

Plant Regeneration Capacity of Calluses Derived from Mature Seed of Five Indonesian Rice Genotypes

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Abstract : Establishment of a suitable system for plant regeneration of rice calluses derived from mature seed is a prerequisite for genetic transformation using callus as the target tissue. Selecting the most suitable medium and assessing the genotype performance for *in vitro* response are essential requirement for this purpose. The experiment with five Indonesian rice genotypes showed that callus-proliferation capacity (CPC) and callus-growth capacity were significantly affected by genotype and CPC by medium. The shoot-regeneration capacity and plantlet-regeneration capacity were affected by the interaction effect between genotype and medium. However for green plant-regeneration capacity, it was affected independently by genotype and medium. Culture media D1 and NB₅ were the most suitable media for callus subculture and plant regeneration, respectively. Genotypes Fatmawati and BP-140 consistently performed best in the callus subculture and plant regeneration.

Key words : Embryogenic calluses, Plant regeneration, Rice, Subculture.

The plant regeneration capacity is one of the primary properties of totipotency of plant cells, which has been widely used for application of plant biotechnology including genetic transformation. For efficient genetic transformation, a highly efficient and robust tissue culture system is a prerequisite since the efficiency of transformation mainly depends on regeneration capacity of genotypes, among others (Alfonso-Rubi et al., 1999). To date, efficient plant regeneration for rice has been reported by some authors (Abe and Futsuhara, 1986; Higuchi and Maeda, 1990; Lee et al., 2002), but despite the availability of protocols for rice regeneration, no procedure appears to be universally adaptable when a new genotype is to be considered for *in vitro* manipulation (Visarada et al., 2002).

In rice, plant regeneration capacity is affected by genotype (Abe and Futsuhara, 1986; Seraj et al., 1997), age and type of explant (Hoque and Mansfield, 2004), nutrient media such as, basal media (Khanna and Raina, 1998; Lee et al., 2002; Lin and Zhang, 2005), plant growth regulators (Pons et al., 2000; Lee et al., 2002), passage in subculture (Lin and Zhang, 2005) and culture conditions. Numerous efforts have been made to improve plant regeneration capacity of rice callus with varying degrees of success. Amino acids such as proline (Yang et al., 1999; Chowdhry et al., 2000) and glutamine (Pons et al., 2000), a specific growth regulator, such as, abscisic acid (ABA) (Higuchi and Maeda, 1990; Kobayashi et al., 1992; Yang et al., 1999) and high concentration of gelling agent (Lai and Liu, 1988; Lee et al., 2002) have been used for

improving the regenerability of rice callus culture.

For *in vitro* regeneration, the use of mature embryos rather than immature tissues as initial explants has distinctive advantages since embryogenic calluses induced from mature seeds are suitable for gene delivery and genetic transformation, actively dividing and capable of regenerating into fertile plants (Jiang et al., 2000). Thus, proliferation of the embryogenic calluses with high regeneration capacity through subculture is prerequisite for the successful development of transgenic rice plants via callus culture. In addition, establishing an efficient plant regeneration system will therefore facilitate the development of transgenic and other biotechnological aims. In this study, we used five culture media in combination with five Indonesian rice genotypes for examining callus proliferation (subculture) and plant regeneration capacities. The objective of this study was to establish a suitable plant regeneration system for five Indonesian rice genotypes using a mature seed as the initial explants.

Materials and Methods

1. Plant materials

Mature healthy dehusked seeds of Fatmawati, Ciapus, BP-23, BP-140 and BP-360-3 (all *indica* subspecies) were soaked with 70% ethanol for 3 min. and surface sterilized in 50% commercial bleach (4.5% sodium hypochlorite) supplemented with 2-3 drops of Tween20 for 45 min. with gently shaking, and then rinsed thoroughly with sterile distilled water three times. The seeds were cultured in Petri dishes (9 cm

Table 1. Composition of media for callus induction, subculture and plant regeneration*.

