New findings in strawberry (*Fragaria* × *ananassa* Duch.) anther culture

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Abstract
In this research, some independent experiments were carried out for investigating the effects of microspore developmental stage, bud pretreatments (cold, heat and Ca), and embryo induction medium on anther culture of some strawberry cultivars. The results showed that the percentages of responsive and embryogenic anthers in cultivar Paros in liquid medium were influenced not only by different developmental stages of microspores, but also by the ratio of mass/volume of the cultured anthers. Moreover, study of the different bud pretreatments in anther culture of four strawberry cultivars demonstrated a highly significant interaction between bud pretreatments and cultivars in the three studied traits including the percentages of responsive, embryogenic and callogenic anthers. The percentage of responsive anthers was increased by low temperature stress (4 °C) and calcium pretreatments. The significant interactions were also observed between embryo induction media and cultivars. Chromosome counting on 30 androgenic plants showed that only one plant was haploid. This plant was obtained from anther culture in cultivar Paros by the use of H1 medium (2 mg l^{-1} IAA and 1 mg l^{-1} BA) and cold pretreatment.

Key words: Strawberry, Microspore developmental stage, Pretreatment, Embryo induction medium.

INTRODUCTION

Strawberry (*Fragaria ananassa* Duch) belongs to the Rosaceae family. Cultivated strawberry is a high–value fruit crop species. Strawberry is appreciated for its unique flavor and nutritious quality (Folta and Davis, 2006). Improvement of the commercial strawberry, which is octaploid (2n=8x=56), has been slow since cultivars are highly heterozygote and conserving useful traits by selfing is difficult due to inbreeding depression (Sayegh and Hennerty, 1989). Traditional breeding efforts for improving strawberry quality and yield are labor intensive, costly and time consuming (Own and Miller, 1996).

Haploid plants have been produced through different *in vitro* methods, which are fast and efficient. Among these methods, androgenesis is the most effective one due to the quantity of microspores. A number of studies have been carried out on the anther culture of strawberry (Niemirowicz–Szczytt *et al.*, 1983; Sayegh and Hennerty, 1989; Quarta *et al.*, 1991; Svensson and Johansson, 1994; Own and Miller, 1996). The first haploid recovery in strawberry through anther culture was reported by Niemirowicz-Szczytt and Zakrzewska (1981) and Niemirowicz-Szczytt et al. (1983). One of the successful research efforts in strawberry anther culture was obtained by Owen and Miller (1996). They achieved the highest shoot regeneration using H1 medium supplemented by 2 mg l^{-1} IAA, 1 mg l^{-1} BA, and 0.2 M glucose. There is a need to improve embryogenesis and plant regeneration in anther culture of strawberry, thus the aims of the present investigation were to study the effects of some important factors including the stages of microspore development, anther pretreat-

ments and embryo induction media on the anther culture of some strawberry cultivars.

MATERIALS AND METHODS

Four strawberry cultivars namely Camarosa, Selva, Paros, and Pajaro were used as donor plants for anther culture. Cultivars were grown in a hydroponic system in a glasshouse. For flowering, the temperature was under 20 °C (day)/12 °C (night), and with a photoperiod of natural light of 12–13 hours.

For cytological determination of microspore developmental stage, some stains including lactophenol blue, orcein, feulgen, and aceto-carmine were investigated. Olympus IX70 inversion microscope was used for monitoring of the microspores.

For surface sterilization, the buds were immersed, respectively in 2.5% (w/v) sodium hypochlorite for 15 min. and 70% ethanol for 30 sec, followed by three rinses with sterile distilled water. Three anther culture media namely H1 (Owen and Miller, 1996), GD (Gresshoff and Doy, 1972) and NN (Nitsch and Nitsch, 1969) were used in this study (Table 1). In all media, pH was adjusted to 5.8. Each replication consisted of one single Petri dish (15×55 mm) containing 5 ml of semi-solid or liquid medium and 10 anthers. The cultures were incubated at 24±1 °C in dark for 30 days. In this study, five independent experiments were conducted. In all experiments, flower buds with microspores at mid-uninucleate stage were used in anther culture except for the first experiment.

Table 1. Composition (mg l^{-1}) of three embryo induction media in strawberry anther culture.

