducted to determine their developmental stages (Fig. 2 A, B and C). Male flower buds having a length of 9 - 10 mm and containing anthers at the mid to late uninucleate microspores were collected in the early morning and exposed to cold temperature at 4 °C for 4 days. The buds were surface sterilized for 60 s in 70% ethanol followed by 20 min in 5.2% sodium hypochlorite solution and 2 - 3 drops of Tween-20 on a gyratory shaker at 150 rpm. Buds were aseptically washed three times with sterile distilled water.

Anther culture

Anthers without filaments from the six aforementioned Cucurbita interspecific hybrids were excised and cultured on MS medium containing 0.8% agar (Murashige and Skoog, 1962) and supplemented with various concentrations and combinations of sucrose at 50, 120 and 150 g l^{-1} and 2,4-D at 1.0 and 5.0 mg l^{-1}. Sucrose and 2,4-D were added to the medium prior to autoclaving at 121 °C and 1.1 kg cm^{-2} pressure for 20 min and the pH was adjusted to 5.8 ± 0.1 before autoclaving as well. Anthers were cultured in 100 × 15 mm sterile Petri dishes containing 30 ml of MS medium. Four replicate-Petri dishes were cultured with five anthers each and the dishes were incubated in dark for 1 week at 35 °C followed by 9 weeks at 25 ± 1 °C. All cultures were

subcultured in the same conditions described above once after 5 weeks of initial culture. For callus differentiation, calli were numbered and transplanted to MS medium supplemented with 0.0494 mg l^{-1} kinetin and 0.05 mg l^{-1} 1-Naphthalene acetic acid (NAA) and grown for 4 weeks before being re-transplanted to MS medium free of PGRs for 4 additional weeks. The cultures were incubated at 25 ± 1 ^{o}C, with a16 h phtoperiod at 40 µ mol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by cool white fluorescent tubes. After 18 weeks of anthers' culture, the percentage of callus which gave plantlets and number of plantlets per callus were recorded.

Sterilization of ovaries and ovule culture

At flowering stage, ovaries from the six Cucurbita interspecific hybrids were picked a day before anthesis as reported perviously (Metwally et al., 1998b; Dumas de Vaulx and Chambonnet, 1986). The buds were exposed to cold pretreatment at 4 ^{o}C for either 0, 7 or 14 days.

Thereafter, peduncles, petals and styles were removed. The ovaries were surface sterilized for 60 s in 70% ethanol and aseptically washed three times with sterile distilled water. The ovules were excised from ovaries and cultured for 4 weeks on MS medium supplemented with 30 g l^{-1} sucrose and 2,4-D at 1 and 5 mg l^{-1} followed by 4 weeks on PGRs-free MS medium. The pH of the medium was adjusted to 5.8 ± 0.1 and the medium was autoclaved as explained above. Four-replicates 100 mm Petri dishes were cultured with 100 ovules each and dishes were incubated at 25 ± 1 ^{o}C under a 16 h photoperiod. After 8 weeks, the numbers of regenerated plantlets, responded ovules, plantlets per ovule and callus formed were recorded.

Plantlets hardening and mitotic studies

Regenerated plantlets from anthers and/or ovules cultures were cultured individually in jars containing PGRs-free MS medium for 4 weeks after what the root system was cut off and shoots were transplanted into jars containing 50 ml of MS medium supplemented with 0.5 mg l^{-1} NaCl and grown for 10 days. The root system was cut off one more time and shoots were cultured for 10 days on fresh medium supplemented with 1 mg l^{-1} NaCl. The plantlets were harvested, washed under tap water and cultured in pots containing sterilized peat moss and the pots were covered with clear plastic bags and kept in growth chamber for 2 weeks. The grown plantlets were grown in a greenhouse for 1 week prior to their transplanting to the field. For mitotic studies, root tips from 20 androgenic as well as 20 gynogenic plantlets were observed using a light microscope. The root tips were fixed for 3 h in 0.05% aqueous colchicine, then for 24 h in a solution of ethanol and glacial acetic acid (3:1; v/v), and finally stained with acetocarmine stain as described by Darlington and Lacour (1976).

