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Effect of Genotype and Culture Media on Callus Formation and Plant Regeneration from Mature Seed Scutella Culture in Rice

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Abstract

In vitro plant regeneration from callus induced from mature seed scutella of four rice genotypes, Lx297, IR64, V19 and IR64-1-1-4 was studied. Three basal media, namely N6, MS and R were tested for callus induction; of these MS was found best for callus induction. The genotype Lx297 was found most responsive and yielded highest percentage (82.50) of callus on MS. Results also showed that callus induced on R medium gave the highest regeneration frequency (13.89%) when transferred to MSK_2 regeneration medium.

Introduction

Efficient plant regeneration from cultured cells and tissues requires successful application of biotechnology in crop improvement. Therefore, the success of cell and tissue culture research depends upon reliable callus culture and plant regeneration procedures. The frequencies of callus induction and plant regeneration in tissue culture of rice are influenced by many factors: culture medium composition, explants source, genotype and environment (Torbert et al. 1998). Among them the genotype and nutrient composition are regarded to be the major sources of variation in *in vitro* culture (Khanna and Raina 1998).

Hartke and Lorz (1989) tested 15 *indica* rice lines and found that seven of them produced embryogenic calli and only four regenerated into plants. Abe and Fustsuhara (1986) tested 66 *indica* and *japonica* cultivars and reported that *japonica* varieties displayed a higher rate of callus induction and regeneration

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than those of *indica*. It is to be noted here that many agronomically valuable rice genotypes are recalcitrant to *in vitro* manipulation because of their poor callus production and regeneration ability. The problem of shoot regeneration has also been encountered during plant regeneration experiments (Chu and Croughan 1990). The rate of success can be enhanced by improving the composition of tissue culture medium, specially by manipulating plant growth regulators (Mandal and Gupta 1995, Zhu et al. 1996), osmotic pressure (Jain et al. 1997) and partial desiccation (Rance et al. 1994).

Various explants have been used for regeneration of rice plant such as mature embryos (Abe and Futsuhara 1984, 1986; Wang et al. 1987, Seraj et al. 1997, Azria and Bhalla 2000) or scutellum-derived callus (Rashid et al. 1996, Toki et al. 1997, Yokoi et al. 1997), immature embryos (Seraj et al. 1997, Li and Liu 1982, Koetje et al. 1989).

The aim of this study was to determine the response of mature scutella of four rice genotypes to various media for best callus formation.

Materials and Methods

The experiment was conducted in the Anther Culture and Genetic Engineering Laboratory of the Philippines Rice Research Institute, Maligaya, Science City of Munoz, Nueva Ecija, the Philippines in the wet season, 2001.

Four rice genotypes, namely Lx297, IR64 (seed-derived), V19 and IR64-1-1-4 (anther culture-derived) were used in this study. Mature dehulled seeds were surface sterilized with 70% ethanol for 1 min rinsed with distilled water and then 50% Clorox for 30 min by vigorous shaking twice. Treated seeds were rinsed twice with distilled water and blot dried onto a filter paper.

For callus induction, dehulled mature surface sterilized seeds were plated in three basal media (Table 1): N6 medium (Chu et al. 1975, Chu 1978, Li 1992), MS and R medium (Barba and Patena 1998). All media used in this study were supplemented with 2.0 mg/l 2, 4-D, 1.0 mg/l NAA, 1.0 mg/l Kn, 3 g/l casamino acid, 500 mg/l glutamine, 2.0 mg/l glycine, 100 mg/l myo-inositol, 5.0 mg/l nicotinic acid, 10.0 mg/l pyridoxine HCl, 10.0 mg/l thiamine HCl, 10.0 mg/l MS Fe source and 5 g/1 glucose, 50 g/l sucrose. The media were solidified with a 0.7% agar. The pH of all media was adjusted to 5.8 before autoclaving at 115_C for 15 min. The media (40 ml) were poured into Magenta vessels and 40 seeds were cultured per vessel per replication per callus induction medium. Three vessels were used per replication.

