ANDROGENESIS INDUCTION THROUGH ANTHER CULTURE IN DAY-NEUTRAL STRAWBERRY (*FRAGARIA* × *ANANASSA* DUCH) CV. 'EVEREST'

Truong Xuan Nguyen[1,2*], Ye-Su Song[1], and Sung-Min Park[1]

[1]Department of Horticulture, Kangwon National University, Chuncheon 200-701, Korea
[2] Institute of Agro-biology, Vietnam National University of Agriculture, Hanoi, Vietnam

Email[*]: truonggianghau@gmail.com

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ABSTRACT

The anther culture method is commonly applied in inducing androgenesis. Our study was conducted to determine the effects of microspore developmental stage, plant growth regulators (PGRs), and interaction between callus age and medium culture on androgenetic plant production in day-neutral strawberry cv. 'Everest'. Moreover, simple sequence repeat (SSR) markers were investigated to identify the homozygous regenerants. Anthers containing microspore at uninucleate stage gave the highest response. The most anther-derived calli were obtained on the Dumas De Vaulx medium (C – medium) containing 0.4 mg L^{-1} benzyl adenine (BA) + 0.1 mg L^{-1} 3-indole acetic acid (IAA) + 2.0 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D). The combination of 1 mg L^{-1} BA and 2 mg L^{-1} IAA produced the maximum rate of plantlet regeneration (10.0%). The interaction between Gamborg medium (B5) and 2-month old callus showed the highest number of shoot regeneration (7.1 shoots). Ploidy analysis of regenerated plants using chromosome observation revealed 50% octoploid (*2n = 8x* = 56), 10% hexaploid (*6x* = 42), 35% aneuploid, and 5% haploid (*n = 4x* = 28). Using 30 SSR primers, we verified the genetic integrity of ten octoploid anther-derived plants with respect to the donor plant. The results showed that no spontaneous doubled haploid originating directly through anther culture was found. This confirmed that all regenerated plants were initiated from somatic cells. Therefore, the *in vitro* anther culture can provide efficient haploid and micro propagation techniques for day-neutral strawberry.

Keywords: Day-neutral strawberry, haploid, microsatellite markers, uninucleate, shoot formation.

Sự tạo cây vô tính thông qua nuôi cấy bao phấn ở cây dâu tây phản ứng trung tính với ánh sáng

TÓM TẮT

Phương pháp nuôi cấy bao phấn được ứng dụng rộng rãi trong tạo cây vô tính. Nghiên cứu của chúng tôi đã tiến hành đánh giá ảnh hưởng của giai đoạn phát triển của hạt phấn, chất điều tiết sinh trưởng, sự tương tác giữa tuổi mô sẹo và nuôi trường nuôi cấy đến sự tạo cây vô tính ở cây dâu tây phản ứng trung tính với ánh sáng. Thêm vào đó, chỉ thị phân tử SSRs cũng được khảo sát để xác định cây tái sinh đồng hợp tử. Hạt phấn ở giai đoạn đơn nhân đã cho sự phản ứng tốt nhất. Hầu hết mô sẹo thu được trên môi trường Dumas De Vaulx (C – medium) chứa 0,4 mg benzyl adenine (BA) + 0,1 mg 3-indole acetic acid (IAA) + 2,0 mg 2,4-Dichlorophenoxyacetic acid (2,4-D). Tổ hợp chất điều tiết sinh trưởng (1 mg BA + 2 mg IAA) trong môi trường MS cho tỷ lệ cây tái sinh cao nhất (10,0%). Sự kết hợp giữa môi trường Gamborg (B5) và mô sẹo 2 tháng tuổi đã cho số mầm tạo thành cao nhất (7,1 mầm). Phân tích độ bội của cây tái sinh bằng đếm nhiễm sắc thể cho thấy 50% là cây bát bội, 10% cây lục bội, 35% cây lệch bội và 5% cây tứ bội (đơn bội). Sử dụng 30 bộ mồi SSRs, chúng tôi nhận thấy 10 cây bát bội được hình thành từ bao phấn giống cây mẹ hoàn toàn về di truyền. Kết quả đã chỉ ra rằng không có cây đồng hợp tử được tạo ra trong nuôi cấy bao phấn. Điều này đã chứng thực rằng tất cả cây tái sinh được hình thành từ tế bào sinh dưỡng. Chính vì vậy, nuôi cấy bao phấn có thể là phương pháp hiệu quả mới trong tạo cây đơn bội và nhân nhanh trên cây dâu tây phản ứng trung tính với ánh sáng.

Từ khóa: Giống dâu tây trung tính, đơn bội, chỉ thị, đơn nhân, tạo chồi.

1. INTRODUCTION

Korea is the fifth biggest strawberry producing country in the world with 203,456 tons (Korea Agro-Fisheries trade Corp, 2011) of both day-neutral (DN) and short-day (SD) cultivars currently used. However, there are undesirable characteristics in SD types when grown in summer seasons with relatively high temperature and long day-length. Using artificial treatment to induce flower initiation is labor intensive and costly (Ruan et al., 2009). Whereas DN types do not enter dormancy in fall because flowering is continuous until temperature is getting cool (Douglas and Thomas, 2005).

Korean researchers have not limited their research only to the development of cultivations (Ruan et al., 2011) but also to the development of new DN hybrid types with conventional breeding methods (Lee et al., 2008). Using conventional breeding method such as hybridization or mutation is very time consuming to release new cultivars (Debnath et al., 2007). In contrast, androgenesis appears to be a more suitable technique in breeding program. Their culture is one of the most widely used to provide breeding materials. The first haploid strawberry plant via anther culture was obtained in three SD cultivars by Owen and Miller (1996). However, Rosati et al. (1975), and Niemirowicz-Szcytt and Zakrzewska (1990) were unsuccessful in this experiment. Subsequently, researchers such as Quarta et al. (1991) also conducted in-depth study in this field. Successful induction of androgenesis with subsequent regeneration of complete haploid plants is limited. Laneri and Damiano (1980) concluded that due to very poor response of strawberry anther culture in vitro conditions, it is classified as one of the most recalcitrant species to regenerate in vitro.

Zhennan et al. (1995) also showed that plant growth regulators and culture medium are two of the main factors affecting androgenesis induction with microspore developmental stage being another important factor for successful androgenesis. Microspore in most plant species at the uninucleate stage is most responsive to induction and it has been shown that highly significant relationship exist between flower bud size, anther color and corresponding microspore developmental stage (Assani et al., 2003; Kozak et al., 2012; Salas et al., 2012). So far, this relationship has not yet been reported in detail in regards to the strawberry crop.

