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PRODUCTION OF HAPLOID PLANT REGENERATION FROM ANTHER CULTURES OF APPLE (*Malus domestica* BORKH)

Rayan, A. O. H. and Nahla A. Awad
Fruit and Ornmental Breeding Department, Horticulture Research Institute, Agricultural Research Center, Giza, Egypt

ABSTRACT

Anther culture in apple (*Malus domestica Borkh*), improve breeding program. Anther at the mid uninucleate stage of pollen development were obtained from two apple genotypes cultivars and stored for one week in refrigerator at 4 ºC. Anther was cultured on MS medium supplemented with 5% sucrose and combination of phytohormones [6-Benzylaminopurine (BA), thidiazuron (TDZ), indol-3-butyric acid (IBA), Naphthalene acetic acid (NAA)] for callus induction and regeneration. Addition of TDZ+IBA to multiplication medium were more effective for increasing the number of initiated shoots compared with BA, NAA. The average percentage of callus formation from Dorsett golden cultivar 31.64% in the first season, while it was 28.38% in the second season. Anna revealed a 27.86% & 25.99% of callus formation in the first &second season, respectively. Regeneration percentage was 24.86%& 23.82% with Dorsett golden, however, it was 20.57% &19.79% with Anna in the first and second seasons, respectively. Positive correlation between media and genotype was showed in first season only for plant regeneration. Moreover, a negative correlation was revealed between media and genotype for callus induction in both seasons. Cytological studies revealed normal cells ranged from 96.90%-97.85% and abnormal cells of about 2.15%-3.01%.

Keywords: anther culture- embryogenesis- 6-Benzylaminopurine (BA), thidiazuron (TDZ), indol-3-butyric acid (IBA), Naphthalene acetic acid (NAA)**.**

INTRODUCTION

Regeneration of haploid plants following anther or ovule culture has been applied to plant breeding and gene transfer programs. However, there are limited applications to woody perennial species.

Improvements in apple production, such as fruit quality, yield, and disease resistance, have been generally achieved through conventional cross-breeding. In apple (*Malus domestica* Borkh), breeding via conventional approaches is difficult due to the high heterozygosity and long life cycle that are common in this economically important rosaceous fruit tree species.

In vitro plant tissue culture has become the most common method not only for propagating virus-free plants but also for improving of woody plants and other crops. The anther culture technology shortage the breeding program by 3 to 5 years in some field crops and fruit trees. Therefore, in recent years, haploid production through anther culture is being used increasingly by breeders to accelerate and facilitate breeding programs in a wide range of crops. Although anther culture seems to be simple in handling, microspore culture has several difficulty. Firstly, the formation of calli and embryos that often formed from somatic tissues of the anther is avoided. Secondly, there is direct access to the microspores, which speeds up the optimization of culture conditions.

The production of doubled haploids would offer new possibilities for genetic studies and breeding especially in perennial fruit species which are characterized by a long reproductive cycle with several years of a juvenile phase, a tendency to allogamy and a high degree of heterozygosity. However, in vitro approaches to induce haploids in apple have limited success until recently in comparison with other plant species (Hofer and Lespinasse 1996). Induction of embryogenesis and limited plant formation has been reported from anther cultures in apple. In spit of plant regenerated, the induction rate of emberyogenesis from cultured apple anthers is still low and highly genotype dependent (Hofer *et al*, 1997). Recently, a protocol for isolated microspore culture was developed in apple (*Malus domestica* Borkh) for one genotype, and successful plant regeneration has been obtained from isolated microspores (Hofer *et al* .1999).

Plants have been produced following anther culture of a number of fruit species. papaw (Litz & Conover 1978), four citrus species (Chen 1985), apples (Zhang *et al*. 1990 and Rayan 2004) and grape (Rajasekaran &Mullins 1979).

The aim of the present study, is the induction of embryogenesis and plant regeneration from apple anther culture. Moreover, studying the influence of genotypes, media composition and culture condition on anther induction.