The culture vessels were sealed using plastic tape and incubated at 27 ± 2_C in total darkness for 13 - 17 days. Thereafter, scutellar-derived calli were

isolated and subcultured in the same medium for one week. One more subculture in the same medium for another week with a lower 2,4-D (0.2 mg/l) gave better growth. Calli were then transferred to the regeneration medium MSK_2 (MS basal salt + 1.0 mg/l MS vitamins + 10.0 mg/l Fe-EDTA + 0.1 g/l myo-inositol + 30 g/l sucrose + 1.0 mg/l NAA + 1.0 mg/l Kn). Regeneration media were solidified with 0.3% agar and 0.2% phytagel. Embryogenic calli were transferred to regeneration media.

Table 1. Composition of the media used.

Component	MS	N6	R
KNO_3	1900	2830	250
NH_4NO_3	1650	-	180
$(NH_4)_2SO_4$	-	463	-
KH_2PO_4	170	400	-
$CaCl_2.2H_2O$	440	166	-
$MgSO_4.7H_2O$	370	185	100
$NH_4H_2PO_4$	-	-	300
$Ca(NO_3).\ 4H_2O$	-	-	90
$MnSO_4.\ H_2O$	16.9	3.3	0.65
H_3BO_3	6.2	1.6	0.10
$ZnSO_4.7H_2O$	8.6	1.5	0.10
KI	0.8	0.8	0.02
$CuSO_4.\ 5H_2O$	0.025	-	-
$Na_2MoO_4.\ 2H_2O$	0.25	0.25	-
$CoCl_2.\ 6H_2O$	0.025	0.025	-

Constituents (mg/l) in common : 3000 casamino acid, 500 glutamine, 2 glycine, 100 myo-inositol, 5 nicotinic acid, 10 pyridoxine HCl, 10 thiamine HCl, 10 MS Fe source, 5000 glucose, 50,000 sucrose, 7000 agar.
Plant growth regulator (mg/l) : Callus induction medium : 2,4-D 2.0 + NAA 1.0 + Kn 1.0.
Regeneration medium : MS basal salt + 1.0 mg/l MS vitamins + 10.0 mg/l Fe-EDTA + 0.1 g/l myo-inositol + 30 g sucrose, 0.5 mg/l NAA + 2.0 mg/l Kn.

For regeneration, cultures were incubated at 28 ± 1_C using 16 h photo-period (General electric cool white fluorescent tubes) for four weeks. When culture-derived plantlets were 2 - 3 cm long, they were transferred to hormone-free MS for root induction. The experiments were carried out in a completely randomized design with three replications.

Data collected were subjected to analysis of variance (ANOVA); and means were separated by Least Significant Difference (LSD) test as well as Duncan's Multiple Range Test (DMRT). SAS statistical program and IRRISTAT program Version 3.1 were used in the analysis of data.

Results and Discussion

Callus induction : Embryogenic callus formation and plant regeneration are shown in Fig. 1. Significant differences were observed among the three genotypes for days first callus observed, per cent callused seed and per cent non-callused seed (Table 2). The genotype IR64 (seed-derived) was excluded from the experiment because it failed to germinate in the callus induction medium due to some factors brought into play during the breaking of dormancy, as a result of seed injury.

