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PRODUCTION OF GYNOGENIC TETRAHAPLOID PLANTS IN STRAWBERRY THROUGH CULTURE OF UNPOLLINATED OVARIES

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ABSTRACT

The present study was conducted in the Tissue Culture Lab., Department of Vegetable Crops, Faculty of Agriculture, Cairo University, Giza, Egypt, during the period from 2005 to 2008. The objective of this work was to produce tetrahaploid plants from strawberry (*Fragaria* × *ananassa* Duch.) by culturing unpollinated ovaries of two different genotypes, viz., Camarosa and Sweet Charlie *in vitro*. Floral buds, 2-4 days before anthesis, were pre-treated at 4˚C for different periods and cultured on different media composition. Germinated ovules were performed and multiplication was induced to produce new shoots. Shoots were rooted and produced whole plantlets for adaptation. Results showed that cold pre-treatment was not efficient for producing the best ovule regeneration and production of tetrahaploid plants. Both cultivars gave nearly similar results under the optimal conditions for ovule regeneration. Determination of ploidy level was carried out and examinations showed that plantlets produced from the ovule culture were tetrahaploid. These results represent a modern tool for breeders to produce tetrahaploid strawberry plants to develop new cultivars in a short period (few months), or using the protocol of ovule regeneration in genetic engineering experiments by transferring new genes to develop better yield or quality of strawberry cultivars.

Keywords: Strawberry, *Fragaria*, *In vitro*, Ovary culture, Gynogenesis, Tetrahaploid and Octaploid.

INTRODUCTION

The cultivated strawberry (*Fragaria* × *ananassa* Duch.) is grown in most arable regions of the world. It is one of the most valuable Vegetable crops in the world; there have been a relatively little application of biotechnology in research or breeding efforts on this crop.

Cultivated strawberry is octaploid (2n = 8x = 56 chromosomes). Traditional breeding efforts to improve strawberry quality and yield are labor intensive, costly, and time-consuming, since many generations of crossing and selection are routinely required for cultivar development. Reducing the ploidy level of breeding material *via* gynogenesis may accelerate plant improvement efforts by more direct exposure of genetic traits at the haploid level, by phenotypic expression of gametes for assessment of ovule-donor potentials and together with chromosome redoubling techniques, by the production of highly homozygous lines for further use as parental lines.

The technologies for haploid and dihaploid production *in vitro* are now widely used to produce new cultivars and for genetic analysis of molecular biological research on many important agricultural crops. Many problems, however, remain and these limit extensive application of the technologies in modern breeding programs (Atanassov *et al*., 1995).

For producing gynogenic plants in vegetable crops, Bossoutrot and Hosemans (1985) produced gynogenic plants in *Beta vulgaris* L. from *in vitro* culture of unpollinated ovules. Predieri *et al*. (1989) obtained regenerated plants from strawberry unpollinated ovaries, but they did not count the chromosome number of these plants. Campion and Alloni (1990), Bohanec *et al*. (1995), and Alan *et al*. (2007) induced haploid plants in onion from *in vitro* culture of unpollinated ovaries. For using anther to produce tetrahaploid plants, Owen and Miller (1996) produced haploid plants from anther culture of three North American strawberry cvs. with regeneration frequency of 0.8 % for the final experiment. Also, Mohamed *et al*. (2002) and Okasha *et al*. (2003) regenerated plants from strawberry anthers but did not find any tetrahaploid plants in the regenerants. Passey *et al*. (2003) tested the pre-pollinated strawberry ovaries as a potential explant source and obtained regenerated plants without any definition of the ploidy level. Metwally *et al*. (1998) and Shalaby (2007) produced haploid plants from *in vitro* culture of unpollinated ovules of *Cucurbita pepo*.

In general, the culture of either unpollinated ovules or ovaries is procedurally similar. Ovary culture is simpler than ovule culture because the dissection of ovaries can be done more efficiently than of ovules. With ovary culture, there is a less danger of injuring the ovule (Keller and Korzun, 1996)

The objective of the present study was to develop a successful protocol for gynogenic tetrahaploid production in strawberry cvs. Camarosa and Sweet Charlie using unpollinated ovaries.

MATERIALS AND METHODS

The experiments of the present study were conducted in the Tissue Culture Lab., Department of Vegetable Crops, Faculty of Agriculture, Cairo University, Giza, Egypt, during the period from 2005 to 2008.

Donor plants

Plants of strawberry cvs. Camarosa and Sweet Charlie grown under greenhouse conditions in the Experimental Farm of Vegetable Crops Dept. were used as a source of the explants (floral buds). Transplants of these cvs. were brought from Strawberry and Non-Traditional Crops Center, Faculty of Agriculture, Ain Shams University, Egypt.

