Identification of Callus Induction Potential of 15 Indonesian Rice Genotypes

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Abstract: The callus induction potential of 15 indica rice genotypes from Indonesia was examined in comparison with that of the japonica rice Nipponbare. Callus was induced from embryos of mature seeds and root segments on MS and CI media. There was genotype × medium × explant interaction for inducing white/cream/yellow callus with an organized structure (callus type I and II) and for callus browning, but not for callus induction ability and diameter of callus. Genotypes significantly differed in inducing high quality of calluses depending on medium and explant used. Four indica types, Fatmawati, Ciapus, BP-23 and BP-360-3, had callus induction-related traits similar to those of Nipponbare. These genotypes would be useful for tissue-culture based research and for crop improvement, particularly for genetic transformation. Culturing seed explant on MS was more suitable for callus induction than either root explant on MS or both explants on CI medium.

Keywords: Callus browning, Callus induction ability, Embryogenic callus, Rice.

Callus induction is one of the substantial steps for selecting the suitability of genotypes for tissue-culture-based research and for plant improvement, particularly for genetic transformation. In rice, different callus types, i.e., type I, II, III and IV, can be induced (Visarada et al., 2002). Type I callus is white and cream colored compact organized callus, type II is a yellow organized callus, type III is a yellow or brown unorganized callus, and the type IV is highly unorganized white, yellow or brown callus. Type-I and-II calluses are embryogenic and can be induced from tissues of various organs such as immature seeds (Masuda et al., 1989), immature embryos (Koetje et al., 1989), and roots (Abe and Futsuhara, 1985; Hoque and Mansfield, 2004). The type-III callus is dark and necrotic. In general, immature embryos and meristematic tissues, having undifferentiated cells, are suitable for callus induction and plant regeneration than mature tissues (Morrish et al., 1987). However, such explants are available only in a restricted period of the growth cycle in the rice plant (Hoque and Mansfield, 2004), and to obtain such explants all year round, we have to grow the plants in a greenhouse. However, embryos of mature seeds are available throughout the year, and are more suitable for rice callus culture. The embryogenic calluses induced by culture of mature seeds are effective used for genetic transformation by particle bombardment (Jiang et al., 2000) or Agrobacterium-mediated transformation (Kumria et al., 2001). The aseptic culture of root explants is also useful since it is relatively easy to provide in any season. In the in vitro culture of rice explants, a significant difference in callus induction has been found among the different genotypes of indica rice (Abe and Futsuhara, 1986; Peng and Hodges, 1989; Seraj et al., 1997) and japonica rice (Yoshida and Oosato, 1998; Ogawa et al., 1999). Indica rice cultivars generally show less induction of callus formation than japonica (Abe and Futsuhara, 1986). Not only callus production, but also somatic embryogenesis and subsequent plant regeneration (Chu and Croughan, 1990) are also inferior in indica rice, which may limit the success in the transformation for indica rice.

Callus induction as well as regeneration potential is affected not only by genotype and the type of explant but also by the composition of the culture medium including plant growth regulators, and by the culture conditions. However, in particular, genotype and type of explant are important factors for the successful embryogenic callus induction and regeneration of rice plants (Rueb et al., 1994). In this study, genotypic differences among 15 Indonesian rice genotypes in callus induction potential was examined, combined with two types of explants (embryos of mature seed and root segment) and two culture media: MS (Murashige and Skoog, 1962) and CI (Potrykus et al., 1979). The success using MS medium (Seraj et al., 1997; Khanna and Raina, 1998; Lee et al., 2002), as well as CI medium (Jiang et al., 2000) for callus induction has already been reported.

The objective of the present study was to identify callus induction potential of 15 Indonesian rice genotypes in vitro. The genotypes identified to have a good induction potential could then be utilized in...
tissue culture-based studies directed for the coming genetic transformation studies with the valuable genes of agricultural interest.

Materials and Methods

Fifteen indica rice genotypes from Indonesia and one japonica rice cv. Nipponbare as a check were used in the experiment since this genotype has been widely used as model in tissue culture and plant improvement studies (Table 1). The first five genotypes listed in the table are broadly cultivated in Indonesia and the following ten genotypes are promising ones bred by Indonesia Institute for Rice Research (Sukamandi, Subang, West Java).

