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ORIGINAL ARTICLE

Production of Haploid and Doubled Haploid Plants from Isolated Microspore Culture of Hot Pepper (*Capsicum annuum* L.)

고추 소포자를 이용한 반수체 및 배가반수체 생산

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ABSTRACT

Haploid/double haploid plants developed from isolated microspores can significantly accelerate plant breeding. Haploid plants can naturally double their chromosomes to create a pure homozygous line of diploid plants. We present a method for producing embryos from isolated microspores of hot peppers (*Capsicum annuumL.*). We analyzed the polyploidization levels of the regenerated plants. The donor plants produced the optimal stage of microspores following short-term growth under low-intensity light, which resulted in high rates of embryogenesis and cotyledonary embryogenesis. To find an efficient culture method, liquid, doubled-layer, and 2-step cultures were tested. Liquid culture yielded the highest number of embryos, whereas the highest efficiency for cotyledonary embryogenesis was afforded by the doubled-layer culture. When normal cotyledonary embryos were transplanted onto a regeneration medium, they developed into complete plants. From these, 208 plants were tested via flow cytometric analysis, and 35.6% and 72.7% of the chromosomes from the Milyang-jare and LV2319 genotypes, respectively, were found to be spontaneous double haploids. These results are the same as those obtained on analyzing horticultural characteristics, including the size of leaves and the size and shape of fruits. The present study provides information on the practical application of isolated microspore culture of hot peppers, factors that affect embryogenesis, and methods for polyploidy testing.

Key Words : Cotyledonary embryogenesis, Culture method, Donor plant, Genotypes, Light conditions

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INTRODUCTION

A haploid plant has only one set of chromosomes and because it can generate pure lines of homozygous double haploid plants, it is very useful for plant breeding (Kim et al., 2010; Popova et al.,

2016). Haploid/double haploid plants are useful for application in breeding programs and studies on genetic engineering, mutations, metabolic changes, embryogenesis, genetic mapping, and marker development (Bhatia et al., 2017; Popova et al., 2016).

Because isolated microspores are unicellular and haploid, they serve as useful material for the production of haploid plants. Compared to an anther culture, an isolated microspore culture lacks the probability of regeneration from anther tissue and is simpler and more efficient, being less labor intensive (Zeng et al,. 2015), which makes it the best method for developing double haploid plants. Despite this fact, the microspore culture method is still difficult because it requires a high degree of skill, which acts as a limiting factor for the advancement of its use (Iricova et al,. 2011)

Although studies on anther, shed-microspore, and isolated microspore cultures are being actively pursued for the generation of red pepper microspore-derived haploid plants, anther cultures are primarily used for the development of new cultivars. However, hot peppers have the disadvantage of being one of the crops with difficult androgenesis, i.e., a low frequency of embryogenesis from anthers, and a low plant regeneration rate (Popova et al., 2016). Moreover, the percentage of double haploid plants generated from hot pepper anther or microspore cultures tends to be low (Dumas de Vaulx et al., 1981; Gyulai et al., 2000; Kim et al., 2013; Supena et al., 2006a).

Factors that are known to affect embryogenesis of hot pepper microspores include genotype, medium composition, culture method, donor plant growth, microspore developmental phase, stress, and specific genes (Irikova et al., 2011; Kim et al., 2013). To develop a method that can increase the efficiency of embryogenesis under growth conditions where season and temperature cannot be controlled, the in the present study, growth under low-/high-intensity light and short-/long-term conditions was attempted. The present study also investigated microspore embryogenesis and development according to culture methods. Furthermore, the correlation between flow-cytometric analysis results and horticultural characteristics were analyzed for their potential in polyploidy testing of microspore-derived plants.

MATERIALS AND METHODS

Experimental materials

In this study, we used three genotypes (Milyang-jare, LV2319, and SNU2) of hot peppers (*Capsicum annuumL.*). Selected seeds were planted to a 32-well tray and grown for 3~4 weeks; after which, seedlings that exhibited emergence of 3~4 true leaves were selected and transplanted into pots with a diameter of 25 cm. The soil used for seedling transplantation was commercially available horticultural top soil. The plants were watered every morning, and fertilizer treatment was applied in 1-week intervals, starting 2 weeks after transplanting the seedlings.

The donor plants were grown for 3~7 months in a greenhouse located at the National Institute of Horticultural and Herbal Science. The peak light intensity in the greenhouse was 1500~2000 μmol m-2s-1. To prevent weakening of the seedlings, buds that had surpassed their optimal period were removed every day, and material was only used up to 16 weeks after transplanting.

To determine the effects of growth period of the donor plant and light conditions on microspore embryogenesis, the plants were grown for 8~12 weeks for the short-term condition and for ≥16 weeks for the long-term condition. A light shield was used to control light intensity to 150 μmol m-2s-1 for the low-intensity light condition.