Evaluation of regenerated strains through androgenesis and gynogenesis

Two hundred dihaploid plants selected from *Cucurbita* interspecific hybrids were planted in greenhouse. The plants were self pollinated at flowering stage and seeds were obtained from vigorous plants (strains) while abnormal plants were excluded. In total, 10 androgenic strains as well as the open pollinated cultivar (Eskandarani) were evaluated. Three seeds of each strain were sown in hills on the north side of ridges (6 × 1 m) and planting space of 30 cm. The experiment

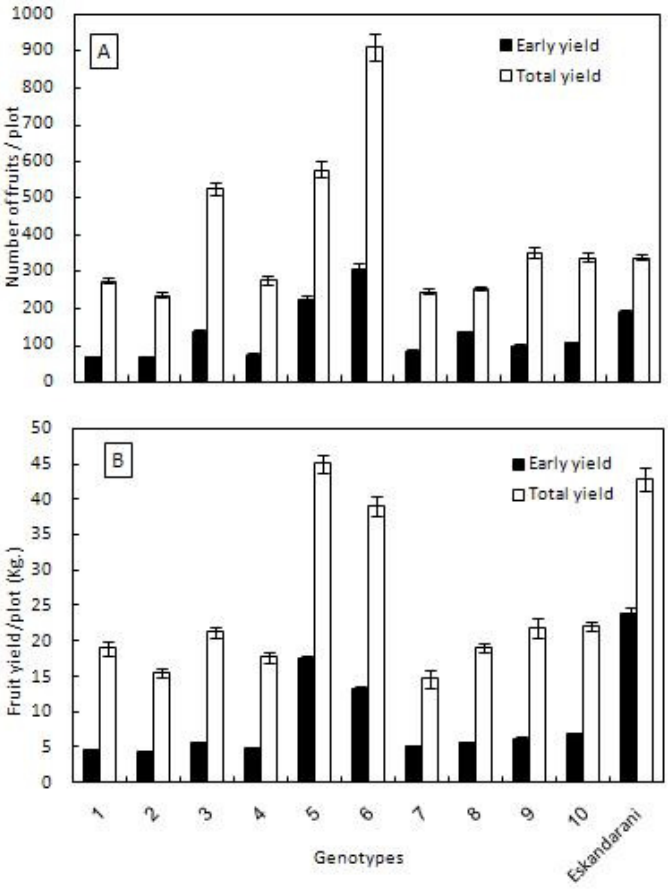

Fig 3. Early and total yield of different strains and open pollinated cultivar 'Eskandarani' A) number of fruits per plot B) fruit yield per plot

was set up in a randomized complete block design with three replicates. The field was immediately irrigated after sowing. Fertilizers were added to the soil as follows: 1) old cattle manure was added at the rate of 47.6 m^{-3} per ha before planting. 2) commercial fertilizers were applied as a mixed fertilizer having 1: 2: 1 ratio with a rate of 238 kg per ha. Ammonium sulphate, super phosphate and potassium sulphate were used as sources of N, P and K, respectively. One half of the fertilizers' mixture was applied 14 and 21 days post-sowing. For evaluation of vegetative traits, three plants were up-rooted from each plot after 45 days of planting. Stem length, number of leaves per plant, fresh and dry weights per plant and leaf area per plant were recorded. Leaf area was determined by LI-3100 area meter (LI-COR, Ing. Lincoln, Nebraska, USA) and dry weights were determined after leaves were dried for 48 h at 70 ^{o}C. During flowering stage, five plants in each plot were labeled, and total number of staminate and pistillate flowers during the whole flowering period, number of days to opening of the first pistillate and staminate flowers, number of pistillate and staminate flowers per plant and sex ratio (male/female flowers) were recorded on labeled plants. For fruit characteristics and yield assessments, the pistillate flowers were labeled in each plot and the fruits were picked 3 days