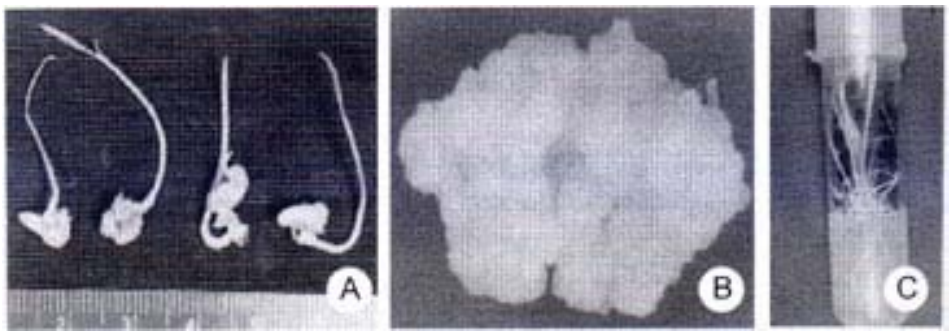

Fig. **1**. Mature seed culture : **A**. 21-day-old seed in culture, **B**. 21-day-old scutellar-derived embryogenic calli and **C**. rooting of *in vitro* proliferated shoots.

Results indicate that the genotype accounts for maximum variation in terms of days for first sign of callus formation, per cent of callused- and non-callused seeds. However, callus induction medium and genotype ∇ callus induction medium were not significant. First callus formation was observed between seven and 11 days of culture (Table 3). Callus induction response was rapid in the genotype Lx297, as callus production was observed within one week of culture, while the genotype V19 responded late (11 days). The highest per cent (80.43) callus was observed in genotype Lx297 over all callus induction media. On the other hand, callusing in the genotype V19 was lower (7.04%). In case of non-callused seed, Lx297 produced less callused seed (19.60%) and V19 produced higher non-callused seed (92.97%). This means genotype Lx297 is better than other genotypes for callus production. The results also indicated a good deal of differences between genotypes. Macabale et al. (2001) reported that the rate of embryogenic callus was higher in the genotype Lx297 (59.4%) than IR64-1-1-4 (37.7%) from mature-seed-scutellar derived calli. Khanna and Raina (1998) reported that the genotype influenced differently both callus induction and regeneration.

The results of the present study revealed no significant differences between the different callus induction media. However, over all MS basal medium was slightly better for callus induction rate (35.89%) than R (35.67%) and N6 (34.83) basal medium. The two most commonly used basal media, MS and N6 (Pandey et al. 1994, Rance et al. 1994, Yoshida et al. 1994, Kunanuvat-chaidach et al. 1995, Rashid et al. 1996, Toki 1997, Yokoi et al. 1997) and a new

Table 2. Mean square values and their significance probability based on ANOVA.

Source of variation	df	Days first callus observed	Callused seed (%)	Non-callused seed (%)
Replication	2	2.33 ns	121.23*	121.15*
Genotype	2	40.11**	13954.01**	13954.22**
CIM	2	0.78 ns	2.55 ns	2.56 ns
Genotype ∇ CIM	4	0.72 ns	7.24 ns	7.25 ns
Error	16	1.04	21.40	21.39
CV (%)		10.9	13.0	7.2

ns = non-significant, * = significant at p = 0.05, ** = significant at p = 0.01.

Table 3. Mean effect of genotypes over all callus induction media.

Genotype	Days first callus observed	Callused seed (%)	Non-callused seed (%)
V19	11.11 a	7.04 c	92.97 a
Lx297	7.00 c	80.43 a	19.60 c
IR64-1-1-4	9.89 b	18.90 b	81.12 b

Means followed by a common letter are not significantly different at the 5% level by DMRT.

medium (R) were tested for callus initiation in all the three genotypes. In general, the frequency of callus initiated of all the genotypes tested was better in MS than N6 and R media. These differences in response of mature seed scutellum to callus induction on MS, N6 and R media suggest that the amount and type of nitrogen may be important in *in vitro* culture of the rice genotype. The amount of nitrogen is higher in MS than that in N6 and R media (Table 1). Similar observations were reported by Azria and Bhalla (2000). They mentioned that the frequency of initiated callus, the size of callus and the quality of callus of all the varieties tested were better in MS than N6. Mandal and Gupta (1995) also reported that MS is better than N6 on callus and plant regeneration in anthers of *indica* rice. The results of the present study on callus induction indicate that the most responsive genotype was Lx297 and the

maximum rate of callus production occurred in MS (82.50%) followed by N6 (80.03%) and R (78.77%) (Fig. 2). IR64-1-1-4 produced more calli in R (20.0%) followed by MS (18.77%) and least in N6 (17.93%). On the other hand, V19 produced more calli in R (8.20%) followed by N6 (6.53%) and least in MS (6.4%).

