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Effect of callus induction media on morphology of embryogenic calli in rice genotypes

K.B.R.S. VISARADA*, M. SAILAJA** and N.P. SARMA**[1]

*National Research Centre for Sorghum, Hyderabad-500030, India**
*Directorate of Rice Research, Rajendranagar, Hyderabad-500030, India***

Abstract

Effects of four culture media on callus induction, regeneration and number of plants per unit culture were studied with mature seeds from five indica rice genotypes as explants. Based on the morphology, the calli were classified into four types as I to IV. Type I and type II are most suited to initiate suspension cultures or as target material for transformation. Number of plants regenerated per unit culture, formation of easily dissociating cell clusters and frequency of type I and type II calli were highest on NBKNB medium. Thus NBKNB medium is suitable for *in vitro* culture of even the hitherto recalcitrant indica genotypes.

Additional key words: medium composition, *Oryza sativa*, regeneration.

Introduction

Rice (*Oryza sativa* L.) cultivars belonging to subspecies *indica* are, in general, less responsive for callus induction as well as regeneration than ssp. *japonica* (Abe and Futsuhara 1984, 1986) which limit the success in the application of genetic transformation techniques for *indica* rice improvement. Embryogenic callus induction is dependent on the interaction between the genotypes and culture conditions. Despite the availability of a plethora of protocols for rice tissue culture, no procedure appears to be universally adaptable when a new genotype is to be considered for *in vitro* manipulation (Maggioni *et al.* 1989). Earlier studies on comparison of callus induction media either used *japonica* genotypes or a single genotype (Poonsapaya *et al.* 1989, Rueb *et al.* 1994, Sivamani *et al.* 1996). Further, adoption of methodology developed at one laboratory to another is often not feasible due to difficulty in simulation of exact conditions of experimentation. Though regeneration, rather than callus induction, is limiting in most of the *indica* rice cultivars, introduction of foreign gene, selection and multiplication of only the transformed sectors are dependent on the embryogenic potential of callus in *in vitro* cultures. Visual selection of embryogenic calli based on morphology enhances the yield of number of plants after transformation. A simple microscopic observation for such calli under aseptic conditions facilitates the selection of only the regenerable types as target tissues. The objective of the present study is to identify a medium for callus induction based on the frequency of not only the regeneration but also plants per unit culture using a few representative *indica* genotypes and study the morphology of callus to enable visual selection of regeneration proficient calli as target tissues for genetic transformation.

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Abbreviations: BAP - N^6-benzylaminopurine; DMRT - Duncan's multiple range test; MS - Murashige and Skoog; NAA - α-naphtaleneacetic acid; T309 - Taipei 309.

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[1]Corresponding author; fax: (+91) 40 4015308, e-mail: npsarma@drr.ap.nic.in,

Materials and methods

Three callus induction media published already along with the one modified in our own laboratory were tested in this experiment (Table 1): NBKNB (Sivamani *et al.* 1996), MSTC (based on Murashige and Skoog 1962), PP (Poonsapaya *et al.* 1989), and GH (Grimes and Hodges 1990). Two concentrations (2 and 3 mg dm^{-3}) of 2,4-dichlorophenoxyacetic acid (2,4-D) were tested for all the media.

