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# Improvement of Shoot Regeneration from Scutella-Derived Callus in Rice

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**ABSTRACT: The optimized *in vitro* culture system was investigated for improvement of regeneration efficiencies by observing the responses of scutella-derived callus of Korean rice (*Oryza sativa* L.). Large variations of callus induction (43.9-93.9%) and shoot regeneration (0-88.7%) were observed among the rice cultivars depending on medium. However, shoot regeneration was significantly improved by selected utilization of basal medium, growth regulators, and carbon sources. N6 basal medium was more efficient for embryogenic callus induction than MS or LS basal medium, while MS was superior to N6 for shoot regeneration. The calli of highly regenerative cultivars grew faster and showed higher rates of green tissue formation (GT) and shoot regeneration (SR) and lower rate of callus browning (CB) than those of recalcitrant cultivars. Although a higher level of kinetin stimulated the GT and SR in highly regenerative cultivars, 10 $mgL^{-1}$ kinetin generally suppressed the GT and SR, while CB was accelerated compared to 2 $mgL^{-1}$ kinetin. Additional benefits of sorbitol combined with maltose (or sucrose) under 5 $mgL^{-1}$ kinetin were certainly confirmed on regeneration efficiencies compared to sucrose alone as carbon source and osmotic regulator. This combination showed high rate of GT and SR with multiple shoots while low rate of CB. With MSRK5SM-Pr medium (5 $mgL^{-1}$ kinetin, 3% sorbitol, 2% maltose, 500 $mgL^{-1}$ proline), the regeneration efficiencies of total 17 out of 24 cultivars were practically improved 160% on average compared to MSRK2S (2 $mgL^{-1}$ kinetin, 3% sucrose) control medium. Especially, the medium was most effective to the cultivars showing a medium level of regenerability such as Daesanbyeo and Dongjinbyeo and Suwon477, enhancing efficiencies more than 300-600% compared to MSRK2S medium.**

***Keywords***: rice (*Oryza sativa* L.), embryogenic callus induction, shoot regeneration, carbon source

Aside from *Arabidopsis* in dicot, rice (*Oryza sativa* L.) has been widely used as a plant material serving as a model system for plant genomics and *in vitro* studies in monocots. Because of its economic importance as a staple food, rice has been a more attractive target for developing transgenic plants. During the last decade, transgenic rice plants have been successfully developed for various purposes by selected choices of target genes combined with different transformation systems such as *Agrobacterium* mediated (Aldemita & Hodges, 1996; Datta *et al.*, 2000; Hiei *et al.*, 1994; Rashid *et al.*, 1996), biolistics (Chen *et al.*, 1998; Jiang *et al.*, 2000), and polyethylene glycol mediated (Datta *et al.*, 2001) transformation both in japonica and indica rice.

Whatever transformation system would be employed, efficient systems for embryogenic callus induction and shoot regeneration have been considered the basic matter in obtaining fertile transgenic rice. Among various tissues of rice, scutella-derived callus of mature seeds has been frequently employed as target materials for genetic transformation by both *Agrobactericum* mediated and biolistics methods because of the manipulative effectiveness (Chen *et al.*, 1998; Hashizume *et al.*, 1999; Hiei *et al.*, 1994; Rashid *et al.*, 1996; Sivamani *et al.*, 1996).

Selected utilization of basal medium, growth regulators, carbon sources, and amino acid are important considerations for improving efficiencies of embryogenic callus induction and shoot regeneration. Especially, shoot regeneration from the callus, the final stage for producing transformants, critically varied among rice cultivars. A number of studies have reported that foreign genes were successfully transformed in callus stage with higher frequencies ranging from 10% to 57%. However, in many cases, unsatisfactory results have been achieved in obtaining transgenic plants due to their unsuitable regeneration systems (Aldemita & Hodges, 1996; Hashizume *et al.*, 1999; Lee *et al.*, 1999; Rashid *et al.*, 1996).

The N6, MS or LS basal media supplemented with 2-6 $mgL^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) for embryogenic callus induction and 0.5-10 $mgL^{-1}$ kinetin and/or 6-benzylaminopurine (BAP) combined with 0.02-1 $mgL^{-1}$ α-naphtaleneacetic acid (NAA) for shoot regeneration have been selectively used depending on rice cultivars (Aldemita & Hodges, 1996; Jiang *et al.*, 2000; Kyozuka *et al.*, 1987; Toki, 1997; Xue and Earle, 1995; Yang *et al.*, 1999).

As carbon source and osmotic regulator, sucrose has been most acceptable for *in vitro* culture of rice. However, maltose (Jain *et al.*, 1995; Lee *et al.*, 1999; Zhang, 1995) and sorbitol (Rashid *et al.*, 1996) were substituted in the media for callus

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induction and shoot regeneration. Even though sorbitol is a major factor for embryogenic callus formation in the monocot plant such as maize (Swedlund & Locy, 1993), proliferation and regeneration of rice callus were suppressed by sorbitol (Yang *et al.*, 1999). Affirmative effects of proline on callus induction, proliferation, and shoot regeneration in transformation of rice have been reported (Jain *et al.*, 1995; Sivamani *et al.*, 1996; Toki, 1997; Yin *et al.*, 1993). However, excessive amounts of proline were not helpful in enhancing regeneration efficiency of japonica rice, Taipei 309 (Yang *et al.*, 1999).

In a practical point of view, consistent and abundant production of regenerated plants is very important to develop transgenic rice. The present study was conducted with two objectives : (1) for improving productivity of embryogenic callus of various type of rices based on cultural morphology of callus by selected utilization of cultural media and (2) for evaluating and improving the regeneration efficiencies of various Korean rice cultivars or lines including japonica, Tongil, japonica x Tongil hybrids, and color rice. Especially, the study was focused on identifying the effect of the regeneration media on regeneration efficiencies by observing responses of callus such as the rates of GT, SR, and CB at different culture periods.