Ingredients	Medium		
	H1	**GD**	**NN**
KNO_3	2022	1000	950
NH_4NO_3	400	1000	720
$MgSO_4 .7H_2O$	246	37	185
$CaCl_2 .2H_2O$	294	-	220
KH_2PO_4	272	300	68
$(NH_4)_2SO_4$	-	-	-
KCl	-	65	-
$Ca(NO_3)_2$	-	241	-
$FeSO_4 .7H_2O$	27.8	27.8	27.8
Na_2 EDTA	37.3	37.3	37.3
$MnSO_4. 4H_2O$	22.3	1.12	25
H_3BO_3	6.2	0.3	10
$ZnSO_4 .7H_2O$	8.6	0.3	10
KI	0.17	0.8	-
$CuSO_4 .5H_2O$	0.025	0.025	0.025
$Na_2MoO_4 .2H_2O$	0.24	0.025	0.25
$CoCl_2 .6H_2O$	0.024	0.025	-
Folic acid	-	-	0.5
Nicotinic acid	0.5	0.1	5
Thiamine·HCl	0.1	1	0.5
Pyridoxine·HCl	0.5	0.1	0.5
Glycine	2	4	2
Biotine	-	0.2	0.05
myo-inositol	100	100	100
Maltose	36032	-	-
Sucrose	-	20000	20000
pH	5.8	5.8	5.8

Experiment 1: Investigation of different stages of microspore development and physical conditions of embryo induction medium

This experiment was carried out using H1 medium containing 2 mg l^{-1} IAA (indole-3-acetic acid) and 1 mg l^{-1} BAP (6-benzylaminopurine,) in cultivar Paros. A factorial experiment on the basis of completely randomized design (CRD) with two factors and three replications was used. The first factor was microspore developmental stage in three levels (tetrad, mid-uninucleate and late-uninucleate stages) and the second factor was physical conditions of embryo induction medium in two levels including liquid and semi–solid medium (containing 0.15 % phytagel).

Experiment 2: Effects of some bud pretreatments (heat, cold and mannitol) on strawberry anther culture

In this experiment, H1 liquid medium containing 2 mg l^{-1} IAA and 1 mg l^{-1} BAP was used. A factorial experiment on the basis of completely randomized design (CRD) with 2 factors and 3 replications was used. The first factor was cultivar in 4 levels (Camorosa, Selva, Paros and Pajaro) and the second factor was pretreatment in 4 levels (cold, cold–mannitol, heat and heat–mannitol). In the case of cold and heat pretreatments, buds were first screwed between wet towels in Petri dishes. In cold–mannitol and heat–mannitol pretreatments, buds were first immersed in 5 ml mannitol solution with a concentration of 0.3 M and then incubated at 4 °C for 4 days or at 30 °C for 1 h, respectively.

Experiment 3: Investigation of calcium and cold combined pretreatment on anther culture in cultivar Paros

This experiment was carried out using H1 liquid medium containing 2 mg l^{-1} IAA and 1 mg l^{-1} BAP in a completely random design (CRD) with 7 treatments and 4 replications. In this experiment, two calcium salts

Table 2. Mean comparison of developmental microspore stage × embryo induction medium interaction in anther culture of cultivar Paros.

Combined treatments		Traits (%)		
Microspore developmental stage	Medium	Responsive Anthers	Embryogenic anthers	Callogenic anthers
Tetrad stage	Liquid	20.00d	3.33b	16.67a
	Semi–solid	80.00b	53.33a	26.67a
Mid uninucleate stage	Liquid	87.27ab	61.51a	25.76a
	Semi–solid	96.67a	66.67a	40.00a
Late uninucleate stage	Liquid	53.33c	20.00b	33.33a
	Semi–solid	93.33ab	73.33a	26.67a

containing $CaCl_2.2H_2O$ and $Ca(NO_3)_2$ with different concentrations (200, 300 and 400 mg l^{-1}) and control (cold pretreatment of the buds) were used. Five ml of the calcium solutions were dispersed in the Petri dishes (15 × 55 mm), followed by the immersion and incubation of the buds at 4°C for 4 days.

