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Varietal Difference Based on Efficiency of Rice Anther Floating Culture

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ABSTRACT : To evaluate the efficiency of anther floating culture according to the maturing group, the varietal difference and classification of fifty varieties was conducted in N6 liquid medium containing 1 mg l^{-1} NAA, 0.25 mg l^{-1} kinetin. The efficiency of callus induction was widely ranged from 0 to 113.4%, but the mean callus induction was not significantly different among maturing groups. The efficiency of anther floating culture showed the highest variation in early-maturing group among three maturing groups. The varieties with the best callus induction were Sambaegbyeo and Jinbuolbyeo, while the recalcitrant variety was Obongbyeo in early-maturing group. The efficiency of plant regeneration showed the highest trends in late-maturing group among three maturing groups. The fifty varieties were classified into three groups (distance=0.78) by cluster analysis based on the callus formation and plant regeneration. Group including only two varieties, Shinunbongbyeo and Sambaegbyeo had the excellent androgenic efficiency, and the medium efficiency of Group was included thirty-six varieties. Whereas twelve varieties, including three Tongil varieties were fell into the bad efficiency of Group . Especially, Tongil varieties containing Japonica rice, Obongbyeo were the recalcitrant genotypes for the anther floating culture.

Keywords : callus formation, classification, genotype, liquid medium, plant regeneration

Plant breeders are deeply interested in haploid production through the anther or microspore culture techniques, because homozygous lines can be obtained by doubling the chromosomes of haploids in one or two generations. In self-pollination species, doubled haploids derived from micro- spores are valuable in speeding up the breeding process. As a means of haploid breeding, the anther or microspore culture technique have been rapidly advanced in rice (Kobayashi *et al.*, 1992; Lee & Lee, 1995; Xie *et al.*, 1995; Castillo & Valles, 2000). Anther culture technique is an important breeding program for developing new varieties of rice in Korea. However, the use of anther or microspore culture has been limited according to genotypes, and the frequencies of green plant were generally low(Choi *et al.*, 1986; Hu, 1983; Castillo & Valles, 2000). In rice, the efficiency of anther or microspore culture was more higher in Japonica than Indica variety, and the frequency of green plant has been largely differed by culture condition among Japonica varieties (Lee & Lee, 1995; Kang *et al.*, 1999). Especially, albino plant and somaclonal variation were problems to solve in haploid breeding of cereal crops. In haploid breeding of rice, the doubled haploids can be obtained by the *in vitro* spontaneous diploidization without colchicine treatment, and they are generally stable (Hu 1983). In recent, the frequency of spontaneous doubled haploids was more than 60% of the regenerated green plants in Japonica rice (Lee and Lee, 1995; Xie *et al.*, 1995). The production of rice haploids has been developed to three methods, anther culture on the solid medium (Lee and Lee, 1995), anther floating culture on a liquid medium (Kang *et al.*, 1999; Lee *et al.*, 2000), and isolated microspore culture (Xie *et al.*, 1995). Of them, the anther floating culture in the liquid medium could be selected the callus originated from microspore without somatic cell, and widely utilized in mutation breeding for the improvement of various plant characters. It would also be particularly useful to develop the automatic process for microspore culture. However, the regeneration of rice green plant in the liquid medium was much less than in anther culture using agar medium (Kang *et al.*, 1999; Lee *et al.*, 2000). Pollen stage, culture media, pretreatments, or genotype were found to be the critical factors in anther floating culture as well as the anther culture on solid medium (Tasy and Chen 1984; Shon *et al.*, 1987; Kobayashi *et al.*, 1992; Xie *et al.*, 1995). And also, the androgenic efficiency in the liquid medium showed a different aspect according to the genotype compared to the solid medium (Kang *et al.*, 1999).

In this work, to evaluate the efficiency of anther floating culture on the liquid medium, we described the varietal difference and classification in the callus induction and plant

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regeneration according to the maturing group of Korean rice varieties.

MATERIALS AND METHODS

Cultivation of Materials

Fifty varieties (fourty-seven Japonica rice and three Tongil rice) with different maturing group were cultivated in field plots with 40 plants spacing 30 cm between rows and 15 cm between plants within the row. Nitrogen application as urea 110 kg/ha, and nitrogen was split with 50% at transplanting, 30% at 2 weeks after transplanting and 20% at panicle initiation stage. Phosphorous (P_2O_5) and potassium (K_2O) were applied at 70 and 80 kg/ha, respectively. Phosphorous was applied with the whole quantity at transplanting, and potassium was split with 80% at transplanting and 20% at panicle initiation stage. The materials for anther culture were randomly collected from 15 plants (one tiller per plant) except border effect at the booting stage before the spikes had emerged from the flag leaf sheath.