## MATERIALS AND METHODS

### Callus induction

To select the efficient medium for embryogenic callus induction, three different basal media, N6, MS, and LS, were tested with three japonica rice, Nagdongbyeo, Dongjinbyeo and Ilpumbyeo, and two Tongil rice, Areumbyeo and Dasanbyeo (Table 1, Figs. 1 and 2). N6 basal callus induction medium was prepared with N6 salts and vitamins (Chu *et al.*, 1975) supplemented with 300 $mgL^{-1}$ casamino acid, 500 $mgL^{-1}$ proline, 500 $mgL^{-1}$ glutamin, 100 $mgL^{-1}$ myo-inositol, 30 $gL^{-1}$ sucrose, 2 $mgL^{-1}$ 2,4-D, and 2.5 $gL^{-1}$ gelrite (pH 5.8). MS or LS callus induction media was prepared with MS (Murashinge and Skoog, 1962) or LS (Linsmaier and Skoog, 1965) salts and vitamins, respectively, in substitution for those of N6. The sterilized seeds were plated on each semi-solid callus induction medium in a petri-dish by embryo faced up and cultured for four weeks at 28°C in dark room.

The study focused on callus induction as follows : 1) total callus induction rates based on the number of seeds plated, 2) the average number of embryogenic callus obtained from 10 seeds, 3) the morphology of the callus, and 4) viabilities of embryogenic callus by subculturing. Embryogenic calli (1-2 mm in diameter) were selected and proliferated for one week under the same conditions as those for callus induction prior to transferring the calli onto the regeneration media.

### Regeneration

Twenty four accessions; 18 japonica including 6 color rices, 3 Tongil-type, 3 japonica x Tongil hybrid were used in this study (Table 2). MSRK2S, MS basal medium containing 2 $mgL^{-1}$ kinetin, 0.5 $mgL^{-1}$ NAA, and 3% (w/v) sucrose, 4 $gL^{-1}$ phytagel (pH 5.8), was used as a control medium for evaluating the regeneration efficiencies of the cultivars. Total eight different media as shown in Table 1 were used to identify the effect of basal media, cytokinins and carbon sources on regeneration. Especially, MSRK5SS-Pr was prepared with MS basal medium supplemented with 5 $mgL^{-1}$ kinetin, 0.5 $mgL^{-1}$ NAA, 500 $mgL^{-1}$ proline, and 4 $gL^{-1}$ phytagel. For this medium, 2% (w/v) sucrose and 3% (w/v) sorbitol were employed as carbon sources instead of 3% (w/v) sucrose. Ten actively growing embryogenic calli were plated on semi-solid

**Table 1.** Compositions of the media for callus induction and shoot regeneration.

| Medium | Composition |
|---|---|
| Callus induction | |
| N6 | N6 salts and vitamins, 300 $mgL^{-1}$ casamino acid, 500 $mgL^{-1}$ proline, 500 $mgL^{-1}$ glutamin, 30 $gL^{-1}$ sucrose, 2 $mgL^{-1}$ 2,4-D, 2.5 $gL^{-1}$ phytagel, pH 5.8 |
| MS | N6 salts and vitamins in N6 callus induction medium was substituted with MS salts and vitamins (Murashinge & Skoog, 1962), pH 5.8 |
| LS | N6 salts and vitamins in N6 callus induction medium was substituted with LS salts and vitamins (Linsmaier and Skoog, 1965), pH 5.8 |
| Regeneration | |
| MSRK2S | MS salts and vitamins, 30 $gL^{-1}$ sucrose, 2 $mgL^{-1}$ kinetin, 0.5 $mgL^{-1}$ NAA, 4.0 $gL^{-1}$ phytagel, pH 5.8 |
| MSRK10S | 2 $mgL^{-1}$ kinetin in MSRK2 was substituted with 10 $mgL^{-1}$ kinetin, pH 5.8 |
| MSRK5SS-Pr | MS salts and vitamins, 20 $gL^{-1}$ sucrose, 30 $gL^{-1}$ sorbitol, 5 $mgL^{-1}$ kinetin, 0.5 $mgL^{-1}$ NAA, 500 $mgL^{-1}$ proline, 4.0 $gL^{-1}$ phytagel, pH 5.8 |
| MSRK2M | Sucrose in MSRK2S was substituted with maltose, pH 5.8 |
| MSRK10M | Sucrose in MSRK10S was substituted with maltose, pH 5.8 |
| MSRK5SM-Pr | Sucrose in MSRK5PLS was substituted with maltose, pH 5.8 |
| MSRB2S | Kinetin in MSRK2S was substituted with 2 $mgL^{-1}$ BAP, pH 5.8 |
| N6RK2S | MS salts and vitamins of MSRK2S were substituted with N6 salts and vitamins, pH 5.8 |

regeneration medium in petridish (10 cm (ϕ)×2cm (height)) and cultured at 25°C under 16 hours of daylight (13.4±1.5 W/ $m^2$) in tissue culture room for eight weeks.

The callus responses related with regeneration were investigated for three major events; 1) green tissue formation (GT), 2) shoot regeneration (SR), and 3) callus browning (CB). Regeneration efficiencies of each cultivar on each medium were evaluated at every two weeks for eight weeks by calculating the ratio of the number of the calli regenerated to those plated on each medium.

### Statistical analysis

All resulting scales of total callus induction rates, productivities of embryogenic callus, and shoot regeneration among the cultivars on the media were statistically analyzed with three replications using Proc GLM of SAS version 6.12 (SAS Institute, Cray, NC) and the means were compared by LSD (Least Significant Difference) test.