Isolation and harvesting of microspores

To use only pollen from the late uninucleate stage to the early binucleate stage, which is the optimal culture period for microspores, buds with purple coloration on 1/4–3/4 of the embryos were collected (Kim et al., 2004). For sterilization, isolation, and harvesting of buds, a blender was used to produce a hot pepper isolated microspore culture system (Kim et al., 2008).

The medium used during isolation and harvesting of microspores was NLNS medium containing 0.37 M mannitol (Kim et al., 2010), which had the same composition as the medium used during pretreatment.

Microspore pretreatment and culture

Isolatedmicrospores were treated for 3 days at a high temperature of 31 ± 1°C. For pretreatment of microspores, isolated microspores were calibrated to a density of 20 × 104per 1mL and 8mL was dispensed onto each 90 × 20 mm culture dish for the high temperature treatment. The medium used for microspore culture was NLNS medium containing 10% sucrose. Microspores were calibrated to a density of 10 × 104 per 1 mL and 1.5 mL was dispensed on each 60 × 15 mm culture dish, and culture was conducted under dark conditions at 25° C. One day after culturing, 1.5 mL of new medium was added to each culture dish. For the experiment on the effects of culture method on normal cotyledonary embryogenesis, liquid and double-layer culture methods were used, as well as a 2-step culture method using the double-layer culture after 1 week of liquid culture.

In the present study, each experiment was performed three times, with five to seven repeated treatments. After 3 weeks of culture, the number of embryos generated was counted from a single culture dish using a dissecting microscope with ×10 magnification and the mean and standard deviation were derived. Normal cotyledonary embryos generated were developed into seedling plants by transplanting onto 1/2MS medium containing 2% sucrose and 0.4% phytagel.

Flow cytometric analysis and analysis of horticultural characteristics of haploid/double haploid plants

To test the polyploidy levels of microspore-derived plants, the correlation between DNA content in leaf cells obtained from flow cytometric measurement and horticultural characteristics of the plants were examined. The materials used for flow cytometry were young leaves of microspore-derived plants and donor plants grown under the same conditions. Horticultural characteristics of the plants measured, included plant length, leaf length and width, flower size, and fruit size and shape.

RESULTS

Microspore embryogenesis and the plant production process

The flower buds were blended with a micro-blender, followed by vortexing and centrifuging to harvest pure microspores with somatic cell debris removed. The microspores were pretreated at a high temperature of 31±1°C, and subsequently, embryogenic microspores were generated after 3 days of culture and proembryos were develoed after 7 days of culture. After 3 weeks of culture, a mixture of globular, heart-shaped, torpedo-shaped, and normal cotyledonary embryos was generated. Among the embryos generated, normal cotyledonary embryos were transplanted onto a regeneration medium; after which, they developed into normal seedling plants. One week after transplantation onto the regeneration medium, the cotyledons changed