Explants and pre-treatments

Closed floral buds (2-4 days before anthesis) were collected from greenhouse plants. The maturity stage of ovaries was determined by the developmental stage of the microspore by staining the anther after crushing it with acetocarmine pigment. The floral buds were collected when microspores were at the late uninucleate or binucleate stages in the first time of the experiment. Subsequently, the floral buds developmental stage could be pre-selected on the basis of their morphology. These buds were exposed to cold pre-treatment (4˚C) for different periods (0, 1, 2, and 4 h) before sterilization.

Culture media

The used culture media were composed of the basal salts and vitamins of Murashige and Skoog (MS) (1962), and Gresshoff and Doy (GD) (1972) with different growth regulators and sugars concentrations as presented in Table 1. pH was adjusted to 5.8 with KOH 0.1N solution before autoclaving at 25±2˚C for 25 min.

Ovary culture and plant regeneration

The cold pre-treated floral buds were used as a source of the explants after sterilization by 5 % commercial bleach for 10 min. and were washed 3 times with distilled-sterilized water. Sepals, petals, and stamens were carefully removed and the receptacle, with the unpollinated ovaries, was sliced, after removing the styles and the stigmas by the back of the blade, and cultured in jars containing the regeneration media (Table 1); one bud / jar. In these experiments 25 floral buds were used per each pretreatment in all used media for each cultivar and the experiments were repeated 4 times. Cultures were sealed and kept in the incubator under dark conditions at 25˚C for 2-3 weeks, followed by 2-3 weeks under light conditions (white-cold light 2000 lux for 16 h/d).

After removing the roots of plantlets which regenerated from ovules on a given medium, they were transferred to the same medium for 4 weeks to examine the multiplication response on these media and increasing the number of shoots followed by transferring all produced shoots to the rooting medium (Table 1).

Cytological studies

Determination of the ploidy level was carried out by using chromosome counts protocol from mitotic cells of root-tips of the plants grown on rooting medium. Root pre-treatment, fixation, hydrolysis and chromosome staining for counting were performed following the protocol described by (Kafkas *et al*., 2002 and Preeda *et al*., 2007).

Acclimatization of the *in vitro* produced plantlets

In vitro produced plantlets were subjected to hardening procedure, as plantlets were washed after removing the rooting medium to remove the residual agar completely from the roots, and were immediately transplanted in plastic cups filled with the appropriate sterilized 1:1 mixture of fertilized peat-moss and sand (v/v). The cups were covered with adaptation bags (plastic clear bags) to keep high humidity and kept in the growth room at 23 ± 2˚C under 16 h photoperiod/day. Plantlets were acclimatized slowly by opening holes in the bags, which were removed gradually within 2 weeks. After the plantlets established a vigorous growth they were transferred into bigger pots with the same mixture.

Statistical procedures

Data recorded in percentage were transformed into arcsines ($\sqrt{x}$) for the purpose of statistical analysis, but the means of the original data were presented (Table 2). Data obtained were statistically analyzed using factorial analysis and mean comparisons were based on LSD test (Snedecor and Cochran, 1969).

Table 1. Components of the studied media for all stages of regeneration from ovules

Component mg/l	MSO	GDO	MGO	MSP	GDP	MGP	R
Micro elements							
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$C_{10}H_{12}N_2NaFeO_8$	36.7	36.7	36.7	36.7	36.7	36.7	36.7
H_3BO_3	6.2	0.3	6.2	6.2	0.3	6.2	6.2
KI	0.83	0.8	0.83	0.83	0.8	0.83	0.83
$MnSO_4.H_2O$	16.9	1	16.9	16.9	1	16.9	16.9
$Na_2MoO_4.2H_2O$	0.25	0.025	0.25	0.25	0.025	0.25	0.25
$ZnSO_4.7H_2O$	8.6	0.3	8.6	8.6	0.3	8.6	8.6
Macro elements							
$CaCl_2$	332.020	-	-	332.02	-	-	-
$Ca(NO_3)_2.2H_2O$	-	208.81	208.8	-	208.81	208.81	208.81
KCl	-	65	65	-	65	65	65
KH_2PO_4	170	300	300	170	300	300	300
KNO_3	1900	1000	1000	1900.000	1000	1000	1000
$MgSO_4$	180.54	17.09	17.09	180.540	17.09	17.09	17.09
NH_4NO_3	1650	1000	1000	1650	1000	1000	1000
Vitamins							
Ascorbic acid	100	100	100	100	100	100	100
Myo-inositol	100	100	100	100	100	100	100
Nicotinic acid	0.5	1	1	0.5	1	1	1
Pyridoxine HCl	0.5	1	1	0.5	1	1	1
Thiamine HCl	0.1	10	10	0.1	10	10	10
Amino acids (mg/l)							
Argenine	-	-	-	100	100	100	-
Glutamine	100	100	100	-	-	-	-
Glycine	2	4	4	2	4	4	4
Natural (g/l)							
Casein	-	-	-	0.500	0.500	0.500	-
Growth regulators (mg/l)							
BA	1	1	1	1.0	1	1	-
GA3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
IBA	1	1	1	1	1	1	0.1
Kinetin	-	-	-	1	1	1	-
Carbohydrates (g/l)							
Glucose	10	10	10	-	-	-	-
Sucrose	20	20	20	30	30	30	30
Gelling agent (g/l)							
Agar	8	8	8	8	8	8	8