Based on the homogeneity of agronomic traits as shown in previous studies, spontaneous haploid can occur during callus induction in anther culture and characterization of doubled haploid (DH) plants (Kernan and Ferrie, 2005). This is because unequivocal identification of DH lines using morphological traits is difficult (Chen et al., 1998). Application of SSRs makers were successfully employed to identify spontaneous haploid plants through anther culture in maize (Pedro et al., 2007), *Pyrus communis* L. (Bouvier et al., 2002), coconut (Perera et al., 2009). In strawberry crop, SSR markers were widely employed to characterize strawberries as well as to identify cultivars and enforce patent protection (Milella et al., 2006; Kasumi and Keita, 2006) because the species has an octoploid genome presenting as many as eight allelic variants for any given locus (Arnau et al., 2003). In contrast, till date, the use of SSRs markers to distinguish homozygous regenerants among anther-derived diploids in strawberry has not been conducted yet.

Our objective was to investigate the effect of microspore developmental stage, plant growth regulators, callus age and culture medium on callus induction and plant regeneration and the use of SSRs markers to identify the origin and fidelity of diploid anther-derived plants.

2. MATERIALS AND METHODS

2.1. Plant materials

A day neutral strawberry cultivar namely Everest was maintained at Kangwon National University (Kangwondo South Korea). Flower buds (1.0 - 1.2 mm in length) with anther

containing microspores at the uninucleate stage were collected and kept in refrigerator at 4°c for 4 days as a cold treatment (Na et al., 2009).

2.2. Sterilization and media preparation

After pretreatment, flower buds were disinfected with 70% ethanol for 15 s and 1% sodium hypochlorite for 10 min followed by three times rinsing with sterilized distilled water. Media were autoclaved at 121°c (108 kPa) for 15 min and pH of the media was adjusted to 5.8 prior to autoclave. Then, 30 ml medium was poured into Petri dish (90 x 15 mm) and petri dishes were sealed by parafilm.

2.3. Callus induction

Anthers with different developmental stage of the microspore (tetrad, uninucleate, binucleate) were cultured on C medium (Dumas De Vaulx, 1981) containing 2M glucose + 0.1 mg L^{-1} BA + 0.4 mg L^{-1} IAA + 2.0 mg L^{-1} 2,4-D to determine the relation between anther color, anther length, and callus induction. Twenty anthers were placed in Petri dishes containing 30 ml medium. Each treatment was repeated five times (five Petri dishes) and arranged in a completely randomized design (CRD). Then cultured anthers were incubated in the dark at 25°C for 2 months.

Anthers containing microspore at uninucleate stage were placed on induction medium consisting of C medium with 2M glucose and different PGRs combinations: Medium I: 0.0 mg L^{-1} BA + 0.0 mg L^{-1} IAA + 0.0 mg L^{-1} 2,4-D; Medium II: 0.1 mg L^{-1} BA + 2.0 mg L^{-1} IAA + 0.4 mg L^{-1} 2,4-D; Medium III: 0.1 mg L^{-1} BA + 0.4 mg L^{-1} IAA + 2.0 mg L^{-1} 2,4-D; Medium IV: 0.4 mg L^{-1} BA + 0.1 mg L^{-1} IAA + 2.0 mg L^{-1} 2,4-D +; Medium V: 1.0 mg L^{-1} BA + 1.0 mg L^{-1} IAA + 0.4 mg L^{-1} 2,4-D; and Medium VI: 2.0 mg L^{-1} BA + 0.1 mg L^{-1} IAA + 0.4 mg L^{-1} 2,4-D. Twenty anthers were placed in Petri dishes containing 30 ml medium. Each treatment was repeated five times (five Petri dishes) and arranged in a completely randomized design (CRD). Cultured anthers were incubated in darkness condition at 25°c for 2 months.

2.4. Plant regeneration

To test the effect of the PGR combinations on plant regeneration, calluses(compact and white-cream) were transferred to MS medium supplemented with 0.2 M glucose, and different combinations of BA (0.0, 0.5, 1.0 or 2.0 mg L^{-1}) and IAA (0.0, 0.5, 1.0 or 2.0 mg L^{-1}).

To investigate the effect of callus age and media on shoot regeneration, calluses with different ages (1, 2, and 3-month-old) were cultured on different medium: MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), and H1 (Owen and Miller, 1996). All media contained 0.2 M glucose, 1 mg L-1 BA, and 2 mg L-1 IAA. Ten calluses were placed in Petri dishes. Each treatment was repeated five times (five Petri dishes), and arranged in a completely randomized design (CRD). The calluses were kept at 25°C, 60 - 70% relative humidity under 16 h photoperiod and light intensity of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

For both experiments, ten calluses were placed per petri dish and each treatment was repeated five times (five petri dishes) and arranged in a completely randomized design. Then, calluses were kept at 25°C, 60 - 70% relative humidity and under 16 h photoperiod with light intensity of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

2.5. Cytological observation

The cytology of all regenerants was evaluated by counting chromosome of active root tip described by Truong et al. (2012) without modification. The root tips were collected from anther derived plants in the early evening (around 5:00 p.m.), pretreated with 2 mM 8-hydroxyquinoline solution for 1 h at room temperature, and incubated at 4°C for 15 h. The root tips were fixed in absolute alcohol-glacial acetic acid solution (3:1, v/v) for 40 min at room temperature, then softened by 1 N HCl for 2 h at room temperature and subsequently at 60°c for 10 min, followed by brief rinsing in distilled water. The fixed root tip (2-3 mm) was placed on a glass slide (Paul Marienfeld GmbH & Co.KG, Germany) and digested by enzyme

solution containing 0.4% cellulase Onozuka RS (Yakult Co. Ltd., Tokyo), 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo), 0.2% macro enzyme R10, 0.07 M KCl, and 1 mM ethylene diamine tetra-acetic acid (EDTA) pH 4.2 at 42° C for 40 min. After that, it was short-rinsed in distilled water and reaffixed in a drop of 3:1 acetic acid and ethyl alcohol at – 20°c for 5 min, then spread by tapping with fine tweezers using a few drops of acetic/alcohol (3:1), air-dried. Air-dried specimens were stained in the 4% Giemsa solution (Kanto chemical Co., INC) diluted with 1/15 M phosphate buffer (pH 6.8) for 3-5 minutes and rinsed briefly with distilled water, air dried. Chromosomes were observed at 10 to 60X magnification under a light microscope (Eclipse E400, Nikon, Japan). Well-spread chromosomes at the metaphase stage were captured using a 3CCD camera (Niko DS-U2/L2 USB) connected to a computer running NIS-Elements F3.00 software (Niko Corp.). Chromosome numbers were calculated from at least 5 cells per root tip of each different regenerant.

