Early Plantlet Regeneration from Five Local Indica Rice Genotypes (*Oryza sativa* L.)

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ABSTRACT

Five local rice genotypes *i.e.* Bg 300, Bg 304, Bg 357, Pokkali and Murungakayan were screened for their callus induction frequency and plantlet regeneration ability. Surface sterilization by rinsing rice caryopses in liquid soap and 1% NaOCl for 30 minutes followed by agitation in 70% ethanol for 1 minute and 2.5% NaOCl for 5 minutes and washing with sterilized distilled water under aseptic conditions was found to be the optimum procedure. Caryopses cultures were maintained on liquid media supplemented with 2% sucrose and various concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid) and TDZ (thidiazuron). A short treatment with either 10 µM or 20 µM 2,4-D for 3 days was found to be the optimum for callusing and subsequent shoot regeneration, irrespective of the genotype. Shoot bud initiation was rapidly accomplished within 15-20 days in all five genotypes. Plantlet regeneration in 33.3% cultures could be achieved in the highest responsive genotype, Bg 300, with 5 µM TDZ treatment for 4 days and subsequent transfer to MS (Murashige and Skoog) medium (1962). However, it could be increased up to 50% by doubling the TDZ concentration (10 µM). Vigorous rooting was observed on hormone free MS medium and more than 90% of plantlets developed successfully when they were transferred to the greenhouse. Among them more than 90% of plants bore panicles. A RAPD (Random Amplified Polymorphic DNA) analysis performed using three random primers, A18, A20 and B18 for the variety Bg 300 revealed that the tissue cultured plants were genetically different from the seed raised original Bg 300 plants. Thus tissue culture could be used to create genetic variability in rice and it helps to widen the gene pool.

KEY WORDS : Rice, NaOCl, Callus Induction, Plantlet Regeneration, 2,4-D, TDZ, RAPD.

INTRODUCTION

Rice (*Oryza sativa* L.), which belongs to the family Graminae (Poaceae), is the single most important food crop in Sri Lanka. It occupies 34 percent of the total cultivated area in Sri Lanka and is the livelihood of more than 1.8 million farm families. More than 30 percent of the total labour force is directly involved in rice or rice-related activities. With a per capita consumption of about 100 Kg/year and a limited annual cultivated rice land of 830,000 ha, Sri Lanka must raise its present average yield level of 3.8MT/ha (Anon, 2003) to achieve self sufficiency in rice. Hence, producing new rice varieties with a higher yielding capacity and good nutritional value is important.

Production of callus and its subsequent regeneration are the prime steps in a crop plant to be manipulated by biotechnological means. A tissue culture system with an efficient degree of somatic embryo production would be useful in genetic manipulation studies aimed at rice improvement. Several potentially useful genes can now be introduced into cultivated rice and prospects for future rice biotechnology are promising (Saharan *et al.*, 2004). The Japonica cultivars were found to be more responsive to regeneration than indica cultivars (Gairi and Rashid, 2004). But it is reported that the callus cultures could be raised easily from any part of the rice plant and induced to regenerate complete plants (Bajaj, 1989).

It has been reported that the following factors affect plant regeneration frequency in rice: genotype, developmental stage of calli in the explants, hormonal composition of the medium, feeder cells, carbohydrate source, partial desiccation or water stress inducing treatments and other medium (Abeyaratne *et al.*, 2004). Various combinations of auxins and cytokinins in the culture medium have been tested for their effect on both callus induction and plant regeneration in rice (Croughan and Chu, 1991). A rapid system has been developed to regenerate shoots within 15 days of non-responsive (re-calcitrant) indica rice varieties using TDZ with a short treatment of 2,4-D (Gairi and Rashid, 2003).

Sri Lanka is a gold mine for rice genes. While there are 120,000 cultivars in the whole world, this small island has 2,800 that have been recorded so far (Shockman, 2003). In this study regeneration ability of few improved and traditional local rice varieties is compared with a combination of 2,4-D and TDZ in the culture medium.