Table 3. Vegetative traits of different strains and open pollinated cultivar (Eskandarani)

Dry weight /plant (g)	Fresh weight /plant (g)	Leaf area /plant (dm^2)	No. of leaves /plant	Stem length (cm)	Genotypes
51.0± 1.02h	663.0± 19.89 i	56.1 ± 1.68g	27.3 ± 0.82d	13.3 ± 0.27e[z]	1
53.7 ± 2.58g	671.0 ± 17.65h	66.3 ± 2.32f	29.7± 0.95c	13.3± 0.33e	2
59.7 ± 1.91f	835.0 ± 26.08g	78.9 ± 3.00c	31.0 ± 1.09b	17.4 ± 0.35e	3
40.3 ± 1.21i	670.3 ± 20.02h	50.5 ± 1.46h	27.3 ± 1.06d	15.3 ± 0.40d	4
100.4± 2.81a	1369.0± 34.23a	108.8± 4.46a	28.7 ± 1.15c	21.2 ± 0.61ab	5
70.7 ± 1.77d	933.3 ± 37.33e	71.6 ± 3.58d	25.7 ± 0.51e	21.3 ± 0.43ab	6
84.6 ± 3.55b	1053.3± 52.67b	78.6 ± 2.12e	34.6 ± 0.71a	117.4± 3.64c	7
37.3 ± 1.64j	305.0 ± 11.96j	25.8 ± 0.49i	9.0 ± 0.27g	7.3 ± 0.15f	8
67.3 ± 2.02e	366.0 ± 11.90h	69.0 ± 1.66e	17.6 ± 0.58f	21.7 ± 0.46ab	9
70.4 ± 2.39d	975.0 ± 35.10c	80.8± 2.30b	17.3 ± 0.71f	22.6± 0.45a	10
75.0 ± 2.78c	943.3 ± 26.22d	59.6 ± 1.52e	25.0 ± 0.73e	21.0± 0.43b	Eskandarani
**	**	**	**	**	F-test[y]

[z] Mean separation within columns by Duncan's multiple range test at 5% level, [y] ** significant at 0.01 level.

Table 4. Flowering traits and fruit characteristics of different strains and open pollinated cultivar (Eskandarani)

Fruit diameter (cm)	Fruit length (cm)	Fruit weight (g)	Sex ratio (male/ female)	Female flowers/ plant (No.)	Male flowers/ plant (No.)	Days to first female flower (No.)	Days to first male flower (No.)	Genotypes
2.4 ± 0.12cd	11.4 ± 0.46bc	69.2± 2.42c	1.1 ± 0.02cd	15.6± 0.30de	18.6 ± 0.74d	53.6 ± 2.68a	55.3 ± 1.66a[z]	1
2.4 ± 0.09cd	10.1 ± 0.51cd	65.0 ± 2.54e	1.3 ± 0.06bc	16.3 ± 0.39d	22.0 ± 1.10c	54.3 ± 1.47a	51.0 ± 1.63b	2
1.9 ± 0.06f	6.8 ± 0.27f	40.6 ± 1.62k	1.0 ± 0.03de	24.7 ± 0.70a	23.3 ± 0.91b	52.2 ± 0.99b	47.6 ± 1.67c	3
2.2 ± 0.08cde	10.2 ± 0.33cd	64.4 ± 1.29f	1.3 ±0.04bc	21.0 ± 0.54b	15.6 ± 0.51f	51.6 ± 1.24b	51.0 ± 1.99b	4
2.7 ± 0.08b	12.6 ± 0.45b	78.0 ± 1.60b	0.9 ± 0.03e	24.7 ± 0.74a	23.3 ± 0.84b	46.3 ± 1.32d	39.3 ± 1.57f	5
2.7 ± 0.09b	8.0 ± 0.22ef	42.8 ± 1.28i	1.1 ± 0.03cd	21.0 ± 0.42b	24.3 ± 0.68b	43.7 ± 1.11c	45.3 ± 0.91d	6
2.4 ± 0.09cd	8.5 ± 0.30def	59.8 ± 2.99h	1.6 ± 0.07a	9.7 ± 0.20f	16.3 ± 0.57ef	52.3 ± 1.57b	51.0 ± 1.05 b	7
2.0 ± 0.06ef	8.7 ± 0.34de	42.1 ± 1.65j	1.1 ± 0.05cd	19.7± 0.79bc	21.6 ± 0.84c	52.5 ± 1.38a	46.0 ± 1.54d	8
2.2 ± 0.07cde	8.4 ± 0.34def	62.0 ± 2.02g	1.1 ± 0.03cd	14.7 ± 0.74e	17.0 ± 0.68e	45.6 ± 1.42d	41.3 ± 1.36e	9
2.85± 0.14b	10.5± 0.21c	65.2 ± 2.35d	1.3 ± 0.04bc	20.0 ± 0.78bc	26.3 ± 0.53a	50.3 ± 1.50c	45.2 ± 1.85d	10
3.5 ± 0.14a	15.6 ± 0.32a	127.0± 3.53a	1.1 ± 0.04cd	18.3 ± 0.59c	19.3 ± 0.40d	35.0 ± 0.88f	38.6 ± 1.12f	Eskandarani
**	**	**	**	**	**	**	**	F-test[y]