2.6. Genetic analysis

Diploid plantlets derived from anther culture were analyzed with SSR markers to determine their source of origin. A set of 30 pairs of strawberry microsatellite specific primers (Table 1) provided by Macrogen company

Table 1. The code number and sequences of SSR primers tested

Primer pairs	Forward (5' to 3')	Reverse (5' to 3')
EMFn_111	GAAGCTCCTCACAAAGTTAAGG	CCTTTGTTGATGTTGTTGTTGA
ChFaM-203	AGGAGAAGACCGGCTGTGTA	TGCCTATAGCTGGCTGTCT
EMFn_110	GACGCTTCGGAGACTGA	CCCCCTTAAAAATAATTAAATCTCC
EMFn_162	AACTGTGTGGTATGCATGTAGC	ATGCACAAAGCAATGCAAGC
EMFn_181	CCAAATTCAAATTCCTCTTTCC	GCCGAAAAACTAACTACCC
EMFn_184	GATGAGAATTGTTTGAGTGAAGG	TGACCAGCGATTCATAACG
MENFn_186	GTAACGACGGCTGCTTCTCC	CGCTCGCTCTTATAAACTTCC
EMFn-225	AAGGAAAAATGCTCAAATACCC	TACGTGCGACGTTAGAGTCC
EMFn_228	TTGCTGAGGATTTGAAAATGG	TGAAGTTTTACTGGCGTTTGC
UFFa_01E03	ACCCCATCTTCTTCAAATCTCA	GACAAGGCCAGAGCTAGAGAAG
EMFn_201	CAGCTCAGAAAAGCTCACAGC	TAGAACGCCAATCACAAACC
EMFn_213	AGCGTGATTTTGCCTTTGTT	CACAGTAAAGAACAGGAGGGAGA
UFFa_19B06	GACGAGTTAACATCAACGACAC	TACTTAGGCTGCTCTATCTG
UFFa_20G06	ACTCAACCACCACATTTCACAC	GAGAAGTTGTCAATAGTCCAGGTG
EMFn_115	TGGAGATGATGGTCAAGACG	GACAAGACCACGAAAACACG
EMFn_136	TTTCTCTTTTGCTCCATAGTTCC	TTCATCAGGATCCAGAAGTCC
UFFa_02F02	CTTTGCAGCTGAAGAACTCTGA	CAGCAGCTGCCTTAGTCTTAGT
UFFa_16H07	CTCTACCACCATTCAAAACCTC	CACTGGAGACATCTAGCTCAAAC
UFFa_03B05	GGAATCCAAGTTACAGGCTTCA	AAGGAGCCTCTCCAATAGCTTC
UFFa_08C11	GGACGTCCCCTTCTTTATTTCT	ACCCCACATTCCATACCACTAC
UFFa_09B11	CTTGGGAGAGAACCAGAAAAAC	TCAGAACCAACTCCAGAGAAGC
UFFa_09E12	CGAGGAAGTAACCTCACAGAAA	GGTGATGGAGAGTGCTGTTAGA
UFFa_09F09	AGAACCATCATCGTCTCTCGTT	GCAATCTCTTCCGGCTTAAAC
UFFA_15H09	TTAGTAGTAGACCTGCCACAAGG	CGGCTTATCTGTAGAGCTTCAA
EMFn_202	CTCTCTCCCTCAACCTCTCG	TGGACCAATATCTCCCTTGC
EMFn_207	TTGGCAAGAATTTATAGCATCG	TCAGGATGTCTTCAGCAAGG
EMFn_226	CGTCAAAGGAACCCTATTTCG	GTGACGGAGGCATCTTGG
UFFa_02H04	ATCAGTCATCCTGCTAGGCACT	TACTCTGGAACACGCAAGAGAA
UFFa_01H06	GGGAGCTTGCTAGCTAGATTTG	AGATCCAAGTGTGGAAGATGCT
UFFa_04H04	ACGAGGCCTTGTCTTCTTTGTA	GCTCCAGCTTTATTGTCTTGCT

company weas used in this study. Genomic DNA of donor plants and regenerated plants from anther culture wase extracted using the CTAB method. The DNA amplifications were carried out in each 20 µl of reaction mixtures (50 ng template DNA, 1.0 µM each of the SSR primer pairs, 1 U Taq DNA polymerase, 2.5 mM dNTP, 2.5 mM $MgCl_2$, 2 µl 10 x PCR buffer, using a thermal cycler (Bio-Rad, C1000 Thermal Cycler). The amplification was performed at 94°c for 5 min, followed 30 cycles of 94°c for 30 s, 55°c for 30 s, 72°c for 1 min, and final extension at 72°c for 5 min. The PCR products were separated on 5.5% polyacrylamide gel followed by silver staining protocol (www.promega.com, 2011).

2.7. Statistical analysis

Data were subjected to analysis of variance (ANOVA) by standard procedures using SAS 9.3 version (Cary, NC). Duncan's multiple range test (DMRT) was used for the determination of significant differences among the means. The $P \leq 0.05$ was considered to be significant.

3. RESULTS

3.1. Effect of microspore developmental stage and plant growth regulator on callus induction

The relationship between anther color, anther length, and developmental stage of microspore and callus induction are recorded in Table 2. The highest callus induction efficiency (88.6%) was produced with anthers at the uninucleate microspore stage (Fig. 1A). In contrast, anthers containing binucleate microspore stage appeared lower (46.9%) efficiency of callus induction. This data indicated that anther length and color were reliable morphological traits for assessing and selecting the appropriate stage for callus induction in anther culture.

Anthers containing uninucleate microspore responded differently to the various PGRs in the induction media except hormone-free treatment (Table 3). The highest value of callus formation was obtained from medium IV with 90%, followed by medium V, medium III, medium VI, medium II with 88.0%, 87.0%, 79.0%, and 51.0%, respectively. Likewise, the callus weight was highest (34.3 mg) in the same combination of BA and IAA (medium IV). Medium I as control treatment gave the lowest weight of callus (0.0 mg). Callus structures were also affected by the levels of auxins and cytokinins. The compact and white-cream to yellow-green calluses were observed in the medium IV and medium V (Fig. 1C). The soft, watery, friable, and brown calluses were found in the medium II, medium III, and medium VI (Fig. 1D). In contrast, anthers that were cultured on hormone-free medium (medium I) failed to produce calluses (Fig. 1B).