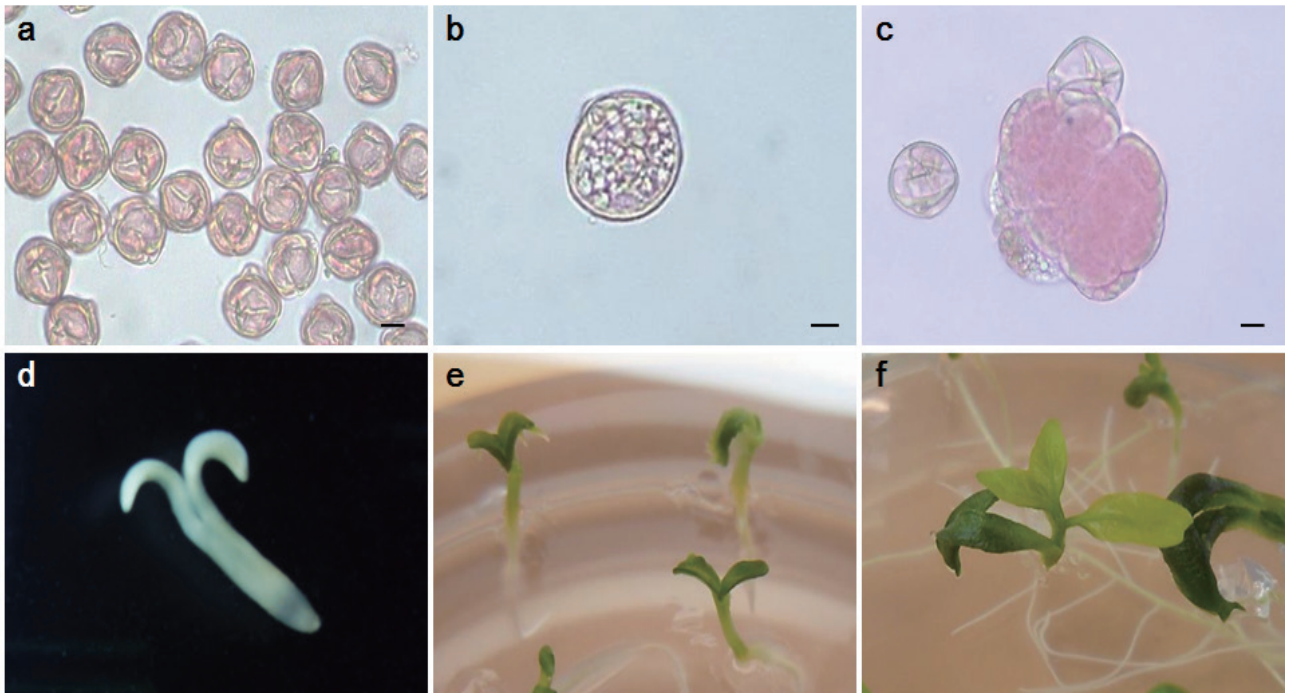


Fig. 1. Microspore embryogenesis and plant regeneration in isolated microspore culture of hot pepper (*Capsicum annuum* L.). a, Freshly isolated microspore at the initial stage of culture. b, Embryogenic microspore after 3 days of culture. c, Multicellular embryo after 7 days of culture. d, Microspore-derived cotyledonary embryo after 3 weeks of culture. e, Embryos after 1 week on regeneration medium. f, Plantlets developed from embryos after 3 weeks on regeneration medium. Scale bars indicate 10 μm in a-c.

to a green color, and after 3 weeks, two to three true leaves developed (Fig. 1).

Effects of growth period and light conditions during donor plant growth on microspore production

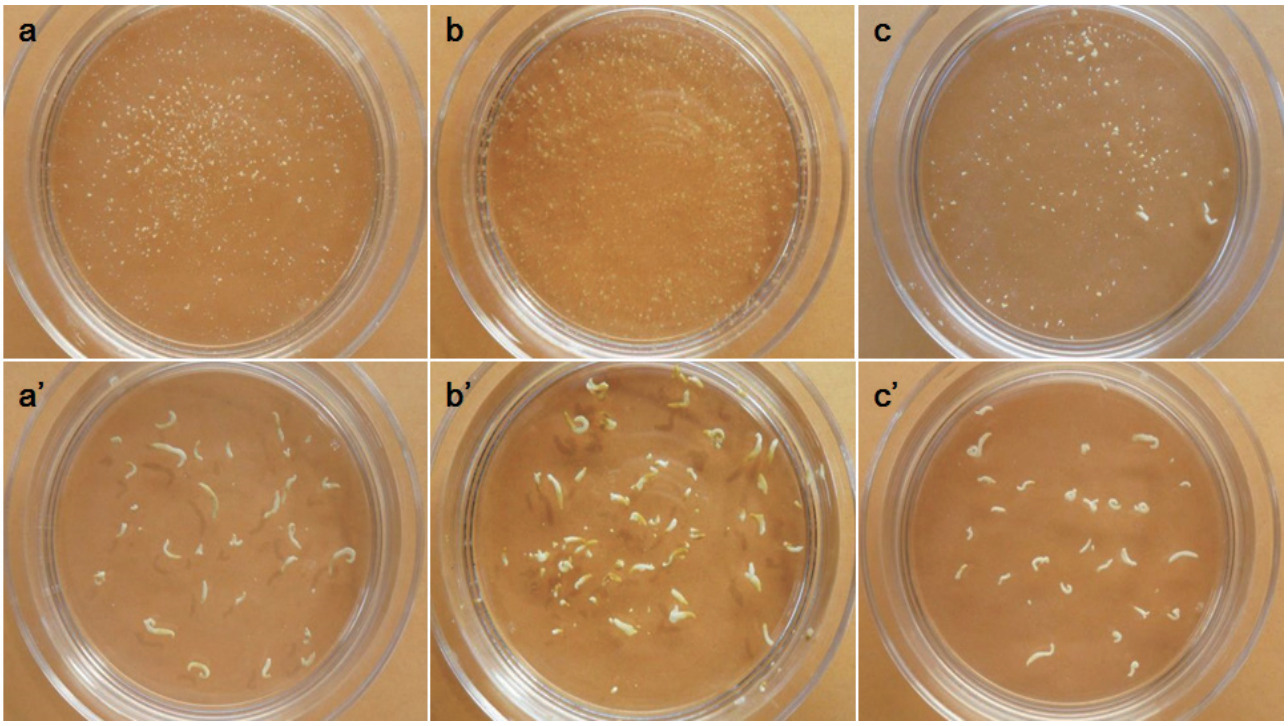
We tested the effects of light intensity and duration of growth of the donor plants on the generation of total and cotyledonary embryos from the induced microspores. The total number of embryos generated from liquid culture of microspores harvested from donor plants grown under low-intensity light and short-term conditions was 115.7, which was ≥1.5 times higher than that of low-intensity light and long-term conditions (Table 1). When double-layer culture was used for the generation of cotyledonary embryos, generation of normal cotyledonary embryos was ≥1.5 times higher for the short-term growth condition. Based on these results, it was determined that shorter growth period of the donor plant was favorable for microspore and cotyledonary embryogenesis.