Mature seeds from field grown rice (*Oryza sativa* L. ssp. *indica*) cultivars Rasi, Seshu, Vibhava, Nagarjuna and Jaya were used for the study. Dehusked seeds were surface sterilized for 5 min in 70 % ethanol followed by 5 min sterilization in 0.1 % mercuric chloride. After rinsing several times in sterile distilled water, the seeds were blotted on sterilized paper towels and plated on corresponding callus induction medium with half of the embryo in contact with the medium. Media were solidified with agar and distributed in presterilized glass Petri plates (90 mm) with 25 - 30 cm^3 in each. Twenty seeds were plated in each Petri plate and percentage of the callus induction was calculated by taking into account the number of seeds giving callus 25 - 30 d after inoculation. All the cultures were incubated in dark at 27 ± 2 °C. 25- to 30-d-old calli were aseptically excised from seed and transferred onto the same medium. 40- to 50-d-old calli were induced on all the four media and further regenerated on MS basal medium supplemented with 2.5 mg dm^{-3} N^6-benzylaminopurine (BAP), 0.5 mg dm^{-3} α-naphtaleneacetic acid (NAA) and 1 mg dm^{-3} kinetin (K) in 150 mm glass culture tubes, each containing 20 - 25 cm^3 of solidified medium and the cultures were incubated under cool fluorescent lamps (irradiance of 132 μmol m^{-2} s^{-1}) at temperature of 25 ± 2 °C. An equal amount of callus (*ca.* 30 mg) was transferred for regeneration. Regeneration frequency was measured as the percentage of calli giving shoots after 30 - 35 d on regeneration medium. Later, the calli were sub-cultured on the same regeneration medium. Plants per unit culture were determined at 60 - 65 d. Shoots with underdeveloped roots were transferred to half strength MS medium containing sucrose to promote the rooting and further they were acclimatized by maintaining a week or two in Yoshida solution (Yoshida *et al.* 1976) and transferred from tube to soil. To minimise the handling and seasonal variations the experiment was conducted twice in wet and dry seasons. Completely randomized block design was adopted for analysis of data. Separation of means was done by DMRT using *MSTATC* statistical software program.

Table 1. Media composition [mg dm^{-3}]. ($FeSO_4 . 7 H_2O$ 27.8 mg dm^{-3} chelated with 37.3 mg dm^{-3} Na_2EDTA was added to all the media).

Component	NBKNB	MSTC	PP	GH
KNO_3	2830	1900	1900	3190
$(NH_4)_2SO_4$	463	-	-	1783
NH_4NO_3	-	1650	1650	-
KH_2PO_4	400	170	170	400
$CaCl_2 . 2 H_2O$	166	440	440	166
$Mg SO_4 . 7 H_2O$	185	370	370	185
KI	0.75	0.8	0.8	0.8
H_3BO_3	3	6.2	6.2	1.6
$MnSO_4 . H_2O$	10	16.9	16.9	3.3
$ZnSO_4 . 7 H_2O$	2	8.6	8.6	1.5
$Na_2MoO_4 . 2 H_2O$	0.025	0.25	0.25	-
$CuSO_4 . 5 H_2O$	0.025	0.025	0.025	-
$CoCl_2 . 2 H_2O$	0.025	0.025	0.025	-
Nicotinic acid	1	0.5	-	0.5
Pyridoxine HCl	1	0.5	-	0.5
Thiamine HCl	10	0.1	0.4	0.1
Glycine	-	2	-	2
Casein hydrolysate	300	300	-	-
Proline	500		-	-
Inositol	-	100	100	-
Tryptophan	-	50	-	-
Sucrose	30000	30000	30000	30000
Kinetin	1	-	-	-
BAP	1	0.2	-	-
NAA	1	-	-	-
Coconut water	-	-	10 %	-

Results and discussion

Rice cultivars adapted to different agroecological regions in the Indian subcontinent belong to the subspecies of *indica*. Vibhava and Seshu were found to be moderate in response to callus induction but showed high regeneration, while, Rasi, Nagarjuna and Jaya had high callus induction but low regeneration in our previous study (Visarada *et al.* 2001).

The nutrient status, particularly the nitrogen source, can effect induction of somatic embryos, and eventually the regeneration, particularly in monocots (Leifert *et al.* 1995). MS and N_6 media are two well known media used to induce callus and regenerate plants in rice genotypes. Although several differences in composition exist between these two media, the critical factor is the ratio of reduced and oxidized nitrogen. Rueb *et al.* (1994) compared four published tissue culture protocols using the *japonica* cultivar T 309 and recommended that of Poonspaya *et al.* (1989). Grimes and Hodges (1990)

studied the influence of the ratio of nitrate nitrogen and ammonical nitrogen (NO_3^-:NH_4^+) on regeneration from the primary callus induced from immature embryos of IR 54 and obtained highest regeneration from the calli induced and regenerated on basal medium containing 31.5 mM nitrate and 13.5 mM ammonium (ratio 70:30). Khanna and Raina (1997) reported highest frequency of regeneration and maximum number of green plants in calli derived from SK-1 medium containing 35 mM KNO_3 and 5 mM $(NH_4)_2SO_4$ with Karnal local cultivar and demonstrated that the composition of basal medium for callus induction was the major determinant of regeneration response. Thus MS medium supplemented with tryptophan and casein hydrolysate, GH medium with increased ratio of oxidized and reduced nitrogen sources, NBKNB medium composed of nitrogen sources as N_6 medium and PP medium as recommended by Rueb *et al.* (1994), are selected for this comparative study. In order to reduce further variation due to culture conditions, interaction of callus induction and regeneration media, and to assess only the callus induction media, all the calli were regenerated on a single MS medium with supplements as described.