Components	Medium MS ^a	Medium CI ^b	Medium D1 ^c	Medium L3 ^d	Medium NB ₅ ^e
<i>Macro elements</i>					
NH ₄ NO ₃	1,650	640		1,700	
(NH ₄) ₂ SO ₄			450		463
KNO ₃	1,900	1,212	2,900	1,900	2,830
CaCl ₂ ·2H ₂ O	440	588	170	400	166
MgSO ₄ ·7H ₂ O	370	247	185	370	185
KH ₂ PO ₄	170	136	400	170	400
<i>Micro elements</i>					
KI	0.83	0.83	7.5	7.5	0.75
H ₃ BO ₃	6.2	3.1	30	30	3
MnSO ₄ ·4H ₂ O	22.3	11.15	100	100	10
ZnSO ₄ ·7H ₂ O	8.6	5.76	20	20	2
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.24	2.5	2.5	0.25
CuSO ₄ ·5H ₂ O	0.025	0.025	0.25	0.25	0.025
CoCl ₂ ·6H ₂ O	0.025		0.25	0.25	0.025
CoSO ₄ ·7H ₂ O		0.028			
FeSO ₄ ·7H ₂ O	27.8	27.8	55.9	41.8	27.8
Na ₂ EDTA·2H ₂ O	37.3	37.3	74.5	55.9	37.3
<i>Organic supplement</i>					
Nicotinic acid	0.5	6.0	1.0	1.0	1.0
Pyridoxine-HCl	0.5	1.0	1.0	1.0	1.0
Thiamine-HCl	0.5	8.5	1.0	1.0	10
Glycine		2.0	2.0	2.0	
Coconut water		100 mL ⁻¹			
Myo-inositol	100	90	100	100	100

* : Concentration in mgL⁻¹.

^a Murashige and Skoog (1962); ^b Potrykus et al. (1979); ^c Lin and Zhang (2005); ^d Lin and Zhang 2005; ^e Chu et al. (1975) for macronutrient and Gamborg et al. (1968) for micronutrient and organic components.

Callus induction medium: MS + 3 mgL⁻¹ 2,4-D + 3% sucrose + 0.8% agar, pH 5.8.

Callus proliferation/subculture medium: all five media + 3 mgL⁻¹ 2,4-D, 500 mgL⁻¹ l-proline, 500 mgL⁻¹ l-glutamine, maltose 3% + Phytigel 0.25%, pH 5.8.

Regeneration medium: All five media + 3 mgL⁻¹ Kinetin + 3 mgL⁻¹ BA + 0.5 mgL⁻¹ IAA + 0.5 mgL⁻¹ NAA + 500 mgL⁻¹ l-proline, 500 mgL⁻¹ l-glutamine, 800 mgL⁻¹ casein hydrolysate + maltose 3% + Phytigel 0.3%, pH 5.8.

Rooting medium: MS + sucrose 3% + agar 0.6%, pH 5.8.

in diameter) containing 20 mL of callus-induction medium and incubated at 27°C in the dark for 30 days.

2. Callus induction, subculture and regeneration

MS (Murashige and Skoog, 1962) medium, gelled with 0.8% agar, supplemented with 3 mgL⁻¹ of 2,4-D and 3% sucrose was used for callus induction.

Five basal media, MS, CI (Potrykus et al., 1979), D1 and L3 (Lin and Zhang, 2005), and NB₅ (micronutrient and organic components of Gamborg et al., 1968; macronutrient of Chu et al., 1975) (Table 1), were used for both subculture of calluses (simply called subculture hereafter) and plant regeneration. All media for subculture were supplemented with 3 mgL⁻¹ 2,4-D, 500 mgL⁻¹ l-proline, 500 mgL⁻¹ l-glutamine, 3% maltose and semi solidified with 0.25% Phytigel (Gellan gum, Kanto Chemical Co., Inc.). Fine, friable,

white/cream/yellow pale globular embryogenic calluses, 2-3 mm in diameter, were selected, and 20 calluses were transferred into each Petri dish containing 20 mL subculture medium (Fig. 1a, 1b) with three replications. They were subcultured at 27°C in the dark for 3 weeks.

