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Effect of Genotype of Donor Plants on the Success of Anther Culture in Sweet Pepper (*Capsicum annuum* L.)

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Abstract - To study the effect of genotype of donor plants on anther culture, anthers of nine hybrid cultivars (Derby, Special, Bossanova, Minipaprika, Fiesta, Boogie, Phenlene, Kufrah, and Clarity) of sweet pepper (*Capsicum annuum* L.) were cultured in a petridish containing C medium (Sibi, Dumas De Vaulx medium) supplemented with 0.1 mg/L 2,4-D and 0.1 mg/L kinetin, 3% sucrose and 0.32% Phytagel. The cultures were incubated in the dark at 35℃ for seven days, and then cultured at 25℃ with a photoperiod of 16 hr. daylength for 40 days. Frequency of callus formation and plant regeneration was varied among cultivars. Callus formation was ranged from 6% in Phenlene to 69.8% in Kufrah. The highest percentage of regenerated plantlets was obtained in cv. Phenlene (2.67%) followed by Bossanova (2.41%). Result of ploidy analysis; chromosome number observation and flowcytometry analysis, showed that haploid plants could be developed from all of these hybrid cultivars except cv. Fiesta, where highest percentage of haploid plants were obtained in Minipaprika (40%) followed by cv. Bossanova (36.1%). Haploid plants derived from these hybrid cultivars contained single set of chromosome (12 in numbers), higher stomata density (numbers), and smaller sized stomata as compare to diploid plants. The mean length of stomata was 26.9 ㎛ in haploid plants and 35.7 ㎛ in diploids.

Key words - Anther cultured plantlets, callus, *Capsicum annuum* L., embryoid, pure lines, regeneration

Introduction

Sweet pepper (*Capsicum annuum* L.) is a most important vegetable crop in Korea, both in terms of cultivated area and economic value. However, the yield of Korean varieties is lower than imported varieties and over all; yield is lower than that of European and American countries. There is clearly a need in Korea for new breeding technique for sweet pepper. One such technique is haploid technology. Anther culture is usually used for production of haploid plants from microspore and is utilized for a rapid large scale production of homozygous lines from which superior lines for hybrid seed production might be selected. The technique of anther culture was first developed in *Datura* by Guha and Maheshwari (1964, 1966). Ever since the first report, the production of haploid in *Capsicum annuum* L. has been reported by many researchers (Dumas *et al*., 1981; Eun *et al*., 1994; Harn *et al*., 1975; Matsubara *et al*., 1992, 1998; Sakata *et al*., 1991; Wang *et al*., 1973). It has long been thought that haploid technology offered the breeder a short-cut method for obtaining homozygous diploid lines in one step, by doubling the chromosomes of haploids. Utility of this technique offered great interest to plant breeders because double haploids can be used immediately as homozygous breeding lines. The efficiency in producing homozygous breeding lines via doubled *in vitro* produced haploids represents significant savings in both time and cost compared with other methods (Bajaj, 1990; Evans *et al*., 1984; Hu and Zeng, 1984). Double haploid as a result of its diploidization, is a good initial material for the genetic improvement of *Capsicum annuum* (Nowak and Nowaczyk, 2005).

Numerous factors; temperature and photoperiod (Kristansen and Andersen, 1993), thermal shock and differences among cultivars (Sakata *et al*., 1991), and donor plant environment (Bajaj, 1990) have been found to influence anther culture response in sweet pepper. Among them the anther culture efficiency was depended on the genotype of parents (Kim *et al*., 1999; Yoon *et al*., 1991). *In vitro* techni-

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ques for the culture of protoplast, anthers, microspores, ovules and embryos have been used to create new genetic variation in the breeding lines, often via haploid production (Brown and Thorpe, 1994) where *in vitro* anther culture of pepper is emphasized as a tool for obtaining double-haploid plants for practical breeding purpose, but several evidences indicates the importance of genotypes for the production of haploid plants from cultured pepper anthers (Qin and Ratino, 1995).

The purpose of the present investigation was to study responses of anther culture to anthers donor sweet pepper cultivars and develop anther culture derived plantlets for initial material for hybrid breeding purpose.

Materials and Methods

Plant material

Sweet pepper (*Capsicum annuum* L) cultivars such as Derby, Special, Bossanova, Minipaprika, Fiesta, Boogie, Phenlene, Kufrah, and Clarity were grown in a sweet pepper grower plastic house and unopened flower buds were collected. Unopened flower buds, which have anthers containing uninucleate microspores, are most suitable for the induction of androgenesis.

