

INTENSIVE PRODUCTION OF DIHAPLOID PLANTS FROM *Cucurbita pepo* L. THROUGH ANTHHER CULTURE TECHNIQUE.

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ABSTRACT

Production of haploid plants using techniques such as anthers culture has long been considered important for genetic studies and plant breeders. Since haploid plants carry only one allele of each gene, recessive characteristics are apparent. These haploid plants can be used to produce homozygous dihaploid plants useful for plant breeding. This study is concerned with the production of dihaploids plants of *Cucurbita pepo* L. Anthers at mid to late uninucleate microspore stage without filament were excised from sterilized buds of three genotypes of summer squash ; i.e. Gabbla, Eskandarani and Rosina F₁. Anthers were cultured on two different induction media. It was found that both media and cultivars affected callus induction and plantlets regeneration. The cultivar Gabbla gave the maximum callus weight (0.450 g) on MS medium supplemented with 5.0 mg /l 2,4-D and 90 g/l sucrose. On the other hand, the cultivar Eskandarani gave the maximum number of plantlets per dish (21.3)and per callus (4.3). Plantlets were acclimatized in the controlled environment. In each genotype, the root tips of plantlets were examined for ploidy level. Results reveal that there was 50% haploid plants in Eskandarani and Gabbla cultivars, while Rosina F₁ had 40% haploid plants.

INTRODUCTION

Haploid is a general term that refers to a plant containing the gametophytic number of chromosomes, that is a single set of chromosomes in the sporophyte. Although in some special cases individual workers obtained haploids spontaneously or experimentally by special methods, until 1964 the large Scale production of haploids in higher plants was only a theoretical possibility. In 1964, Guha and Maheshwari reported direct development of embryos from microspores of *Datura innoxia* by the culture of excised anthers as cited by Kurt and Evans (1998). This technique involved the isolation and sterilization of flower buds, with culture on a medium at a culture room. And the development of this technique for the induction of callus from cultured anthers and the production of haploid plants from calli played a very important role in increasing efficiency in plant breeding programs. By doubling chromosomes in haploid plants it is possible to obtain completely homozygous lines in a short time thereby providing a method for speeding up and increasing the selection efficiency (Yetisir and Sair, 2003).

The most important factor appears to be the genotype response to androgenesis in the development of callus from anthers and microspores. It appears that optimum media and pretreatment conditions vary from genotype to other (Gorecka *et al.*, 2005).

In this study three summer squash (*Cucurbita pepo* L.) cultivars; i.e., Eskandarani, Gabbla, and Rosina F₁ were used for anther culture in order to evaluate their capacity for callus production and plantlets regeneration by using two different media and four days cold pre-treatment.

MATERIALS AND METHODS

Cucurbita pepo L. Eskandarani and Gabbla cultivars as well as Rosina F₁ were used in the present study. Donar plants were grown under greenhouse conditions in the Experimental Farm of Kafr El-Sheikh, Faculty of Agriculture during 2004 – 2005 in order to obtain male flower buds for the laboratory work. Flower buds of 9-10 mm length (Fig. 1) with anthers containing microspores with mid to late uninucleate stage were harvested (Fig. 2). The stages of the microspores were determined by microscopic observation of anthers (9-10 mm) stained with a drop of acitocarmine. Buds were collected in the morning and exposed to a cold temperature pretreatment at 4 °C for 4 days as reported by Tipirdamz (2004).

Before isolating anthers, buds were sterilized with 70% ethanol for 2 min. and 25% Clorox for 20 min under a laminar flow cabinet. Then they were rinsed three times with sterile distilled water. The anthers without filament were excised and plated onto two different induction media.

Preparation of media:

Anthers were plated onto a solid MS medium (Murashig and Skoog, 1962) containing 90 g/l sucrose and 5.0 mg/l 2, 4-D (2, 4-dichloro phenoxy acetic acid) and MS medium containing 90 g/l sucrose and 5.0 mg/l 2, 4-D and 1.0 mg/l Kin (kinetin), both being referred to as induction medium. The media components were pH adjusted by 1N NaOH and 1 NaHCl to 5.6 and 5.8 Agar 8.0 g/l was added before autoclaving. All media were autoclaved at 121°C for 20 min. and 1.1 Kg/cm²

Five anthers were cultured on every Petri-dish containing 30 ml media. The dishes were sealed with parafilm and incubated in the dark at 35°C for 1 week. Then they were incubated in dark at 25± 1°C For 4 weeks. Calluses were sub-cultured under the same conditions for additional 4 weeks. After 9 weeks on induction media, the following data were recorded :

- 1 – Average callus weight (g).
- 2 – Average callus diameter (cm).

