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***Tissue Culture Note***

# Studies on callus induction, plant regeneration and transformation of Javanica rice cultivars

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**Abstract** The purpose of this study was to develop a reproducible efficient procedure for the transformation of Javanica rice cultivars from Indonesia. Five rice cultivars cultivated in Indonesia now were examined for their capacity on the callus growth, plant regeneration and transformation. Their potential was affected by genotype and medium. Regarding the quality of callus, type I calli produced higher plant regeneration frequency than type II calli. So type I calli were inoculated with *A. tumefaciens* harboring binary plasmid pAFT14, which had a hygromycin resistance (*hpt*) gene and a *gus* gene. In this study, we examined two media for two steps in the tissue culture process for transformation, i.e. callus inducing and plant regeneration. The results show that C-modified medium was the suitable media for callus induction in the most Javanica rice cultivars. *Agrobacterium*-mediated transformation system has been extended to five Javanica rice cultivars. Among them, Rojolele consistently gave the best performance.

**Key words:** Callus, Javanica rice, transformation.

Successful genetic transformation in rice is possible when efficient and reproducible plant regeneration protocols are available for the particular cultivar. High efficiency tissue culture system for Japonica rice has already been established by the numerous researchers including the pioneer work of Hiei et al. 1994. In Indica rice, several applicable tissue culture methods for subculture and regeneration have been reported (Peng and Hodges 1989; Aldemita and Hodges 1996; Rashid et al. 1996; Khanna and Raina 1998). However, only few tissue culture methods have been reported for Javanica rice. Abe and Futsuhara (1986) have reported that Javanica rice cultivars displayed low capacity for callus growth and plant regeneration. Therefore, identification and screening of Javanica rice cultivars suitable for callus growth and plant regeneration are prerequisites for genetic transformation programs.

Lee et al. (2002) found that the number, colour, size, shape and the appearance of the embryogenic calli varied among the rice genotypes depending on the type of basal medium, indicating that induction of high-quality rice callus is influenced by genotype, medium, and the kind of explants as well as by their interaction. Induction of embryogenic calli in rice is considered as the most critical step for the success in plant transformation. Visarada et al. (2002) found that genotype showing moderate callus induction showed high regeneration ability.

The purpose of this study was to develop a reproducible and an efficient procedure for callus induction and plant regeneration of embryogenic calli from mature seeds of selected Javanica rice cultivars from Indonesia for future genetic transformation studies. Callus induction is the first step in rice transformation to obtain embryogenic calli (Rachmawati et al. 2004). In particular, genotype and the composition of culture medium are important factors for successful embryogenic callus induction and regeneration of plant. Significant genotypic variations in Japonica and Indica rice have been reported (Abe and Futsuhara 1986; Rashid et al. 1996; Khanna and Raina 1998).

In the present study, genotypic differences in callus induction, subculture, and plant regeneration of Javanica rice cultivars were examined using C-modified medium (Rachmawati et al. 2004) and MS2 medium (Dong et al. 1996) (Table 1). We also examined the efficiencies of transformation at two phases: i.e. at the generation of stably transformed callus and at the regeneration of plantlets from transformed callus. Mature seeds of five Javanica rice cultivars, namely Bulu, Menthik, Rojolele, Situpatenggang and Tenggulang were used as the explants source of *in vitro* experimental materials. The five cultivars tested are broadly cultivated in Indonesia. Bulu, Menthik and Rojolele are local rice cultivars which have an aromatic fragrance and a delicious taste. Situpatenggang and Tenggulang are new rice cultivars

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tolerant to drought (gogo rice). The callus induction rate was calculated after 4 weeks of culture as the percentage of the seeds producing callus.

As results, significant difference was found in callus induction rate and quality of callus induced among the five genotypes of Javanica rice (Table 2). Although a considerable variation was observed, all genotypes of rice can induce callus on both media. Two types of calli, embryogenic and non-embryogenic ones, were obtained from the embryo scutellum. The embryogenic calli (type-I) were relatively dry, milky white in color, compact and nodular in appearance (Figure 1A, B). In contrast, non-embryogenic calli (type-II) were soft and watery, mostly white but some brown in color (Figure 1C, D). Four weeks after induction, the calli were subcultured onto fresh medium, which had the same composition as that used for callus induction. Following the subculture, the type-I calli grew about 5 to 10-fold in volume 30 days after transfer. However, the type-II calli turned brown and died.

