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Comparison of the Efficiency to One-Step and Two-Step Anther Culture in Rice (Oryza sativa L)

Jeong Hoon Min[1], Jin Soo Sim[1], Hyun-Suk Lee[2], Young-Hie Park[2], Gyu-Hwan Park[3] and Kyung-Min Kim[4]

Abstract

Anther culture method has commonly been performed two-step culture of callus induction during the plant regeneration stage. Two-step culture was generally required to a much longer duration of culture for regeneration. There are still needed to be solved the disadvantage of the two-step culture technique. Anther of F_1 plants derived from Drimi2*[3]/*AR*1 of new plant type and improved rice cultivars were compared with method efficiency using one-step and two-step culture method. The results showed that one-step culture was more effective than two-step culture. Callus induction averaged 8.2% for one-step culture and 6.1% for two-step culture. The proportion of plants that survived after colchicine treatment was similar as 17.5% for one-step culture and 16.7% for two-step culture. However, there was a highly significant difference when results of plant regeneration were compared. Frequency of regenerated plant averaged 13.6% for one-step culture, which was 7.1% higher than two-step culture (6.5%). These results provide valuable data that may be used to establish an efficient plant regeneration system for rice cultures.

Keywords: Anther culture, One-step culture, Two-step culture, Plant regeneration, Rice

Introduction

Anther culture is an actively developed method of great potential. It shortens the breeding term and increases selection efficiency by producing doubled haploid plants from hybrids (Yan *et al*., 1996). Production of double haploids through anther culture is a rapid approach to homozygosity that shortens the time required for the development of new rice cultivars as compared to conventional methods (Xa and Lang, 2011). Haploids are also valuable for the detection and repair of desirable recessive traits introduced through mutation (Chen *et al*., 2001) or hybridization (He *et al*., 2006). Such doubled haploid techniques provide opportunities to develop green spot in anther culture. However, it is important to enhance the efficiency of anther culture. The frequency and period of plant regeneration can be increased by the concentration and combination of plant growth regulators (Yi *et al*., 2003; Chung and Sohn, 1996; Yamamoto *et al*., 1995; Henry *et al*., 1994). However, there are some difficulties of anther culture to be solved more applicable to rice breeding. These are as follows: a tremendous number of anthers have to be inoculated on a callus formation medium, callus induction is transferred to the plant regeneration medium, which is labor intensive (Yamamoto *et al*., 1995). Anther culture, regenerated plant have commonly been performed two-step culture of callus induction during the plant regeneration stage (Kim *et al*., 2004; Yi *et al*., 2003; Henry *et al*., 1994). Two-step culture was generally required to a much longer duration of culture for regeneration.

[1] Undergraduate, Division of Plant Biosciences, School of Applied BioSciences, College of Agriculture and Life Science, Kyungpook National University, Daegu, 702-701, Korea.
[2] Post-Doc. Follow, Division of Plant Biosciences, School of Applied BioSciences, College of Agriculture and Life Science, Kyungpook National University, Daegu, 702-701, Korea.
[3] Professor, Department Ecological & Environmental System, College of Ecological & Environmental Science, Kyungpook National University, Sangju, 741-711, Korea.
[4] Professor, Division of Plant Biosciences, School of Applied BioSciences, College of Agriculture and Life Science, Kyungpook National University, Daegu, 702-701, Korea. E-mail: kkm@knu.ac.kr

The advantages of incorporating anther culture in rice breeding are shortening the breeding cycle by immediate fixation of homozygosity, increase selection efficiency, widening genetic variability through the production of gametoclonal variants, and allowing the early expression of recessive genes (Gioi and Tuan, 2002). The one-step culture technique has been developed, in which anther plants was generated from inoculated anthers without transferring the callus to a regeneration medium. Application of anther culture of F_1 hybrid of tongil×transgenic plant was used in this experiment to evaluate the culture efficiency of one-step and two-step culture. The aim of our investigations is to establish culture methods for developing haploid production which effect callus induction and regenerated plant to rice breeding.