3.2. Effect of plant growth regulators, callus age and medium on plant regeneration

Various combinations of BA and IAA had a considerable effect on plant regeneration (Table 4).

Table 2. Effect of microspore developmental stage on callus response on C medium supplemented with 0.4 mg L^{-1} + 0.1 mg L^{-1} + 2.0 mg L^{-1} in strawberry day-neutral cv. 'Everest', observed after 2 months

Microspore developmental stage	Length of anther (mm)	Color of anther	callus formation [y] (%)	Callus weight [y] (mg)
Tetra	0.7 – 0.9	Greenish-yellow	78.3±4.08b	22.3±3.55c
Uninucleate	1.1 – 1.2	Yellow	88.6±9.01a	32.4±2.56a
Binucleate	1.4 – 1.5	Bright-yellow	46.9±6.61c	27.0±3.83b

Note: [y] Values are the means ± standard error (n = 5). Values followed by the same letter within a column are not significantly different (P ≤ 0.05); Duncan's Multiple Range Test (DMRT)

Table 3. Effect of plant growth regulators on anther culture response on C medium in day-neutral strawberry cv. 'Everest', observed after 2 months

Culture medium	Plant growth regulators (mg L^{-1})			Callus Formation [y] (%)	Callus weight [y] (mg)
	BA	IAA	2,4-D		
Medium I	0.0	0.0	0.0	0.0±0.00e	0.0±0.00e
Medium II	0.1	2.0	0.4	51.0±5.70d	18.4±0.55c
Medium III	0.1	0.4	2.0	87.0±3.54ab	21.1±0.71b
Medium IV	0.4	0.1	2.0	90.0±5.52a	34.3±2.20a
Medium V	1.0	1.0	0.4	88.0±2.07ab	20.4±1.46b
Medium VI	2.0	0.1	0.4	79.0±6.99b	12.5±1.80d

Note: [y] Values are the means ± standard error (n = 5). Values followed by the same letter within a column are not significantly different (P ≤ 0.05); Duncan's Multiple Range Test (DMRT)

Out of seven combinations of hormones, the calluses cultured on MS medium containing 2.0 mg L^{-1} IAA and 1.0 mg L^{-1} BA (Fig. 1f) gave the highest shoot regeneration (5.0%) and the number of shoot per callus (4.0). In the combination of 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} BA, low regeneration rate (2.0%) and low number of shoot per callus (2.1 shoots) were observed. Controversially, calluses cultured on the media with 2.0 mg L^{-1} IAA + 0.5 mg L^{-1} BA induced green, hard without shoot occurrence (Table 4). On the media without hormones (control) or 0.5 mg L^{-1} IAA + 1.0 mg L^{-1} BA or 0.5 mg L^{-1} IAA + 2.0 mg L^{-1} BA or 1.0 mg L^{-1} IAA + 2.0 mg L^{-1} BA, calluses turned yellowish brown, exhibited slow growth and finally died (Table 4, Fig. 1D).

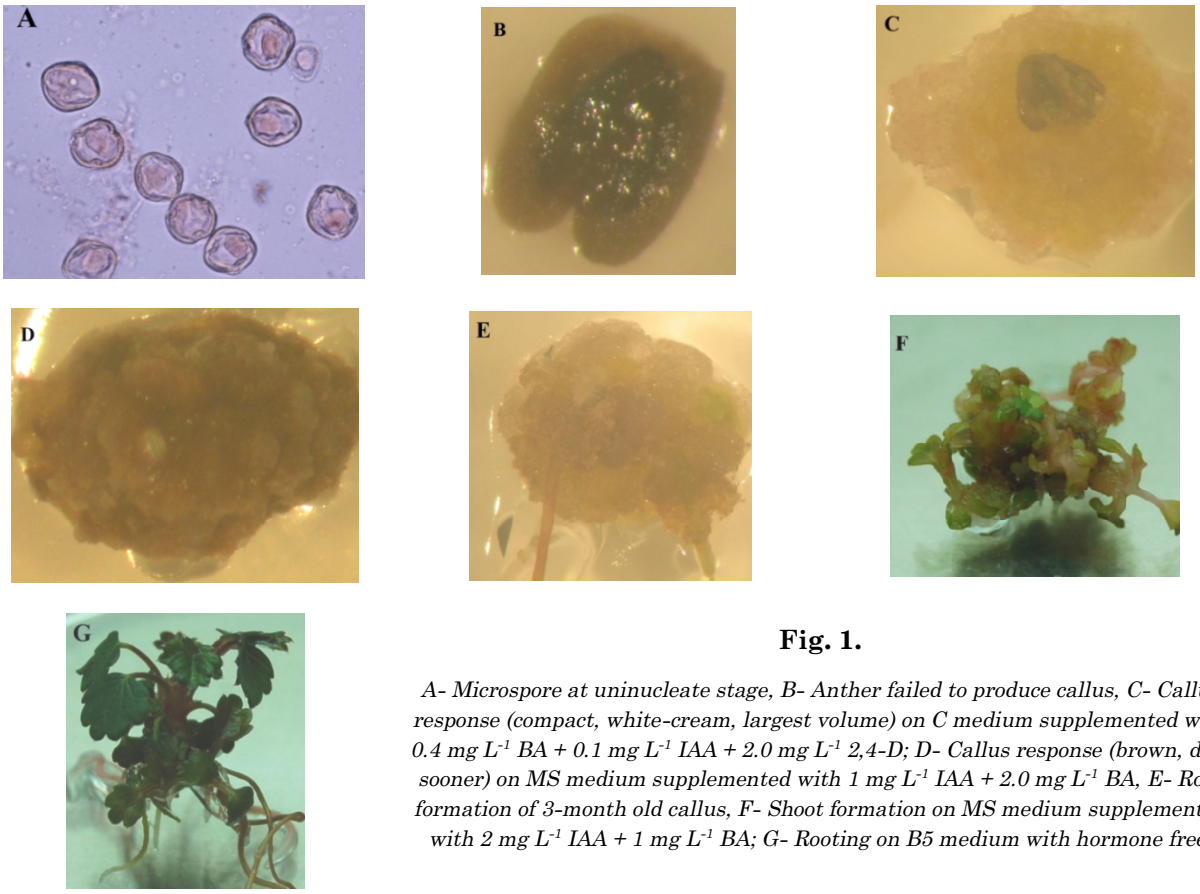

Fig. 1.