In general, callus induction rate of the five Javanica rice cultivars on C-modified medium was higher than that on MS2 medium (Table 2). This finding is consistent with the results reported by Ogawa (2000) that C-medium is applicable to a wider range of cultivars including Indica, Japonica and Javanica subspecies. The

Table 1. Composition of media for callus induction and subculture.

| Composition | MS2* | C-Modified* |
|---|---|---|
| *Macronutrient components* | | |
| $KNO_3$ | 1900 | 2020 |
| $NH_4NO_3$ | 1650 | — |
| $(NH_4)_2SO_4$ | — | — |
| $KH_2PO_4$ | 170 | 272 |
| $CaCl_2 \cdot 2H_2O$ | 440 | 147 |
| $MgSO_4 \cdot 7H_2O$ | 370 | 245 |
| $NaH_2PO_4 \cdot 2H_2O$ | — | — |
| *Micronutrient components* | | |
| $FeSO_4 \cdot 7H_2O$ | 27.8 | 19 |
| $Na_2EDTA$ | 37.3 | — |
| $MnSO_4 \cdot H_2O$ | 22.3 | 1.6 |
| $H_3BO_3$ | 6.2 | 2.8 |
| $ZnSO_4 \cdot 7H_2O$ | 8.6 | 2.2 |
| KI | 0.8 | — |
| $CuSO_4 \cdot 5H_2O$ | 0.025 | 0.20 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.25 | 0.13 |
| $CoCl_2 \cdot 2H_2O$ | 0.025 | — |
| *Organic components* | | |
| Glycine | 2.0 | — |
| Thiamine | 0.4 | 1.0 |
| Pyridoxine | 0.5 | 10.0 |
| Nicotinic acid | 0.5 | 1.0 |
| Myoinositol | 100 | 100 |
| Alanine | — | 445 |
| Proline | — | 1151 |
| *Carbon source* | | |
| Sucrose | 30000 | — |
| Maltose | — | 15000 |
| *Phytohormone* | | |
| 2,4-Dichlorophenoxyaceticacid | 2.0 | 2.0 |

*: Concentration in mg $l^{-1}$. Both media adjusted at pH 5.8

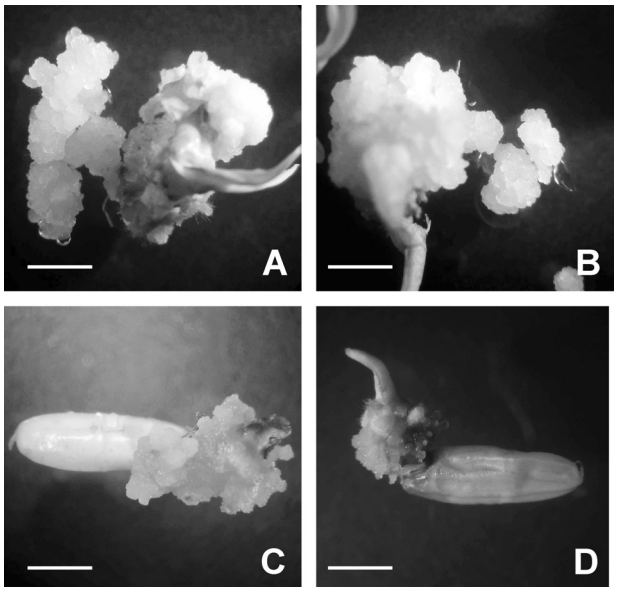


Figure 1. Morphological phenotypes of initiated calli. A, B: embryogenic calli (type-I) and C, D: non-embryogenic calli (type-II). Bars=5 mm.

Table 2. Callus induction from mature seeds of 5 Javanica rice cultivars after 4 weeks of culture.

| Cultivar | Callus induction medium | Percentage of callus-inducing seeds | | |
|---|---|---|---|---|
| | | Type-I* | Type-II** | Total |
| Bulu | MS2 | 22.7±9.8 | 25.3±12.8 | 48.0±7.3 |
| | C-Modified | 34.7±9.8 | 25.3±5.5 | 60.0±8.1 |
| Menthik | MS2 | 20.0±6.6 | 28.0±5.5 | 48.0±3.6 |
| | C-Modified | 28.0±5.5 | 24.0±3.6 | 52.0±8.7 |
| Rojolele | MS2 | 5.3±5.5 | 25.3±7.3 | 30.6±5.9 |
| | C-Modified | 52.0±5.5 | 20.0±6.6 | 72.0±8.6 |
| Situpatenggang | MS2 | 24.0±3.6 | 32.0±8.6 | 56.0±3.6 |
| | C-Modified | 28.0±5.5 | 30.6±7.6 | 58.6±9.8 |
| Tenggulang | MS2 | 10.6±3.6 | 21.4±8.7 | 32.0±8.6 |
| | C-Modified | 34.7±5.9 | 29.3±5.9 | 64.0±7.3 |