MATERIALS AND METHODS

The experiments were carried out at the Plant Reproductive Biology Laboratory of the Institute of Fundamental Studies (IFS), Hantana Road, Kandy and at the Biotechnology Laboratory of the Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka during the 6 months period commencing from 12th November, 2004.

Genotype of Rice (Oryza sativa L.)

The material used for this experiment was mature indica rice seeds from healthy mother plants of five common local genotypes grown under the greenhouse conditions at IFS, Kandy (Table 1).

Table 1. Details of different genotypes of rice used in the experiment

Variety	Type	Duration (Months)
Bg 300	Improved	3
Bg 304	Improved	3
Bg 357	Improved	3 ½
Pokkali	Traditional (Indian variety)	>4
Murungakayan	Traditional	>4

Surface Sterilization

Mature seeds of rice were dehusked manually and examined under the stereo microscope for insect damages. Seeds were sterilized by washing in Teepol (1:1, v/v) for 5 min followed by 3 rinses in tap water. Seeds were then shaken with 70% ethanol for 1 min in the laminar air flow chamber. Finally they were treated with different concentrations (1%, 2%, 2.5%,3.5% and 5% v/v) of commercial bleach (5.25% sodium hypochloride) for different agitation periods (5, 10 and 30 min) followed by 3 rinses in sterilized distilled water (Table 2).

Table 2. Concentration and agitation period of NaOCl of different treatments

Treatment	Concentration of NaOCl	Agitation Period (min)
T1	1%	10 (in the LAFC*)
T2	2%	10 (in the LAFC*)
T3	1%	30 (outside)
	2.5%	5 (in the LAFC*)
T4	3.5%	10 (in the LAFC*)
T5	5%	10 (in the LAFC*)

i.e. LAFC*– Laminar Air Flow Chamber

For each treatment 12 seeds from each genotype were used. Each experiment was repeated three times. Three seeds were cultured in a baby food jar (6.5 x 4.5 cm, capped with plastic caps and wrapped with 2 strips of parafilm), containing 10 ml of solid MS medium, incorporated with 2% sucrose and 0.6% agar. Cultures were incubated at 25±2 °C in complete darkness. The presence of contaminants, the nature of contaminants (bacteria or fungi) and the number of seeds germinated were recorded after 10 days.

Culture Media

Liquid culture medium supplemented with 2,4-D in 4 different concentrations *i.e.* 10 µM (2.2 mg/l), 20 µM (4.4 mg/l), 40 µM (8.8 mg/l) and 80 µM (17.6 mg/l) and 2% sucrose was used for callus induction. Liquid culture medium supplemented with either 5 µM (1.1 mg/l) or 10 µM (2.2 mg/l) TDZ and 2% sucrose was tested for shoot induction. Murashige and Skoog (1962) culture medium supplemented with 2% sucrose and 0.6% agar was used as the regeneration medium.

Callus Culture Initiation

For each treatment, 24 cultures from five different genotypes, each with four caryopses were raised. Each experiment was repeated three times. Initially the caryopses were introduced to baby food jars containing 10 ml of liquid culture medium supplemented with 2,4-D. Six cultures for each 2,4-D concentration were raised. The cultures were maintained in a culture room at 25 ± 2 ^{0}C and 24 h light (fluorescent tubes 36W/54, 6500 K; 15 µmol m^{-2} s^{-1}) with continuous shaking for 3 days. Then they were transferred to hormone free solid MS medium for 7 days followed by liquid culture medium containing TDZ for 4 days giving similar conditions as above. For each TDZ concentration three cultures were maintained. Subsequently the seeds were subcultured on hormone free solid MS medium for regeneration and maintained at 25 ± 2 ^{0}C and 24 h light.