A- Microspore at uninucleate stage, B- Anther failed to produce callus, C- Callus response (compact, white-cream, largest volume) on C medium supplemented with 0.4 mg L^{-1} BA + 0.1 mg L^{-1} IAA + 2.0 mg L^{-1} 2,4-D; D- Callus response (brown, died sooner) on MS medium supplemented with 1 mg L^{-1} IAA + 2.0 mg L^{-1} BA, E- Root formation of 3-month old callus, F- Shoot formation on MS medium supplemented with 2 mg L^{-1} IAA + 1 mg L^{-1} BA; G- Rooting on B5 medium with hormone free,

Table 4. Effect of IAA and BA on callus response on MS medium in day-neutral strawberry cv. 'Everest', observed after 2 months

Plant growth regulators (mg L^{-1})		Callus growth response	Shoot formation[y] (%)	No. Of shoot/ callus[y]
IAA	BA			
0.0	0.0	Brown	0.0±0.00c	0.00±0.00c
0.5	1.0	Yellow - brown	0.0±0.00c	0.00±0.00c
0.5	2.0	Brown, necrotic	0.0±0.00c	0.00±0.00c
1.0	0.5	Green - red	2.0±0.36b	2.10±1.00b
1.0	2.0	Brown	0.0±0.00c	0.00±0.00c
2.0	0.5	Green	0.0±0.00c	0.00±0.00c
2.0	1.0	Green - red	10.0±0.82a	4.00±0.96a

Note: [y] Values are the means ± standard error (n = 5). Values followed by the same letter within a column are not significantly different (P ≤ 0.05); Duncan's Multiple Range Test (DMRT)

The effects of various media cultured with different ages of the calluses on plant regeneration are shown in Table 5. The effect of media on plant regeneration was highly significant (P ≤ 0.01). Plant regeneration was highest (10.0%) on B5 medium and the lowest (5%) on MS medium. Effect of callus age on plant regeneration rate was also highly significant (P ≤ 0.01). Similarly, callus age significantly affected the number of shoot per callus (P ≤ 0.01). Shoot number per callus was also highest (7.1 shoots) when 2-month-old callus was cultured on B5 medium followed by H1 medium at the same callus age (5.0 shoots). However, shoots were not produced from both 1-month-old calluses (Table 5) and 3-month-old calluses, while the roots occurred from 3-month-old calluses (Fig 1E). Shoots with green leaves were then transferred to hormone free B5 medium, and root induction was 100% (Fig. 1g) after 4 weeks of culture.

Table 5. Effect of media and callus age on plant regeneration rate and shoot number per callus in strawberry cultivar 'Everest', observed after 2 months

Treatment	Shoot formation (%)	No. of shoot/ callus
MS		
1-month-old	0.0±0.00d	0.0±0.00d
2-month-old	3.0±0.56c	4.1±0,79c
3-month-old	0.0±0.00d	0.0±0.00d
B5		
1-month-old	0.0±0.00d	0.0±0.00d
2-month-old	10.0±1.32a	7.1±0.92a
3-month-old	0.0±0.00d	0.0±0.00d
H1		
1-month-old	0.0±0.00d	0.0±0.00d
2-month-old	6.0±0.87b	5.0±1.06b
3-month-old	0.0±0.00d	0.0±0.00d
Medium	**	**
Callus age	**	**
Medium x Callus	**	**

*Note: **Highly significant at P = 0.01. [y] Values are the means ± standard error (n = 5). Values followed by the same letter within a column are not significantly different (P ≤ 0.05); Duncan's Multiple Range Test (DMRT)*

3.3. Cytological evaluation and genetic fidelity of anther-derived plants

Twenty regenerants derived from anther culture were checked for the ploidy levels by counting chromosomes of the root tips (Table 6). Among them, one plantlet was haploid (5%) with 27 chromosomes (Fig. 2A), ten plantlets were octoploid (55.0%) with 56 chromosomes (Fig. 2G), two plantlets showed hexaploid (10.0%) with 42 chromosomes (Fig. 2B), eight plantlets were aneuploid (35.0%) ranging from 49 to 55 chromosomes (Fig.2C, D, E, and F).

Therefore, to confirm whether diploid plantlets originated from either spontaneous doubling chromosomes of microspore or initiated from somatic tissue (nucellus and integuments) during anther culture in the present study, a total of 30 SSRs markers were screened to detect somaclomal variants using DNA samples from the 11 diploid anther-derived plants and donor plants. Among 30 tested SSRs markers, seven primers pairs (EMFn_111, ChFaM-203, EMFn_228, EMFn_213, EMFn_136, UFFa_09E12, UFFa_09F09, UFFa_02H04) did not amplify any band, 20 primers pairs (EMFn_110, EMFn_181, EMFn_184, MENFn_186, UFFa_01E03, EMFn_201, UFFa_19B06, EMFn_115, EMFn_136, UFFa_02F02, UFFa_16H07, UFFa_03B05, UFFa_08C11, UFFA_15H09, EMFn_202, EMFn_207, EMFn_226, UFFa_01H06, UFFa_04H04) produced from 3 to 6 bands, two primers (UFFa_20G06, UFFa_09B11) showed maximum bands (8 bands, Fig. 3A and Fig. 3B). These results indicated that regenerated plants and the donor plants had identical banding patterns for all of the SSRs primers tested, and there was no morphological and cytological variation, confirming that anther-derived plants were originated from somatic tissue in the anther culture of day-neutral strawberry.