Experiment 4: Effects of embryo induction media and cultivar on strawberry anther culture

A factorial experiment on the basis of completely randomized design (CRD) with 2 factors and 4 replications was used. The first factor was cultivar (Camorosa, Selva, Paros and Pajaro) and the second factor was liquid medium (Table 1) in five levels including: H1 medium with 2 mg l^{-1} IAA and 1 mg l^{-1} BAP, (Owen and Miller, 1996); GD medium with 2 mg l^{-1} IAA and 1 mg l^{-1} BAP (GD1); GD medium with 2 mg l^{-1} NAA (α-naphthalene acetic acid) and 5 mg l^{-1} kinetin, (Svensson and Johansson, 1994); NN medium with 2 mg l^{-1} IAA and 1 mg l^{-1} BAP (NN1); NN medium with 0.4 mg l^{-1} IAA and 3.2 mg l^{-1} kinetin (Owen and Miller, 1996).

In all experiments, three traits including the percentages of responsive, embryogenic and callogenic anthers (Figure. 1) were investigated after 30 days.

For chromosome counting, the root tips (1.5 cm) were treated by 0.002 M 8-Hydroxyquinoline at room temperature for 5 h. They were then fixed in absolute ethanol: glacial acetic acid (3:1) for 24 h and stored at 4 °C. Prior to chromosome counting, the root tips were hydrolyzed in 1M HCl for 15 min at 60 °C stained for 6 days in 4% (w/v) Haematoxylin at room temperature, and squashed in 45% (v/v) acetic acid. Slides were analyzed using a BX50 Olympus microscope and microscopic photographs were taken by a connected DP12 digital camera. Data analysis was carried out using SPSS (V 16) software. Comparison of means was carried out based on the LSD method.

RESULTS AND DISCUSSION

Determination of microspore developmental stages

Lactophenol blue was better than other the dyes for determination of microspore developmental stage, probably due to its lower molecular weight. Sepal length was used as morphological marker (Douglas, 1985), so that microspores with sepal length between 1–3 mm, 4–5 mm and 5–9 mm evaluated as tetrad, mid and late-uninucleate stages, respectively.

Experiment 1: Investigation of different stages of microspore development and physical conditions of embryo induction medium

Analysis of variance showed that the main and interactive effects of the studied factors were significant for two out of three studied traits (percentage of responsive anthers and embryogenesis). Therefore, mean comparison was carried out only for interactive effects (Table 2).

In all studied uninucleate stages of the microspores, the best percentage of responsive anthers was observed in semi–solid medium, however, in mid-uninucleate stage the difference between semi–solid and liquid media was not significant (96.67% and 87.27%, respectively). It seems that the used gelling agent does not play a key role in this developmental stage of microspores. The similar results were obtained for embryogenesis trait. These results are similar to the previous report which showed the semi–solid medium was better than liquid medium (Owen and Miller 1996).

In mid-uninucleate stage, no significant differenc was observed between two media. However, in tet-

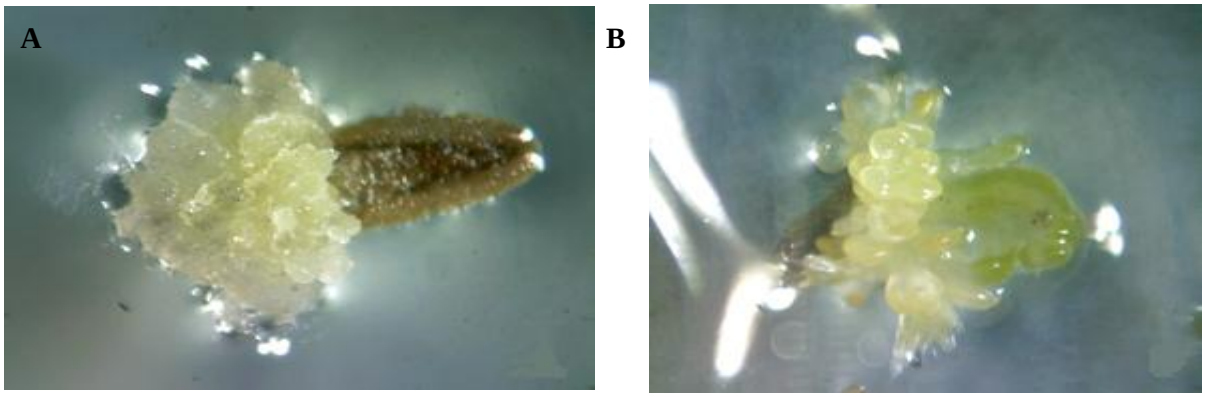

Figure 1. Callus **(A)** and embryo **(B)** formation in strawberry anther culture.