In the long-term growth and high-intensity light condition, the degree of microspore development could not be predicted by the percentage of petals and sepal, or coloration of the anther. Even very small buds contained mostly late binucleate stage pollen that was in a stage beyond the optimal microspore culture stage, and thus, culture could not be attempted. In older plants, it was difficult to harvest only microspores in the optimal stage, and culture efficiency was greatly reduced (Supplementary Fig. 1). These results demonstrated that growing donor plants under low-intensity light is most suitable for induction of microspore embryos and generation of cotyledonary embryos.

Table 1. **Embryo development according to growth period and light condition of donor plant of embryos in isolated microspore culture of hot pepper**

Light condition – Donor plant age	Culture method	No. of embryos/plate			
		Globular &Heart	Torpedo & Cotyledonary	ELS	Total
High – Young	Liquid	39.4±11.8	0.0±0.0	19.5±4.5	58.9±9.7
Low – Young		85.5±9.8	0.0±0.0	30.2±7.8	115.7±11.6
Low – Old		53.1±9.7	1.7±1.5	20.8±5.5	75.6±10.7
High – Young	Double-layer	6.5±3.5	21.1±4.4	9.5±3.9	37.1±6.8
Low – Young		13.0±4.1	19.6±5.8	38.9±8.4	71.5±9.3
Low – Old		4.3±2.5	9.7±3.0	8.1±3.3	22.1±5.9

ELS indicates embryo-like structure.



Supplementary Fig. 1. Results of microspore-derived embryo development after 3 weeks of culture. Culture method; a-c, Liquid culture, a'-c', Double-layer culture. a, a', High light/young plant, b, b', Low light/young plant, c, c', Low light/old plant.

Microspore embryogenesis and cotyledonary embryo production according to culture method and genotypes

To investigate the effects of culture method on embryogenesis and development, pretreated microspores were subjected to liquid, double-layer, and 2-step cultures. The double-layer culture is a method in which a layer of liquid medium containing microspores is placed on top of a solid medium, whereas the 2-step culture is a method in which the double-layer culture is performed for 1 week after liquid culture. The results from using these three types of culture methods indicated that the highest number of embryos were generated from the liquid culture method for the Milyang-jare and LV2319 genotypes, whereas generation of normal

cotyledonary embryos was highest using the double-layer culture method (Table 1, Fig. 2).

For the Milyang-jare genotype, the number of induced embryos was highest from the liquid culture with 95.6, followed in order by the 2-step and double-layer culture methods. The number of