## RESULTS

### Callus induction

The growth rate and morphology of embryogenic calli varied depending on the culture media and rice cultivars used (Figs. 1 and 2). The calli from Nagdongbyeo and Dongjinbyeo, japonica rice, were steadily induced at higher rates ≥90% regardless of the media used, while 43.9-79.4% in Tongil rice depending on medium (Fig. 2). The calli of the japonica cultivars grew faster with numerous independent globular structures showing relatively more yellowish than those of Tongil rice (Fig. 1A and B, Fig. 2A). Morphology of the embryogenic callus was slick and yellowish-compact type with globular shape (Fig. 1C). Aside from Tongil rice, Ilpumbyeo showed lowest callus induction rates ranging from 45.6-66.1% depending on the media (Fig. 2A). The callus from the cultivars grew rather slow as un-separated clusters and the embryogenic calli were comparably whitish, semi-translucent, soft, and friable (Fig. 1E).

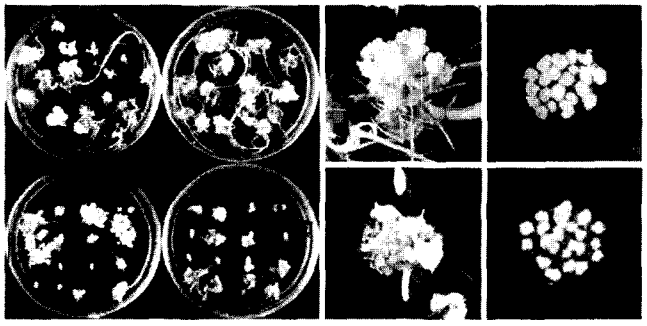

**Fig. 1.** Comparison of callus induction from hulled mature seeds of japonica and Tongil rice on N6 basal callus induction medium containing 2 $mgL^{-1}$ 2,4-D at four weeks. A : Nagdongbyeo, Dongjinbyeo, Ilpumbyeo, and Areumbyeo from left top to right bottom. Calli induced from Nagdongbyeo (B) and Areumbyeo (D) and their embryogenic calli (C and E), respectively.

### Effect of media on callus induction

N6 basal callus induction medium found to be the most effi-

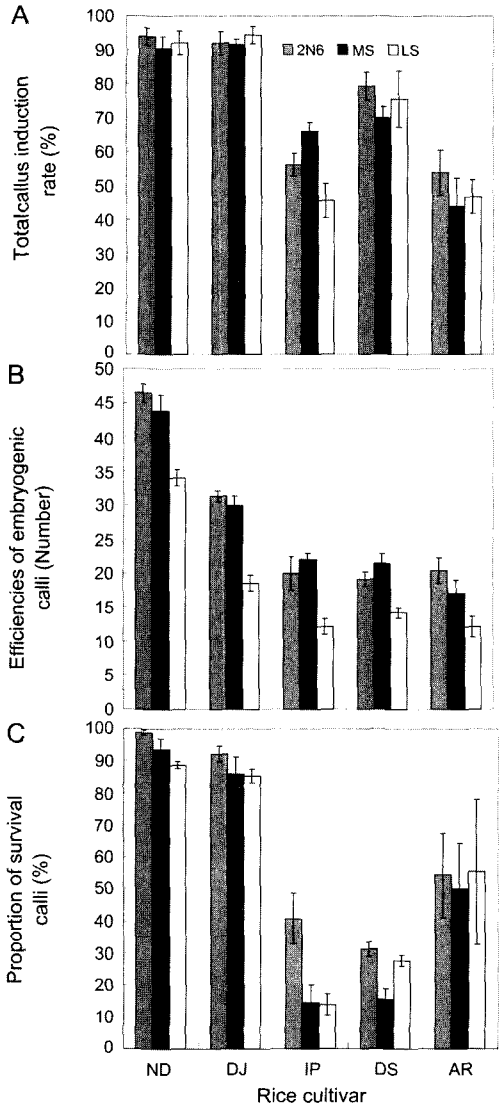


**Fig. 2.** Effect of basal media on callus induction and proliferation among the rice cultivars A : Total callus induction rates based on the number of the seeds, B : Average number of embryogenic calli produced by 10 seeds, C : The rate of survived calli by subculturing. The error bars indicate standards errors in tree replications of the experiments.
ND=Nagdongbyeo, DJ=Dongjinbyeo, IP=Ilpumbyeo
DS=Dasanbyeo, AR=Areumbyeo.

cient for callus induction except in some cultivars (Fig. 2). Average callus induction rate and yields (number of calli selected per 10 seeds) of embryognic callus of five cultivars on N6 basal medium were 75.1% and 27.5, respectively, which showed comparably higher level than those on MS and LS medium (Fig. 2A and B). However, variation of callus induction rates and yields of embryogenic calli among the cultivars on each medium were greater than that among the media in each cultivar. After one week of subculturing, the calli showing weak viability were eliminated and the healthy embryogenic calli could be proliferated and utilized for regeneration because direct transfer to the regeneration medium showed callus browning. In general, the proportion of growth calli in each cultivar on N6 basal medium was significantly higher than that on MS or LS media (Fig. 2C). Efficiency of embryogenic callus induction of Nagdongbyeo on N6 basal medium was 6 folds higher than recalcitrant cultivars, Ilpumbyeo and Dasanbyeo.