Irrespective of the media used, callus initiation was early in the genotypes Rasi and Nagarjuna, and was delayed in the genotypes Vibhava and Seshu. The genotypes Rasi, Nagarjuna and Jaya were found to be superior to Vibhava and Seshu in callus induction (Table 2). Significant differences were observed among genotypes as well as media in respect of callus induction. Concentration of 2,4-D (2 and 3 mg dm^{-3}) either alone or in interaction with medium did not have much influence on the callus induction. Interaction of genotype, media and concentration of 2,4-D was highly significant. Mean callus induction on NBKNB and MSTC was 68.5 % and 68.6 %, respectively, whereas it was less on PP (40.6 %) and GH (31.6 %) (Table 3). Duncan's multiple range test for the effect of media on callus induction revealed that NBKNB and MSTC are equally superior to PP and GH media, whereas differences between NBKNB and MSTC and PP and GH were not significant.

Regeneration of calli induced on different media was highly dependent on genotype ($P < 0.01$), the medium ($P < 0.01$), and their interaction. Concentration of 2,4-D in the induction medium did have a bearing on the regeneration of the callus ($P < 0.05$). On transfer to regeneration medium the calli induced on NBKNB medium, multiplied rapidly yielding a large mass of callus, while, on MSTC the proliferation of the callus was slow. In comparison, however, most of the calli induced on PP and GH media turned necrotic on transfer to regeneration medium. Regeneration from the calli induced on GH medium was very low (4.3 %) and necrosis was very high (Table 3). Ranking on the basis of regeneration frequency of the calli induced on different media revealed no significant difference among NBKNB

Table 2. Response of *indica* genotypes *in vitro* to different cultivation media (NBKNB, MSTC, PP, GH) and 2,4-D concentration [mg dm^{-3}].

Genotype	NBKNB		MSTC		PP		GH	
	2,4-D 2	2,4-D 3	2,4-D 2	2,4-D 3	2,4-D 2	2,4-D 3	2,4-D 2	2,4-D 3
	callus induction [%]							
Vibhava	60.8 ± 8.3	38.8 ± 13.3	61.6 ± 5.9	58.8 ± 2.9	35.9 ± 13.5	30.8 ± 4.9	20.1 ± 3.5	28.0 ± 10.1
Seshu	46.4 ± 2.7	57.1 ± 8.6	53.8 ± 3.9	66.9 ± 6.1	4.5 ± 5.4	17.3 ± 4.0	6.6 ± 5.8	24.6 ± 10.4
Nagarjuna	67.7 ± 8.4	81.4 ± 6.1	73.8 ± 7.2	64.0 ± 7.2	59.4 ± 6.2	46.5 ± 11.9	52.8 ± 9.3	38.9 ± 4.6
Rasi	78.6 ± 9.9	94.1 ± 4.2	85.6 ± 6.6	75.8 ± 4.6	63.0 ± 4.0	45.1 ± 7.1	41.9 ± 6.0	37.3 ± 11.3
Jaya	65.6 ± 14.3	78.1 ± 5.9	57.7 ± 7.8	82.2 ± 5.4	47.5 ± 15.9	62.4 ± 6.0	47.3 ± 11.7	35.7 ± 7.6
	regeneration [%]							
Vibhava	68.1 ± 2.0	86.1 ± 10.4	51.7 ± 6.0	71.6 ± 3.6	59.0 ± 10.1	43.6 ± 11.7	0.0	8.3 ± 11.8
Seshu	75.9 ± 4.5	68.2 ± 22.8	51.8 ± 6.9	54.8 ± 2.3	51.1 ± 25.9	51.1 ± 25.9	11.1 ± 15.7	0.0
Nagarjuna	49.8 ± 4.9	58.6 ± 6.0	39.4 ± 11.3	68.0 ± 9.3	0.0	25.0 ± 4.1	17.8 ± 1.6	18.7 ± 4.6
Rasi	22.2 ± 15.5	27.7 ± 2.7	16.2 ± 6.3	23.5 ± 4.5	22.2 ± 15.5	27.8 ± 7.8	26.1 ± 5.5	3.7 ± 5.2
Jaya	44.4 ± 11.3	26.7 ± 2.4	16.9 ± 1.4	64.3 ± 15.2	18.6 ± 15.3	36.1 ± 30.7	0.0	8.3 ± 11.8
	number of plants [culture^{-1}]							
Vibhava	24.4 ± 2.7	21.1 ± 1.3	4.7 ± 1.1	6.5 ± 1.0	10.7 ± 1.9	6.1 ± 2.1	0.0	0.0
Seshu	14.1 ± 0.5	13.9 ± 1.0	7.4 ± 1.4	8.4 ± 2.6	0.0	0.0	0.0	0.0
Nagarjuna	5.9 ± 0.3	8.0 ± 0.5	5.4 ± 0.6	6.5 ± 1.0	0.0	8.0 ± 3.0	0.0	0.0
Rasi	1.8 ± 0.7	8.8 ± 3.8	10.1 ± 1.1	6.3 ± 0.6	3.5 ± 1.4	0.8 ± 0.8	0.0	0.0
Jaya	2.2 ± 1.3	1.9 ± 0.7	2.6 ± 0.8	3.8 ± 0.6	0.0	0.8 ± 0.5	0.0	0.25 ± 0.1