For plant regeneration, the above-mentioned media supplemented with 3 mgL⁻¹ Kinetin, 3 mgL⁻¹ BA, 0.5 mgL⁻¹ IAA, 0.5 mgL⁻¹ NAA, 500 mgL⁻¹ l-proline, 500 mgL⁻¹ l-glutamine, 800 mgL⁻¹ casein hydrolysate, 3% maltose and 0.3% Phytigel were used. The pH of all media was adjusted to 5.8 before autoclaving. Calluses grown and proliferated from the same medium were transferred to the corresponding regeneration medium. Subcultured clusters of calluses ca. 10-13 mm in diameter were transferred into Petri dish containing 20 mL regeneration medium, 10 clusters in each dish

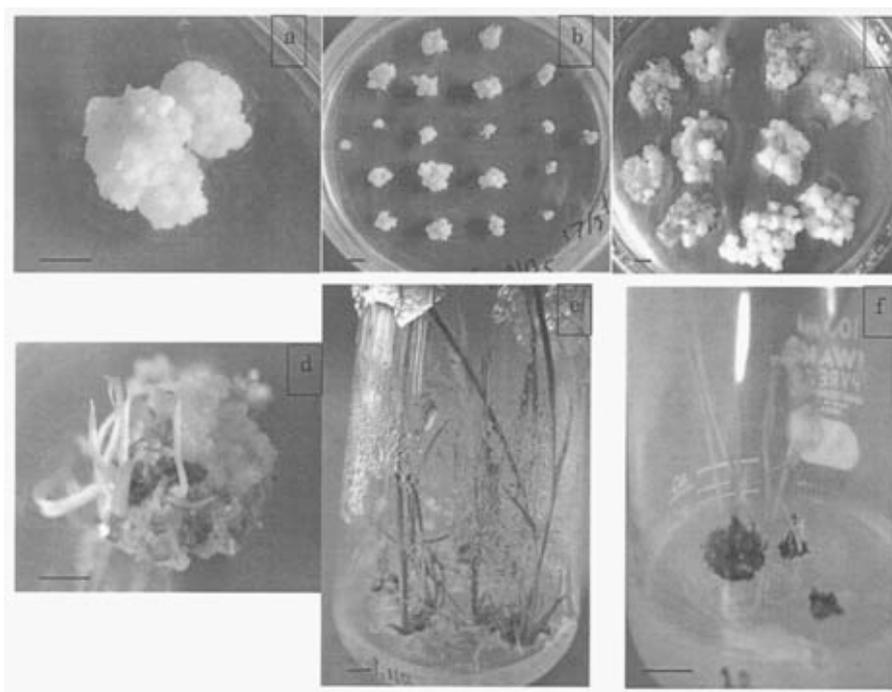


Fig. 1. Plant regeneration from mature seed-derived calluses. a) an embryogenic calluses of genotype Fatmawati on the D1 subculture medium; b) 20 clusters of calluses of Fatmawati on the NB₅ subculture medium after 21-day culture; c) 10 clusters of calluses of Fatmawati on the NB₅ subculture medium showing shoot regeneration after a 14-day culture; d) plantlets of BP-140 growing on D1 regeneration medium; e) and f) plantlets of Fatmawati regenerated on medium NB₅ and D1, respectively.

(Fig. 1c) with five replications, and incubated under a 16-h photoperiod of fluorescent light, with 8-h darkness, at 27°C, for 3-5 weeks.

Regenerated shoots, when their height reached over 2 cm, were transferred to a plant growth regulator-free MS medium for root development (Fig. 1d). When leaf height reached 10 cm, the green plants were transferred onto the soil and grown in a greenhouse for further growth.