### Regeneration responses of the callus

On regeneration media, embryogenic callus tended to show positive response related with regeneration for GT and SR and negative response for CB. Generally, the callus having high regeneration potential formed GT on the surface of callus with fast growth for 7-10 days of cultivation (Fig. 3A) and, consequently, 0.2-1.0 cm size shoots emerged at 14th day (Fig. 3C). Only in Heugnambyeo, the purple color tissue was observed prior to GT formation (Fig. 3B), followed by SR wherein green leaf with purple color were found at the edge of the leaves (Fig. 3D). However, the calli of low regenerative cultivars frequently showed CB with relatively slow growth and finally died. The ratio of the three responses and their changes varied depending on rice cultivar and kind of medium used (Fig. 3E, F and G). As shown in Fig. 4, positive correlation was found between the ratio of GT and SR, while negative between CB and GT (or SR).

### Effect of basal media and cytokinins

Regeneration responses of scutella-derived calli of rice were compared among the N6 or MS basal regeneration media combined with kinetin or BAP (Fig. 5). The MS basal

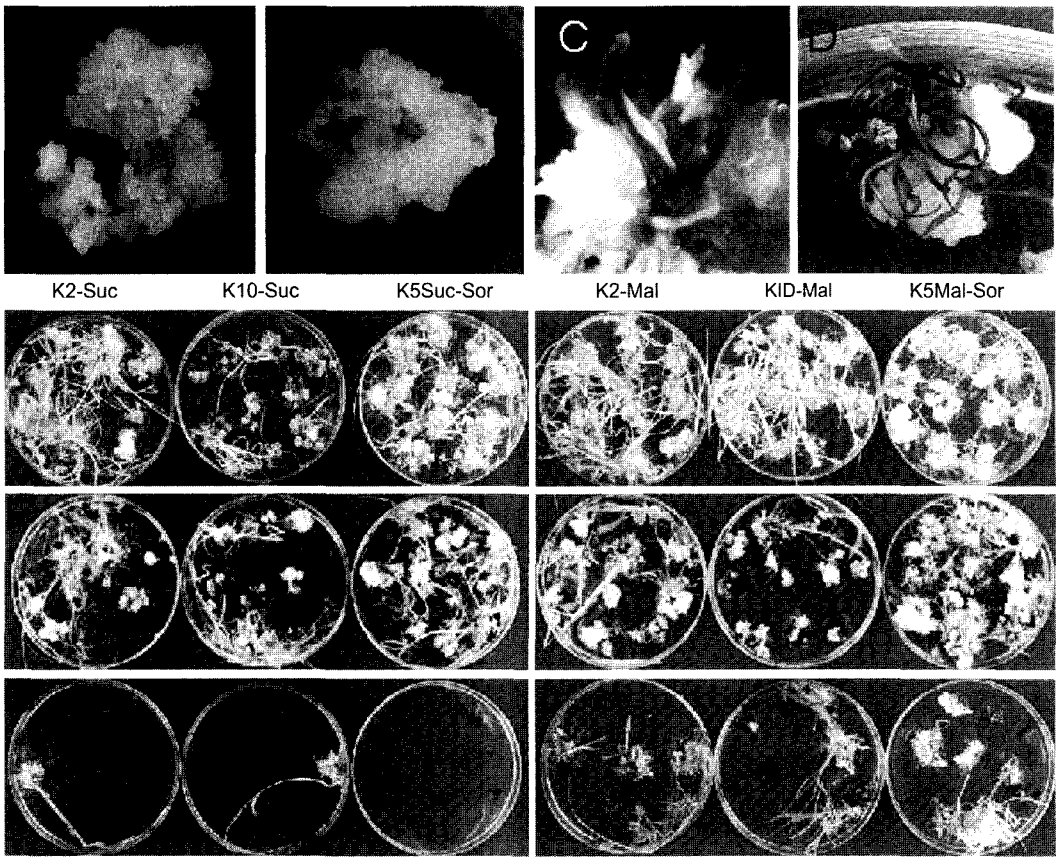


**Fig. 3.** Regeneration responses of scutella-derived calli of rice on various MS basal regeneration medium. A and B : Green (Nagdongbyeo) or purple (Heugnambyeo) color tissue formed on MSRK2S at 7 days and their shoot regenerated (B and E) at 14 days. Comparison of regeneration efficiencies on different MS regeneration media (E : Nagdongbyeo, F : Dongjinbyeo, and G : Areumbyeo on MSRK2S, MSRK10S, MSRK5SS-pr, MSRK2M, MSRK10M, and MSRK5SM-pr from the left, respectively).

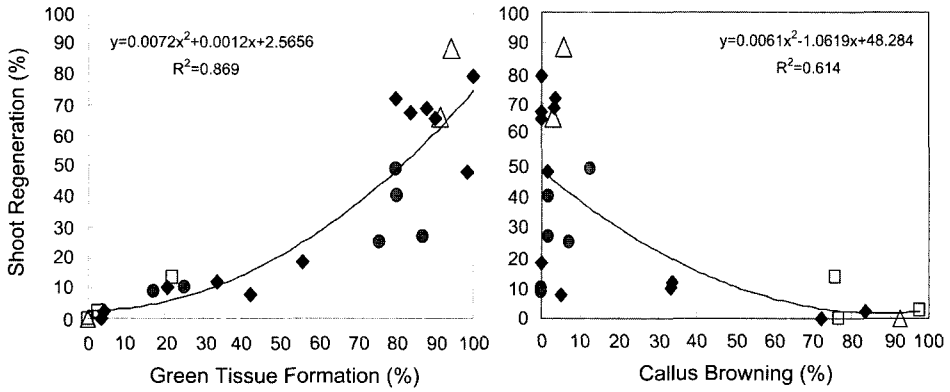


**Fig. 4.** Correlations among the regeneration responses of Korean rices cultivars. △ : japonica, × : Tongil, □ : Tongil rice, ◆ : japonica rice, ● : color rice. Genotype of all color rice was included in japonica rice.