Mature healthy seeds were dehusked manually and soaked in 70% ethanol for 3 min. with gentle agitation followed by rinsing with sterile distilled water. The seeds were then surface sterilized in 50% commercial bleach (5% sodium hypochlorite) for 30 min. with agitation and rinsed with sterile distilled water three times. The root explants were obtained from the seedlings grown on MS-basal medium. The embryos of mature seeds (seed explants) and root segment (root explants, from 5- to 7-day-old seedling) were aseptically cultured on callus induction medium; eight explants were placed on a Petri dish (9 cm in diameter) containing 20 mL MS or CI medium supplemented with 2 mg L\(^{-1}\) 2,4 dichlorophenoxyacetic acid (2,4-D) (Table 2). Anticipate the contamination that would arise, we cultured additional explants of each genotype on both media. The pH of medium was adjusted to 5.8 before autoclaving, and the cultures were kept at 25±1\(^{\circ}\)C under dark condition. After a 30-day culture, explants were examined for callus induction ability (CIA : total number of explants

| No. | Genotypes | Parents             |
|-----|-----------|---------------------|
| 1.  | Fatmawati | BP68G-MR-4-3-2/Maros|
| 2.  | Gilirang  | B6672/Memberamo      |
| 3.  | Ciapus    | Memberamo//IR-66154-221-2-2/Memberamo |
| 4.  | Cimelati  | Memberamo//IR-66160/Memberamo |
| 5.  | IR-64     | IR-5657/IR-2061      |
| 6.  | BP-23     | IR-64/IRBB7//IR64    |
| 7.  | BP-140    | IR-66738-18-1-2//Barumun/IRBB7 |
| 8.  | BP-360-2  | B10182/Memberamo//IR-66160/Memberamo//Memberamo |
| 9.  | BP-360-3  | B10182/Memberamo//IR-66160/Memberamo//Memberamo |
| 10. | BP-205D-KN-78-1-8 | Dacava line 85/Memberamo |
| 11. | BP-135    | IR-65598-27-3-1//Suban//Barumun |
| 12. | BP-138    | Muncul//IR-64/TB154E-TB-1 |
| 13. | BP-143    | Memberamo//IR-65598//Memberamo |
| 14. | B10597F   | IR-BB7/Cincklonik     |
| 15. | BP-355E   | Memberamo//IR-665600/Memberamo//IR-65598/Cibodas |
| 16. | Nipponbare | (check) Yamabiko/Sachikaze |

Table 1. Genotypes used in this experiment.

Table 2. Composition of media used in this study.

| Components         | Medium MS* | Medium CI* |
|--------------------|------------|------------|
| Inorganic salts    |            |            |
| Macro elements     |            |            |
| NH\(_4\)NO\(_3\) | 1650       | 640        |
| KNO\(_3\)         | 1900       | 1212       |
| CaCl\(_2\)2H\(_2\)O | 440        | 588        |
| MgSO\(_4\)7H\(_2\)O | 370        | 247        |
| KH\(_2\)PO\(_4\)  | 170        | 136        |
| Micro elements     |            |            |
| KI                 | 0.83       | 0.83       |
| H\(_3\)BO\(_3\)   | 6.2        | 3.1        |
| MnSO\(_4\)4H\(_2\)O | 22.3       | 11.15      |
| ZnSO\(_4\)7H\(_2\)O | 8.6        | 5.76       |
| Na\(_2\)MoO\(_4\)2H\(_2\)O | 0.25       | 0.24       |
| CuSO\(_4\)5H\(_2\)O | 0.025      | 0.025      |
| CoCl\(_2\)6H\(_2\)O | 0.025      | –          |
| CoSO\(_4\)7H\(_2\)O | –          | 0.028      |
| FeSO\(_4\)7H\(_2\)O | 27.85      | 27.8       |
| Na\(_2\)EDTA2H\(_2\)O | 37.25     | 37.3       |
| Organic supplement |            |            |
| Nicotinic acid     | 0.5        | 6.0        |
| Pyridoxine-HCl     | 0.5        | 1.0        |
| Thiamine-HCl       | 0.5        | 8.5        |
| Coconut water      | –          | 100mL\(^{-1}\) |
| Glycine            | 2.0        | 2.0        |
| Carbon source      |            |            |
| Sucrose            | 30000      | 20000      |
| Mannitol           | –          | 36430      |
| Phytohormone (2,4-D) | 2.0       | 2.0        |