3. Evaluation and experimental design

For the callus subculture, two variables: a) callus-proliferation capacity (CPC in %): (number of callus clusters proliferated)/(number of callus clusters on the subculture medium)⁻¹ × 100; and b) callus-growth capacity (CGC in mm²) shown by the size of callus clusters after a 21-day culture, were examined. The size of callus clusters is the average of multiplication of the longest and the shortest diameter of the cluster of callus.

For plant regeneration, three variables were examined: a) shoot-regeneration capacity (SRC in %): (number of callus clusters developing green shoot buds)/(number of callus clusters on the regeneration medium)⁻¹ × 100; examined after a 3-week culture; b) plant-regeneration capacity (PRC in %): (number of callus clusters developing green plantlets)/(number of

callus clusters on the regeneration medium)⁻¹ × 100; examined after 4-5-week culture; and) green plant-regeneration capacity (GRC) shown by the number of green plantlets per replication; examined when plantlets were transferred to soil.

A completely randomized factorial design was used in this study. Data were then analyzed statistically by the analysis of variance (ANOVA) and differences among means were evaluated by Duncan's multiple range test (DMRT).

Results

In the subculture, callus-growth capacity (CGC) was affected independently by medium ($p < 0.01$) and genotype ($p < 0.01$), but callus-proliferation capacity (CPC) was affected independently by genotype ($p < 0.01$) (Table 2). The interaction effect between genotype and medium was not significantly detected for these traits (CGC and CPC). In the five genotypes, CGC varied with the genotype but CPC did not. In addition, medium accounted for the greatest variation in the proliferation and growth of calluses (Table 2). Since the interaction effect is not significant for both traits, the effect of genotype on CGC and that of medium on CPC and CGC are presented in Fig. 2 and 3, separately. Among five genotypes, Fatmawati and BP-140 had the highest CGC (Fig. 2). All media

Table 2. Analysis of variance for callus proliferation (CPC, CGC) and plant regeneration variables (SRC, PRC, GRC).

Source of variation	df ^a	df ^b	CPC	CGC	SRC	PRC	GRC
Genotype (g)	4	4	259.50 ^{ns}	2202.08 ^{**}	7893.50 ^{**}	3381.50 ^{**}	2383.82 [*]
Medium (m)	4	3	5786.17 ^{**}	4855.48 ^{**}	1260.00 ^{ns}	2366.33 ^{**}	4964.35 ^{**}
Genotype x Medium (g x m)	16	12	39.92 ^{ns}	267.25 ^{ns}	2667.50 ^{**}	1075.50 ^{**}	1142.02 ^{ns}
Error	50	80	191.33	298.77	715.50	215.50	762.67
Total	75	100					

Data show mean square value. ns: non-significant, *: significant at $p = 0.05$, **: significant at $p = 0.01$, respectively.

^a: for callus subculture, calculated on the basis of combination of 5 genotypes and 5 media with 3 replications.

^b: for regeneration, calculated on the basis of combination of 5 genotypes and 4 media with 5 replications (medium L3 was excluded from the analysis because of no variation over replications).

CPC (callus-proliferation capacity in %): number of callus clusters proliferated)/(number of callus clusters on the subculture medium)⁻¹ × 100.

CGC (callus-growth capacity in mm²) is the average of the multiplication of the longest and the shortest diameter of the cluster of callus after a 21-day culture.

SRC (shoot-regeneration capacity in %): (number of callus clusters developing green shoot buds)/(number of callus clusters on the regeneration medium)⁻¹ × 100.

PRC (plant-regeneration capacity in %): (number of calluses clusters producing green plantlet)/(number of callus clusters on the regeneration medium)⁻¹ × 100.

GRC (green plant-regeneration capacity in %): number of green plantlets per replication.

except for L3 had high CPC (Fig. 3i). Medium D1 had the highest CGC among the five media and L3 had the lowest CGC (Fig. 3ii).