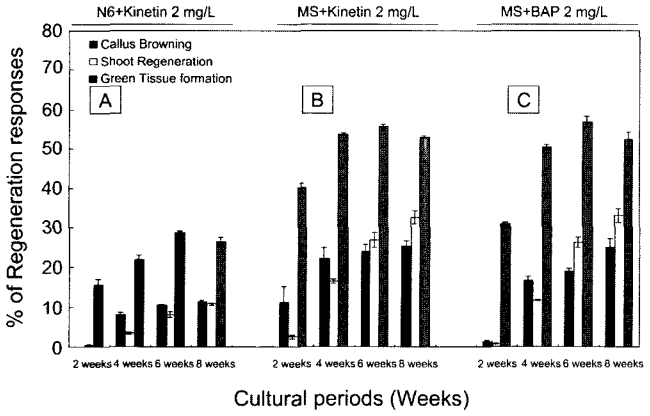


**Fig. 5.** Effect of basal media and cytokinins on regeneration responses of scutella-derived calli of 24 rice cultivars. A : N6 basal medium supplemented with 2 $mgL^{-1}$ kinetin, B : MS basal medium supplemented with 2 $mgL^{-1}$ kinetin, C : MS basal medium supplemented with 2 $mgL^{-1}$ BAP

regeneration medium showed significantly higher rates of GT (53.1%), SR (32.5%), and CB (25.4%) compared to those of N6 basal medium representing 26.5%, 10.8%, and 11.5% of GT, SR, and CB, respectively (Fig. 5A and B). This indicated that MS basal medium was superior to N6 basal medium for shoot regeneration although negative response of the callus was also stimulated on the medium together with positive responses.

Regeneration efficiency of each cultivar varied depending on the type of cytokinin (Table 2). The same concentration (2 $mgL^{-1}$) of kinetin and BAP tended to show equivalent effect on regeneration of calli as follows ; 53.1% and 52.4% of GT, 32.5% and 33.0% of SR, and 25.4% and 25.5% of CB on MSRK2S and MSRB2S, respectively (Fig. 5B and C). However, 2 $mgL^{-1}$ kinetin stimulated the positive responses (53.8% of GT and 16.7% of SR) compared to 2 $mgL^{-1}$ BAP (50.5% of GT and 11.8% of SR) within four weeks (Fig. 5). Furthermore, 14 out of the 24 cultivars showed higher level of SR on MSRK2S than on MSRB2S for eight weeks of cultivation (Table 2). Thus, utilization of kinetin seemed to be more appropriate for regeneration of calli than BAP.

### Effect of kinetin and carbon source

Regeneration responses of rice calli were greatly affected by critically different concentrations of kinetin and kinds of carbon sources (Table 2, Figs. 3 and 6). Although high concentration of kinetin stimulated either GT or SR in highly regenerative cultivars such as Nagdongbyeo, Donganbyeo, and Ilmibyeo, 10 $mgL^{-1}$ kinetin combined with 0.5 $mgL^{-1}$ NAA affected negatively to the shoot regeneration rather than positively compared to 2 $mgL^{-1}$ kinetin. At eight weeks of cultivation in all cultivars tested, the rates of GT (53.0%) and SR (32.5%) on MSRK2S were significantly higher than that (46.8% and 30.7% of GT and SR, respectively) on MSRK10S. However, the rate of CB (24.5%) on MSRK2S was significantly lower than that (33.2%) on MSRK10S (Fig. 6).

The beneficial effects of maltose on shoot regeneration from the callus were increasing positive response and decreasing negative response at all regeneration media even under 10 $mgL^{-1}$ kinetin (Figs. 3 and 6). Generally, in most of the cultivars that have high (or medium) regeneration potential such as Nagdongbyeo and Dongjinbyeo and even in recalcitrant cultivar such as Ilpumbyeo and Areumbyeo, maltose positively stimulated the regeneration responses and the growth of calli increasing GT and SR but decreasing CB (Fig. 6). GT and SR were improved (4.6-13.9% and 1.7-11.8%) and CB were decreased (8.3-17.2%) by substitution of sucrose to maltose depending on the concentrations of kinetin.

### Effect of sorbitol and proline

To identify the additional effect of sorbitol and proline on

**Table 2.** Comparison of regeneration efficiencies of rice calli among the cultivars on different regeneration medium at eight weeks of cultivation.