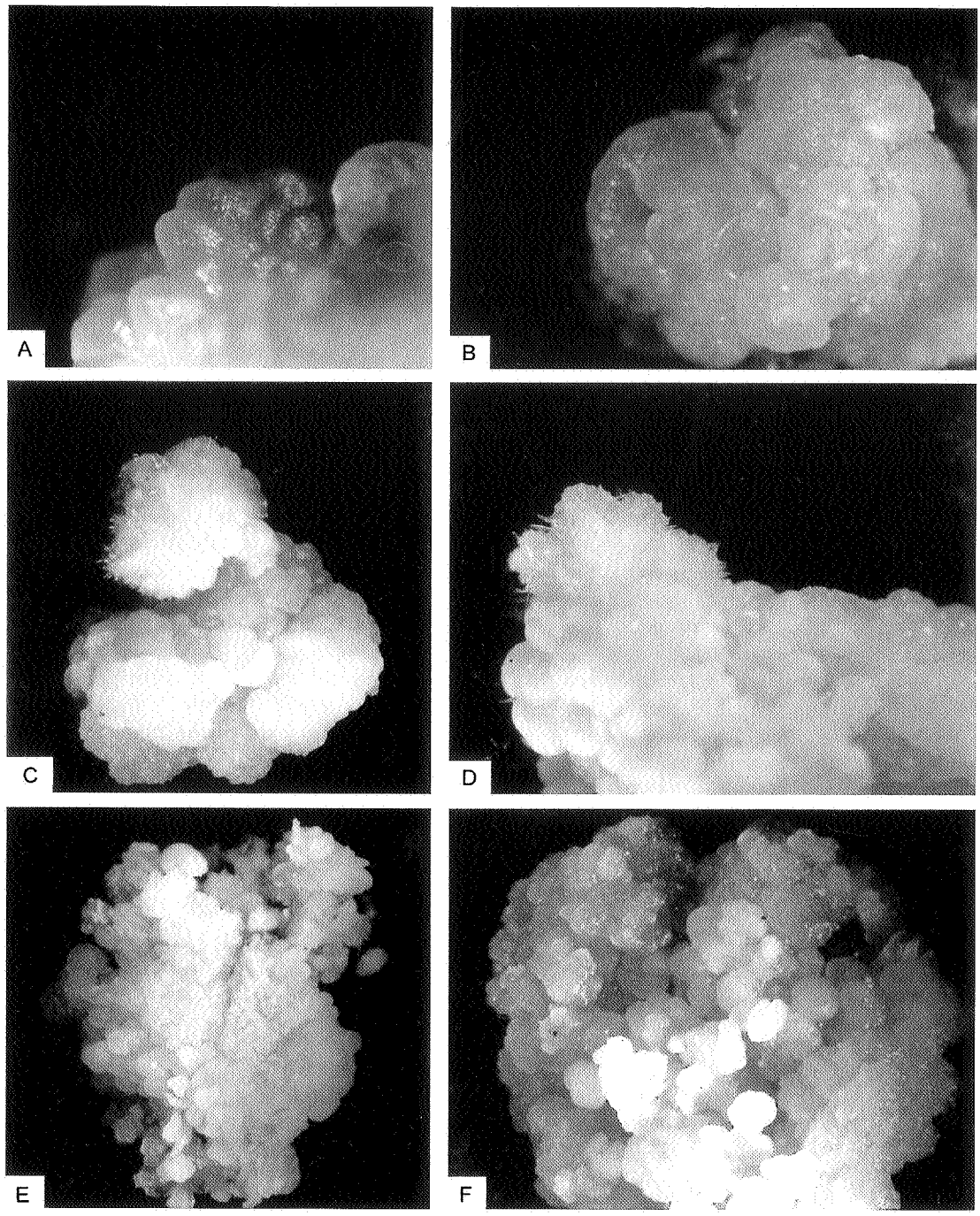

Fig. 1. Morphology of callus in *indica* genotypes: *A* - pre-globular proembryo, *B* - cup shaped somatic embryo, *C*, *D* - type I white or cream compact calli, which have excellent regeneration, *E*, *F* - type II yellow organized calli, which have good regeneration.

Table 3. DMRT ranking of different media for tissue culture response by rice genotypes. Means followed by same letter within the column are not significantly different at 5 % level of probability by DMRT.

Medium	Callus induction	Regeneration	Number of plants [culture^{-1}]	Rate of callus proliferation	Friability of the calli
NBKNB	68.5^{A}	53.9^{A}	9.3^{A}	high	high
MSTC	68.6^{A}	45.7^{A}	5.5^{B}	medium	medium
PP	40.6^{B}	29.8^{AB}	1.4^{C}	medium	low
GH	31.6^{B}	4.3^{B}	0.1^{D}	low	high