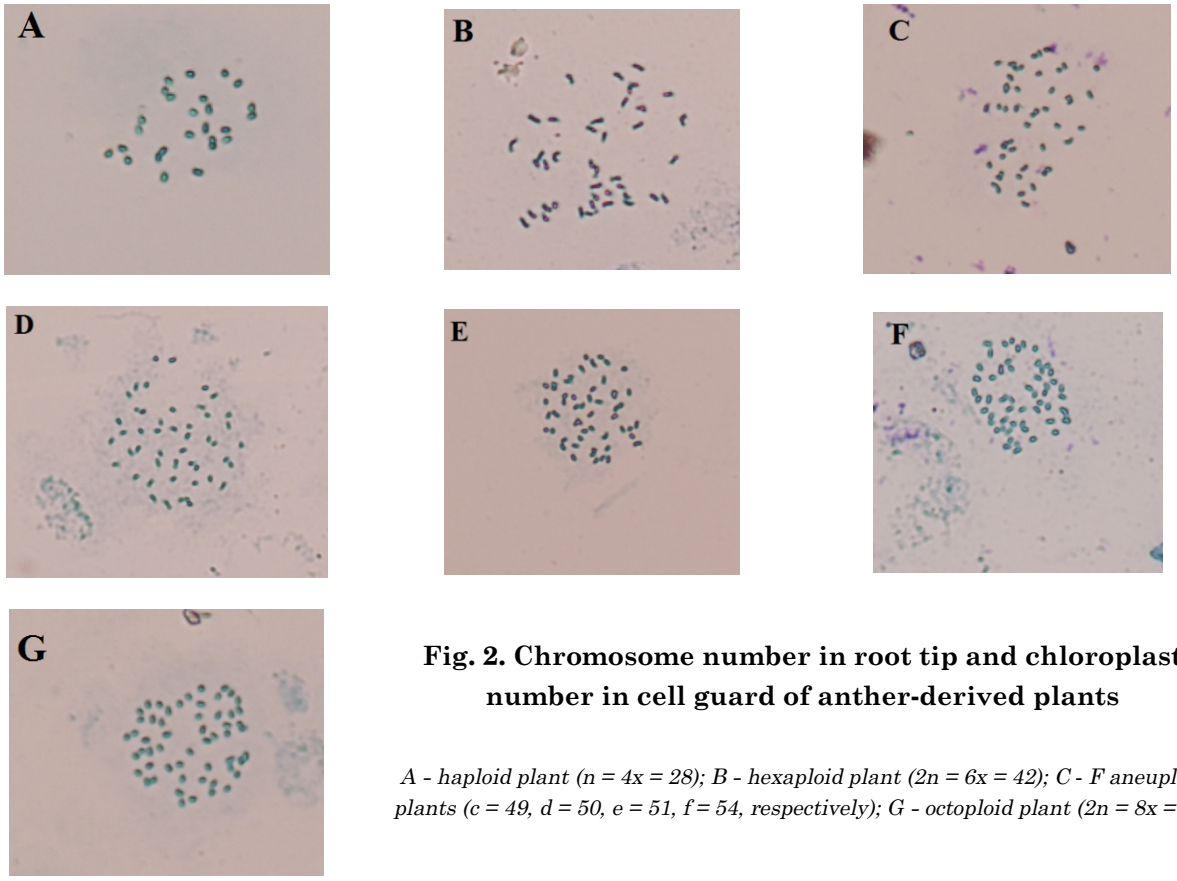

Fig. 2. Chromosome number in root tip and chloroplast number in cell guard of anther-derived plants

A - haploid plant (n = 4x = 28); B - hexaploid plant (2n = 6x = 42); C - F aneuploid plants (c = 49, d = 50, e = 51, f = 54, respectively); G - octoploid plant (2n = 8x = 56)

Table 6. Chromosome and chloroplast count of regenerated plants

Ploidy of regenerants	No. Of chromosomes	Ploidy Ratio (%)	No. Of chloroplast
Haploid (*4x*)	27 ± 1.51	1 (5.0)	28.1 ± 1.39
Hexaploid (*6x*)	41.7 ± 0.13	2 (10.0)	43.4 ± 2.02
Octoploid (*8x*)	56.0 ± 0.00	10 (50.0)	55.2 ± 1.81
Aneuploid	52.2 ± 0.96	7 (35.0)	51.1 ± 1.13

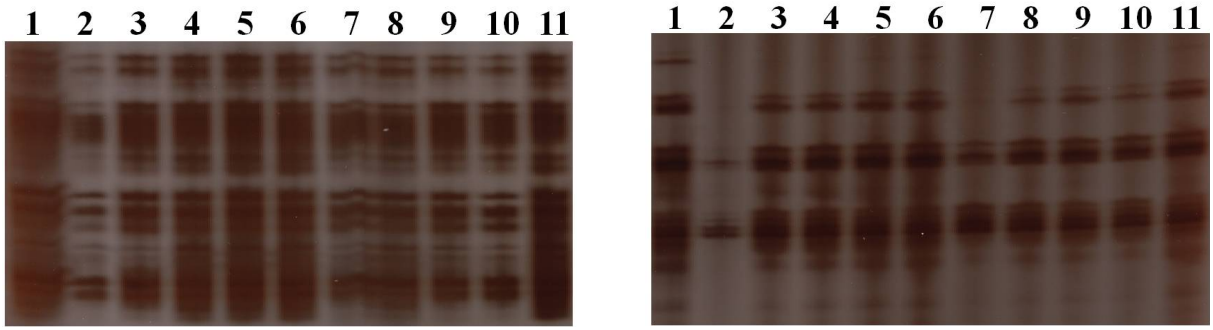

Fig. 3. SSRs primers profiles of donor plants and anther-derived plants using primer UFFa_20G06 (left), UFFa_09B11 (right). Lanes: 1 the donor plant, 2 – 11 regenerated plants

4. DISCUSSION

Selection of optimal age of the anther is very important in the induction of androgenesis because microspores respond only at a specific developmental stage. Generally, the microspores around the mitosis are most responsive. Assani et al. (2003) and Peixe et al. (2004) reported that the anther gave best response when the pollens were at uninucleate stage. Basically, bud size, anther length, anther color are used as an index of the microspore stage. In strawberry, pale green to yellow anthers seemed to be suitable for callus induction (Niemirowicz-Szczytt, 1990). Our results were similar to this finding.

In this study, the plant growth regulators in the culture medium also affected anther callus induction. Niemirowicz-Szczytt and Zakrzewska (1990) reported that a combination of 0.4 mg L^{-1} BA, 0.1 mg L^{-1} IAA and 2.0 mg L^{-1} 2,4-D was good for inducing callus in strawberry anther culture just as the case in our study. Jiajun and Kai-hua (2008) concluded that MS medium supplemented with 2.0 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D produced higher calluses in strawberry cv. 'Dajiangjun' than that of cv. 'Xiangfei'. On the other hand, Perera et al. (2009) showed that a combination of auxin and cytokinin was more beneficial than cytokinin alone for callus induction in coconut anther culture. They stated that kinetin and 2-Isopentyl adenine enhanced the production of callus when 100 μM 2,4-D was present in the culture medium for coconut anther culture. In this experiment, callus formations decreased when BA concentration exceeded 1 mg L^{-1} and this finding was similar to those reported by Nehra et al. (1990). In contrast, Quarta et al. (1991) used only 23 μM kinetin in both solid and liquid media (GD medium) and received higher callus induction rate in strawberry anther culture.