Then they were transferred to differentiation medium (Metwally *et al.*, 1998) which consists of MS medium supplemented with 0.23 µM Kin and 0.27 µM NAA (2- α -naphthalene acetic acid). After that the calluses were incubated in a Precision Growth Chamber at 25 ± 1°C under 16-h photoperiod (3000 Lux) for 4 weeks. Then they were transferred to growth regulator- free MS medium for 4 weeks, Metwally *et al.* (1998), (Fig. 3) then the following data were recorded:

- 1 -Number of callus producing plantlets / dish.
- 2 -Number of plantlets/callus.
- 3 - Number of plantlets/dish.

Data were tested by analysis of variance and Duncan's multiple range test was used for the comparison among treatment means (Duncan, 1955).

Acclimatization of plantlets and cytological studies:

After plantlets were obtained, they were transplanted individually into jars containing MS medium without growth regulators, and kept under 16-h photoperiod for one month, plantlets produced an extensive root system. The roots were cut and transferred to MS medium free –hormones with 1.0 g/l NaCl and kept in 16-h photoperiod for 3-5 days (Fig. 4). Good root system was obtained (2-3 roots at 1-2 cm in length). Finally plants were washed under tap water to remove agar traces and cultured in autoclaved sterilized pots containing peatmoss and covered with clear plastic bags to maintain humidity in the greenhouse for 1-2 weeks (Fig. 5) then transplanted to soil.

For cytological study root tips from 10 plants for each genotype were fixed in colchicine 0.05% for 2-h then in 3:1 v/v solution of ethyl alcohol and glacial acetic acid for 24-h finely stored in 70% ethyl alcohol until examination. Root tips were examined microscopically after being stained with acetocarmine.

RÉSULTS AND DISCUSSION

I- Callus dimension :

1- Effect of cultivars :

Data presented in Table (1) show that, variable responses were expressed by each of the three cultivars tested on two different induction media. The analysis showed that cultivars significantly affected callus induction ability measured as the weight and the diameter of formed callus. It is clear from the data that the highest weight of callus was obtained from Gabbla cultivar (0.395 g). On the other hand, Eskandarani cv. and Rosina F₁ gave lower values (0.290 and 0.246 g), respectively. The highest diameter of callus were 1.9 and 1.8 cm resulted from Eskandarani cv. and Rosina F₁ consecutively. However, Gabbla cv. gave the lowest callus diameter (1.5 cm).

Table 1: Effect of cultivars on callus induction and plantlets regeneration from squash anthers culture .

Cultivar	Average callus weight(g)	Average callus diameter(cm)	No. of callus producing plantlets/ dish#	No. of plantlets / callus	No. of plantlets / dish
Gabbla	0.395 a	1.5 b	2.00 a	1.9 b	9.4 b
Eskandarani	0.290 b	1.9 a	2.13 a	3.5 a	17.4 a
Rosina F ₁	0.246 c	1.8 a	1.38 b	0.9 c	4.5 c
F. test	**	**	**	**	**

Every dish contained 5 callus

**Indicate highly significant differences at p<0.01 according to F test .

Means followed by a common letter are not significantly different at the 5 % level according to Duncan 's test .

2-Effect of media :

By using two media data in Table (2), show that there was significant differences between the two media. Callus weight was 0.320 g for the first

medium (MS medium supplemented with 5.0 mg/l 2, 4-D and 90g/l sucrose), and 0.301g for the second medium (MS medium supplemented with 5.0 mg/l 2, 4- D and 1.0 mg/l Kin and 90 g/l sucrose), while callus diameter was 1.7 and 1.8 cm for the first medium and second medium, respectively.

Table 2: Effect of media on callus induction and plantlets regeneration from squash anthers culture .

Media	Average callus weight (g)	Average callus diameter (cm)	No. of callus producing plantlets/ dish#	No. of plantlets / callus	No. of plantlets / dish
MS + 90 g/L sucrose + 5 mg/l 2,4-D	0.320 a	1.7 b	1.8 a	2.5 a	12.4 a
MS + 90 g/L sucrose + 5 mg/l 2,4-D + 1 mg/l Kin	0.301 b	1.8 a	1.8 a	1.7 b	8.4 b
F. test	**	**	N.S.	**	**

Every dish contained 5 callus

**Indicate highly significant differences at p<0.01 according to F test .

N.S. Indicate nonsignificant differences according to F-test .

Means followed by a common letter are not significantly different at the 5 % level according to Duncan 's test .