Anther culture procedure and culture media

Fifteen Japonicas according to maturing group (each 5 varieties of early-, medium-, and late-matured group) were used to investigate the efficiency of anther floating culture by heading date. The target tillers were pre-treated in a low temperature incubator (10°C) for 10 days, before the spikes had emerged from the flag leaf sheath. After pretreatment, the spikes with the leaf sheaths removed were surface-sterilized with 70% (v/v) ethanol for 30 sec. and then surface-dried on a clean bench. Anthers at mid- to late-uninucleate microspore stages were inoculated in the liquid callus induction medium which consisted of basal N6 medium (Chu *et al.*, 1975) containing 1 mg l^{-1} (w/v) NAA, 0.25 mg l^{-1} (w/v) kinetin. The plant regeneration medium was supplemented with 1 mg l^{-1} (w/v) NAA, and 1 mg l^{-1} (w/v) kinetin and 0.8% (w/v) Gelrite (Phytagel, Sigma Ltd.) based on the callus induction medium. Both media were adjusted to pH 5.8. One hundred anthers were plated in each Petri dish (65×15 mm) containing 5 ml liquid medium. The Petri dishes were sealed with parafilm (50×15 mm, Whatman Ltd.) and placed in the dark at 25±1°C for 50 days. From 30 days after culture, the induced calli (about 3~5 mm size) were transferred to the plant regeneration medium (87×15 mm Petri dish containing 20 ml solid medium) every 10 days. The callus were maintained with an 18h photoperiods with a light intensity of 27 μmol^{-2} s^{-1} at 25±1°C. The regenerated plants were grown in the glasshouse. All experiments were tested in a completely randomized design with three replications (100 anthers per replication). Callus induction, plant regeneration and ploidy of regenerants were investigated, and data analysis was compared by Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5%.

Varietal classification of the efficiency of anther floating culture

To investigate the effect of genotype for anther floating culture, a total of fifty varieties, forty-seven Japonica variety and three Tongil varieties (Tongil type was developed by crossing between Indica and Japonica), were cultured in the same condition mentioned above. The efficiency of anther floating culture were evaluated based on the percentage of callus induction and plant regeneration. Varietal groups were classified by average linkage cluster analysis using squared Euclidean distance for similarity measure. Data analysis was performed using the SAS statistical software (V 6.12, SAS Institute Inc.).

RESULTS AND DISCUSSION

Anther floating culture by maturing group

The efficiency of anther floating culture showed the different aspects depending on genotype (Table 1). The efficiency of callus induction according to maturing group in liquid medium was ranged from 0 to 113.4%, but the mean callus induction was not significantly different among maturing groups. The efficiency of plant regeneration showed the highest trends in late-maturing group among three maturing groups, but the mean value of plant regeneration was not significantly different among maturing groups, too. The varieties with the highest efficiency for anther floating culture were Sambaegbyeo and Jinbuolbyeo in early-maturing group. In early-maturing group, Obongbyeo was a recalcitrant variety for the callus induction, and Joryeongbyeo and Odaebyeo were recalcitrant varieties for the plant regeneration. These results indicated that the efficiency of anther floating culture was more affected by genotype than maturing group although early maturing varieties showed the highest varietal difference among maturing groups. The androgenic efficiency was affected by growth regulators, gelling agents, physiological status of donor plants and genotype and so on in solid medium as well as in liquid medium (Miah *et al.*, 1985; Choi *et al.*, 1986; Lee *et al.*, 1988; Rout & Sarma, 1991; Lee & Lee, 1995; Kang *et al.*, 1999). In this work, when the callus induced from liquid medium transferred to the solid regeneration medium, most callus were brownish in one week, and the low efficiency of plant regeneration was caused by browning of callus (Tsay & Chen

Table 1. Variation of callus formation and plant regeneration in anther floating culture of Japonica rice according to maturing group.