| Cultivar and remarks | | Medium: 2 mgL$^{-1}$BAP | 2 mgL$^{-1}$ kinetin | | 10 mgL$^{-1}$ kinetin | | 5 mgL$^{-1}$kinetin | | Mean$^{\dagger}$ LSD 0.05 |
|---|---|---|---|---|---|---|---|---|---|
| | | MSRB2S | MSRK2S | MSRK2M | MSRK10S | MSRK10M | MSRK5SS-Pr | MSRK5SM-Pr | |
| Japonica | Daesanbyeo | 8.8 d$^{\ddagger}$ | 7.9 d | 21.4 c | 4.9 d | 8.9 d | 31.8 b | 52.6 a | 19.5 h 9.09 |
| | Donganbyeo | 57.8 c | 71.9 bc | 90.9 a | 88.1 ab | 85.1 ab | 74.1 abc | 87.8 ab | 79.4 b 17.11 |
| | Dongjinbyeo | 5.2 c | 18.3 bc | 32.5 b | 13.7 c | 33.4 b | 61.1 a | 58.7 a | 31.9 g 15.86 |
| | Dongjinbyeo 1 | 36.7 cd | 48.1 bc | 60.6 b | 31.7 d | 62.3 b | 95.0 a | 93.3 a | 61.1 e 14.34 |
| | Ilmibyeo | 71.3 bc | 68.9 c | 67.7 c | 77.2 bc | 72.4 bc | 94.3 a | 84.8 ab | 76.7 bc 14.20 |
| | Ilpumbyeo | 8.8 abc | 11.8 a | 9.4 ab | 2.8 bc | 3.1 bc | 1.4 c | 2.2 bc | 5.6 jk 7.73 |
| | Jongnambyeo | 49.8 d | 65.2 c | 80.2 b | 65.0 c | 84.9 ab | 93.3 a | 84.2 ab | 74.7 bcd 9.87 |
| | Manchubyeo | 50.0 b | 79.2 c | 90.0 a | 70.0 ab | 80.0 a | 75.0 a | 75.0 a | 74.2 cd 21.60 |
| | Nagdongbyeo | 49.3 d | 67.4 bc | 83.6 ab | 63.6 cd | 80.5 ab | 81.0 ab | 96.5 b | 74.6 bcd 16.56 |
| | Odaebyeo | 0.0 c | 2.1 c | 7.0 b | 2.1 c | 10.0 a | 0.0 c | 0.0 c | 3.0 kl 2.48 |
| | Suwon 461 | 1.8 ab | 0.0 b | 3.6 ab | 0.0 b | 2.3 ab | 4.1 a | 3.6 ab | 2.2 kl 3.91 |
| | Shindongjinbyeo | 14.1 ab | 10.0 bc | 6.3 bc | 1.6 c | 21.3 a | 7.5 bc | 10.0 bc | 10.1 ij 9.25 |
| Color rice$^{\P}$ | | | | | | | | | |
| | Heugjinjubyeo | 0.0 b | 8.8 b | 23.5 a | 1.7 b | 6.0 b | 23.4 a | 22.1 a | 12.2 i 9.69 |
| | Heugnambyeo | 22.0 b | 25.0 b | 28.8 b | 18.9 b | 29.1 b | 50.3 a | 47.8 a | 31.7 g 13.89 |
| | Jeukjinjubyeo | 91.0 a | 48.7 c | 63.1 c | 60.5 c | 65.7 bc | 85.2 a | 82.9 ab | 71.0 d 17.62 |
| | Suwon 477 | 39.8 b | 10.0 c | 65.0 a | 21.7 bc | 31.7 b | 73.3 a | 66.7 a | 44.0 f 21.53 |
| | Sanghaehanghyeulla | 49.8 b | 26.7 cd | 36.7 c | 21.7 d | 10.0 e | 78.3 a | 80.0 a | 43.3 f 11.66 |
| | SR19685-5-3-1-1-1 | 21.1 e | 40.0 de | 66.7 b | 43.3 cd | 61.7 bc | 95.0 a | 73.3 b | 57.3 e 19.58 |
| Tongil | Andabyeo | 1.3 cd | 0.0 d | 8.3 a | 3.3 bc | 10.0 a | 0.0 d | 5.0 b | 4.0 kl 2.71 |
| | Areumbyeo | 79.3 a | 2.5 d | 35.0 b | 0.0 d | 17.5 c | 0.0 d | 12.5 cd | 21.0 h 14.02 |
| | Dasanbyeo | 11.8 ab | 13.6 ab | 14.8 a | 0.0 b | 15.2 a | 0.0 b | 17.8 a | 10.4 ij 14.47 |
| Japonica x Tongil | AC line$^{\#}$ | 64.5 c | 88.7 ab | 92.5 a | 82.4 b | 92.5 a | 84.0 b | 88.7 ab | 84.8 a 7.06 |
| | SC line$^{¥}$ | 58.3 c | 65.7 bc | 76.9 bc | 63.5 bc | 78.3 bc | 83.7 a | 87.0 a | 73.3 cd 15.87 |
| | Nonganbyeo | 0.0 a | 0.0 a | 0.0 a | 0.0 a | 0.0 a | 0.0 a | 0.0 a | 0.0 1 0.00 |
| Mean$^{\phi}$ | | 33.02 d$^{\pounds}$ | 32.52 d | 44.35 b | 30.73 d | 40.08 c | 49.67 a | 51.35 a | 40.24 2.62 |
| LSD 0.05 | | 11.12 | 14.99 | 14.58 | 12.58 | 11.08 | 9.97 | 15.92 | 4.85 |

$^{\dagger}$and$^{\phi}$; Mean proportion of the regeneration efficiencies of each cultivar on all media tested and 24 rice cultivars on each medium, respectively.
The mean proportion followed by the same letter are not significantly different at P=0.05 (LSD) within column (rice cultivars)$^{\S}$ and row (regeneration medium)$^{\pounds}$, respectively.
$^{\ddagger}$The value within row followed by same letter are not significantly different among the medium in each rice cultivar at P=0.05 (LSD).
$^{\P}$All color rice cultivars or lines were included in japonica rice.
$^{\#}$AC and $^{¥}$ SC lines were developed by anther and somatic culture from progenies crossed between Milyang 23 (Tongil rice) and Gihobyeo (japonica rice).

shoot regeneration of calli, 3% (w/v) sucrose (or maltose) was substituted with 3% (w/v) sorbitol combined with 2% (w/v) sucrose (or maltose) and 500 mgL$^{-1}$ proline under 5 mg L$^{-1}$ kinetin. During four weeks, the rate of GT (66.5%) on MSRK5SS-Pr was significantly higher than that on MSRK2S (53.8%) or MSRK10S (51.7%), respectively, while the rate of SR (10.3%) was delayed (Fig. 6C and F). Later than four weeks of cultivation, however, the rate of SR on MSRK5SS-Pr was increased up to 41.2% with multiple shoots (Fig. 3, Fig. 6C). The each rate of GT and SR on MSRK5SS-Pr at eighth week were increased in 12.7% and 17.2%, respectively, and the rate of CB was decreased in 6.2% compared to those on MSRK2S medium. This result indicated that the growth of callus was stimulated and multiple shoots of regenerated plants could be obtained in higher frequency by supplementation of both proline and sorbitol in combination with sucrose (or maltose) under 5 mgL$^{-1}$ kinetin (Fig. 3, Table 2). The supplementation of sorbitol and proline into the medium was attributed to be more effective to obtain regenerated plants of rice rather than utilization of the mediua containing sucrose or maltose alone without proline.