Materials and Methods

Preparation of Explants

The materials was grown F_1 plants of crosses between 'Drimi 2' (Junam//Samgang/Nagdong Doubled haploid///Cheongcheong/Nagdong Doubled Haploid) as a resistant donor of the brown plant hopper (BPH; *Nilaparvata lugens*) and 'CNDH-32' (Cheongcheong/Nagdong Doubled Haploid), a resistant donor of the white-backed plant hopper (WBPH; *Sogatella furcifera*) were grown during the summer of 2013 on the field of Kyungpook National University Research Facilities in Gun-wi, Gyeongbuk, Korea. Each line with 25 plants per row was planted at 30×15 cm distance. Spikes with flag leaf sheath of these F_1 plants were sampled 18 to 20 days before heading. Wrapped panicles in moistened water and cold pretreated at 12℃, 15days in darkness. After cold treatment for development stage of pollen microspore was determined with distance between auricles of leaf and spike or panicle (2-5cm) and apical position (1/2-1/3) inside panicles (Yi *et al*., 2003). Spikes were surface sterilized with 94% ethanol, was flame sterilized for a few seconds. Anthers of spiklet in the middle spikes were cut and inoculated aseptically and cultured on solidified medium.

Callus Induction and Plant Regeneration

One-step culture media for callus induction consisted of N_6 medium 1 mg/L NAA, 2 mg/L kinetin, 40 g/L maltose, and 4 g/L gelrite (pH 5.8). The media were autoclaved at 121°C for 15 min. One-step culture was incubated for 20 days at 26 ± 1°C (2500 lux, 14-h light). Regenerated plants of 1 to 2 cm in height were transferred to test tubes containing the same medium for further development. Plantlets length of 10 to 12cm were then transferred in potted soils and grown to maturity in the Genetic Modified Organism (GMO) greenhouse. Two-step culture media for callus induction consisted of N_6 medium, 2 mg/L NAA, 0.5 mg/L kinetin, 40 g/L maltose, and 5 g/L gelrite (pH 5.8). The calli were transferred to the N_6-Y_1 medium supplemented with 40 g/L maltose, 0.2mg/l 1-naphthaleneacetic acid (NAA), 5 mg/L kinetin, and 5 g/L gelrite (pH5.8) at 26±1°C for 4 weeks in the darkness. The calli of 1 mm in diameters were transferred from the callus induction media to regeneration media (10 calli/petri dish). Media for regeneration consisted of 1 mg/L IAA, 2 mg/L kinetin, 40 g/L maltose, and 4 g/L Gelrite. The samples were then incubated under a 14 hours light/10 hours dark photoperiod at 26 ± 1°C for 21 days. Regenerated plantlets, about 1 to 2 cm in length, were subcultured in MS medium containing 1 mg/L NAA, 5 mg/L kinetin, 2 g/L casein, 30 g/L sucrose, and 6 g/L gelrite, to incubate under a 14 hour light/10 hour dark photoperiod at 26 ± 1°C up to a root length of 3 cm and grown as described above.

Acclimatization and Colchicine of Regenerated Plantlets

The regenerated plantlets were placed in a box of 1 g/L HYPOneX® Professional 20-20-20 (HYPOneX COPERATION, USA) for 7 days for root growth. Haploid plantlets derived from anther culture were treated with 0.2% colchicine (Sigma Co.) for 1 day after the hardening of regenerated plantlets (3 to 5 leaf stage). The probability of ploidy plantlets were investigated survival ratio in 10 days after transplanting on the soil and fertility. The doubled haploid and haploid plants were measured culm length, panicle length, panicle number in three replications.

Results and Discussion

Rice anthers were cultured using both one-step and two-step culture methods. Callus induction in one-step culture was, on average, 2.1% higher than that two-step culture. The (Drimi2*3/AR1)-2, 3, 6 genotype in one-step culture showed a higher frequency of callus induction, while the (Drimi 2*3/AR1)-1 genotype in two-step culture showed a higher frequency of callus induction (Table 1). In this study, one-step culture resulted in higher callus induction than two-step culture in the Drimi 2*3/AR1 genotype. In practical application of anther culture to rice breeding, this method is a strong advantage of the one-step culture method.