In the plant regeneration experiment, all five genotypes tested were capable of generating shoot bud and subsequently regenerating plantlets. However, the regeneration capacity significantly varied with the genotype (Table 2). Irrespective of medium used, plantlet regeneration was early in genotype Fatmawati. In general, shoot regeneration was poorly synchronized; it took 15- to 20 days for rice calluses to regenerate shoots on regeneration medium. SRC and PRC were significantly affected by the interaction effect between genotype and medium ($p < 0.01$) but GRC was not (Table 2). Genotype accounted for the greatest variation in SRC and PRC. Conversely, medium contributed to give the highest variation in GRC (Table 2). Fatmawati showed no difference in SRC on different media and had a high PRC on NB₅ (Table 3). Fatmawati and BP-140 showed the highest PRC on NB₅ (Table 3) and the highest GRC on the average (Fig. 4i). Ciapus cultured on CI and NB₅; BP-23 on MS; BP-140 on NB₅, CI, and DI; BP-360-3 on DI and CI, had high SRC (Table 3). However, Fatmawati and BP-140 cultured on NB₅, and BP-360-3 cultured on DI had a high PRC. Ciapus, BP-23 and BP-360-3 produced a relatively low PRC (Table 3) and low GRC (Fig. 4i). Medium NB₅ gave the highest GRC for all five genotypes and MS, CI and D1 gave similarly low GRC (Fig. 4ii).

The appearance of plantlets greatly varied with the culture medium. For instance, on NB₅ medium, Fatmawati produced numerous green healthy plantlets

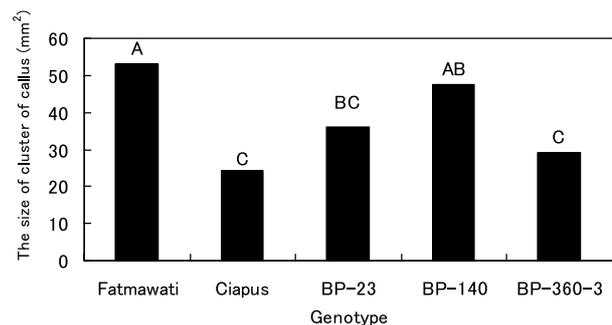


Fig. 2. Comparison for genotype on CGC in the subculture after 21-day culture. Values with the same alphabet show no difference by DMRT at the 0.05 probability level. Each value is the average of genotype performance across five media with three replicates and each consists of 20 clusters of seed-derived callus. CGC (callus-growth capacity) is shown by the size of callus clusters in mm². The size of callus cluster is the average of the multiplication of the longest and the shortest diameter of the cluster of callus.

with many tillers (Fig. 1e), while, on D1, it produced light yellow/brownish plantlets with few tillers (Fig. 1f).

Discussion

Plant regeneration is indispensable for plant transformation technology and other biotechnology aims. In this study, a suitable regeneration system for mature seed derived calluses of five *indica* subspecies has been established. Stages from callus proliferation till regeneration have been investigated. After the