3-Effect of cultivars-media interaction:

As for interaction effect, results in Table (3) show that the differences among treatment means were significant for each of callus weight and diameter.The highest record of callus weight (o.450 g) was obtained from Gabbla cv. anthers grown on MS medium supplemented with 5.0 mg/l 2, 4-D and 90 g/l sucrose. On the other hand, the highest record of callus diameter was obtained from Eskandarani cv. (1.9 cm) and Rosina F₁ (1.9 cm) anthers grown on MS medium supplemented with 5.0 mg/l 2, 4- D and 1.0 mg/l Kin and 90 g/l sucrose. The lowest value of callus diameter (1.4 cm) resulted from Gabbla cv. anthers grown on MS medium supplemented with 5.0 mg/l 2, 4- D and 90 g/l sucrose.

Table 3: Effect of interaction between cultivars and media on callus induction and plantlets regeneration from squash anthers culture .

Cultivar	Medium@	Average callus weight(g)	Average callus diameter (cm)	No. of callus producing plantlets/ dish#	No. of plantlets / callus	No. of plantlets / dish
Gabbla	1	0.450 a	1.4 d	2.5 a	2.7 b	13.5 b
	2	0.340 b'	1.5 d	1.5 b	1.1 c	5.3 c
Eskandarani	1	0.280 d	1.8 bc	2.3 a	4.3 a	21.3 a
	2	0.300 c	1.9 a	2.0 ab	2.7 b	13.5 b
Rosina F1	1	0.230 e	1.7 c	0.8 c	0.5 d	2.5 d
	2	0.263 d	1.9 ab	2.0 ab	1.3 c	6.5 c
F. test		**	*	**	**	**

Every dish contained 5 callus

* Indicate significant differences at p<0.01 according to F test .

** Indicate highly significant differences at p<0.01 according to F test .

Means followed by a common letter are not significantly different at the 5 % level according to Duncan 's test .

@ Medium 1 = MS medium + 90 g/L sucrose + 5 mg/L 2,4-D

@ Medium 2 = MS medium + 90 g/L sucrose + 5 mg/L 2,4-D + 1 mg/L Kinitin.

As it is obvious from the results, variable responses were expressed by genotypes tested on both different induction media, and anthers pass their normal pathway of development with the result that individual pollen grain divided repeatedly to form callus. Calluses varied from anther to anther with shape, texture and size. This difference is strongly indicative of a genetic component in each genotype and this result corresponds with that obtained by Ashok Kumar *et al.* (2003) in cucumber, and Metwally (1993 and 1994) in tomato and pepper, and Metwally *et al.* (1998) in squash, indicating that callus induction varied according to the plant genotype and media content.

II- Plantlets regeneration:

1- Effect of cultivars :

Data presented in Table (1) show that the differences among cultivars were highly significant for number of callus producing plantlets per dish. The differences among genotypes were also highly significant for number of plantlets per callus and number of plantlets per dish. Eskandarani cv. gave the highest number of plantlets per dish (17.4) and highest number of plantlets per callus (3.5), and Rosina F₁ gave the lowest number of plantlets per dish (4.5) and the lowest number of plantlets per callus (0.9). The differences among cultivars could be attributed to the genetic architecture of the cultivars. These results indicate significant genotype dependence in the androgenic response of *Cucurbita pepo* L., revealed by Shail and Robinson (1987) in squash varieties, Qin-x and Rotino, (1995), Christopher and Rajam, (1996) in their researches on pepper genotypes for the induction of haploids, Cao *et al.*, (1995) on broccoli, cabbage and Chinese cabbage-petsai cultivars, indicating haploid induction of vegetable crop genotypes varied according to the plant genotypes.

2- Effect of media:

Data presented in Table (2) Show that the differences between the two induction medium for number of callus producing plantlets per dish were not significant. However, the number of plantlets per dish and number of plantlets per callus were highly significant.

3- Effect of media-cultivars interaction:

Data presented in Table (3) shows that the differences for number of callus producing plantlets per dish were highly significant due to the interaction effect between cultivars and media. There were highly significant differences among treatment means for number of plantlets per dish and number of plantlets per callus. The highest number of plantlets per dish and number of plantlets per callus were 21.3 and 4.3 respectively, which resulted from Eskandarani cv. anthers grown on MS medium supplemented with 5.0 mg/l 2, 4-D and 90 g/l sucrose. The lowest number of plantlets per dish and number of plantlets per callus were 2.5, 0.5 respectively which resulted from Rosina F₁ anthers grown on MS medium supplemented with 0.5 mg/l 2, 4-D and 90 g/l sucrose.

Root-tips from 10 androgenic plantlets from each genotype were cytologically examined under a light microscope. Results (Fig. 6) reveal that there was 50 % haploid ($2n = x = 20$) and 50 % dihaploid ($2n = 2x = 40$) in Eskandarani and Gbbla cvs, while Rosina F₁ had 40 % haploid and 60%

dihaploid. Haploid plants confirm derivation from microspores as opposed to somatic origin.