Also, one-step culture is not only shorten the breeding period but also reduces the emergence of undesirable variants, in addition to being labor-saving due to no transfer callus. There was observed a similar tendency in another one-step culture method (Yamamoto *et al*., 1995). After incubation for 15 days, callus induction was too small to observe; however, after 30 days, calli could be visually observed. Globular pro-embryo callus emerged from anther was observed at 15 days under a microscope (Fig. 1). The process of callus induction commences when cells from the outer region divide forming a small group of anther. The calli for one-step light condition were showed white and originate from compact and regular shaped callus, while calli for two-step dark condition formed outer region consisting of less-compacted, loosely-disposed and irregular shaped callus. Petri dish with cultured anthers showed the induction of androgenetic structures which give rise to plantlets in the same medium (Fig. 1). The plant regeneration of one-step culture was 13.6% and that of two-step culture was only 6.5%. The plant regeneration of one-step culture was 7.1% higher than that of two-step culture (Table 2). Chen *et al*., (1991) reported that frequency of anthers producing callus, capacity of differentiation was tightly related to donor genotype. The regenerated plants of one-step culture grew in a tangle with calli, while the regenerated plants of two-step culture grew near brown calli (Fig. 1). This differs from some previous reports that callus induction and regenerated plant are inherited independently (Zhu *et al*., 1992). Therefore, the selection for higher callus induction should be sufficient to improve anther culture efficiency and one-step culture (Yan *et al.,* 1996). In Drimi 2*3/AR1, 70 regenerated plantlets achieved acclimatization after one-step culture, while only 16 regenerated plants were acclimatized after two-step culture. Sixty-three regenerated plantlets were treated with colchicine and 12 regenerated plantlets were processed in the two-step culture. After colchicine treatment, plant survival rate was 17.5% (11 plants) for one-step culture and 16.7% (2 plants) for two-step culture (Table 3). After colchicine treatment, regenerated plants (11 plants for one-step culture and 2 plants for two-step culture) were transferred to seedling plug for 20 days. Among all of the transplanted plants, the non-haploid, colchicine-treated plants grew poorly and remained short, while the double haploid colchicine-treated plants grew well and became large and fresh plants (Fig. 3). Also This result was efficient more than Kang *et al*., (1999) result in doubled-haploid plants.

Haploid plant was smaller in length, had narrower leaf size, shorter petioles, and smaller panicles than the diploid plants. Similarly, haploid rice plants showed smaller culm length and panicle length. However, tiller number and effective tillering number remained the same (Table 4). One-step and two-step cultures showed little difference in terms of average culm length, panicle length, panicle number including effective tiller number. In haploid plants, one-step and two-step cultures showed an average of 3.1 cm, 5.2 cm, 1.9, and 0.1 for culm length, panicle length, panicle number including effective tiller number, respectively. In double haploid plants, these same factors differed by only 1.3 cm, 2.7 cm, 3.1, and 4, respectively (Table 4). Generally phenotypic traits were not significantly different for plants of Drimi 2*3/AR1. In spite of a number of anther culture varieties, callus formed was usually transferred to a plant regeneration medium, which is labor intensive and time consuming. One-step culture was partially solved by modifying anther culture methods. It was reported that skillful method shortens the breeding term and increases selection effiency by producing doubled haploid plants from hybrids. The efficiency of double haploid rice from anthers cultures *in vitro* was a rapid approach to homozygosity that shortens the time required for the development of new rice cultivars. Techniques of anther cultures provide opportunities green plant body in methodological. It is important to save time and energy because it increases the efficiency of anther culture. In this study, we reported it is more effective to produce anther culture compared to one-step culture and two-step culture. Frequency of callus induction showed average one-step culture in 8.2%, frequency of two-step culture in 6.1%, one-step culture higher 2.1% than two-step culture. In anther culture method, two-step culture was a little higher than one-step culture in callus induction. On the contrary in this study, one-step culture's higher than two-step culture's in callus induction. It is considered that due to the difference between the genotype. Plant regeneration showed average one-step culture in 13.6%, two-step culture in 6.5%, one-step culture's frequency higher 7.1% than two-step culture's frequency. The regenerated plants after colchicine treatment were similar as 17.5% of one-step culture and 16.7% of two-step culture. It is considered that due to the genotypic similarity. In the phenotype traits, there was little difference between the one-step culture and the two-step culture of haploid and diploid plants. It was also considered that due to the genotypic similarity.