Maturing group	Variety	No. of callus /100 anthers[a]	Plant regeneration (%)[b]		
			Green	Albino	Total
Early	Sambaegbyeo	113.4a	27(15.9)	0(0.0)	27(15.9)
	Jinbuolbyeo	101.4a	22(9.4)	2(0.9)	24(10.3)
	Joryeongbyeo	56.0b	1(0.8)	0(0.0)	1(0.8)
	Odaebyeo	36.4bc	0(0.0)	0(0.0)	0(0.0)
	Obongbyeo	0.0d	0(0.0)	0(0.0)	0(0.0)
	Mean	**61.4**^{ns}	**10.0(5.2)**^{ns}	**0.4(0.2)**^{ns}	**10.4(5.4)**^{ns}
Medium	Hwaseongbyeo	82.8a	14(6.8)	13(6.3)	27(13.0)
	Donghaebyeo	75.2a	7(3.7)	0(0.0)	7(3.7)
	Sinseonchalbyeo	68.2a	11(8.4)	1(0.8)	12(9.2)
	Gancheokbyeo	66.6a	11(8.3)	0(0.0)	11(8.3)
	Hwajinbyeo	17.0b	2(5.9)	2(5.9)	4(11.8)
	Mean	**62.0**^{ns}	**9.0(6.6)**^{ns}	**3.2(2.6)**^{ns}	**12.2(9.2)**^{ns}
Late	Gyehwabyeo	82.0a	10(4.9)	1(0.5)	11(5.4)
	Daeyabyeo	68.6ab	13(8.9)	4(2.7)	17(11.6)
	Mangeumbyeo	62.8ab	11(16.2)	2(2.9)	13(19.1)
	Yeongdeogbyeo	52.0bc	26(23.6)	3(2.7)	29(26.3)
	Daecheongbyeo	30.8c	3(3.9)	3(3.9)	6(7.8)
	Mean	**59.2**^{ns}	**12.6(11.5)**^{ns}	**2.6(2.5)**^{ns}	**15.2(14.0)**^{ns}

[a] Callus induction medium: liquid N6 medium+1 mg l^{-1} NAA+0.25 mg l^{-1} kinetin. [b] Plant regeneration medium: N6+1 mg l^{-1} NAA+1 mg l^{-1} kinetin+0.8% Gelrite, ( ): Percentage of induced callus. Data followed by the same letter in each column were not significantly different at the 5% level based on Duncan's multiple range test, ns: non-significant among mean values.

1984; Kang *et al.*, 1999; Lee *et al.*, 2000). Sometimes, the androgenic efficiency could be increased by the culture condition using a liquid medium in cereal crops (Kobayashi *et al.*, 1992; Xie *et al.*, 1995; Castillo & Valles, 2000). The reason was known that the anther floating culture allowed better nutrient availability to the developing callus or embryo, eliminated the competition among embryogenic pollen grains, and also some toxic inhibitors released directly into the liquid medium (Wernicke & Kohlenbach 1976; Sunderland & Roberts, 1977).

On the other hand, the ploidy of regenerated plants was very important, because the stable doubled haploid was valuable in speeding up of breeding time. The doubled haploids could spontaneously be obtained in vitro without colchicine treatment. In this work, the frequency of doubled haploid was very low in early- and late-maturing group (Fig. 1). The highest frequency of doubled haploid was 28.3% in medium-maturing group. Similar results were reported by Lee *et al.* (2000) and Kang *et al.* (1999). Hu (1983) also reported that the frequency of doubled haploid was too low in treatment of chemical mutagen. While, Lee & Lee (1995) reported that the frequency of doubled haploid was more than 60% for regenerated plants in high concentration of agar or Gelrite medium. In this work, the varieties with high

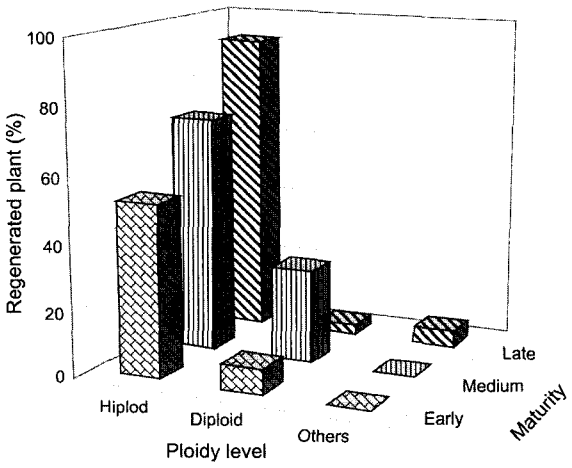

Fig. 1. Variation of ploidy level according to the maturing group of the regenerated plant by rice anther floating culture.

androgenic potential (Sambaegbyeo and Jinbuolbyeo) could be selected through the evaluation of androgenic efficiency in liquid medium. Accordingly, the evaluation of practical variety containing various genotype will be obtained an important information in the breeding strategy through anther floating culture.