MATERIALS AND METHODS

1-Plant material:

Experiments were carried out with Dorsett golden and Anna apple trees. Flower buds were taken in February and the anthers were used as explants for callus initiation and plant regeneration. Anthers at the mid-uninucleate stage of pollen development were collected from wood bud as a described by (Hofer *et al.* 1997). Before culturing the anthers on the initiation medium, the anthers (flowering bud) were incubated for one week at (4˚C). This treatment is essential for anther growing. The buds were sterilized in 70% ethanol for 1 minute, and then transferred to 20% Cholorax solution for 2 minutes. Sterilized buds were rinsed 3 times with sterilized double distilled water.

2- Inoculation of anthers:

In a laminar air flaw cabinet the anthers were aseptically excised by fine sterilized forceps from buds and plated in Petri dishes 60x10 mm containing callus induction medium (2-3 anther/cm). The dishes were sealed with Para film and incubated in darkness at 26˚ C for 3-4 weeks. The anthers that produced calluses were counted after 4-5 weeks from planting. Anthers were cultured on modified MS medium Murashig and Skoog (1962). The MS basal medium supplemented with 4 different combinations.

a- MS+2mg/l TDZ +0.1 mg IBA. **b**- MS+2mg/l BA +0.1 mg IBA.
c- MS+2mg/l TDZ +0.1 mg NAA. **d**- MS+2mg/l BA +0.1 mg NAA.

All media were supplemented with 50 g/l sucrose and 0.7% agar. The pH was adjusted to 5.6 by Na OH and HCl 1N and autoclaved at 121˚C for 20 min and then poured into 60x10 mm Petri dishes. The initiated calluses were transferred to fresh growth media every four weeks.

3- Regeneration medium: -

For plant regeneration the maintained calluses were transferred to regeneration media. The regeneration media were MS medium supplemented with thiamine HCl 0.4 mg/l, myo-inositol 100 mg/l and 4 different combinations of BA, TDZ, IBA and NAA.

a- MS+4mg/l TDZ +0.5 mg IBA. **b**- MS+4 mg/l BA +0.5 mg IBA.

c- MS+4mg/l TDZ +0.5 mg NAA. **d**- MS+4 mg/l BA +0.5 mg NAA.

Numbers of shoots per callus piece were recorded after two months from transferring the callus to regeneration medium.

4-Cytological analysis:-

Root–tip were collected from regenerated plants 2-3 root tip/plant growing media . Root-tip were pretreated by chilling in liquid MS media, then fixed in Conroy's fluid hydrolyzed with 1N HCL at 60 for 10 min and stained with 1% acetocarmine. At least 250 metaphase cells were examined.

5-Statistical analysis: -

Randomized complete design with sex replications was used in analysis of the recorded values of callus induction and regeneration of two genotypes per /100 anthers callus induction. LSD test was used to determine the significant differences between genotypes, media and interaction between genotypes and media types. Data analysis was performed by MSTAT-C (1990) Computer statistical analysis program.

RESULTS AND DISCUSSION

In the present study different combinations of phytohormones and sex replications for each of these combination were used to check anthers ability for callus initiation and regeneration from two apple genotypes (Dorsett golden and Anna apple). Friable ceramic white calli were produced on anthers of each genotype within four weeks of culturing on all MS modified media. Obtained calli were tested for plant regeneration ability by plating them on MS regeneration medium. The influence of genotype, medium composition and genotype x medium interaction on callus formation and regeneration was evaluated as a percentage of the anthers formed calluses relative to the total number of planted anthers. Analysis of variance of anther response for callus initiation and regeneration show a significant difference, which were observed between genotypes in the first and second season (Tables 1& 2).