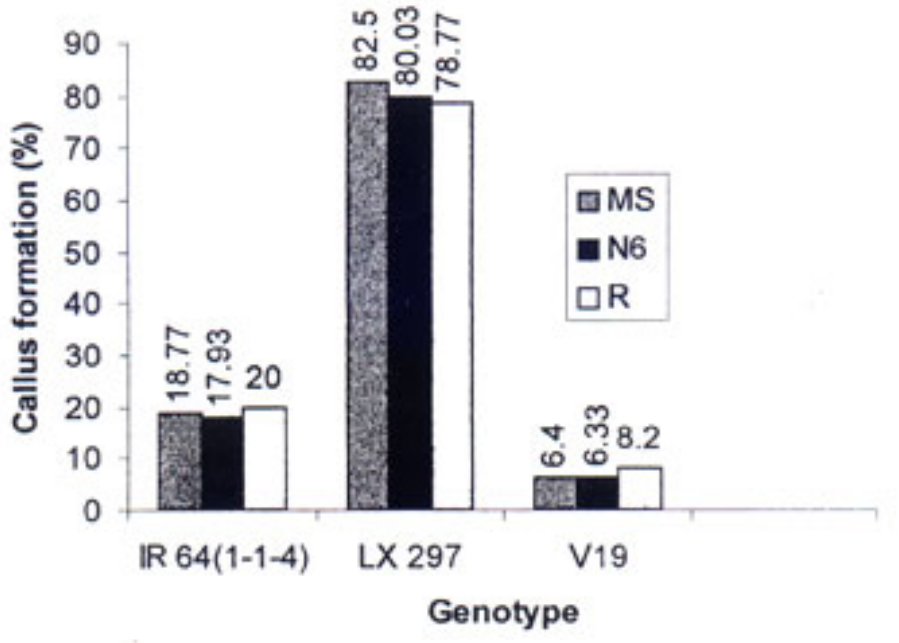

Fig. **2**. Percentage of callus induction in three rice genotypes.

Plant regeneration : Only the responsive genotype (Lx297) was subjected to MS based regeneration medium MSK_2. The results show that Lx297 calli, induced on MS gave highest regeneration frequency (13.89%) on transfer to MSK_2 (Table 4). Both organogenesis and embryogenesis have been shown to be affected by the type and amount of cytokinin in the regeneration medium (Yoshida et al. 1994). In the present study, shoots regenerated on Kn supplemented medium at a higher concentration, namely 2.0 mg/l (Table 1). Shoot differentiation was observed within 2 - 3 weeks on the regeneration medium, compared to 30 - 40 days reported by Yoshida et al. (1994).

Considering green spots, roots and necrosis in regeneration medium it was found that R-MSK_2 media combination performed better in embryogenic calli (green spot, 36.11%) as well as production of non-embryogenic calli (calli with root + necrosis = 69.44%) than N6-MSK_2 and MS-MSK_2 media (Table 4). When

Table 4. Mean effect of callus induction regeneration media for regeneration ability of mature seed scutellar derived calli of the rice genotype Lx297.

Callus induction medium	No. of regenerates (%)	No. of calli with green spots (%)	No. of calli with roots (%)	No. of calli with necrosis (%)
N6-MSK_2	10.65	13.43	10.42	25.93
MS-MSK_2	1.85	12.14	17.14	17.48
R-MSK_2	13.89	36.11	22.22	47.22
S.E.D.	5.56	9.78	12.42	11.89
5% LSD	22.15	38.33	48.67	46.61

three media composition were compared as regards their ability to induce green spots, rooting and necrosis in embryogenic calli, R-MSK_2 was found to be the best followed by N6-MSK_2 and MS-MSK_2.

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