rad and late-uninucleate stages, our results showed that the percentage of responsive anthers and embryogenesis in semi–solid medium were significantly higher than those in liquid medium. A high significant difference between the anthers cultivated in the different stages of microspore development was not expected, because it was demonstrated that all microspores are not in the same stage in one anther. In liquid medium, the anthers with the microspores at the mid-uninucleate stage produced more responsive anthers and embryogenesis in comparison to the cultured anthers at the tetrad and late-uninucleate stages. These different results in liquid medium may be due to the differences in the density (mass/volume) of cultured anthers at the different stages of microspore development. All cultured anthers at the tetrad stage were placed in the bottom of the medium. On the other hand, the cultured anthers at the mid-uninucleate stage had the optimum density, because they were floated in the surface of medium. Therefore, the anthers cultured in the mid-uninucleate stage had the better gas exchange in comparison to cultured anthers at the tetrad stage and also they had a better nutrient supply in comparison to anthers cultured in the late-uninucleate stage. Therefore, it seems that the ratio of mass/volume of the cultured anthers can be another important factor for embryo formation in liquid medium. This new and important factor had not already been investigated by the researchers. If the hypothesis of effect of density on anther culture is true, density is effective on the percentage of responsive anthers and embryogenesis via changes in oxygen, carbon dioxide and ethylene concentrations, pH and nutrients absorption. This factor could also be investigated in the isolated microspores culture for increasing embryo production.

Experiment 2: Effects of some bud pretreatments (heat, cold and mannitol) on strawberry anther culture

Analysis of variance showed that all studied effects (cultivar, bud pretreatment and their interactions) were statistically significant for three studied traits, therefore, only the interaction effects were compared (Table 3). A highly significant interaction was observed between cultivar and pretreatment, as they have been reported by previous studies (Niemirowicz–Szczytt *et al.*, 1983; Quarta *et al.*, 1991). The cold pretreatment was more effective than other used pretreatments. It had the highest positive effect to start embryogenesis in all studied cultivars, except in cultivar Pajaro. The cold pretreatment can increase microspore division (Svensson and Johansson, 1994). In cultivar Pajaro, the heat pretreatment increased the percentage of embryogenesis. Integrated pretreatments (cold–mannitol and heat–mannitol) decreased the percentage of responsive anthers and there was not a significant difference between integrated pretreatments in all cultivars.

Experiment 3: Investigation of calcium and cold combined pretreatment on anther culture in cultivar Paros

Analysis of variance showed significant differences between Ca pretreatments in the three studied traits. By increasing $Ca(NO_3)_2$ concentration, the percentage of responsive anthers decreased significantly, while opposite results were observed by increasing $CaCl_2.2H_2O$ concentration. The highest percentages of responsive anthers were obtained by using 400 mg l^{-1} $CaCl_2.2H_2O$, 200 and 300 mg l^{-1} $Ca(NO_3)_2$ treatments (87.5, 81.7 and 70 %, respectively). Also, the use of 400 mg l^{-1} $CaCl_2.2H_2O$ produced the highest embryogenesis (77.5

Table 3. Mean comparison of cultivar × bud pretreatment interaction on strawberry anther culture.