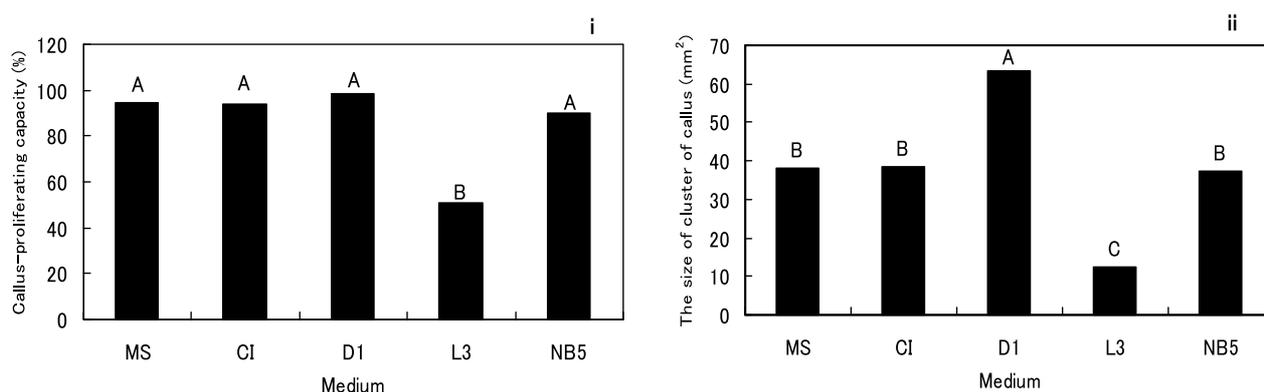


Fig. 3. Comparison for medium on (i) CPC, (ii) CGC in the subculture after 21-day culture. Values with the same alphabet show no difference by DMRT at the 0.05 probability level. Each value is the average of the value from five genotypes with three replicates and each consists of 20 clusters of seed-derived calluses. CPC (callus-proliferation capacity in %): number of callus clusters proliferated) (number of callus clusters on the subculture medium)⁻¹ × 100. CGC (callus-growth capacity in mm²) is the average of the multiplication of the longest and the shortest diameter of the cluster of callus.

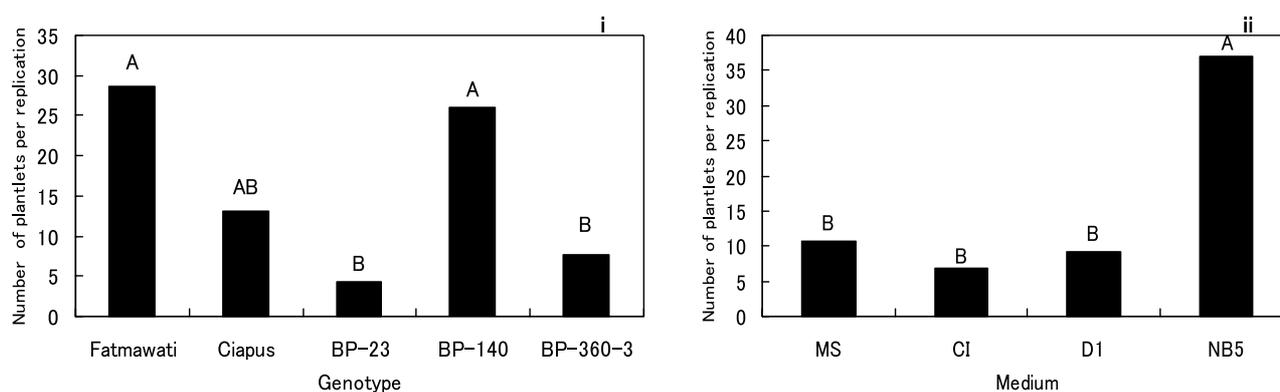


Fig. 4. Comparison for genotype (i) and medium (ii) on GRC in the regeneration. Values with the same alphabet show no difference by DMRT at the 0.05 probability level. Values in (i) show the mean for four media, and the values in (ii) show the means for five genotypes with five replicates and each consists of 10 clusters of seed-derived calluses. GRC (green plant-regeneration capacity in %): number of green plantlets per replication. Medium L3 was excluded from the analysis because of no variation over replication.

step of callus induction, in our view, selecting the most suitable medium and assessing the genotype capacity for callus proliferation and regeneration in order to improve its regenerability are essential for the success of genetic transformation using callus as target material. The result of the present experiment indicated that the callus-proliferation capacity (CPC) was affected only by medium, while the callus-growth capacity (CGC) was independently influenced by genotype and by medium (Table 2). The significant effect of genotype on CPC was not detected (Table 2) due to the fact that the genotypes used excluding BP-140 had been selected as the most responsive ones in callus induction and quality-related callus traits (Carsono and Yoshida, 2006, *in press*). However, the genotypes and media tested significantly differed

in CGC, with Fatmawati and BP-140 were the most responsive genotypes (Fig. 2) and D1 was the most suitable medium for callus subculture (Fig. 3ii). The genotypic differences in CGC may have resulted from the difference of the activity of the genes that control the callus proliferation process, such as those involved in plant hormone metabolism (Henry et al., 1994; Ezhova, 2003). Some differences in composition existed among five media seem to cause the difference in promoting proliferation and growth of callus (Table 1).