On the other hand, the total of 50 varieties were evaluated

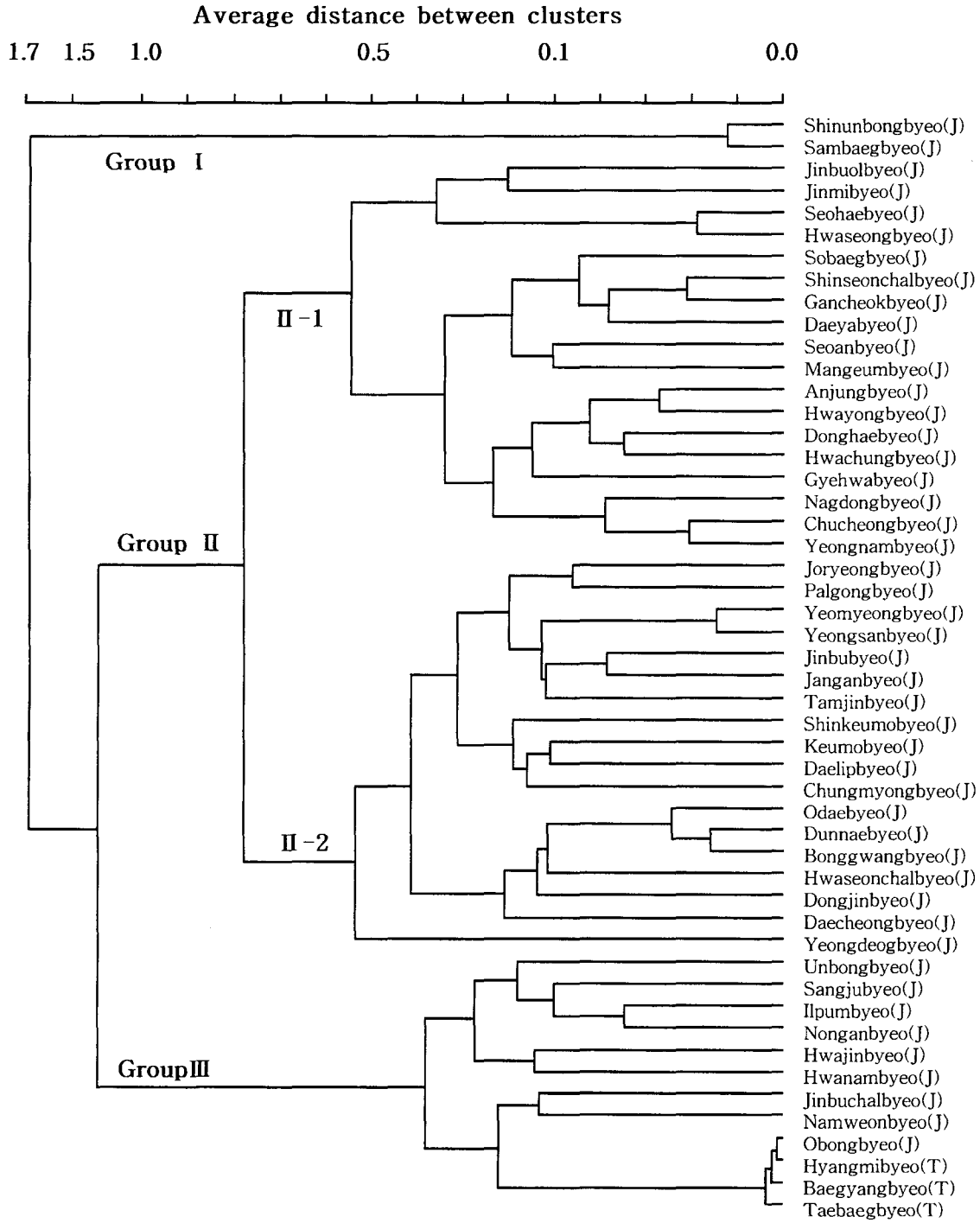

Fig. 2. Classification of rice varieties according to the callus formation and plant regeneration in rice anther floating culture by cl uster analysis. J: Japonica, T: Tongil variety developed by crossing Indica and Japonica. Group I: excellent, Group II-1: good, Group II-2: medium, Group III: bad.

for callus formation and plant regeneration in the same liquid medium. When compared with the androgenic response, callus induction and plant regeneration showed an obvious difference according to genotype. The tested varieties were classified into three groups (distance=0.78) by cluster analysis based on the callus formation and plant regeneration (Fig. 2). Two varieties, Shinunbongbyeo and Sambaegbyeo belong to the early maturing group, were classified as the excellent group (Group I), and thirty-six varieties, including Jinjuolbyeo, Jinmibyeo, Hwaseongbyeo and so on, were fell

Table 2. Efficiency of anther floating culture according to maturing group in Japonica type.