*: Concentration in mgL\(^{-1}\). Both media did not contain Myoinositol.
with calluses per total number of explants cultured × 100%), average diameter of callus (ADC in cm), % of white or cream or yellow calluses that belong to callus type I or II (WYC: number of white or cream or yellow calluses with compact or friable structure per number of explants cultured ×100%), and % of callus browning (CBW: number of callus browning per number of explants cultured ×100%). A completely randomized factorial design with three replications was employed in this study. Analysis of variance (ANOVA) and Dunnett’s pairwise multiple comparison t-test for genotypes were performed by using SPSS 10 software.

**Results**

All genotypes induced calluses, but CIA varied with the genotypes. Total number of explants with calluses was 76 for Nipponbare; on the average per dish was 6.3 (79.17%). Fig. 1 shows the induced callus of some genotypes. B10597F had poor callus induction ability. Phenotypically, calluses derived from mature seed were relatively hard, and sometimes dry, but those derived from root segment were highly mucilaginous, soft and covered with a translucent sticky substance. In some roots, calluses were produced on a few sites of the segment. Most of the root segments with hairs had a high ability to induce callus.

A significant interaction was detected for genotype × medium × explant (p<0.01 for WYC and CBW), medium × explant (p<0.01 for CIA, WYC and CBW), genotype × explant (p<0.01 for CIA, WYC and p<0.05 for CBW), and genotype × medium (p<0.05 for WYC) (Table 3). For WYC and similarly for CBW, except for the genotype × medium interaction, main- and interaction effects were significant. For CIA, the medium × explant interaction accounted for the greatest variation. In general, explant accounted for the greatest variation in ADC, WYC, and CBW, but not in CIA. We found no significant interaction among the genotype × medium × explant for CIA and ADC. Medium × explant and genotype × explant interaction

![Fig. 1. Calluses of the genotypes B10597F (A), BP-360-3 (B) and Nipponbare (C), derived from seed explant (A, B, C followed by subscript number 1 and 2) and root explant (with subscript number 3 and 4), cultured on MS (with subscript number 1 and 3) and CI (with subscript number 2 and 4) media.](image-url)
for ADC, genotype × medium interaction for both CIA and ADC were also not significant. The main effect of each factor for these traits was significant (p < 0.01, Table 3).

Although a significant genotype × medium × explant interaction was obtained for WYC and CBW as previously mentioned, we mainly analyzed the genotypic difference among the traits observed. In comparison with Nipponbare, Fatmawati, Ciapus, BP-23, and BP-360-3 had similarly superior callus induction-related traits irrespective of the medium and explants used according to Dunnett’s pairwise multiple comparison t-test at 0.05 probability level (Table 4). On the other hand, BP-135 was the only genotype showing significantly inferior performance in all traits as compared with Nipponbare.

In the overall analysis, MS medium and seed explant were superior for CIA, ADC, and WYC, whereas CI medium and root explant were superior for CBW.

**Discussion**

It is well known that the genotype (Abe and Futsuhara, 1986), the interaction between genotype and medium (Khanna and Rainma, 1998) and also the interaction between genotype and explant (Hoque and Mansfield, 2004) have significant effects on callus induction. In this study, the effect of genotype × medium × explant interaction on WYC and CBW was significant, but that on CIA and ADC was not (Table 3). The WYC and CBW are believed to be related to the quality of callus. White/cream/yellow calluses as reported by Visarada et al. (2002) are embryogenic calluses and are also associated with at least two other types of calluses, i.e. 1) a soft, transparent, unorganized callus, which is nonmorphogenic or may form only roots, and 2) soft mucilaginous callus, which often form roots. Maeda (2000) and Maeda et al. (2002) reported that white and green patches are often seen on the surface stratum of callus with high regeneration ability. Moreover, in sorghum, Kaeppler and Pedersen (1997) reported that the production of a high quality callus depends mainly on the genotype. Lee et al. (2002) found that the number, colour, size, shape and appearance of the embryogenic calluses 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 explant as well as by their interactions.