In conclusion, using the improved anther-culture method allows efficient haploid-plants production and doubled haploid lines in squash in order to develop new varieties.

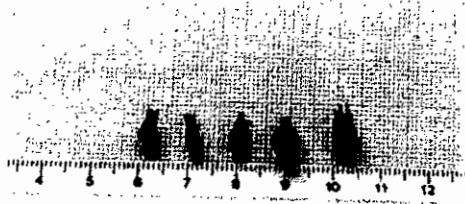


Fig. 1 : Length of flower buds



Fig. 2: Uninucleate stage in microspores from squash anthers



Fig. 3 : Embryos and plantlets formation from squash anther culture.

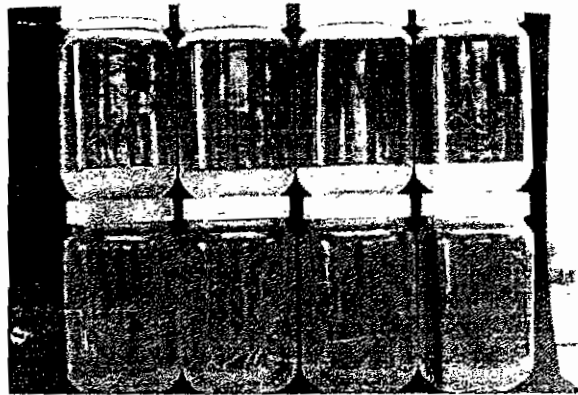


Fig. 4 : Regenerated plants on MS medium without hormones .



Fig. 5 :Regenerated plants in pots covered with plastic bags.

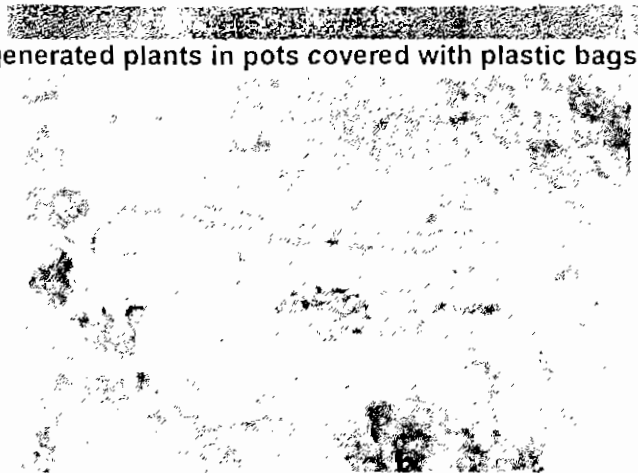


Fig. 6 : Mitotic metaphase in a root tip of haploid anther- derived squash plants $2n = x = 20$.

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الإنتاج المكثف للنباتات الأحادية المتضاعفة من قرع الكوسة عن طريق زراعة المتوك

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تعد تقنية زراعة المتوك بهدف إنتاج نباتات أحادية المجموعة الكروموسومية هامة و ضرورية للدراسات الوراثية و لمربي النبات، حيث تسمح هذه النباتات بظهور الصفات المتحيزة بحالة أصيلة و ذلك لأنها تحمل أليل واحد من كل جين. و تستخدم النباتات الأحادية عادة في إنتاج نباتات متضاعفة متجانسة تماما تفيد في برامج تربية النباتات.

تتضمن هذه الدراسة إنتاج نباتات من قرع الكوسة أحادية متضاعفة. تم جمع البراعم الزهرية لثلاثة أصناف من قرع الكوسة (اسكندراني، جبلة، الهجين روزينا) و عندما تكون حبوب اللقاح في الطور أحادي النواة. و بعد التعقيم يزال الخيط تماما و تزرع على بيئات تكوين الكالس.

لوحظ تأثير معنوي لكل من البيئة و الأصناف على تكوين الكالس و تكشف النباتات، حيث أعطى الصنف جبلة أعلى وزن للكالس ٠,٤٥٠ جم على بيئة MS المزودة بـ ٥,٠ ملجم / لتر من 2,4-D و ٩٠ جم/لتر سكروز، بينما أعطى الصنف اسكندراني أكبر عدد من النباتات / طبق (٢١,٣) و أكبر عدد من النباتات / كالس (٤,٣).

تمت أقلية النباتات الناتجة، كما تم إجراء الدراسة السيتولوجية للقمم النامية للجذور لتحديد عدد كروموسوماتها. و كانت نسبة النباتات الأحادية ٥٠% في الصنفين الاسكندراني و جبلة بينما كانت هذه النسبة في روزينا جبل أول ٤٠%.