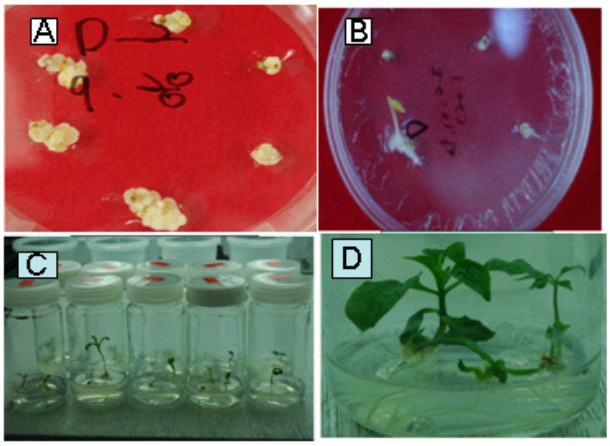

Fig. 1. Plantlets regenerated from the anther culture of Sweet pepper (*Capsicum annuum* L.) cv. Special. A: Callus formed from the anthers sown in C1 medium. B: Embryos developed after 6 weeks of culture. C: Plantlets regenerated after transferring to hormone-free MS medium. D: Plantlets ready for transplanting for acclimatization. Size of Petri dish and Glass bottle is 85 x 15 mm and 13 x 7 cm, respectively.

Flower buds sterilization

Flower buds were soaked in 70% ethanol for 30 seconds. Subsequently, they were sterilized for 15 min with 15% sodium hypochlorite containing two drops of tween 20 as a spreader followed by rinsing three times with sterile distilled water. First an incision was made on one side of the flower bud and the stamens were gently taken out with a pair of fine forceps and collected in a sterile petri dish. The filament from the stamen was then carefully removed and five anthers are normally transferred to each culture vessel. During excision of anthers special care was taken to ensure that they are not injured in any way. Damaged anthers were discarded, as they often tend to produce callus from parts other than pollen.

Culture media for anther culture

Demas De Vaulx media (C media) was supplemented with 0.1 mg/L 2,4-D, 0.1 mg/L kinetin, 3% sucrose, adjusted to pH 5.8, and solidified with 0.32% phytagel. Media autoclaved at 1.2 kg/cm^2, 120℃ for 15 min were dispensed in 5 mL aliquots into petri dishes (size 85x15 mm), and the petri dishes were sealed with parafilm in clean bench. Hormone-free MS medium was composed of MS basal medium supplemented with 3% sucrose.The pH of the medium was adjusted to 5.8, and solidified with 0.2% Gelrite.

High temperature treatments

The cultures were then incubated at 35℃ for one week

Fig. 2. Plantlets (regenerated from the anther culture of Sweet pepper (*Capsicum annuum* L.) cv. Special) transferred to small pots (8 x 8 cm) and big pot (24 x 25 cm) containing horticulture soil media, subsequently.

under dark condition, followed by incubation at 25℃ under 16 h photoperiod with 20 μmol · m^{-2} · s^{-1} of fluorescent light for 40 days.

Plant regeneration from embryoids

The cotyledonary embryos grown from the embryoids were transferred to hormone-free MS medium under the same conditions as the anther culture.

Acclimatization

Regenerated plantlets were transferred to plastic plug (6 cm x 6 cm) containing a mixture of sterilized vermiculite supplemented with one-fourth strength of MS inorganic salts and acclimatized under 16 h photoperiod at 25℃, 20 μmol · m^{-2} · S^{-1} of fluorescent light. The plantlets were covered with a vinyl film for the first 30 days, after which they were transferred to 8 cm x 8 cm diameter plastic pots containing horticulture soil (Bio Sangtho, Seminis, Korea) and transferred to plastic house and later transplanted on 24 cm x 25 cm plastic pots for its growth.

Ploidy level test

To analyze the ploidy level, chromosome numbers of cells were investigated on root tip cells. About 1 centimeter long root tips were excised from plants and placed in freshly prepared fixative aceto-alcohol (glacial acetic acid: absolute ethy alcohol = 1:3, v/v) for 8 to 12 hours in 4℃. Root tips were washed thrice with distilled water and cut 1 millimeter small portion of growing tip with a sharp dissecting blade on a glass slide for slide preparation and stained with 2% aceto-orcein solution following the method described by Tyagi and Kancherla (1991). Macerated root tips were squashed in a drop of aceto-orcein stain on a clean glass slide, covered with glass cover slip, heated and pressed to spread the chromosomes. Prepared slides were observed under bright field illumination. Likewise, for the flow cytometry analysis, newly growing leaves were crushed slightly using sharp razor blade in a nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA; Partec, Munster, Germany). After filtration through a 30-um nylon sieve, a staining solution containing the dye (4,6-diamdino-2-phenylindole-2HCl (solution B of the kit)) was added. The mixture was analyzed using PAS flow cytometry (Partec company, Germany). Histograms of DNA content were evaluated using Partec software package. Routinely, at least 10,000 nuclei were observed for each leaf of each individual plant.