1- The effect of genotypes on callus induction and plant regeneration from anthers:

Data in Tables (1, 2 &Fig.1) show that the ability of anthers of Dorsett Golden and Anna apples genotypes for callus induction and regeneration was significantly differed in the first and second season. Callus formation was observed after 4weeks from anther culture, while regeneration was showed after two months. Percentage of induced callus cultures from Anna genotype ranged from 22.26% to 32.75% and 22.75% to 29.62%, in the first and second season respectively. While regeneration ranged from 18.64% to 22.00% and 19.13% to 20.83% in the first and second season respectively.

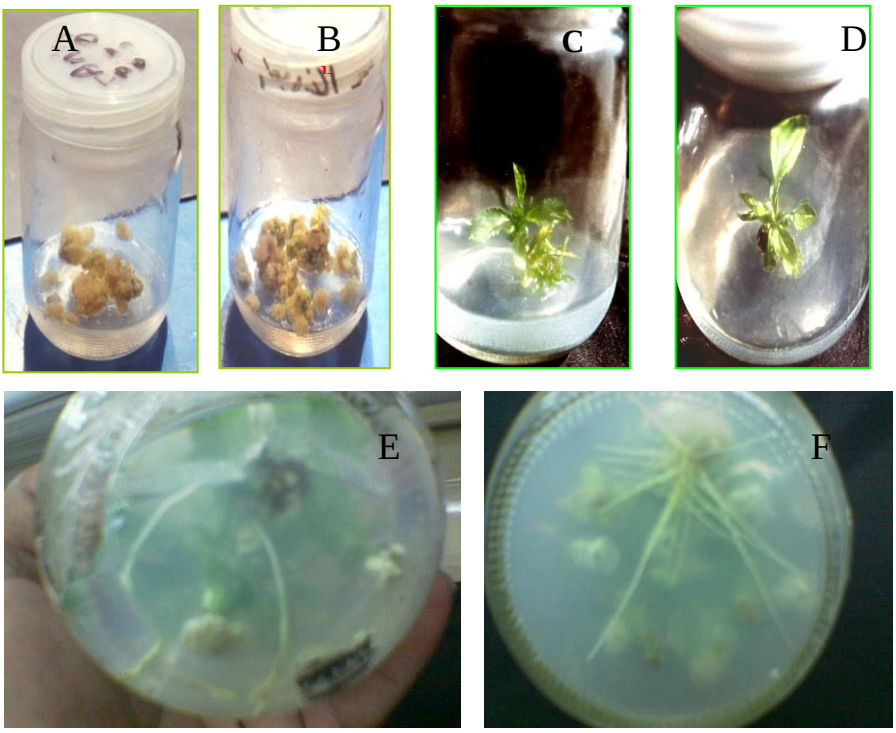

Fig.(1): Callus cultures from (A) Anna and (B) Dorsett Golden genotype. Regenerated shoots from (C) Anna and (D)Dorsett Golden after 8 weeks of planting callus. Root formation after 20 weeks of planting callus (E) Anna and (F) Dorsett Golden

Meanwhile, callus formation with Dorset golden ranged from 26.22% to 40.43% and 25.16% to 32.70%.However, regeneration ranged from 23.07% to 25.80% and 22.60% to 25.47% in the first and second season, respectively. The results revealed a highly significant variation between genotype in their ability for callus initiation and plant regeneration. In apple, a marked effect of genotype on anther culture response has been reported by (Todorovic *et a*l., 1992; Hofer *et al.* ,1997; Hidano *et al.,*1995; Hofer *et al*., 1999 and Rayan ,2004)].

2- The effect of media on callus induction and plant regeneration from anthers:

The composition of media is one of the most important factors that affect callus induction and plant regeneration. The type and concentration of phytohormones in the media played the main role on callus induction and plant regeneration Tables (1, 2&Fig.1).The obtained results showed that the MS_1 medium supplemented with.2 mg/l TDZ + 0.1 mg/l IBA, 4 mg/l TDZ + 0.5 mg/l IBA was the best media for callus induction and plant regeneration,

respectively. The ability of callus for plant regeneration on MS_1 was higher than that of MS_2, MS_3 and MS_4. The percentage of callus induction on MS_1 medium from Anna was 32.75%& 29.62% in the first and second seasons, respectively. While,the percentage of callus formation on MS_2, MS_3 and MS_4 from Anna cultivars were (27.82&28.09% and 22.26%, respectively) in the first season and (27.50%,24.08%& 22.75%, respectively) in the second season. On the other hand percentage of callus formation from Dorsett golden was superior in MS1 medium (40.34% &32.70% in the first and second season , respectively) rather than the other tested medium.The same effect of medium was observed for regenerated plants from callus with both of the Anna and Dorsett golden cultivars. Regeneration on Ms_1 medium for Anna 22% &20.83% for the first and seasons, respectively. Meanwhile, it was 25.80% and 25.47% for Dorsett golden in the first &second seasons, respectively. These results reflect the influence of the TDZ& the IBA hormone in the stimulation of callus induction and regeneration with a high percent. Contrarily, NAA & BA did not have the ability to induce and regenerate a high percentage, this was clear in MS_4 which contain both of NAA& BA, it recorded a low percentage of callus induction &regeneration comparable to MS_1&MS_2.

The effectiveness of media composition on the ability of callus initiation has been reported by (Todorovic *et a*l.,1992; Hofer *et al*.,1997 &1999; Witte *et al.*,1999 and Rayan 2004).

3- Genotypes x Media interaction on callus induction and plant regeneration from anthers:

Negative correlation coefficient between genotype and medium for callus induction in both of the two studied seasons was observed. On the other hand, the correlation was positive for plant regeneration in the first season, meanwhile, it was negative in the second season. However, data showed that the genotype Dorsett Golden has higher mean number of total callus induction 40.34 /100 anthers in the first season and 32.70/100 anthers in second season on MS_1 media. The number of Anna anthers produced calluses were 32.75/100 anthers in first season and 29.62/100 anthers in second season also on MS_1 media. The genotype Dorsett Golden has higher mean number of total regenerated plants produced from 186 calli (25.80%) in the first season and 157 calli (25.47%) in the second season compared by Anna 150 calli (22.00%) in the first season and 144 calli (20.83%) in the second season in the Ms_1 media. These results are in a good agreement with the results of Zimmermann & Broome (1981), and Matthewes *et al*. (1998).

Cytological analyses:-

Fig.(2) revealed chromosome number in root-tip cells of regenerated plants. A total of 250 cells examined in these plants reveled a remarkable variation in chromosome number as compared with normal haploid apple cells (1N=17). Fig (2) show normal haploid and abnormal cells in root-tip. The average percentage of normal haploid cells ranged between 96.90%-97.85%, a low level of abnormal cells (Aneuploid) with an average of 2.15%-3.10% were recorded.This is in a good agreement with (Abdel-Rahem & Ragab (1993), Ragab *et al*. (1997) and Rayan (2004).