### Regeneration efficiencies of Korean rices

Efficiencies of SR critically varied depending on rice cultivars and the types of the regeneration medium ranged from 0% to 96.5% (Table 2). In general, japonica rice showed higher rate of SR compared to Tongil types. However, AC and SC line, which were developed by anther and somatic culture from the progenies of Milyang 23 (Tongil) x Gihobyeo (japonica) cross, showed highest levels of SR representing average 67.4% and 64.2%, respectively, on all media tested.

Among the japonica rice, SR of Nagdongbyeo, Donganbyeo, Manchubyeo, Ilmibyeo, and Jongnambyo were higher than an average of 50% on all media tested. Daesanbyeo, Dongjinbyeo, and Dongjinbyeo 1, showed midium levels of SR representing 7.9%, 18.3%, and 48.1%, on control medium,

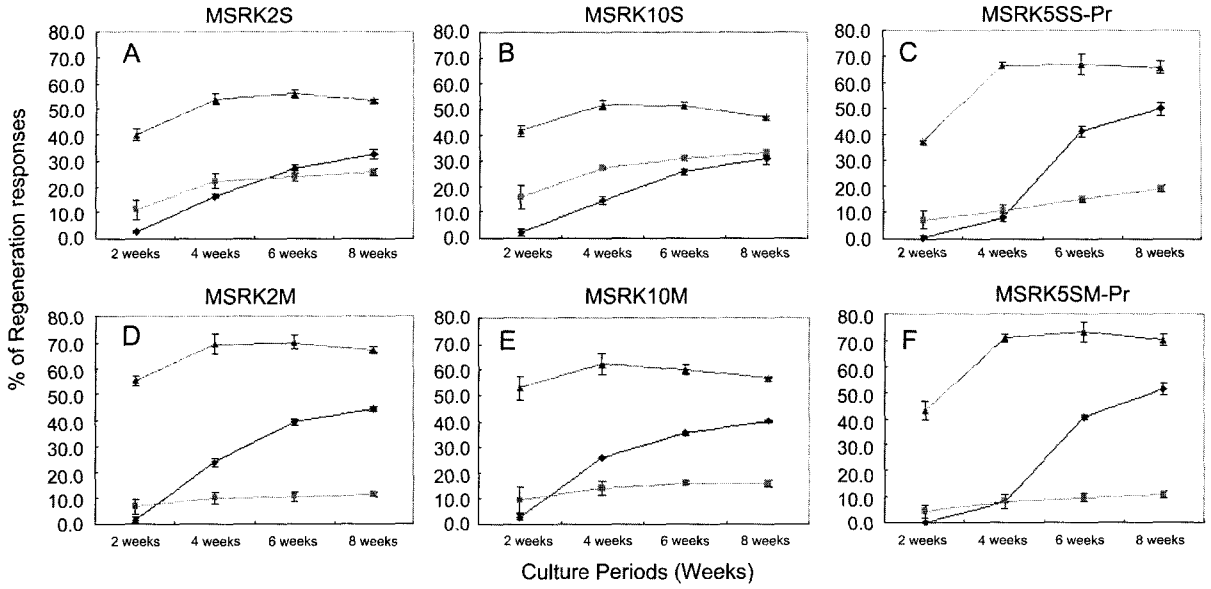


**Fig. 6.** Comparison of regeneration responses of the calli on MS basal regeneration media supplemented with different concentrations of kinetin and carborn sources. A, B, C, D, E, and F : MSRK2S, MSRK10S, MSRK5SS-pr, MSRK2M, MSRK10M, MSRK5SM-pr, respectively. -▲ -: Green tissue formation, -◆ -: Shoot regeneration, -■ -: Callus browning

respectively. In the same manner of highly regenerative cultivars, embryogenic calli of the cultivars were induced with independent globular shape in high frequencies (data not shown). However, regeneration responses of the cultivars were more severely affected by the type of the regeneration medium compared with those of the highly regenerative cultivars. Especially, these cultivars certainly confirmed the effectiveness of MSRK5SS-Pr (or MSRK5SM-Pr) containing both maltose and sorbitol combined with proline on stabilities of SR with active growing of callus, otherwise low level of CB. The SR rates of the cultivars were improved more than three folds on average, maximum six folds in Daesanbyeo, when compared with MSRK2S control medium.

Ilpumbyeo, Heugjinjubyeo, Odaebyeo, Suwon 461, and Shindongjinbyeo showed poorest *in vitro* cultural responses representing extremely low rates of GT (0-22.1%) and SR (0-7.6%) with slow growth of callus and high rates of CB (34.1-49.5%), even though they were included in japonica rice.