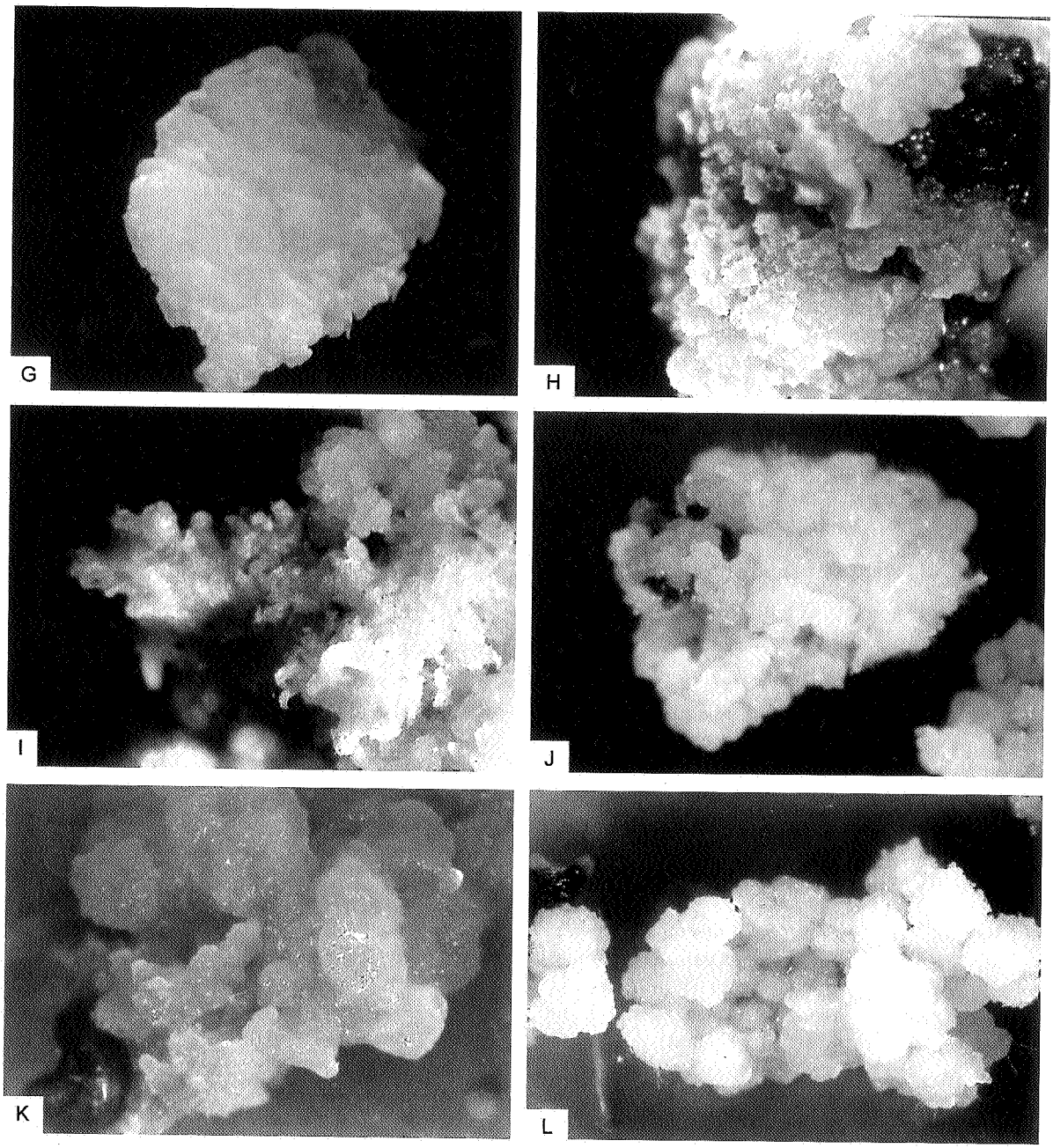

Fig. 2. Morphology of callus in *indica* genotypes: *G*, *H*, *I* - type III yellow or brown unorganized calli, which turn brown, *K*, *L*, *M* - type IV rhizogenic calli produce only roots.

(53.9 %), MSTC (45.7 %) and PP (29.8 %) (Table 3). Regeneration and plants per unit culture were superior only when 3 mg dm^{-3} 2,4-D was used in the genotype Nagarjuna. Vibhava and Seshu showed higher regeneration and greater number of plants per culture though the callus induction frequency was low (Table 2). The genotype Jaya had a low regeneration from the calli induced on NBKNB. Nevertheless, the calli induced on MSTC with 3 mg dm^{-3} 2,4-D showed higher regeneration (64.3 ± 15.3 %) and greater number of plants per culture (3.8 ± 0.6) (Table 2). Thus the regeneration in the genotypes like Nagarjuna and Jaya, which were observed to be less regenerable in our earlier study, could be improved substantially, while in Rasi it continued to be low.

Number of regenerants per culture is strongly dependent on genotype, medium and their interaction. The media were ranked distinctly by DMRT for this trait. NBKNB medium followed by MSTC yielded greater number of plants per culture (Table 3). Lai and Liu (1986) observed that the callus induced on MS had consistently low regeneration ability than that on N_6. Raval and Chattoo (1993) made a comparative evaluation of MS and N_6 and found that the latter without any supplements is superior to MS medium for callus growth and further that N_6 medium supplemented with casein hydrolysate showed less callus growth than MS. Addition of tryptophan to MS medium stimulated plant regeneration (Koetje *et al.* 1989). Casein hydrolysate was found to enhance the embryogenic callus growth and plant regeneration (Maeda *et al.* 1986, Moura *et al.* 1997). Especially, the casein hydrolysate (enzymatic) but not casein hydrolysate (acid) is promotive, as was confirmed by the present experimentation. Therefore, MS medium supplemented with these organic additives was used for comparison. Besides genotypic variation, other sources of variation could be due to different culture methods. Poonsapaya *et al.* (1989) demonstrated the importance of even the type of culture vessel and geographical location of the experiment in the reproducibility of the results. In the present study regeneration of calli induced on GH medium was low probably because of vast difference in nitrate and ammonical nitrogen between the induction medium (N_6) and regeneration medium (MS). In low regenerating genotypes, Nagarjuna and Jaya, the regeneration as well as plants per unit culture were enhanced substantially when calli were induced on NBKNB.