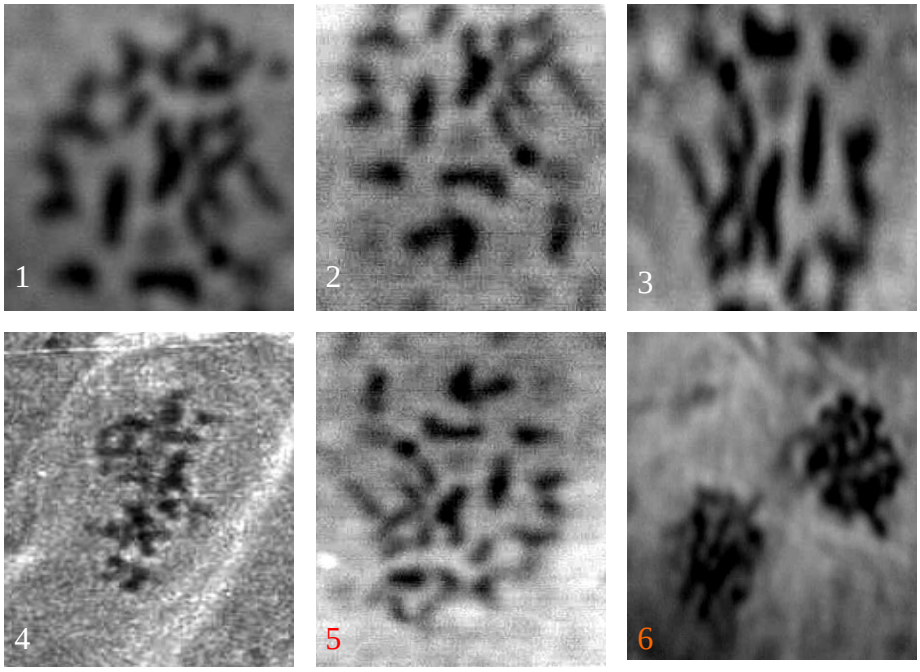

Fig 2: Chromosome variations observed in root-tips of regenerated plants of apple anther callus cultures. 1, 2 Normal haploid 3, 4, 5 aneuploid and 6 telephase stage.

In conclusion, haploid apple plants produced from anther culture are useful for producing of double haploids which introduce the possibility for breeding and genetically studies especially in subtropical fruit species which are characterized by a long reproductive cycle that includes several years of juvenile phases, attendance to allogamy and a high degree of heterozygous.

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انتاج نباتات أحادية من متوك التفاح بزراعة الأنسجة في المعمل

أحمد عثمان حمادة ريان و نهلة عبد الفتاح عوض
قسم تربية الفاكهة- معهد بحوث البساتين- مركز البحوث الزراعية

تمثل زراعة المتوك مصدرا خصبا للتباينات الوراثية وانتاج العد يد من السلالات النقية والأصناف في عدد من الأنواع النباتية المهمة في العالم.

تناولت هذه الدراسة مدى استجابة التركيب الوراثي لتكوين الكالوسات وإستيلاد النباتات في صنفين من أصناف التفاح المنزرعة في جمهورية مصر العربية وقد شملت الدراسة صنفي التفاح (دورست جولدن و آنا). اختلفت الأصناف المختبرة في قدرتها على تكوين الكالوسات وإستيلاد النباتات من المتوك خلال موسمين متتاليين اختلافا كبيراً وقد كان تأثير التركيب الوراثي عالي المعنوية وقدرت الاستجابة لتكوين الكالوسات وإستيلاد النباتات على أساس النسبة المئوية للكالوسات والنباتات المتكونة. أظهرت النتائج قابلية الصنف درست جولدن على تكوين الكالوسات وإستيلاد النباتات أفضل من الصنف آنا.

حيث كان متوسط إنتاج الكالوسات في الصنف دورست جولدن٣١,٦٤% في الموسم الأول وفى الموسم الثاني كانت ٣٨.٢٨% وفى الصنف الأنا كانت في الموسم الأول ٢٧,٨٦% وفى الموسم الثاني كانت٩٩.٢٥ %. كذلك اختلفت الأصناف اختلافا كبيراً في قدرتها على إستيلاد النباتات وكانت قابلية الصنف دورست جولدن على إستيلاد النباتات أفضل من الصنف آنا حيث كان متوسط إستيلاد النباتات في الصنف دور ست جولدن ٢٤,٨٦% في الموسم الأول وفى الموسم الثاني كانت ٢٣,٨٢% بينما في الصنف أنا كان متوسط إستيلاد النباتات في الموسم الأول ٢٠,٥٧% وفى الموسم الثاني كانت ١٩,٧٩%.