In previous research, Owen and Miller (1996) indicated that the best regeneration was obtained from MS medium containing 2.0 mg L^{-1} IAA + 1.0 mg L^{-1} BA in three American short-day cultivars of strawberry. Whereas, Savel'ev et al. (2010) reported that nine strawberry cultivars produced 10% plant regeneration when calluses were sub-cultured on MS

medium supplemented with 2 mg L^{-1} BA and 1 mg L^{-1} IAA. Zhennan et al. (1995) highlighted that the optimum medium for plantlet regeneration in anther culture was the MS medium supplemented with 2.0mg L^{-1} BA, 2.0 mg L^{-1} Zeatin, and 4.0 mg L^{-1} IAA, which gave highest shoot regeneration rate (40.0%). Nehra et al. (1990) also mentioned that the combination of 10 μM BA and 1 μM αNAA showed better shoot regeneration with minimum callusing from immature leaf in day-neutral strawberry cultivars. However, in the current experiment, shoot formation did not occur when BA concentration exceeded 2 mg L^{-1}. Na et al. (2009) reported that the highest shoot regeneration rate (23%) of short-day strawberry cultivar, namely 'Sulhyang' was obtained when anther-derived calluses were cultured on MS medium supplemented with 4 mg L^{-1} Thidiazuron (TDZ), whereas our study failed to record such levels (data not shown).

Jiajun and Kai-hua (2008) indicated that the physiological condition and developmental stage of explants played an important role in regeneration. Naveed et al. (2009) reported that the highest plant regeneration frequency in wheat embryogenic-callus culture (95% by cv. 'Chakwal-97') was obtained from 26 days old callus, whereas 48 days old callus showed the lowest (13%). Decreasing of regeneration capability with increasing age of callus is a critical problem for callus maintenance and regeneration in *Miscanthus × giganteus in vitro* culture (Kim et al., 2010) and this finding was similar to our results. On the other hand, Singh et al. (2011) reported that the decrease of peroxidase activity can be highly correlated with increasing callus age and affected plant regeneration rate in *Narigi crenulata* (Roxb) culture. In strawberry, the plant regenerated from 8-week-old callus did not show any distinct morphological variants while a significant proportion of deformed leaf shape (6 - 13%) and yellow leaf (21 – 29%) was obtained among plants regenerated from 16 and 24-week-old callus (Nehra et al., 1992). In our results, the 3-month-old callus was degenerated and formed roots (Fig. 1E) as reported by Niemirowicz-Szcytt (1990).

Spontaneous chromosome doubling occurs in haploid cell during anther culture, but another possibility can occur from anther wall or connective tissue of donor plant or from unreduced microspores. It is difficult to distinguish doubled haploids from normal somatic diploid using flow cytometry analysis and cytogenetic observation, because they contain same nuclei and same chromosomes (Diao et al., 2009). The use of SSR markers, known as microsatellites, could identify spontaneous doubled haploid or homozygous plants, because SSR markers are co-dominant, meaning that both alleles of a heterozygous locus can be detected. To date, SSRs have been successfully used for confirmation of homozygous, spontaneous doubled haploids in coconut (Perera et al., 2008), maize (Pedro et al., 2007), *Cucumis melo* L. (Malik et al., 2011). Our results (Fig. 2) showed that there was no morphological and cytological variation, confirming that there is genetic fidelity between regenerated plants and donor plants in the anther culture of day-neutral strawberry.

5. CONCLUSIONS

Altogether, the present results illustrate the possibility of using anther culture to produce haploid plants. However, previous studies indicates the yield of haploid plantlets is low (< 5%). For future application, an isolated microspore culture technique should be used to increase the haploid plant production and to avoid the involvement of anther wall tissue. The step of chromosome doubling is still necessary to make this method readily useable in day-neutral strawberry breeding programs.

REFERENCES

Arnau G., Lallemand J. and Bourgoin M. (2003). Fast and reliable strawberry cultivar identification using inter simpe senquence repeat (ISSR) amplification. Euphytical, 129: 69-79.

Assani A., Bakry F., Kerbellec F., Haicour R., and Foroughi-Wehr B. (2003). Production of haploid from anther culture of banana [*Musa balbisiana* (BB)]. Plant Cell Rep., 21: 511-516.

Bouvier L., Guerif P., Djulbic M., Durel C.E., Chevreau E., and Lespinasse Y. (2002). Chromosome doubling of pear haploid plants and homozygosity assessment using isozyme and microsatellite markers. Euphytica, 123: 255-262.

Chen Y., Hausner G., Kenaschuk E., Procunier D., Drib-Nenki P., and Penner G. (1998). Identification of microspore-derived plants in anther culture of flax (*Linum usitatissimum* L.) using molecular markers. Plant Cell Report., 18: 44-48.

Debnath S.C., Jaime A., and Silva T.D. (2007). Strawberry culture *in vitro*: Applications in genetic transformation and biotechnology. Fruit, vegetable and cereal science and biotechnology Vol.1: 1-12.

Diao W., Jia Y., Hio S., Zhang X., Lou Q., and Chen J.F. (2009). Efficient embryo induction in cucumber ovary culture and homozygous identification of the regenerants using SSR markers. Scientia Horticulturae., 119: 246-251.

Douglas V., and Thomas R.F. (2005). Complex segregation analysis of day-neutrality in domestic strawberry (*Fragaria × ananassa* Duch). Euphytica, 145: 331-338.

Dumas De Vaulx R., Chambonnet B., and Pochard E. (1981). Culture *in vitro* d'antheres de piment (*Capsicum annuum* L.): Amelioration des taux d'obtention de plantes chez differents genotypes par des traitements a +35oC. Agronomie, 1: 859-864.

Germanà M.A (2006). Doubled haploid production in fruit crops. Plant Cell, Tissue and Organ Culture, 86: 131–146.

Grimes H.D., and Hodges T.K. (1990). The inorganic NO_3:NH_4 ratio influences plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.). J Plant Physiol., 136: 362-367.

Jaijun X., and Kaihua N. (2008). Study on factors of affecting callus induction of strawberry. Gansu Agr. Sci. and Techn., 12: 5-8.

Kasumi S., and Keita H. (2006). Development and characterization of simple sequence repeat markers (SSR) to identify strawberry cultivars (*Fragaria × ananassa*. Duch). Japan. Soc. Hort. Sci., 75: 399-402.

Kim, H.S., Zhang G., Yuvik J.A., and Widholm J.M. 2010. *Miscanthus × giganteus* plant regeneration: effect of callus types, ages and culture methods on regeneration competence. GCB Bioenergy, 2: 192-200.

Korea Agro-Fisheries Trade Corp. (2011). Korean Strawberry Fact Book. Research Centre of Fruit Vegetable for Export. The University of Seoul, 3-4.

Kozak K., Galek R., Waheed M.T., and Sawicka-Sienkiewicz E. (2012). Anther culture of *Lupinus angustifolius*: callus formation and the development of multicellular and embro-like structures. Plant Growth Regul., 66:145-153.