Fig. 3. Plant appearance of anther culture derived (cv. Boogie). A: Diploid with vigorous, big leaf size, longer internodes and taller, B: Haploid with small leaf size, shorter internodes, weak and dwarf. This trend of morphological appearance was similar in other experimental cultivars.

Results and Discussion

The Percentage of calli formed from anthers in cv. Kufrah, Clarity, Special, Boogie, Derby, Fiesta, and Phenlene were 69.8%, 59.9%, 43.4%, 39.6%, 35.2%, 14.1% and 6.0%, respectively. These result supports to Matsubara *et al*. (1998), who had obtained embryoid and callus formation in all cultivars, however, in the result the frequency of embryoid formation was higher in cv. Cheongyang and cv. Fushimi Amanaga. In this study, when the calli were transferred to the media for induction of plantlets, the percentage of plantlets regenerated was ranged from 0.04% in cv. Clarity to 2.67% in cv. Phenlene (Table 1). The present study is in agreement with the previous report where the efficiency of embryogenesis depending in the genotypes was varied from 0.8% to 12.0% in cultured anthers (Eun *et al*., 1994 and Sakata *et al*., 1991) where they found a significant difference in the responses of anther culture among cultivars. Similarly, Qin and Ratino (1995) reported the importance of genotypes for the production of haploid plants from cultured pepper anthers. They repeatedly observed that various cultivars exhibited

Table 1. Frequency of callus induction, plant regeneration by culture of anthers from nine sweet pepper hybrid cultivars

Cultivars	No.of anthers cultured	No. of callus formed	No. of plants regenerated	Haploid plant (%)
Derby	8400	2960 (35.2)[z]	98 (1.16)[y]	32.8
Special	14000	6080 (43.4)	83 (0.59)	20.0
Bossanova	1700	-[x]	41 (2.41)	36.1
Minipaprika	500	-	11 (2.2)	40.0
Fiesta	1095	153 (14.1)	26 (2.4)	0.0
Boogie	6100	2420 (39.6)	109 (1.78)	21.9
Phenlene	750	45 (6.0)	20 (2.67)	25.0
Kufrah	8160	5700 (69.8)	12 (0.15)	21.3
Clarity	5040	3020 (59.9)	2 (0.04)	0.0

Numbers in parentheses are: [z] Percent of callus formed relative to the total number of anthers cultured, [y] Percent of regenerated plants relative to the total number of anthers cultured.
-[x] data not collected

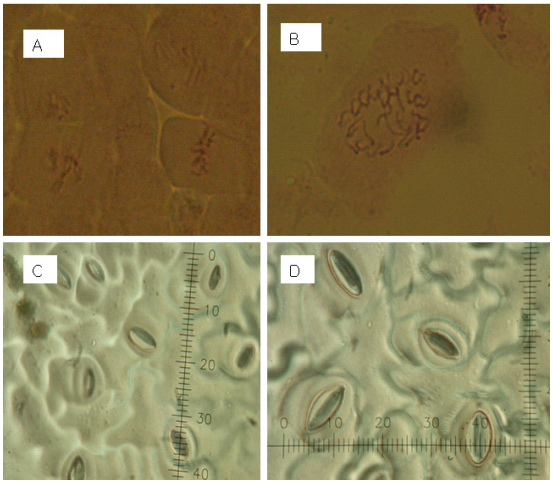

Fig. 4. Cytological differences between haploid and diploid plants: Chromosomes observed (1000X) in root tip cells of A: Haploid and B: Diploid plants stained with aceto-carmine Stomata density and size; C: Epidermal peel from regenerated haploid, B: Epidermal peel from regenerated diploid plants.

different growth responses in culture. Bajaj (1977) reported that out of 21 cultivars of *Triticum aestivum*, haploid tissue could be obtained from anthers of only ten cultivars. Similarly, Chahal and Gosal (2002) reported that the genotypic differences for the response to anther culture existed at genera, species or even varietals levels. One of the reasons for failure in anther culture is that most workers restrict themselves to one cultivar and abandon the work if unsuccessful. It is highly desirable that a general survey of various cultivars should be undertaken.