Callus browning is one of the major problems in *in vitro* culture. Abe and Futsuhara (1991) and Ogawa et al. (1999) argued that callus browning is genetically controlled, which correspond to the present study. A decrease in the rate of callus growth is occasionally related to the appearance of a brown colored area (Maeda et al., 2002). A low 2,4-D concentration (Mitsuoka et al., 1994) and high temperature (Hoque and Mansfield, 2004) seem to cause callus browning or regeneration of albino plants. However, in the present study, 2 mg L\(^{-1}\) 2,4-D and 25°C were used for callus induction, which are supposed to be an optimum for callus induction. However, the result of callus browning in this study should be considered carefully, since explants of the genotype that could not produce the calluses were not examined. If we examined the browning of the culture including the browning of explants, the result would be slightly different, particularly in the genotypes that had low ability in callus induction ability, i.e., Gilirang, BP-138, BP-143, particularly in the genotypes that had low ability in callus induction ability, i.e., Gilirang, BP-138, BP-143, BP-105, and B10597F (Table 4).

In the present study, we provided root explants from 5- to 7-day-old seedlings; although Hoque and Mansfield (2004) found that the younger explants (3- to 5-day-old seedlings) of *indica* rice were most efficient in both callus induction and plant regeneration. This

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**Table 3. Analysis of variance for callus induction performance.**

| Source of variation       | df | Callus induction ability (CIA) | Average diameter of callus (ADC) | Callus white/yellow (WYC) | Callus browning (CBW) |
|---------------------------|----|--------------------------------|---------------------------------|--------------------------|-----------------------|
| Genotype (g)              | 15 | 2669.92**                     | 0.04**                          | 2337.67**                | 557.29**              |
| Medium (m)                | 1  | 8138.02**                     | 0.16**                          | 2929.69**                | 3987.63**             |
| Explants (e)              | 1  | 14179.69**                    | 1.87**                          | 19300.13**               | 9847.00**             |
| Genotype × Medium (g × m) | 15 | 276.91**                      | 0.01*                           | 462.67*                  | 291.45*               |
| Genotype × Explants (g × e)| 15 | 617.19**                      | 0.02*                           | 1079.64**                | 397.35*               |
| Medium × Explants (m × e) | 1  | 17347.00**                    | 0.02*                           | 6018.88**                | 6302.08**             |
| Genotype × Medium × Explants (g × m × e)| 15 | 319.23**                     | 0.01**                          | 610.89**                 | 435.76**              |
| Error                     | 128| 243.33                        | 0.01                            | 263.67                   | 191.24                |
| Total                     | 192|                               |                                 |                          |                       |

Data show mean square value, ns : nonsignificant, * : significant at p = 0.05, ** : significant at p = 0.01, respectively.
is because a large variation was found in root length in the 340 5-day-old seedlings, in some genotypes root length was only a few centimeters.

Medium composition plays an important role in callus induction (Khanna and Raina, 1998; Ogawa, 1999). The nutrient, particularly the nitrogen source, affects the callus induction of somatic embryos, particularly in monocots (Leifert et al., 1995). MS medium was suitable for obtaining high CIA, ADC and WYC, while CI medium was suitable for obtaining low CBW, although the difference was slight (Table 4). MS medium, compared with CI medium, has a higher concentration of macro and micro elements, but lower content of organic supplement, only one carbon source (i.e. sucrose) and no coconut water (Table 2). Such differences might cause the differences in tissue culture response.

Although genotype significantly contributed to the variation of traits observed, the contribution was not as large as we expected (Table 3). This may be due to the similar genetic background of the materials used. Eight of them are derived from the same parent, i.e., cv. Memberamo (from crossing between B6555 and IR-19661), and twelve of them are also derived from the IR-series (breeding lines) developed by IRRI (Table 1), thus all genotypes carry a genetic composition (female/male parent) from IR-series.