كذالك أستخدم في هذه الدراسة أربعة بيئات لتكوين الكالوسات وإستيلاد النباتات من المتوك وكان الوسط الغذائي MS_1 أفضل من MS_2, MS_3, MS_4 وقد أوضحت الدراسة أن بيئة إنتاج الكالوسات تلعب دوراً مهماً في نجاح زراعة المتوك في التفاح وقد أعطت بيئة MS_1 أعلى نسبة لإنتاج الكالوسات وإستيلاد النباتات في الموسمين. كذلك أظهرت النتائج عدم وجود تفاعل معنوي بين التركيب الوراثي والبيئة على إنتاج الكالوسات فى حين أن هذا التفاعل معنوى فى إستيلاد النباتات من المتوك فى الموسم الاول فقط.

كذلك تم دراسة السلوك الكروموسومى في قمم الجذور للنباتات الناتجة وكانت النتائج المتحصل عليها أن الخلايا المنتظمة تراوحت بين ٩٦,٩٠% إلى ٩٧,٨٥% وان هناك نسبة من الخلايا الغير منتظمة تراوحت بين ٢,١٥% إلى ٣,١٠%.

Table (1): Effect of genotype, medium composition and interaction between genotype X medium composition on callus formation of apple anther in the first and second seasons(2005/2006 &2006/2007).

Genotype	2005/2006				2006/2007		
	Media type	No. of cultured anthers	No. of formed anthers callus	Percentage formed anthers callus	No. of cultured anthers	No. of formed anthers callus	Percentage formed anthers callus
Anna	MS_1	458	150	32.75	486	144	29.62
	MS_2	442	123	27.82	480	132	27.50
	MS_3	420	118	28.09	479	115	24.08
	MS_4	442	100	22.26	457	104	22.75
	Mean	**440.5**	**122.75**	**27.86**	**475.5**	**123.75**	**25.99**
Dorsett golden	MS_1	461	186	40.34	480	157	32.70
	MS_2	449	133	29.62	470	141	30.00
	MS_3	432	130	30.09	477	120	25.16
	MS_4	450	118	26.22	451	115	25.49
	Mean	**448**	**141.75**	**31.64**	**469.5**	**133.25**	**28.38**
Mean for media	MS_1	459.5	168	36.56	483	150.5	31.15
	MS_2	445.5	128	28.73	475	136.5	28.73
	MS_3	426	124	29.10	478	117.5	24.58
	MS_4	446	109	24.43	454	109.5	24.11
Grand Mean		**444.25**	**132.25**	**29.76**	**472.5**	**128.5**	**27.14**
L.S.D $_{0.5}$		G= 0.597	M= 0.845	GXM=N.S	G = 0.476	M=0.673	GXM=N.S

Table (2): Effect of genotype, medium composition and the interaction between genotype x medium composition on regeneration ability from apple anther callus culture in the first & second seasons (2005/2006 & 2006/2007).

Genotype	2005/2006				2006/2007		
	Media type	No. of cultured callus	No. of Regenerated plants	Percentage of Regenerated plants	No. of cultured callus	No. of Regenerated plants	Percentage of Regenerated plants
Anna	MS_1	150	33	22.00	144	30	20.83
	MS_2	123	25	20.32	132	26	19.96
	MS_3	118	22	18.64	115	22	19.13
	MS_4	100	21	21.00	104	20	19.23
	Mean	**122.75**	**25.52**	**20.57**	**123.75**	**24.5**	**19.79**
Dorsett golden	MS_1	186	48	25.80	157	40	25.47
	MS_2	133	33	24.81	141	32	22.69
	MS_3	130	30	23.07	120	29	24.16
	MS_4	118	30	25.42	115	26	22.60
	Mean	**141.75**	**35.25**	**24.86**	**133.25**	**31.75**	**23.82**
Mean for media	MS_1	168	40.5	24.10	150.5	35	23.25
	MS_2	128	29	22.65	136.5	29	21.24
	MS_3	124	26	20.96	117.5	25.5	21.70
	MS_4	109	25.5	23.39	109.5	23	21.00
Grand Mean		**132.25**	**30.25**	**22.87**	**128.5**	**28.13**	**21.89**
L.S.D $_{0.5}$		G = 0.906	M = 1.281	G X M = 1.812	G=2.413	M=N. S	G X M= N. S