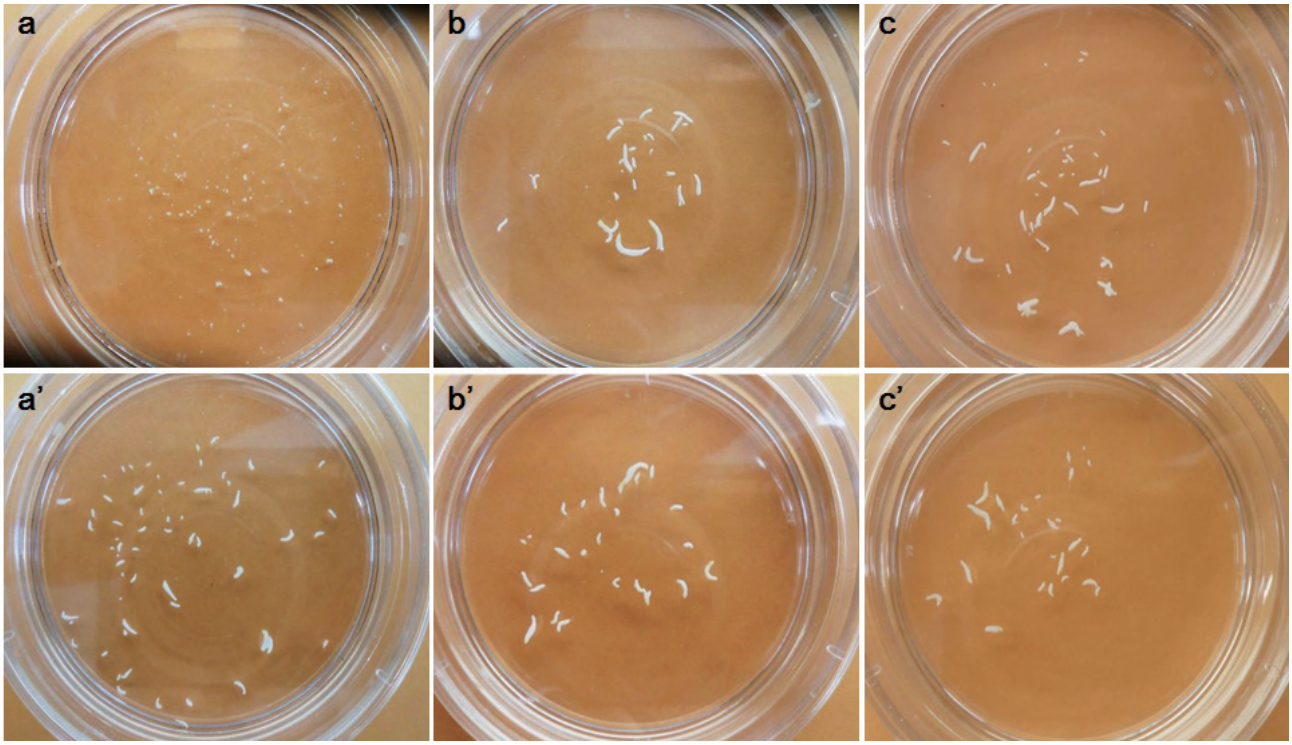


Fig. 2. Comparison of embryos development from microspore cultured in culture method with different genotype. Genotype is a–c Milyang-jare, a'–c' LV2319. Culture method a, a' Liquid culture. b, b' Double layer culture. c, c' 2-step culture.

normal cotyledonary embryos generated was highest from the double-layer culture with 23.8, followed by the 2-step culture with 14.0. However, there was no cotyledonary embryo generated from the liquid culture.

On the other hand, for the LV2319 genotype, the number of induced embryos was highest from liquid culture with 55.9, followed by the double-layer culture with 35.8, and the 2-step culture with 30.5. The number of normal cotyledonary embryos generated was highest from double-layer culture with 17.0, followed in order by the 2-step and liquid cultures.

When isolated microspores from Milyang-jare, LV2319, and SNU2 genotypes were cultured, multiple embryos and normal cotyledonary embryos were successfully obtained from Milyang-jare andLV2319, whereas only a small number of embryos in early-stage of development were obtained from SNU2. Moreover, the results from the liquid culture revealed large differences in embryogenesis with the total number of induced embryos being 95.6 and 55.9 from Milyang-jare andLV2319, respectively. Because the increased number of induced embryos created greater nutrient competition, the Milyang-jare genotype was unable to generate normal cotyledonary embryos, whereas LV2319 had fewer induced embryos, which allowed for multiple cotyledonary embryos to generate even in the liquid culture (Table 2). The results of the present study demonstrated that liquid culture was suitable for embryogenesis, whereas the double-layer culture

was suitable for producing normal cotyledonary embryos. In addition, the efficiency of embryogenesis based on genotype should be considered.

Regeneration of microspore-derived plants

When normal cotyledonary embryos generated by culture were transplanted onto regeneration medium, approximately 95% developed into

Table 2. Effect of culture method on the production of embryos in isolated microspore culture of hot pepper

Genotype	Culture method	No. of embryos/plate			
		Globular &Heart	Torpedo & Cotyledonary	ELS	Total
Milyang-jare	Liquid	76.6±18.3	-	19.0±5.1	95.6±21.6
	Double layer	4.5±1.3	23.8±4.4	4.8±1.5	33.0±4.2
	2-step	24.0±6.1	14.0±3.9	13.3±4.4	51.3±7.8
LV2319	Liquid	14.1±4.5	8.0±4.5	33.7±10.1	55.9±13.4
	Double layer	4.5±3.1	17.0±3.2	14.3±4.2	35.8±7.1
	2-step	6.0±2.3	12.8±3.3	11.8±3.9	30.5±7.0
SNU2	Liquid	0.3±0.2	-	0.2±0.1	0.5±0.3
	Double layer	0.8±0.6	-	-	0.8±0.6
	2-step	-	-	0.3±0.2	0.3±0.2

ELS indicates embryo-like structure.

complete plants. Two weeks after transplantation onto 1/2MS medium with 2% sucrose, complete cotyledons emerged and embryos became erect, whereas during the 5th week of culture, three to five true leaves emerged. During the 8th week of culture, seven to eight true leaves had emerged, and the plants were transplanted into soil and allowed a 2-week acclimation period. Complete plants were planted into large pots and grown in a greenhouse (Fig. 3).