Kim M., Jang I.C., Kim J.A., Park E.J., Yoon M., and Lee Y. (2008). Embryogenesis and plant regeneration of hot peper (*Casicum annuum* L.) though isolated microspore culture. Plant Cell Rep., 27: 425-434.

Kumar H.G., Murthy H.N., and Paek K.Y. (2003). Embryogenesis and plant regeneration from anther culture of *Cucumis sativus* L. Scientia Horticulture 98: 213-222.

Malik A.A., Cui L., Zhang S., and Cheng J.F. (2011). Efficiency of SSR markers for determining the origin of melon plantlets derived through unfertilized ovary culture. Horticultural Science, 38: 27-34.

Miella A., Saluzzi D., Lapelosa M., Bertino G., Spada P., and Martelli G. (2006). Relationships between an Italian strawberry ecotype and its ancestor using RAPD markers. Genet. Resour. Crop Evol., 53: 1715-1720.

Mordhorst A.P., and Lorz H. (1993). Embryogenesis and development of isolated barley (*Hordeum vulgare* L.) microspore are influenced by the amount and composition of nitrogen sources in culture media. J Plant Physiol., 142: 485-492.

Lanneri V., and Damiano C. (1980). Strawberry anther culture. Ann Ist Sper Frutti, p.403-416. In: Y.P.S. Bajaj (ed). Biotechnology in agriculture and forestry 12 – Haploid in crop improvement I. Springer-Verlarg.

Lee J.N., Lee E.H., Im J.S., Na C.W., and Yae B.W. (2008). Breeding of new ever-bearing strawberry 'Goha' for summer culture. Kor. J. Hort. Sci. Technol., 26(4): 413-416.

Na H., Hang G., Kim D.Y., and Yoon M.K. (2009). Callus Formation and Plant Regeneration via Embryogenesis from anther culture of strawberry (*Fragaria × ananassa* Duch.). Abstracts of Kor. J. Hort. Sci. Technol., 27 (SUPPL. II).

Naveed, I.R., Bano A., Rashid H., Khan M.H., and Chaudhry Z. (2009). Effect of age of embryogenic callus on plant regeneration in local cultivars of wheat *(Triticum Aestivum* L.*)*. Pak. J. Bot., 41: 2801-2806.

Nehra N.S., Stushnoff C., and Kartha K.K. (1990). Regeneration of plant from immature leaf-derived

callus of strawberry *(Fragaria × ananassa*). Plant Science, 66: 119-126.

Niemirowicz-Szczytt K. (1990). Strawberry (*Fragaria × ananassa* Duch.): *In vitro* production of haploid, pp 403-416. In: Y.P.S. Bajaj (ed). Biotechnology in agriculture and forestry 12 – Haploid in crop improvement I. Springer-Verlarg.

Niemirowicz-Szczytt K., and Zakrzewska Z. (1980). *Fragaria × ananassa* anthers culture. Bull Acad Pol Sci, p. 403-416. In: Y.P.S. Bajaj (ed). Biotechnology in agriculture and forestry 12 – Haploid in crop improvement I. Springer-Verlarg.

Owen H.R., and Miller A.R. (1996). Haploid plant regeneration from anther cultures of three North American cultivars of strawberry *(Fragaria × ananassa* Duch*)*. Plant Cell Reports, 15: 905-909.

Pedro R.B., Guimarães C.T., Paiva L.V., Duarte J.M., Maluf W.R., and Edilson P. (2007). Androgenetic haploids and SSR markers as tools for development of tropical maize hybrids. Euphytica, 156: 95-102.

Peixe A., Barroso A.P., and Pais M.S. (2004). Induction of haploid morphogenic calluses from *in vitro* cultured anthers of *Prunnus Armeniaca* cv. 'Harcot'. Plant Cell, Tissue and Organ Culture, 77: 35-41.

Perera P.I.P., Perera L., Hocher V., Verdeil J.L., Yakandawala D.M.D., and Weerakoon L.K. (2008). Use of SSR markers to determine the anther-derived homozygous lines in coconut. Plant Cell Report. 27: 1697-1703.

Perera P.I.P., Yakandawala D.M.D., Hoccher V., Verdeil J.L., and Weerakoon L.K. (2009). Effect of growth regulators on microspore embryogenesis in coconut anther culture. Plant Cell Tissue Organ Culture, 96: 171-180.

Promega Corporation (2011). Technical manual: GenePrint® STR Systems (Silver stain detection). http.//:www.prome.com.

Quarta R., Nati D., and Paoloni F.M. (1991). Strawberry anther culture. Acta Horticulture 300. *In vitro* culture, p. 335-339.

Rosati P., Devreux H., and Laneri V. (1975). Anther culture of strawberry, p. 403-416. In: Y.P.S. Bajaj (ed). Biotechnology in agriculture and forestry 12 – Haploid in crop improvement I. Springer-Verlarg.

Ruan J.W., Murti R., Lee Y.H., and Yeoung Y.R. (2009). Strategy for late summer and autumn fruit production system of June bearing strawberry cultivars by high land short day treatment. Kor. J. Hort. Sci. Technol., 27 (Suppl. I): 55. (Abstr.).

Ruan J., Yeoung Y.R., and Larson K.D. (2011). Influence of cultivar, planting date, and planting materials on yield of day-neutral strawberry cultivars in highland area of Korea. Hort. Environ. Biotechnol., 52(6): 567-575.

Salas P., Rivas-Sendra A.P., and Seguí-Simarro J. (2012). Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther culture. Euphytica, 184: 235-250.

Savel'ev N.L., Oleinikova O.Y., Il'ina N.S., Turovtseva E.S., and Chivilev V.V. (2010). *In vitro* androgenesis of fruit and berry plants. Russian Agricultural Science, 36: 15-17.

Silva T.D. (2010). Indica rice anther culture: can the impasse be surpassed. Plant Cell Tissue Organ Culture, 100: 1-11.

Singh N., Meena M. K., and Patni V. (2011). Effect of plant growth regulators, explants type and efficient plantlet regeneration protocol through callus induction in *Naringi crenulata* (Roxb.) Nicolson and its biochemical investigation. African Journal of Biotechnology, 10: 17769-17777.

Truong X. N., Song Y.S., and Park S.M. (2012). Haploid plant production through anther culture in day-neutral strawberry (*Fragaria × ananassa* Duch) cv. Albion. Journal of International Society for Southeast Asian Agricultural Sciences, 18(1): 173-184.

Zhennan X., and Shuyuan W. (1995). Studies on factors affecting strawberry anther culture efficiency. Acta Agriculturae Shanghai. 11: 27-30.