Maturing group	Efficiency of anther floating culture			No. of variety
	High	Medium	Low	
Early	Shinunbongbyeo Sambaegbyeo Jinbuolbyeo Sobaegbyeo	Joryeongbyeo Yeomyeongbyeo Jinbubyeo Shinkeumobyeo Keumobyeo Odaebyeo Dunnaebyeo	Unbongbyeo Sangjubyeo Jinbuchalbyeo Namweonbyeo Obongbyeo	16
RCI[a] (Mean, %)	64.6-114.4 (98.5)	35.6-56.0 (45.5)	0.0-20.0 (9.2)	
Medium	Seohaebyeo Hwaseongbyeo Anjungbyeo Hwayongbyeo Donghaebyeo Shinseonchalbyeo Gancheokbyeo Jinmibyeo Seoanbyeo	Palgongbyeo Janganbyeo Chungmyongbyeo Daelipbyeo Hwaseonchalbyeo Bonggwangbyeo	Hwajinbyeo Nonganbyeo	17
RCI (Mean, %)	62.8-92.8 (76.6)	34.2-59.4 (46.1)	11.0-17.0 (14.0)	
Late	Gyehwabyeo Hwachungbyeo Nagdongbyeo Daeyabyeo Chucheongbyeo Yeongnambyeo Mangeumbyeo	Yeongsanbyeo Yeongdeogbyeo Tamjinbyeo Dongjinbyeo Daecheongbyeo	Hwanambyeo Ilpumbyeo	14
RCI (Mean, %)	62.8-82.0 (70.7)	30.8-52.8 (44.8)	14.0-22.6(18.3)	
Total mean (%)	81.9	45.5	13.8	
No. of variety (%)	20(42.6)	18(38.3)	9(19.1)	47

[a]RCI: range of callus induction.

into the medium group (Group II), whereas twelve varieties, including Namweonbyeo, Obongbyeo, and Tongil type, and so on, fell into the bad group (Group III). Especially, Tongil varieties didn't formated the callus in anther floating culture. The group II could be sub-divided into two groups. The group II-1, including Jinbuolbyeo, Sobaegbyeo, Anjungbyeo, and so on, was showed the good efficiency for anther floating culture (75.5% of mean callus induction and 7.9% of mean plant regeneration), and the group II-2 was included Joryeongbyeo, Odaebyeo, Yeongdeogbyeo and so on with 45.5% of mean callus induction and 7.5% of mean plant regeneration.

For the breeding convenience, when forty-seven Japonica varieties were also divided the three degree (high, medium, low) by maturing group based on the callus induction, the varietal efficiency of rice anther floating culture was shown in Table 2. Most varieties showed a good androgenic response except nine varieties. Twenty varieties (42.6%) were the high efficiency of callus induction, and the range of callus induction was 62.8% to 114.4%. Eighteen varieties (38.3%) with medium efficiency of callus induction showed the range of callus induction from 30.8% to 59.4%, and others (19.1%) were less than 22.6% in callus induction. The varietal difference in the efficiency of anther floating culture showed broadly in the early maturing group, and the mean callus induction of high efficiency varieties was 98.5% (4 varieties), but that of low efficiency ones were 9.2% (5 varieties).

The varietal difference of androgenic efficiency was reported by some researchers (Choi *et al.*, 1986; Quimio & Zapata, 1990; Kim *et al.*, 1991; Kwon & Sohn, 2000), and most Indica or Tongil type varieties had the much lower androgenic efficiency than Japonica ones as our result. Recently, the QTL analysis was conducted to increase the anther culture efficiency in hybrids between a high efficiency variety and a recalcitrant variety (Kim *et al.*, 2000; Kwon *et al.*, 2002). In this study, there was a distinct varietal difference in the anther floating culture, and the earley-

maturing group showed the most drastic variation in callus induction. Of them, we were interested in Shinunbongbyeo and Sambaegbyeo with the excellent efficiency of anther floating culture. In the future, these varieties can broadly be utilized to increase the breeding efficiency for *in vitro* mutation breeding, *in vitro* selection for disease or stress tolerant traits, and breeding materials. Moreover, the doubled haploids originated from microspores by the anther floating culture will contribute to the development of new variety in some crops, because the anther floating culture technique can eliminate the somatic callus or embryoid originated from anther wall or filament.

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