Data were expressed as the average of five replicates, each with 15 mature seeds
Callus appearance Type-I* : relatively dry, compact and a milky white color
Type-II** : watery, soft and brown color

induction rate of type-I calli from all the five Javanica rice cultivars on C-modified medium was higher than that on MS2 medium, especially for Rojolele. On C-modified medium, the induction rate of Rojolele was 10 times higher than one on MS2. On the other hand, the induction rate of type-II calli of the five cultivars on MS2 medium was almost the same as that on C-modified medium (Table 2).

To determine the regeneration ability, the initiated calli were transferred to a regeneration medium which was composed of macronutrient, micronutrient and organic components of MS, 2.0 mg $l^{-1}$ 6-benzylaminopurin (BAP), 1 mg $l^{-1}$ alpha-naphtaleneacetic acid (NAA), 300 mg $l^{-1}$ casamino acid, 3% maltose, 3% sorbitol and 0.4% phytagel, pH 5.8 and cultured for 4 weeks. We compared plant regeneration ability between two types of calli (type-I and type-II) obtained from two callus induction media. The plant regeneration frequency is defined as the percentage of calli yielding at least one plantlet per total number of calli inoculated after 4 weeks of culture.

In the plant regeneration experiment, all five cultivars tested were capable of regenerating plantlets. However, plant regeneration frequency varied among five cultivars (genotypes) and between two different phenotypes of calli. We also observed that type-I calli produced much higher plant regeneration frequency than type-II calli. Type-I calli of Rojolele obtained from C-modified medium showed the highest regeneration frequency of 83% compared to those from MS2 which gave 43% of regeneration frequency (Figure 2A). Consequently, regeneration frequency of green plantlets from calli produced on C-modified medium was higher than that from calli produced on MS2 medium (Figure 2B). On the other hand, the regeneration frequency of type-II calli was approximately 10% irrespective of the medium used for callus induction (data not shown). Type I calli of Situpatenggang (STP) initiated on MS2 and C-modified media gave similar plant regeneration frequency (Figure 2A). In relation to regeneration response, Rojolele was found to be the best genotype followed by Situpatenggang, Menthik, Bulu, and Tenggulang. The results have established that, for the media and cultivars tested in this study, the callus growth rate and the quality of the calli are dependent on both the medium used and the cultivar. As reported by Khanna and Raina (1998), the quality of initiated calli played a significant role in callus proliferation and subsequent plant regeneration.

Since the type-I calli grew vigorously and showed much higher regeneration potential than the type-II calli, the type-I calli were chosen as explants for transformation experiments. Hiei et al. (1994) also reported previously that choosing a vigorously growing callus as starting material is important for rice transformation mediated by *Agrobacterium*. Additionally, the quality of calli might be a key factor for the success

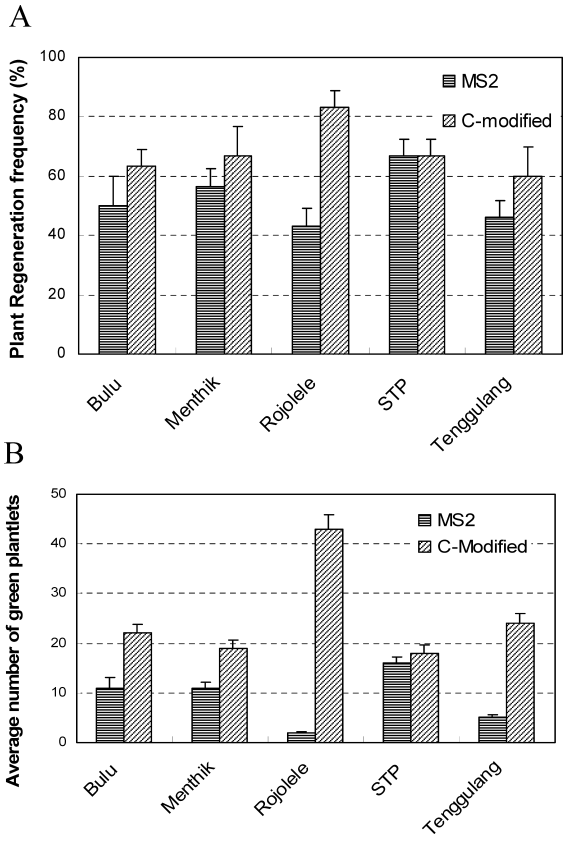


Figure 2. Plant regeneration of type-I calli (A) and induction of green plantlets from calli produced on MS2 and C-modified media (B) of five Javanica rice cultivars. The initiated calli from callus induction media MS2 and C-modified were transferred into regeneration medium. The regeneration frequency is defined as the percentage of calli yielding at least one plantlet. Average number of green plantlets per 100 seed cultured are shown.