Vain *et al.* (1989) designated two types of callus cultures, such as type I and type II to identify the embryogenic callus and nonembryogenic callus in maize. Likewise in the present study variation in callus morphology was studied and subsequently four types of calli were designated to facilitate the identification of regeneration proficient calli in the early stages.

Initially the callus surface was smooth and undulated (Fig. 1*A*). In 15-d-old calli there was no difference in the organization. Differentiation into various morphological forms commenced at day 30. With further sub-culturing of calli an increased number of phenotypes were distinct. Formation of pre-globular pro-embryos preceded organized somatic embryos (Fig. 1*B*). Based on the variation in the morphology, the callus can be classified to four types(Figs. 1, 2): type I - white or cream colored compact organized callus, type II - yellow organized callus, type III - yellow or brown unorganized callus, and type IV - rhizogenic callus. First two types gave rise to potentially regenerating tissues. Type III callus turned dark and necrotic. Type IV was highly unorganized white, yellow or brown and gave rise only to the roots. The globular surfaces were either smooth or hairy in texture and cream to white in color. Hairy surfaced calli were frequent on NBKNB medium and scant with MSTC. GH medium yielded mostly yellow to white callus of smooth surface whereas PP medium induced calli of smooth surface with yellow colour. Hairy surfaced calli turned green faster and transformed to shoots than the other types thereby promoting regeneration in all the *indica* genotypes.

During subculture, no apparent selection of calli was made to arrive at an unbiased conclusion. Incremental growth of the calli cultured on NBKNB medium was highest followed by MSTC among the media studied. Calli grown on PP medium were larger than those on GH medium. Marked differences in the colour and organization of the calli were observed. In GH the embryogenic regions were intermingled with large brown patches of calli. On NBKNB, the brown regions were less and rhizogenic calli were more, than on all the other media. Calli induced on NBKNB and GH comprised of loosely arranged cell clumps. In the genotypes Vibhava and Seshu, suspension cultures could be established easily from the calli originated on NBKNB and GH media, whereas, many a times, the calli induced on MSTC turned necrotic, when initiated for suspension cultures.

Earlier studies though described the embryogenic and nonembryogenic callus, a detailed description of the callus phenotype to distinguish in the early stages is not provided (Narciso and Hattori 1996, Heyser *et al.* 1983, Chowdhry *et al.* 1993, Bhaskaran and Smith 1990, Dinghou and Komamine 1994, Krishnaraj and Vasil 1995, Maeda *et al.* 1986). Jones and Rost (1989), studied the ontogeny of somatic embryos from tissue cultures of mature caryopsis of rice and observed appearance of small translucent nodules initially some of which later enlarged and organized into somatic embryos. Our observations confirm this and also the elongated, leaf like scutella which failed to develop (Fig. 2*F*). Embryogenic callus is associated with at least two other types of callus tissue *1*) soft, transparent, unorganized callus, which is nonmorphogenic or may form only roots (Fig. 2*J,L*) and *2*) soft mucilaginous callus which is often associated with roots (type IV, Fig. 2*K*) (Vasil and Vasil 1984).

Development and propagation of embryogenic calli is a prerequisite for successful transformation. Bec *et al.* (1998) adopted preliminary histological analysis of the target sample before undertaking transformation because they found that organized clustered proembryo structures as target tissues are unfavourable. Sivamani *et al.* (1996) described an elegant selection procedure in which sibling calli of regenerating calli were selected for transformation. Selection for regeneration proficient calli based on morphology, however, is easier and requires less time than selection of sibling calli. The present study confirms that type I and II calli are most suited for transformation (Visarada and Sarma, unpublished results).

NBKNB medium increased the production of

embryogenic calli and formation of easily dissociating, friable clumps and is broadly adaptable to a wide range of *indica* genotypes. These friable clumps offer an additional advantage in using small calli as targets for biolistics and also for selecting only the transformed sectors after. Selection agent permeates the small cell clumps more effectively, thereby minimizing the non-transformed escapes (Jain *et al.* 1996). Number of plants regenerated, formation of easily dissociating cell clusters and greater frequency of white to yellow organized calli were highest in calli induced and sub-cultured on NBKNB medium compared to the other media studied. Based on these observations, NBKNB medium was selected for callus induction of *indica* rice genotypes in subsequent studies.

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