Combined treatments		Traits (%)		
Cultivar	Pretreatment	Responsive anthers	Embryogenic anthers	Callogeneic anthers
Paros	Heat	60.00bc	6.67f	53.33b
	Heat–mannitol	36.67f	13.33e	23.33de
	Cold	92.96a	10.37ef	82.63a
	Cold–mannitol	43.33ef	10.00ef	33.33c
Selva	Heat	16.67gh	0g	16.67ef
	Heat–mannitol	6.67hij	0g	6.67gh
	Cold	46.67def	33.33b	13.33fg
	Cold–mannitol	13.33ghi	0g	13.33fg
Pajaro	Heat	20.00g	6.67f	13.33fg
	Heat–mannitol	0j	0g	0h
	Cold	6.67hij	0g	6.67gh
	Cold–mannitol	3.33ij	0g	3.33h
Camarosa	Heat	56.67bcd	30.00bc	26.67cd
	Heat–mannitol	53.33cde	20.00d	33.33c
	Cold	66.67b	46.67a	20.00def
	Cold–mannitol	50.00cde	26.67c	23.33de

Table 4. Means of calcium pretreatments in anther culture of cultivar Paros.

Combined treatments		Traits (%)		
Calcium salts	Concentration (mg l^{-1})	Responsive anthers	Embryogenic anthers	Callogenic anthers
Control	-	55.00c	15.00e	40.00ab
$Ca(NO_3)_2$	200	81.7ab	30.15cd	51.55a
	300	70.00b	40.00bc	30.00bc
	400	48.41c	24.32de	24.09c
$CaCl_2.2H_2O$	200	48.41c	24.32de	24.09c
	300	54.04c	42.69b	11.35d
	400	87.50a	77.50a	10.00d

%). Results also showed that by increasing $CaCl_2.2H_2O$ and $Ca(NO_3)_2$ concentrations, the callogenesis decreased significantly and the highest callogenesis was observed at 200 mg l^{-1} $Ca(NO_3)_2$ (Table 4).

Overall, results showed that some Ca pretreatments especially 400 mg l^{-1} $CaCl_2.2H_2O$ had a high positive effect in strawberry anther culture to induce embryogenesis. Increasing cytosolic calcium can lead to calcium bonding with calmodolin. This complex (calcium–calmodolin) causes transition of G_2 to M in cell cycle (Pauls *et al.*, 2006). An investigation on the effects of calcium ions on the isolated microspore culture in *Brassica napus* showed that calcium signal has had an important role in the embryogenesis induction of microspores (Pauls *et al.*, 2006). Also a report on the somatic embryogenesis of wheat showed that $Ca(NO_3)_2$ had an inhibitory effect on the somatic embryogenesis while $CaCl_2$ and $CaSO_4$ had a positive effect on the somatic embryogenesis (Mahalakshmi *et al.*, 2007).

Table 5. Means of cultivar × medium interaction in strawberry anther culture.

Combined treatments		Traits (%)		
Cultivar	Embryo induction medium	Responsive anthers	Embryogenic anthers	Callogenic anthers
Paros	GD	40.00ef	10.00efg	30.00de
	GD1	60.00c	33.33b	26.67ef
	NN	54.54cd	15.76ef	38.79bcd
	NN1	23.33gh	6.67fg	16.67fgh
	H1	93.33a	20.00cde	73.33a
Selva	GD	96.67a	16.67def	83.33a
	GD1	73.33b	30.00bc	43.33bc
	NN	23.33gh	13.33ef	10.00hij
	NN1	93.33a	60.00a	33.33cde
	H1	50.00cde	26.67bcd	23.33efg
Pajaro	GD	3.33ij	0g	3.33ij
	GD1	60.00c	13.33ef	46.67b
	NN	13.33hi	0g	13.33ghi
	NN1	46.67de	20.00cde	26.67ef
	H1	0j	0j	0j
Camarosa	GD	43.33def	16.67def	26.67ef
	GD1	73.33b	33.33b	40.00bcd
	NN	26.67g	10.00efg	16.67fgh
	NN1	33.33fg	16.67	16.67fgh
	H1	86.67a	53.33a	33.33cde

Concerning plant regeneration, the embryos obtained from all applied pretreatments were not regenerated or they had weak plant regeneration (data not shown).