The chromosome numbers in haploid and diploid plants were 12 and 24, respectively (Fig. 4). In this study, result showed that haploid plants could be developed from all of these hybrid cultivars except cv. Fiesta. The highest percentage (40%) of haploid plants was obtained from cv. Minipaprika followed by cv. Bossanova (36.1%) whereas the lowest was from cv. Special (20%) (Table 1). This result is close to the result of Dumas *et al*. (1981) who reported up to 50% haploid, depending on cultivars and culture methods. Yoon *et al*. (1991) had also obtained 47% haploid and 53% diploid plants in their pepper anther culture. They further mentioned that efficiency of anther culture depended on the genotype of donor plants. Spontaneous chromosome doubling during callus proliferation causes straight regeneration of double haploids (Chahal and Gosal, 2002; Keller and Amstrong, 1983) where frequencies of haploids/double haploids vary with the plant species and the cultural conditions.

In the present study, the average stomata length of diploid and haploid plants differed markedly, with diploid stomata being longer than haploid stomata (Fig. 4). The mean length of stomata in haploid and diploid plants was 26.9 ㎛ and 35.7 ㎛, respectively, that is 56% higher than haploid plant (Table 2). An identical difference was also reported by Przywara *et al*. (1988) in Kiwifruit (*Actinidia deliciosa*). They had reported that the mean stomatal length 24±1.7 ㎛ for haploids and 33±2.4 ㎛ for diploids.

Table 2. Morphological and cytological characteristics of anther culture derived plantlets from seven hybrid cultivars (Derby, Special, Bossanova, Minipaprika, Boogie, Phenlene and Kufrah)

Ploidy level of plant	Plant height (cm)	Leaf Size		Fruit size (gm)	Internodes (cm)	Stomatal length (㎛)
		Length (cm)	Width (cm)			
Haploid	58.0±17.5 (39) [z]	7.1±1.4 (390)	3.8±0.96 (390)	25.3±11.1 (786)	2.8±0.96 (390)	26.9±4.2 (170)
Diploid	74.1± 23.3 (111)	9.4±1.4 (1110)	5.5±0.9 (1110)	67.1±29.1 (1696)	3.9±1.4 (1110)	35.0±3.1 (435)

[z] Numbers in parentheses are number of samples used for calculation

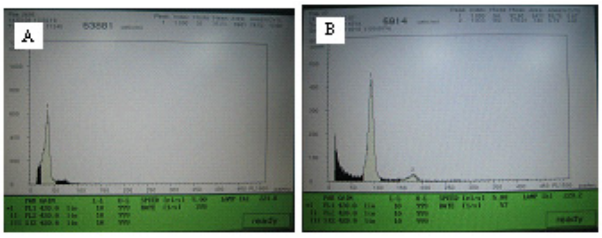

Fig. 5. Histogram shown by flow cytometry analysis of A: Haploid plant, B: Diploid plant.

Isogenic diploids can be obtained by chromosome diploidization. Although it is possible by conventional inbreeding and back crossing to obtain pure lines, it is a time-consuming process. These fertile homozygous plants can be used for producing the inbred lines required to utilize hybrid vigor (Nowak and Nowaczyk, 2005). Double haploid plants obtained in this study will be a good initial material for the genetic improvement of *Capsicum annuum* L., especially for heterosis breeding. This anther culture system has been used to produce many double haploid plants of bell pepper in breeding programs (Abak *et al*., 1982; Caranta *et. al*., 1996; Daubeze *et al*., 1990; Dumas and Pochard, 1986; Hendy *et al*., 1985; Pochard *et al*., 1983).

In morphological characters, haploid plants had poor vigor, thinner stem, shorter internodes, and smaller size of leaf and fruits as compare to diploid plants. In the present study, haploid fruits failed to produce seeds with some exception (Fig. 3). In cytological characters, mitotic cell contain 12 numbers of chromosomes and density of stomata numbers were higher in haploid plants. However, the size of stomata in leaves was smaller as compare to diploid plants (Fig. 4). In average, diploid plants had higher plant height, bigger leaf size, longer internodes and bigger fruit size as compare to haploid plant. Plant height measured on diploid and haploid plant was 74.1 cm and 58.0 cm, respectively. Similarly, leaf length; 9.4 and 7.1, leaf width; 5.5 and 3.8 cm, respectively. Likewise, internodes length of diploid plants was longer than that of haploid plant (2.8 cm) and fruit size was also bigger than that of haploid plant (25.3 gm) (Table 2). It supports the statement of Chahal and Gosal (2002) who had reported that cell size and vigor of the plant increased as the ploidy level increased. Most of the regenerated plants showed variation in their characters compared with that of their mother plants in terms of its vegetative, fruit, and yield parameters.

Hence this study conclude that the success of anther culture depend on the genotype of donor plant where cv. Phenlene and Bossanova were found having more percentage of regenerated plants among the nine hybrid lines as a source of anthers and these anther culture derived dihaploid plants will be initial material for varietal improvement activities especially for hybrid (heterosis) breeding.

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