Analysis of microspore-derived haploid/double haploid plants: Analysis of horticultural characteristics and flow cytometric analysis

For polyploidy of regenerated plants derived from isolated microspores of hot peppers, the percentage of haploids and double haploids were analyzed by phenotype and flow cytometry. A total of 208 individuals of microspore-derived plants (87 and 121 from Milyang-jare and LV2319 genotypes, respectively) were analyzed to differentiate haploids

Fig. 3. Production process of microspore-derived plant. a-d; Plant developed from embryos after on regeneration medium. Regeneration period; a, b, 2 weeks, c, 5 weeks, d, 8 weeks on regeneration medium. e, Plants that developed after 2 weeks of growth in soil. f, Growth of haploid and doubled haploid in the greenhouse.

from double haploids. Investigation of horticultural characteristics during the period when the fruits turned red indicated that there were no major differences in total plant length, whereas there were substantial differences in stem length (length to the first branching stem, clean stem height), leaf length, and leaf width (Table 3). In addition, there were substantial differences in fruit length and width. Haploids were similar to the fruit that occurred without fertilization, and there was no seed. Conversely, double haploids produced fruits that were same size and shape as the donor plants and had normal seed development (Fig. 4).

Classifications of haploid and double haploid plants based on flow cytometric analysis and horticultural characteristics were in complete agreement. In particular, among horticultural characteristics, fruit morphology and seed development allowed haploid and double haploid plants to be easily differentiated from each other.

DISCUSSION

The growth conditions of the donor plants not only affect the percentage of embryogenic microspores and changes pollen morphology, but can also cause physiological and biochemical changes that affect the culture environment (Ferrie et al., 2014; Kim and Jang, 2001). Amino acids and growth regulators within anthers change according to culture

Table 3. **Characterization of haploid and doubled haploid (DH) plants from microspore-derived embryos**

Genotype	Ploidy level	Rate (%)	Plant height (cm)	Clean stem height (cm)	Clean stem width (mm)	Leaf length (cm)	Leaf width (cm)	Flower length (cm)	Fruit length (cm)	Fruit width (cm)	Fruit shape
Milyang-jare	H	64.4	30.5±6.9	4.4±2.6	7.6±2.0	9.7±1.7	2.5±0.5	1.8±0.2	3.9±0.4	1.4±0.3	Circular, Cordate
	DH	35.6	30.8±9.4	6.3±3.7	7.3±1.5	12.2±2.6	3.3±0.6	1.9±0.2	7.9±1.9	1.8±1.2	Moderately triangular
LV2319	H	27.3	31.5±7.9	5.6±3.5	7.9±1.9	10.1±2.9	2.4±0.8	1.9±0.6	6.5±3.1	1.2±0.3	Circular, Cordate
	DH	72.7	31.1±9.5	7.1±2.7	8.3±1.5	13.5±2.8	3.2±0.7	2.1±0.4	10.1±2.3	1.9±0.4	Narrowly triangular, Hornshaped