Conclusion

The efficiency of double haploid rice from anthers cultures *in vitro* was a rapid approach to homozygosity that shortens the time required for the development of new rice cultivars. Techniques of anther cultures provide opportunities green plant body in methodological. It is important to save time and energy because it increases the efficiency of anther culture. In this study, we reported it is more effective to produce anther culture compared to one-step culture and two-step culture. Frequency of callus induction showed average one-step culture in 8.2%, frequency of two-step culture in 6.1%, one-step culture higher 2.1% than two-step culture. In anther culture method, two-step culture was a little higher than one-step culture in callus induction. On the contrary in this study, one-step culture's higher than two-step culture's in callus induction. It is considered that due to the difference between the genotype. Plant regeneration showed average one-step culture in 13.6%, two-step culture in 6.5%, one-step culture's frequency higher 7.1% than two-step culture's frequency. The regenerated plants after colchicine treatment were similar as 17.5% of one-step culture and 16.7% of two-step culture. It is considered that due to the genotypic similarity. In the phenotype traits, there was little difference between the one-step culture and the two-step culture of haploid and diploid plants. It was also considered that due to the genotypic similarity.

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Fig. 1: Microphotograph of Callus and Regeneration Plant in One-Step Anther Culture and Two-Step Anther Culture

Fig. 2: Comparison to one-step and two-step anther culture method in rice. One-step culture (➞); Anthers inoculated directly on a pertirdish and plant regenerated before acclimatization treatment. Two-step culture (⇨); calli after 30 days on callus induction medium and then transfer to a regeneration medium, and plant regenerated before acclimatization treatment. Acclimatization treatment and then transfer plastic pot (→). Plants (135 days after transplating) after colchicine treatment. A; Haploid plant, B: Doubled Haploid plant.

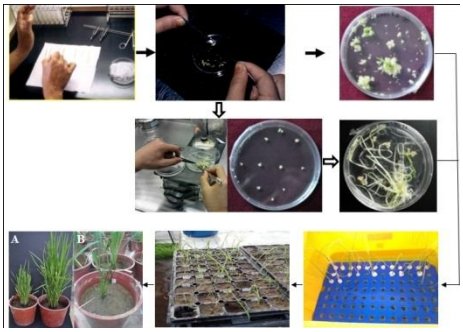

Fig. 3: Plants (135 Days after Transplanting) After Colchicine Treatment. A: Haploid, B: Doubled Haploid

Table 1: Callus Induction Frequency of One-Step and Two-Step Culture

Genotype	No. of anthers inoculated		No. of calli from anthers (%)	
	One-step	Two-step	One-step	Two-step
(Drimi 2*3/AR1)-1	459	963	27±3.3[a] (5.9)	116±2.7 (12.0)
(Drimi 2*3/AR1)-2	206	360	23±6.4 (11.2)	14±3.5 (3.9)
(Drimi 2*3/AR1)-3	596	505	94±5.5 (15.8)	44±4.2 (8.7)
(Drimi 2*3/AR1)-6	225	324	12±2.5 (5.3)	8±1.7 (2.5)
(Drimi 2*3/AR1)-7	311	248	8±2.3 (3.2)	8±2.5 (3.2)
Total	1,797	2,400		
Average			32.8 (8.2)	38.0 (6.1)

[a]mean ± standard deviation.

Table 2: Plant Regeneration Frequency of One-Step and Two-Step Culture

Genotype	Culture method	No. of anthers inoculated(A)	No. of plants regenerated(B)	B/A (%)
(Drimi 2*3/AR1)-3	One-step	596	81±12.3[a]	13.6
	Two-step	505	33± 8.3	6.5

[a]mean ± standard deviation.

Table 3: Plantlets Derived From Anther Culture Transferred to Pots in Drimi 2*3/AR1

Culture method	No. of plants accumulated	No. of plants treated with colchicine (A%)	No. of plants in pots (B%)	No. of plants survived (C%)
one-step	70±8.4[a]	63(90.0)	13(20.6[b])	11(17.5[c])
two-step	16±5.6	12(75.0)	3(25.0)	2(16.7)

[a]mean ± standard deviation, [b]B/A, [c]C/A.

Table 4: Phenotypic Traits of One-Step and Two-Step Culture Plant after Colchicine Treatment

Culture method	Ploidy level	Culm length (cm)	Panicle length (cm)	No. of tillering	No. of effective tillering
one-step	haploid	28.0±0.0[a]	10.5±0.0	19.0±0.0	8.0±0.0
	double haploid	45.9±8.5	15.7±2.3	17.1±9.1	7.9±5.7
two-step	haploid	24.9±0.0	9.8±0.0	14.0±0.0	9.0±0.0
	double haploid	44.6±0.0	13.0±0.0	14.0±0.0	5.0±0.0

[a]mean ± standard deviation.