post-flowering. Fruit weight, fruit length and fruit diameter were recorded. Fresh fruits were picked at 2 days intervals, where the number and weight of fruits were recorded. Early yield (fruits yield of the first five pickings) and total yield (fruits yield of all pickings) in terms of fruit number and weight were recorded.

Statistical analysis

Data were subjected to ANOVA and Duncan's multiple range test using SAS program (Version 6.12, SAS Institute Inc., Cary, USA).

References

Afza R, Shen M, Zapata FJ, Xie J, Fundi HK, Lee KS, Bobadilla M, Kodym A (2000) Effect of spikelet position on rice anther culture efficiency. Plant Sci, 153: 155–159.

Arafeh MO (2006) Production of double haploid squash plants. M.Sc. Fac. of Agric., Cairo Univ., Egypt

Bajaj YPS (1990) In vitro production of haploid and their use in cell genetic and plant breeding. In: Bajaj YPS (Ed.), Biotechnology in Agriculture and Forestry 12: Haploid in Crop Improvement I. Springer Verlag, Berlin, pp. 1–44.

Bridgen MP (1994) A review of plant embryo culture. Hortscience 29: 1243-1245.

Bueno MA, Gómez A, Boscaiu M, Manzanera JA, Vicente O (1997) Stress-induced formation of haploid plants through anther culture in cork oak (*Quercus suber*). Physiol Plantarum 99: 335–341.

Campion B, Azzimont MT, Viani EM, Chiavi S, Falavigna A (1992) Advanced in haploid plants induction in onion (*Allium cepa* L.) through *in vitro* gynogenesis. Plant Sci, 86: 97-104.

Darlington CD, Lacour LE (1976) The Handling of Chromosomes, 6th ed. Allen and Unwin, London.

Doctrinal M, Sangwan RS, Sangwan-Norreel BS (1989) *In vitro* gynogensis in *Beta vulgaris* L. Effect of plant growth regulators, temperature, genotypes and season. Plant Cell Tiss Org, 17: 1–12.

Dumas de Vaulx R, Chambonnet D (1986) Obtention of embryos and plants from in vitro culture of unfertilized ovules of Cucurbita pepo L. In: Horn W, Jensen CJ, Odenbach, W, Schieder O (Eds.), Genetic Manipulation in Plant breeding. Proceedings of the International Symposium organised by EUCARPIA, September 8–12, 1985. Walter de Gruyter & Co., Berlin, pp. 295–297.

Evans DA, Sharp WR, Flick CE (1981) Growth and behaviour of cell culture: Embryogenesis and

organogenesis. In: Thorpe TA (ed.) Plant Tissue culture. Methods and Applications in Agric. Acad. Press, New York, pp. 45-113.