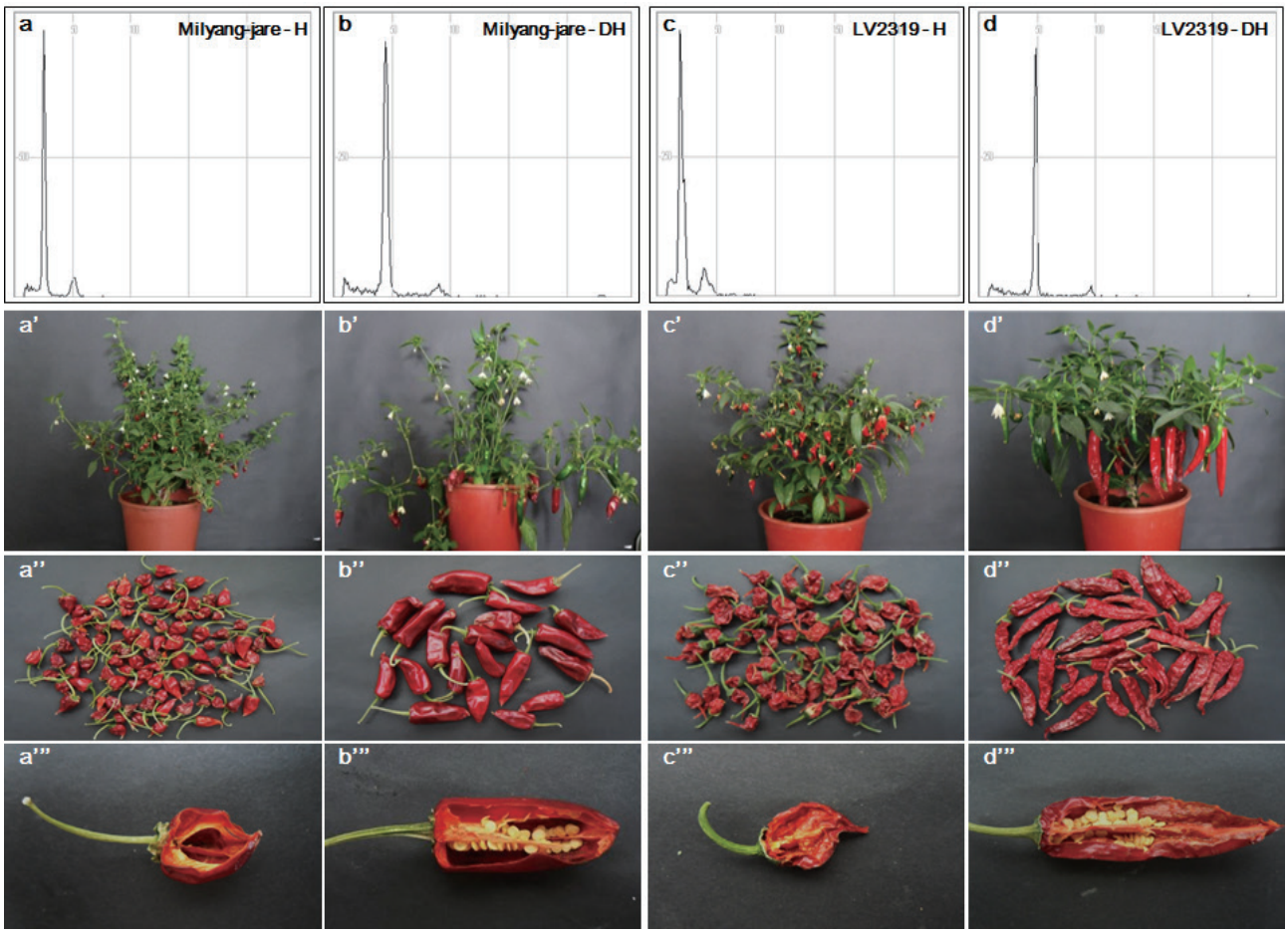


Fig. 4. Flow cytometric analysis and plant characterization of microspore-derived regeneration plant. Genotype. a and b group is Milyang-jare, c and d group is LV2319. a-d, Flow cytometric analysis data. a and c; Haploid, b and d; Doubled haploid. a'-d', Microspore mediated plant in fruiting stage. a''-d'', Fruits harvested from regenerated plants a'''-d''', Fruit and seed development.

conditions, and this affects androgenesis efficiency (Ellialtıoğlu et al., 2001). The physiological state of the donor plant depends on growth conditions, including light, temperature, light cycle, and light intensity, and they affect pollen and flowering quality (Ercan et al., 2006; Kim and Jang, 2001). Moreover, when aged donor plants are used as the material, embryogenesis does not occur or tends to decrease (Kristiansen and Andersen, 1993).

Because the donor plants age faster under high-intensity light, microspore viability and embryogenesis efficiency decrease; with a longer growth period, microspore development of buds is not uniform, which results in the production of a mixture of microspores in various stages of development. When culturing microspores from hot peppers, it is very important to select buds that contain microspores that are in the optimal stage for culture. However, a growth chamber or phytotron that could completely control growth conditions of the donor plant would be high-cost burden, whereas microspore embryogenesis efficiency can be sufficiently increased by applying the simple and low-cost method of growing the donor plant for a short period and controlling light intensity by installing a shield.

During anther/microspore culture, the quality of the calluses and embryos change according to the physical morphology of the culture medium. Generally, solid culture produces excellent embryo

quality and high regeneration efficiency and conversion into green plants (Hu and Kasha 1997; Immonen and Anttila 2000; Zhou and Konzak 1989; Zhou et al., 1991). Although liquid culture has generally been used to culture microspores isolated from hot peppers, double-layer culture is more favorable than liquid culture for effectively producing cotyledonary embryos (Kim et al., 2010, 2013)

In our study, the results with Milyang-jare were similar to those of previous studies (Kim et al., 2013), whereas the LV2319 genotype did not exhibit large differences in embryogenesis and cotyledonary embryogenesis according to the culture method. It is suspected that cotyledonary embryos were generated from liquid culture because there was no nutrient competition because of fewer induced embryos. When culturing microspores from hot peppers, it is important to select the proper culture method because embryogenesis efficiency may vary according to the genotype used. Moreover, to apply hot pepper microspore culture to various genotypes, it is necessary to identify the characteristics of the test material and select the appropriate culture method according to the genotypes used and the study objectives.