MSO: Murashige and Skoog (1962) medium with regeneration components; GDO: Gresshoff and Doy (1972) medium with regeneration components; MGO: Murashige and Skoog micro elements plus Gresshoff and Doy (macro elements and vitamins) with regeneration components; MSP: Murashige and Skoog medium with another regeneration components; GDP: Gresshoff and Doy medium with another regeneration components; MGP: Murashige and Skoog micro elements plus Gresshoff and Doy (macro elements and vitamins) with another regeneration components and R: Rooting medium.

RESULTS AND DISCUSSION

Effect of media and pre-treatments

Ovaries of strawberry cvs. Camarosa and Sweet Charlie were collected when the microspores in the floral buds were at the late uninucleate or binucleate stages (Fig. A.1) The same stage was used to collect onion (Campion and Alloni, 1990) and strawberry floral buds (Mohamed *et al*., 2002). This stage was tested under four different cold pre-treatments (0, 1, 2, and 4 h) at 4˚C with seven different media (Table 1) to examine ovule regeneration ability in this study. After putting the cultures (explants cultured in jars of media) in the dark for 2-3 weeks at 25˚C, the optimum percentage of responding ovaries was achieved with MGO medium (Table 2) associated with zero cold pre-treatment which, significantly, were the best conditions for the two studied cultivars. These results agree with those of Predieri *et al*. (1989) who obtained regeneration from the ovaries of strawberry without cold pre-treatment, and also with Campion and Alloni (1990) and Bohanec *et al*. (1995) using unpollinated onion ovules. The same medium, i.e., MGO medium associated with 1 h cold pre-treatment also gave a high response for both cvs. but significantly ranked second (Table 2). It was evident that cold pre-treatments were not effective for ovule culture response in most cases. The response started through 2-3 weeks of incubation in dark at 25˚C by increasing the volume of the ovaries which turned to green color (Fig. A.2-9). The detached tissue of the receptacle gave hard callus (Fig. A.11-12) and some of the ovaries regenerated small plantlets (small shoots with roots) (Fig. A.10-13). Predieri *et al*. (1989) reported that most regeneration of strawberry ovaries originated from the area where the ovary had been detached from the receptacle and the internal parenchymatic ovary tissues close to the ovule and that was obvious through the histological examinations. After this period in dark the cultures were exposed to light (16 h/d photoperiod) for 3-4 weeks using the same media. The response of the ovaries after the dark-light photo period seemed to be like ovule germination by forming small plantlets from the ovaries and dark-light photo period conditions with the selected medium enabled large number of ovaries to develop whole plantlets. Dark-light period was important for regeneration of strawberry anther culture to produce more shoots (Owen and Miller, 1996) and squash anther culture (Metwally *et al*., 1998), meanwhile, Bossoutrot and Hosemans (1985) produced gynogenic plants in *Beta vulgaris* L. from *in vitro* culture of unpollinated ovules without dark treatment. On the contrary, Predieri *et al*. (1989) found that the dark has negative effect on strawberry ovaries culture.

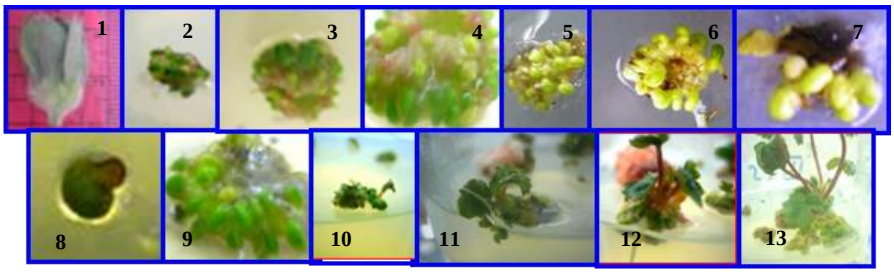

Fig. A. 1: Strawberry floral bud; 2-9: Increasing the volume of the ovaries and turning to green color; 10-13: regenerated plantlets from ovaries; 11-12: hard callus with red color.