For callus proliferation, 2,4-D is an essential element as reported by Inoue and Maeda (1980) and Mitsuoka et al. (1994). In the present study, however, 3 mg L⁻¹ of 2,4-D was supplied equal to all media tested. Suggesting the differences in genotype's response

Table 3. Comparison for genotypes and media on SRC and PRC using DMRT.

Genotype	SRC (%)				PRC (%)			
	MS	CI	D1	NB ₅	MS	CI	D1	NB ₅
Fatmawati	56A a	54AB a	80A a	98A a	20A b	32A b	22AB b	60A a
Ciapus	20A b	68AB a	12 C b	40 B ab	6A a	4 B a	6 B a	14 B a
BP-23	52A a	20 C b	26 BC b	30 B b	4A a	4 B a	2 B a	10 B a
BP-140	44A b	84A a	62AB ab	96A a	8A b	22AB b	18AB b	70A a
BP-360-3	34A b	46 BC ab	80A a	20 B b	8A b	6 B b	34A a	4 B b

Data are the means of five replicates, each with 10 clusters of callus.

The same capital alphabet (between genotypes within a medium) and small alphabet (between media within a genotype) in column and row, respectively, show no difference by DMRT at the 0.05 probability level.

SRC (shoot-regeneration capacity in %): (number of callus clusters developing green shoot buds)/(number of callus clusters on the regeneration medium)⁻¹ × 100.

PRC (plant-regeneration capacity in %): (number of calluses clusters producing green plantlet)/(number of callus clusters on the regeneration medium)⁻¹ × 100.

may not be associated with 2,4-D. This also suggests that the genetic and non-genetic factors (medium, growth regulator, and culture condition) clearly influence both CPC and CGC. However, we could not determine exactly what nutrient is a critical factor for callus proliferation/propagation, since the nutrients are too numerous and demands as well as uptake by the rice cell are diverse. It is likely that the basal composition of medium influenced embryogenic callus proliferation, as has been previously reported by some researchers (Rueb et al., 1994; Lin and Zhang, 2005). The result of present experiment, however, is not in accordance with the result of Lin and Zhang (2005), who reported that L3 was the best medium for subculture of three *indica* genotypes.

For plant regeneration, we identified a significant interaction between genotype and medium in SRC and PRC, but not in GRC that was affected independently by genotype and by medium (Table 2). This indicates that, for SRC and PRC of each genotype shows a different response with regard to regeneration media used, with Fatmawati and BP-140 being the most responsive (Table 3). Fatmawati is a new plant type, released in 2003 in Indonesia, with many valuable agronomic characters such as low tillering capacity, large panicle and high palatability. On the other hand, BP-140 is a promising line, thought not released yet. Both genotypes would be useful for breeding program through *in vitro* approach.

Each genotype had nearly the same GRC on the four regeneration media tested. Khanna and Raina (1998) found the interaction effect between genotype

and medium in SRC and plant regeneration frequency of mature seed-derived calluses of three *indica* types, although we did not. Such different findings may be attributed to the differences in genotypes, media and method of calculation used.

Generally, medium NB₅ was the most suitable medium for plant regeneration in this study. Medium NB₅ probably may promote the propagation of embryogenic calluses and thus improve the regeneration capacity. This result is in accordance with Sivamani et al. (1996) and Visarada et al. (2002) who used NB₅ with a slight different in composition of plant growth regulators used.

From this experiment, it is clear that medium is only suitable for specific developmental stage of callus. It also indicates that the composition of basal medium used for callus proliferation is not always optimal for plant regeneration since the nutritional requirements of the two phases of development (callus proliferation and regeneration) may vary. Our finding corresponds to Khanna and Raina (1998) and, finally, we conclude that both callus proliferation and plant regeneration capacities were genotype- and medium-dependent.

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