## DISCUSSION

Even though a great deal of progress has been made in *in vitro* studies of rice, the results have been unsatisfactory in producing transformants due to recalcitrant nature of genotypes to regeneration both in indica and japonica rice (Aldemita & Hodges, 1996; Hashizume *et al.*, 1999; Lee *et al.*, 1999; Rashid *et al.*, 1996). The most desirable strategy to improve the regeneration efficiency is the employment of optimized cultural medium considered as follows; 1) obtaining embryogenic callus showing suitable morphologies, 2) maintaining and proliferating the calli with viability, 3) enhancing the frequencies of green tissue formation and shoot regeneration with multiple shoot, and 4) reducing callus browning.

For obtaining transgenic plants of rice with scutella-derived embryogenic callus, N6 (Aldemita & Hodges, 1996; Hiei *et al.*, 1994; Rashid *et al.*, 1996) or MS basal medium (Datta *et al.*, 2000) have been selectively used depending on genotype of rice. A number of reports have emphasized the importance of the type of calli for regeneration efficiency in many monocot plants such as maize (Tomes & Smith, 1995) and rice (Lee *et al.*, 2002; Zhang, 1995). In the present study, beneficial effects of N6 medium containing 2 $mgL^{-1}$ 2,4-D were indicated in the callus induction rate and the morphology and viability of the embryogenic callus (Figs. 1 and 2). However, for regeneration, MS basal medium was superior to modified N6 basal medium under 2 $mgL^{-1}$ kinetin and 0.5 $mgL^{-1}$ NAA (Fig. 5). Variation in tissue culture responses on different basal media seemed to be mainly due to different inorganic nitrogen sources (Hashizume *et al.*, 1999; Yin *et al.*, 1993). The ratio of $KNO_3/NH_4NO_3$ plays an important role in suspension culture of rice cell and high concentration of $NH4^+$ was harmful to the cultured cell increasing the ratio of cell death (Yin *et al.*, 1993).

Additionally, the types and concentrations of cytokinins combined with NAA have been considered to be important factors on shoot regeneration of both indica (Xue & Earle, 1995) and japonica rice (Kyozuka *et al.*, 1987, Lee *et al.*,

2002; Su *et al.*, 1992). Supplementation of higher level of BAP (or kinetin) was more effective on regeneration of rice (Lee *et al.*, 2002; Xue & Earle, 1995; Yang *et al.*, 1999). However, 10 $mgL^{-1}$ cytokinin under restricted level of NAA tended to reduce the regeneration frequencies (Kyozuka *et al.*, 1987; Su *et al.*, 1992). In this study, GT and SR might be stimulated with 10 $mgL^{-1}$ kinetin in Nagdongbyeo, Donganbyeo, and etc., which are highly regenerative cultivars (Table 2). However, in general, 10 $mgL^{-1}$ kinetin showed inhibitory effects on regeneration of calli decreasing GT and SR, while increasing CB. Furthermore, it is generally known that kinetin and BAP show equivalent effect on regeneration of rice, however, significant differences of regeneration efficiencies in some cultivars were observed between the supplementations of BAP and kinetin (Table 3).

Since shoot regeneration from the calli is accompanied with growth of calli, minimizing callus browning was important. Furthermore, for producing more regenerated plants, frequencies of regeneration of calli and number of regenerated plants per callus should be enhanced. Most *in vitro* culture in rice have essentially relied on sucrose as carbon source. But there are some exceptions where other types of carbon sources have been assessed for improving regeneration efficiencies. Jain *et al.* (1995) reported that maltose either, alone or in combination with sucrose, was beneficial for plant regeneration. Sorbitol has been used for restoring and enhancing the plant regeneration ability of rice callus (Kishor and Reddy, 1986) since it seemed to serve osmoticum that imparted beneficial effect on embryogenesis in cereal crops (Swedlund and Locy, 1993).

Although affirmative effects of maltose on regeneration of rice were certified rather than sucrose even under 10 $mgL^{-1}$ kinetin, the greatest enhancement of regeneration efficiencies was observed in the media supplemented with sorbitol and proline combined with maltose (or sucrose) (Table 2, Fig. 6). When only sorbitol was offered to the medium, growth of callus was delayed compared to the use of either sucrose or maltose (Kishor and Reddy, 1986). However, if sorbitol was supplemented in combination with sucrose or maltose, the growth rate of callus was critically stimulated. From this point of view, MSRK5SM-Pr was satisfied with these items as described above. With the media, more than three folds of regenerated plants with more than 30% higher efficiencies based on the number of the calli plated could be obtained in Nagdongbyeo compared to MSRK2S (data not shown). Especially, this medium was most effective for moderately regenerative cultivars, such as Dongjinbyeo, Suwon477, and etc., enhancing regeneration efficiencies more than 300% on average compared to MSRK2S control medium (Table 2).

Regeneration efficiencies varied depending on genotypic background of the rice cultivars; higher efficiencies in japonica rice while lower in Tongil rice, which in fact were included in genetically different group from japonica rice (Song *et al.*, 2002). Among the japonica x Tongil hybrid, the poorest regeneration response of callus was observed in Nonganbyeo, which has been improved for japonica rice but is genetically close to Tongil rice. However, highest efficiencies of shoot regeneration were observed in other two hybrids rice, AC and SC lines. It might be due to genetic factors involved in *in vitro* culture ability as shown in previous studies with two different genotypes, of which regeneration ability were significantly different such as Nipponbare (japonica) x Kasalath (indica) and Milyang 23 (Tongil) x Gihobyeo (japonica) (Taguchi *et al.*, 1997; Kwon *et al.*, 2001, 2002). However, the present result demonstrated that improved *in viro* culture system allowed to overcome the recalcitrance in regeneration and to enhance the regeneration efficiencies in many rice cultivars. Thus, the present study suggested not only useful index of the regeneration efficiencies of various genotype of rice but also a practical method for improving the efficiencies by observing responses of calli on different use of carbon sources and growth regulators.

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