Fig. B. Multiplication which induced large number of shoots on the main.

Fig. C. Transferring shoots to the rooting medium (R) to obtain whole plantlets.

Fig. D. Steps of plantlets adaptation.

Fig. E. Chromosome counting (cv. Sweet Charlie)

1. Control octaploid plant (2n = 8X = 56 chromosomes) (40*10 X).

2. Tetrahaploid plant (2n = 4X = 28 chromosomes) (100*10X).

Table 2. Regeneration of strawberry cvs. Camarosa and Sweet Charlie from *in vitro* ovary culture

Medium	Cold pre-treat (h)	Ovule regeneration (%)		Shoot multiplication (means of shoots number/shoot)		Plantlets adaptation (total recovery plantlets)	
		Camarosa	Sweet Charlie	Camarosa	Sweet Charlie	Camarosa	Sweet Charlie
MSO	0	35 d-f	41 cd	0.00 i	0.00 i	10 e-j	15 e-i
	1	7 m-q	17 hi	0.00 i	0.00 i	1 ij	1 ij
	2	4 p-t	6 o-s	0.00 i	0.00 i	0 j	0 j
	4	4 q-u	4 r-u	0.00 i	0.00 i	0 j	0 j
GDO	0	29 e-g	49 c	0.00 i	0.00 i	2 h-j	4 g-i
	1	12 i-l	19 h	0.00 i	0.00 i	0 j	0 j
	2	5 o-s	6 o-s	0.00 i	0.00 i	0 j	0 j
	4	4 p-t	2 uv	0.00 i	0.00 i	0 j	0 j
MGO	0	85 ab	88 a	2.75 c	1.75 i	104 a	63 a
	1	37 de	73 b	2.25 cd	2.25 i	41 b-d	45 bc
	2	19 h	28 fg	2.00 d-f	1.50 i	18 c-f	18 d-g
	4	9 k-o	8 n-r	0.00 i	0.00 i	4 g-j	5 f-j
MSP	0	12 i-m	16 h-j	2.25 cd	2.25 e-g	23 c-e	17 d-h
	1	8 l-p	6 o-s	1.25 gh	1.25 cd	2 h-j	5 f-j
	2	4 p-t	5 o-s	1.00 h	1.00 gh	1 ij	2 h-j
	4	2 t-v	0 v	0.25 i	0.00 i	2 ij	0 j
GDP	0	27 g	33 d-g	0.00 i	0.00 cd	17 d-h	14 e-i
	1	5 o-s	11 j-n	0.00 i	0.00 gh	2 h-j	4 g-j
	2	4 p-t	4 p-t	0.00 i	0.00 h	0 j	0 j
	4	5 o-s	4 r-u	0.00 i	0.00 i	0 j	0 j
MGP	0	7 m-q	12 i-m	8.25 a	8.50 i	25 c-e	25 c-e
	1	5 o-s	4 p-t	3.50 b	2.25 i	6 e-j	9 e-j
	2	4 p-t	0 v	1.50 fg	1.50 i	2 h-j	1 ij
	4	4 p-t	0 v	1.00 h	1.00 i	0 j	0 j
R	0	5 o-s	13 h-k	0.00 i	0.00 a	0 j	0 j
	1	4 q-u	5 o-s	0.00 i	0.00 c-e	0 j	0 j
	2	0 v	3 s-u	0.00 i	0.00 fg	0 j	0 j
	4	0 v	0 v	0.00 i	0.00 h	0 j	0 j

MSO: Murashige and Skoog medium with regeneration components; GDO: Gresshoff and Doy medium with regeneration components; MGO: Murashige and Skoog micro elements plus Gresshoff and Doy (macro elements and vitamins) with regeneration components; MSP: Murashige and Skoog medium with another regeneration components; GDP: Gresshoff and Doy medium with another regeneration components; MGP: Murashige and Skoog micro elements plus Gresshoff and Doy (macro elements and vitamins) with another regeneration components and R: Rooting medium.