In Table 3, we found a high contribution of the explant to the variation of traits observed. The seed explant was suitable for obtaining high CIA, ADC, and WYC, and root explant for low CBW (Table 4). In addition, four genotypes i.e., Fatmawati, Ciapus, BP-23, and BP-360-3 have been identified as excellent genotypes for high CIA, ADC, WYC and low CBW compared with Nipponbare (Table 4). This shows that the seed explants of these superior genotypes have a high callus induction potential which depends on the activity of genes that determine and maintain the meristematic activity of the cells, level of hormones, and sensitivity to hormones, as well as on the activity of other genes that control different stages of plant morphogenesis (Ezhova, 2003). Fatmawati, released as a new cultivar in Indonesia in 2003, and BP-360-3 are new plant type (NPT) with low tillering capacity.

Table 4. Comparison of callus induction-related traits of 15 genotypes with those of Nipponbare and overall means for media and explants.

| No. | Genotype         | Callus induction ability (% CIA) | Average diameter of callus (cm ADC) | Callus white/yellow (%) WYC | Callus browning (%) CBW |
|-----|------------------|---------------------------------|-------------------------------------|----------------------------|-------------------------|
| 1.  | Fatmawati        | 69.79**                        | 0.29**                              | 44.79**                    | 11.46**                 |
| 2.  | Gilirang         | 59.38*                         | 0.19*                               | 34.38*                     | 19.79**                 |
| 3.  | Ciapus           | 80.21**                        | 0.26*                               | 50.00*                     | 14.58**                 |
| 4.  | Cimelati         | 67.71**                        | 0.24*                               | 36.46*                     | 20.83**                 |
| 5.  | IR-64            | 79.17**                        | 0.24*                               | 57.29**                    | 8.33**                  |
| 6.  | BP-23            | 86.46**                        | 0.30**                              | 65.63**                    | 6.25**                  |
| 7.  | BP-140           | 90.63**                        | 0.33**                              | 64.58**                    | 25.00*                  |
| 8.  | BP-360-2         | 67.71**                        | 0.20*                               | 33.33*                     | 25.00*                  |
| 9.  | BP-360-3         | 82.29**                        | 0.33**                              | 52.08**                    | 17.71**                 |
| 10. | BP-205D-KN-78-1-8| 69.79**                       | 0.25**                              | 44.79**                    | 23.96**                 |
| 11. | BP-135           | 46.88*                         | 0.19*                               | 28.13*                     | 22.92*                  |
| 12. | BP-138           | 57.29*                         | 0.26**                              | 31.25*                     | 17.71**                 |
| 13. | BP-143           | 61.46*                         | 0.20*                               | 34.38*                     | 13.54**                 |
| 14. | B10597F          | 33.33*                         | 0.15*                               | 15.63*                     | 14.58**                 |
| 15. | BP-355E          | 70.83**                        | 0.29**                              | 45.83**                    | 27.08*                  |
| 16. | Nipponbare (check)| 79.17**                       | 0.38                                | 57.29                      | 6.25                   |

| Explant          | Callus induction ability (% CIA) | Average diameter of callus (cm ADC) | Callus white/yellow (%) WYC | Callus browning (%) CBW |
|------------------|---------------------------------|-------------------------------------|----------------------------|-------------------------|
| Seed explant     | 77.47                           | 0.36                                | 53.52                      | 24.35                   |
| Root explant     | 60.29                           | 0.16                                | 33.46                      | 10.03                   |
| MS medium        | 75.39                           | 0.29                                | 47.40                      | 21.75                   |
| CI medium        | 62.37                           | 0.23                                | 39.58                      | 12.63                   |

ns : nonsignificant, * : significant compared to Nipponbare’s performance using Dunnett’s pairwise multiple comparison t-test at 0.05 probability level with right-sided (>control) for CIA, ADC and WYC, and left-sided (<control) for CBW. Values are means of combination of 2 media and 2 explants with 3 replicates. The difference between seed explant and root explant or between MS medium and CI medium are significant (Table 5).
and large panicles. Ciapus is also a new cultivar released in 2003 and BP-23 is a promising line, though not released yet. These genotypes should be further examined for their plant regeneration ability or may be used as genetic transformation materials. We believed that these genotypes with good callus induction-related traits would be useful for plant regeneration, though Visarada et al. (2002) found that genotypes showing moderate callus induction showed high regeneration ability. Since these genotypes are similar to Nipponbare in callus induction ability, further works especially on genetic transformation experiments may be promising.

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