in transformation (Lin and Zhang 2005). The type-I calli derived from mature seeds of each cultivar were infected for 5 min with *Agrobacterium tumefaciens* strain EHA101 containing the binary vector pAFT14 for transformation as described previously (Rachmawati et al. 2004). After 3 days of co-cultivation, the infected calli were washed with sterile water containing 250 mg $l^{-1}$ carbenicillin and transferred to C-modified medium containing 250 mg $l^{-1}$ carbenicillin and 50 mg $l^{-1}$ hygromycin. Subsequently, hygromycin-resistant calli were transferred to regeneration medium. Small pieces of hygromycin-resistant calli were subjected to histochemical assay of GUS activity. Moreover, the regenerated plantlets were analyzed by PCR to confirm the presence of the *hpt* and *gus* genes (data not shown). The hygromycin-resistant calli were obtained in all the five cultivars examined, but genotypic difference were found in the frequencies of both hygromycin-resistant and GUS-positive calli formation (Table 3).

The overall transformation efficiency of the five cultivars revealed that Rojolele rice cultivar has a high transformation efficiency (23%), similar to that of the

Table 3. Efficiency of transformation by *Agrobacterium tumefaciens* strain EHA101 (pAFT14) and regeneration of transgenic plants in five Javanica rice cultivars.

| Rice cultivar | Experiment | Number of calli | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cocultivated (A) | $Hyg^R$ (%)[a] | $GUS^+$ (%)[b] | $Hyg^R$ showing regeneration | Plantlet regenerated | $GUS^+$ plant (B) | Efficiency (B/A, %) |
| Bulu | 1 | 100 | 64 (64.0) | 17 (26.5) | 13 | 13 | 10 | 10.00 |
| | 2 | 80 | 41 (51.0) | 12 (29.3) | 10 | 10 | 7 | 8.75 |
| Menthik | 1 | 100 | 59 (53.6) | 15 (25.4) | 11 | 14 | 8 | 8.00 |
| | 2 | 80 | 36 (45.0) | 5 (13.8) | 4 | 4 | 3 | 3.75 |
| Rojolele | 1 | 100 | 85 (85.0) | 35 (41.2) | 25 | 27 | 23 | 23.00 |
| | 2 | 80 | 68 (85.0) | 27 (39.7) | 24 | 24 | 18 | 22.50 |
| Situpatenggang | 1 | 80 | 53 (66.0) | 12 (22.6) | 10 | 11 | 3 | 3.75 |
| | 2 | 100 | 46 (46.0) | 7 (15.2) | 11 | 8 | 5 | 5.00 |
| Tenggulang | 1 | 80 | 40 (50.0) | 7 (17.5) | 6 | 7 | 6 | 7.50 |
| | 2 | 80 | 27 (33.7) | 5 (18.5) | 5 | 5 | 4 | 5.00 |

[a] Percentage $Hyg^R$ calli=( Number of hygromycin resistant calli/Number of calli plated on hygromycin-selection medium )×100
[b] Percentage $GUS^+$ calli=( Number of selected calli showing GUS expression/Number of calli showing hygromycin resistance )×100

Japonica rice cv. Nipponbare (Rachmawati et al. 2004). Whereas transformation efficiency of four other Javanica rice cultivars was in the range of 3 to 10% (Table 3). The differences in the transformation efficiency among the cultivars are likely due to differences in the sensitivity of the genotypes to *Agrobacterium* infection and in the regeneration frequency. As reported earlier, *Agrobacterium*-mediated transformation of higher plants is highly dependent upon species, genotype and competency of the target plant tissue, host recognition, and other factors (Birch 1997; Hiei et al. 1994; Toki 1997).

This study emphasizes the importance of tissue culture conditions for transformation efficiency and demonstrates that the success in genetic transformation studies depends on the genotype and tissue culture conditions. C-modified medium has been proved to be applicable for callus induction of many Javanica rice cultivars. *Agrobacterium*-mediated transformation system has been extended to five Javanica rice cultivars. Therefore, the *Agrobacterium*-mediated transformation system might be used to develop transgenic rice with economically important genes either to increase production or to improve nutritional quality.

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