The MGO medium showed increase in responding ovaries, which may be due to its components of lower macro nutrients, higher organic and inorganic nitrogen, higher vitamins, usage of BA, IBA and GA_3, and two different sources of sugar (sucrose and glucose). The results agree with those of Bohanec *et al*. (1995) who obtained high regeneration frequency from onion ovaries. Usage of BA agree with reports of Bossoutrot and Hosemans (1985) on *Beta vulgaris* L. unpollinated ovules, Predieri *et al*. (1989) on strawberry ovaries, Owen and Miller (1996) and Mohamed *et al*. (2002) on strawberry anther culture. Also, usage of BA, IBA, and GA_3 confirmed results obtained by Passey *et al*. (2003) who obtained 12 % regeneration frequency from pre-pollinated ovaries of the strawberry cv. El Santa.

After producing plantlets, these plantlets were used for multiplication which started with removing the roots of the plantlets and culturing the shoots in fresh medium of the same medium used before (one shoot/jar) for 4 weeks and incubated under 16 h/d photoperiod at 22 ± 2˚C.

After 4 weeks, the MGP medium, significantly, was determined as the best medium for multiplication which induced large number of shoots, viz., (8.25 and 8.5 shoots) (Fig. B) per each cultured shoot induced from ovaries pre-treated with zero cold temperature for cvs. Camarosa and Sweet Charlie, respectively (Table 2).

After multiplication, the shoots were transferred to the rooting medium (R medium, Table 1) to obtain whole plantlets again for adaptation (Fig. C). A total of 537 plantlets were obtained before adaptation. Plantlets were washed to remove the residual agar and cultured in small pots filled with sterilized soil mixture (1 peat-moss : 1 sand) and covered with clear plastic bags for 10 days in the growth room at 22 ± 2˚C with 16 h/d photoperiod. Adaptation was performed gradually by opening holes in the clear plastic bags till removing them followed by transferring the plantlets to the greenhouse (Fig. D). The MGO medium was, significantly, the best for producing recovery plants in adaptation stage for both cultivars under zero cold pre-treatment followed by the same medium with 1 h cold pre-treatment (Table 2). The total number of the plantlets decreased to 53 plantlets through the adaptation.

Ploidy level:

Regenerated plantlets were evaluated for ploidy level *via* chromosome counting. The chromosome number of these plantlets were determined (Fig. E) according to the protocol described by (Kafkas *et al*., 2002, and Preeda *et al*., 2007) and found that the plantlets produced from the ovule germination were tetrahaploid (2n = 4x = 28 chromosomes).

Our results, i.e., establishment of a successful protocol for producing gynogenic strawberry plants is important for breeders to obtain new dihaploid lines in a short period (few months) or using it as suitable material for applying the new technology of genetic engineering by transferring new genes to improve the yield and the quality of strawberry cultivars.

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انتاج نباتات رباعية أنثوية المنشأ فى الفراولة من خلال زراعة مبايض غير ملقحة

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أجريت هذه الدراسة فى مختبر زراعة الأنسجة، قسم الخضر، كلية الزراعة، جامعة القاهرة، الجيزة، مصر، خلال الفترة من ٢٠٠٥ حتى ٢٠٠٨. وكان الهدف من هذا البحث هو انتاج نباتات رباعية من الفراولة الثمانية (*Fragaria* × *ananassa* Duch.) -معملياً- من خلال المنشأ الأنثوى بزراعة مبايض غير ملقحة لتركيبين وراثيين مختلفين هما Camarosa، و Sweet Charlie. عوملت براعم زهرية قبل تفتحها بـ ٢-٤ يوم بالبرودة على ٤°م لفترات مختلفة ثم زرعت على بيئات مختلفة التركيب. تم الإستيلاد من بعض البويضات وهذه تم دفعها لإنتاج أفرع جديدة. تم إجراء تجذير لهذه الأفرع لإنتاج نباتات كاملة للأقلمة. أظهرت النتائج ان معاملة التبريد المبدئية لم تكن فعالة لإنتاج أفضل إستيلاد من البويضات وإنتاج نباتات رباعية. أعطى كلا الصنفين نتائج متشابهة تقريباً فى ظل الظروف المثلى للإستيلاد من البويضات. تم تحديد مستوى التضاعف وأظهرت الفحوص أن النباتات الناتجة من البويضات كانت رباعية. هذه النتائج تمثل وسيلة حديثة للمربين لإنتاج نباتات فراولة رباعية من الثمانية تستخدم فى إنتاج أصناف جديدة خلال فترة وجيزة (بضعة اشهر)، أو استخدام هذا البروتوكول للإستيلاد من البويضات كمادة مناسبة فى تجارب الهندسة الوراثية عن طريق نقل جينات جديدة لتحسين محصول وجودة الفراولة.