Experiment 4: Effects of embryo induction media and cultivar on strawberry anther culture

Analysis of variance showed that the main effects of cultivar, liquid medium and their interactions were significant for three studied traits; therefore mean comparisons were carried out only for interaction effects (Table 5). Results for the percentage of responsive anthers showed that among two media GD (containing 2 mg l^{-1} NAA and 5 mg l^{-1} kinetin), that previously had been used in strawberry anther culture (Quarto *et al.*, 1991) and GD1 (containing 2 mg l^{-1} IAA and 1 mg l^{-1} BAP), GD1 medium was better than GD in all studied cultivars except for cultivar Selva. Similar results were obtained when two other media including NN (containing 0.4 mg l^{-1} IAA and 3.2 mg l^{-1} kinetin) (Owen and Miller, 1996) and NN1 (containing 2 mg l^{-1} IAA and 1 mg l^{-1} BAP) were compared. The NN1 medium was better than NN in cultivars Selva, Pajaro and Camarosa. These results seem to be due to different combinations of plant growth regulators. In the suitable media (GD1 and NN1), BAP was used as cytokinine. Among 3 media H1, GD1 and NN1 that had the same hormone combination, H1 medium was better than two others in cultivars Paros and Camorosa, which might be due to the high ratio of potassium nitrate to ammonium nitrate (Own and Miller, 1996). The highest percentages of responsive anthers in cultivars Paros and Camarosa were obtained by H1 medium (93.33 and 86.67%, respectively) while the GD and NN1 media produced the highest responsive anthers in cultivar Selva (96.67 and 93.33%, respectively). In cultivar Pajaro, GD1 medium was determined as the best medium for producing responsive anthers (60%).

For embryogenesis trait, the GD1 medium was better than GD medium in all cultivars. Also, the NN1 medium was more efficient than NN in all cultivars present for cultivar Paros. This positive effect of NN1 medium may be due to folic acid that was present in the NN1 medium. The cell cycle could be induced by folic acid (George *et al.*, 2008).

The highest embryogenesis in cultivars Selva and Pajaro were obtained by NN1 medium (60 and 20%,

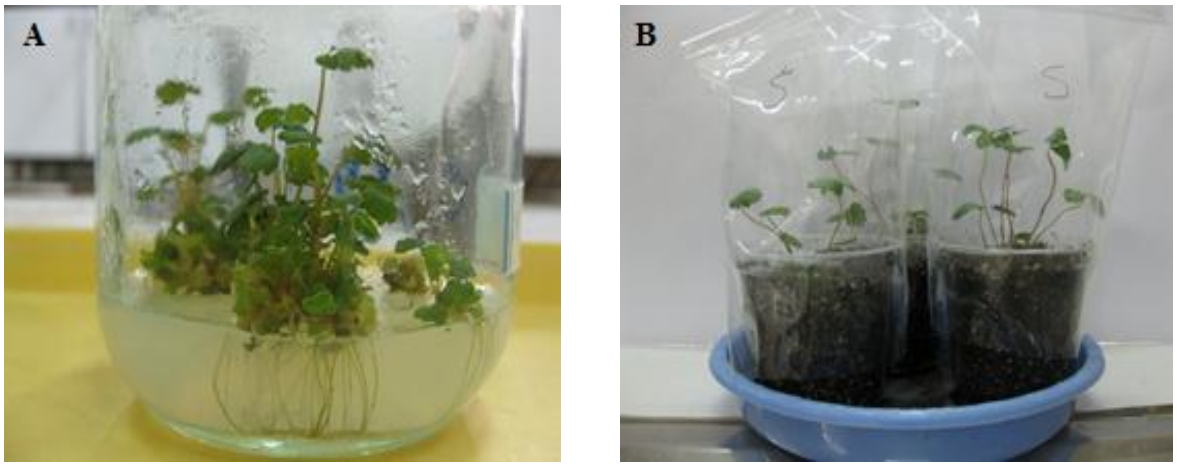

Figure 2. Regenerated plants from strawberry anther culture **(A)** and the plants in the acclimatization stage **(B)**.

respectively) while the best embryogenesis in cultivars Paros and Camarosa were obtained by GD1 and H1 media (33.33 and 53.33%, respectively).

Chromosome counting

From all regenerated plants, 30 plants were acclimatized (Figure. 2) and used for chromosome counting. Results showed that only one plant was haploid. This plant was obtained from anther culture of cultivar Paros on H1 medium (containing 2 mgl^{-1} IAA and 1 $mg\ l^{-1}$ BA) and by the use of cold pretreatment.

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