Ferrie MR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants (pp. 309–344). Kluwer Academic Publishers, Dordrecht, The Netherlands

Ferrie MR, Taylor DC, Mackenzie SL, Rakow G, Raney JP, Keller WA (2008) Microspore mutagenesis of Brassica species for fatty acid modifications: a preliminary evaluation. Plant Breeding 127: 501–506.

Forster BP, Thomas WTB (2005) Doubled haploids in genetics and plant breeding. Plant Breed Rev, 25:57–88.

Gorecka K, Krzyzanowska D, Gorecki R (2005) The influence of several factors on the efficiency of androgensis in carrot. J Appl Genet, 46: 245-269.

Hanna AB (1994) Genetic evaluation of some economic characters in onion (*Allium cepa*). Ph.D. Thesis, Fac. Agric. Moshtohor, Zagazig Univ., Egypt

Hassan AM, Sherif HS, Nazeem HR, Abdel-Migid AA (1984) Genetic behavior of some economic characters in Squashes (*Cucurbita sp.*). Annals of Agric Sci-Moshtohor 22: 175-186.

Kiviharju E, Pehu E (1998) The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. Plant Cell Tiss Org, 54: 97–104.

Kurtar ES, Sari N, Abak K (2002) Obtention of haploid embryos and plants through irradiated pollen technique in squash (*Cucurbita pepo* L.). Euphytica 127: 335–344.

Kwack SN, Fujieda K (1988) Somatic embryogenesis in cultured unfertilized ovules of *Cucurbita moschata*. J Jpn Soc Hortic Sci, 57: 34-42.

Lux H, Herrmann L, Wetrel C (1990) Production of haploid sugar beet (*Beta vulgaris* L.) by culturing unpollinated ovules. Plant Breeding 104: 177-183.

Metwally EI (1985) Inheritance studies on squash crop. PhD thesis, Fac. Agric. Tanta Univ., Egypt

Metwally EI, Moustafa SA, El-Sawy BI, Shalaby TA (1998a) Haploid plantlets derived by anther culture of *Cucurbita pepo*. Plant Cell Tiss Org, 52: 171-176.

Metwally EI, Moustafa SA, El-Sawy BI, Haroun SA, Shalaby TA (1998b) Production of haploid plants from in vitro culture of unpollinated ovules of *Cucurbita pepo* L. Plant Cell Tiss Org, 52: 117–121.

Metwally EI, Moustafa SA, El-Sawy BI, Shalaby TA (1998c) Horticultural characters for the diploid plants derived from anther culture in *Cucurbita pepo* L. Cucurbitaceae 98: 287-290.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum 15: 473–497.

Navorro-Alvarez W, Beanzigeri PS, Eskridge KM, Shelton DR, Guslefson VD, Hugo N (1994) Effect of sugar in wheat anther culture media. Plant Breeding 112: 53-62.

Robinson RW, Boetter MA, Shail JW (1978) Gynoecious sex expression in Cucurbita resulting from an interspecific cross. Cucurbit Genetics Coop Rpt, 1: 31-32.

Robinson RW, Walters DS (1997) Cucurbits. Cambridge University Press, UK. pp 39

Shail JW, Robinson RW (1987) Anther and ovule culture of *Cucurbita*. Cucurbit Genetics Coop Rpt 10: p 92.

Shalaby TA (1996) Producing double haploid plants through ovule and anther culture technique in *Cucurbita pepo* L. M.Sc. Thesis, Fac. Agric., Tanta Univ. Egypt

Shalaby TA (2007) Factors affecting haploid induction through *in vitro* gynogenesis in summer squash (*Cucurbita pepo* L.). Sci Hortic-Amsterdam 115:1-6.

Veilluex RC (1994) Development of new cultivars via anther culture. Hortscience 29: 1238–1241.

Wang M, Bergen SV, Duijn BV (2000) Insights into a key developmental switch and its importance for efficient breeding. Plant Physiol, 124: 523–530.