Theoretically, plants produced by microspore culture should only have half of the chromosomes possessed by the somatic tissues. However, microspore cultures of Brassicaceae crops produce haploids, diploids, and polyploids, as well as mixed ploids (haploids and diploids coexisting in a single plant) plants (Yuan et al., 2015). It is essential to conduct polyploidy testing to use microspore-derived plants in genomic studies and breeding programs (Bhatia et al., 2017).

Quick and accurate methods for testing polyploidy include flow cytometric analysis, chromosome count at the root or root tip, chloroplast count in guard cells, and phenotype analysis. Flow cytometry is fast, simple, and accurate, but it requires expensive equipment (Supena et al., 2006a), whereas counting the number of chromosomes in root tips and buds may be appropriate, but technically challenging (Jansen, 1974; Wang, 1998). Relatively simple methods include counting the number of chloroplasts in guard cells and performing comparative analysis on the characteristics of haploid and polyploid plants by examining their horticultural characteristics (An et al., 2011; Luitel and Kang, 2013; Supena et al., 2006a). However, chloroplast counts in guard cells may produce different results depending on the growth conditions of the plant and the location from which the leaf was collected (An et al., 2011; Asahi and Toyama, 1982; Possingham and Saurer, 1969; Olszewska et al., 1983). Thus, to accurately determine polyploidy of microspore-derived plants, it is necessary by attempt two different types of analyses.

We increased the accuracy of polyploidy test results by using horticultural characteristics and flow cytometric analyses. In particular, by using fruit morphology and seed development of haploid and double haploid plants, we were able to completely classify double haploid plants.

To use a haploid for breeding, the transition to double haploid must take place. To artificially increase the frequency of double haploid plant production, colchicine, a compound that effectively inhibits chromosomal migration during mitosis by microtubule depolymerization, is widely used. In addition, caffeine, nitrous oxide, trifluralin, and amiprophos-methyl (AMP), which are antimicrobial herbicides, can be used to produce double haploid plants (Hansen and Andersen, 1998b; Hansen et al., 1988; Thomas et al., 1997).

Plants that have a high percentage of spontaneous double haploids may be especially useful for breeding. This is because such plants offer the advantage of not requiring colchicines for chromosome doubling, which can save time and effort (Ari et al., 2016; Yuan et al., 2015). The

mechanism of spontaneous chromosome doubling is unclear, but changes in microfilaments and microtubules in the gametophyte production stage during microspore mitosis are suspected to be the cause (Yuan et al., 2015). In addition, the number of spontaneous double haploids can be increased by early binucleate stage microspore culture, low and high temperature treatment, and osmotic stress (Kasha et al., 2001; Li and Devaux 2003; Yuang et al. 2011). Therefore, to increase the production of double haploids when culturing microspores, it would be advantageous to add compounds such as colchicines or choose the right stage of microspores or pretreatment methods.

When microspores isolated from hot peppers were transfered to a regeneration medium, approximately 95% of the embryos developed into complete plants, but only 16.5% of those were double haploid plants (Kim et al., 2013). The chromosome doubling rates of hot pepper microspore-derived embryos have varied among studies, with rates of 65% (Dolcet-Sanjuan et al., 1997), 35.6% (Dumas de Vaulx et al., 1981), 32.6% (Gyulai et al., 2000), and 14%-51% (Supena et al., 2006b). Moreover, there is a difference in the percentage of microspore-derived haploids/double haploids based on different genotypes (Ari et al., 2016). According to a study by Supena et al., the double haploid production rate among six genotypes was 33.8%, but the four genotypes with the lowest efficiency had a rate of 14-33%, whereas for the top two genotypes it was 41-51% (Supena et al., 2006b).

The results in the present study showed that hot pepper microspore-derived plants were only haploids and double haploids, whereas the Milyang-jare and LV2319 genotypes exhibited over a 2-fold difference in spontaneous double haploid production efficiency being 35.6% and 72.7%, respectively, which is a greater difference than that of a study by Kim et al. (2013) that also used the Milyang-jare genotype. The LV2319 genotype had a high double haploid production efficiency of 72.7%, despite no artificial polyploidization. Genotypes that show ≥60% spontaneous doubling, such as the LV2319 genotype, may be used for breeding and as study material without the need for artificial induction of double haploids (da Silva Dias, 2003).

Microspore embryogenesis offers tremendous opportunities in breeding and life sciences, and in particular, an isolated microspore culture is a system that has broad applicability in androgenesis and sporophytic embryogenesis studies. Despite such advantages, the number of crops and genotypes that have succeeded in isolated microspore culture is limited. The growth conditions of the donor plant and the three types of culture methods used in this study could be employed to produce multiple embryos and plants, although the natural double haploid production percentages varied according to genotype, and the double haploid classification method may be useful for selection and classification of breeding material.

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