Shoot Development and Rooting

Regenerated plants were carefully removed from the regeneration medium and transferred to culture tubes (19 x 2.3 cm Pyrex test tube; covered with cotton plug) containing 10 ml of liquid MS supplemented with 2% sucrose. A sterile paper wick was used to place the plant in the medium without submerging and left for 1 week in the culture room under 24 h light. Then they were transferred to liquid MS supplemented with 1% sucrose for 1 week followed by liquid MS without sucrose for another week. Subsequently, the plants were transferred to ½MS medium (liquid) and kept inside a growth chamber for 1 week. Later, they were transferred to small plastic pots containing a mixture of loam and compost and kept inside a growth chamber under humid conditions for 5 days. Finally, the well-hardened plants were transferred to the green house.

Confirmation of the Genetic Basis of the Tissue Cultured Plants

Leaf samples collected from four different callus-derived plants of Bg 300 and seed raised parent Bg 300 plant were subjected to RAPD analysis at the Biotechnology Laboratory, Wayamba University of Sri Lanka, in order to confirm the genetic basis of the regenerated plants.

Leaf samples were collected from 4 weeks old seedlings of four callus-derived plantlets and one seed derived plant of Bg 300. They were transferred to ice and stored on ice until further use.

Leaf material (50 mg) was ground quickly with 400 µl of extraction buffer [100 mM Tris-HCl (pH 8.00), 50 mM EDTA, 500 mM NaCl, 1% w/v SDS]. After the addition of another 400 µl of extraction buffer, the liquid phase was transferred to 1.5 ml-eppendorf tube and placed on ice until further use. This was mixed well by slowly inverting the tubes with equal volume of chloroform for 5-10 minutes and spun at 7500 rpm, for 4 minutes and 30 seconds in a micro-centrifuge. Then the supernatant was transferred to another fresh tube and mixed gently with equal volume of 100% ethanol. It was spun at 7500 rpm for 4 minutes and 30 seconds. The DNA pellet was washed twice with 70% ethanol for 1 minute at 1000 rpm and dried at room temperature. The DNA pellet was resuspended in 50 µl of autoclaved deionized

distilled water and stored at 4 ^{0}C. Quality and quantity of the DNA was assessed by agarose gel electrophoresis.

Three random primers A18, A20 and B18 (Operon Company, Almaeda, USA) were used for PCR (Polymerase Chain Reaction). The DNA amplification reactions were performed in a volume of 20 µl containing approximately 50-100 ng genomic DNA, 2 µl each of dATP, dGTP, dCTP, dTTP (2mM), 0.5 µl of a primer, 0.4 µl *Taq* polymerase (5U/µl), 1.45 µl $MgCl_2$ (25 mM), 2 µl 10X Mg free buffer and 11.65 µl of autoclaved deionized distilled water. The mixture was gently mixed and centrifuged prior to adding 2 drops of mineral oil. The amplification was performed in a thermocycler programmed as follows: 45 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. Amplification products were loaded in 1% agarose gel for electrophoresis in 0.5X TBE buffer. Gels were stained with ethidium bromide and photographed under UV light using Polaroid film.

Statistical Analysis

The data were analyzed using the SAS statistical software package (SAS/STAT Software, 1999) at 0.05 probability level.

RESULTS

Surface Sterilization

Gradual reduction in contamination of rice caryopses was observed with the increment in chlorox concentration (Figure 1). The data were analyzed in 'Analysis of Variance' (ANOVA) procedure (Pr=0.05). The model (Pr>F, <0.0001) and the treatment effect (Pr>F, <0.0001) were significant, while the varietal effect (Pr>F=0.7967) was not significant (R^2=0.959859, CV=21.58520). A significant reduction in contamination percentage was obtained with treatment number 3 (1% chlorox, 30 min outside and 2.5% chlorox in LAFC) and 5 (5% chlorox, 10 min in LAFC) respectively (Least Significant Difference = 4.923). The contamination percentage was <5% with these two treatments.

More than 90% of the contaminants were bacteria and the rest were fungi and no contamination was observed caused by both sources. The contamination pattern clearly showed that the contaminants arose from the explanted seed material, thus the contamination may be seed borne.

Germination

Days taken for germination varied between 3 to 6 days in five genotypes (Figure 2). The traditional varieties, Pokklai and Murungakayan took 5-6 days for germination while other three varieties germinated within 3-4 days.

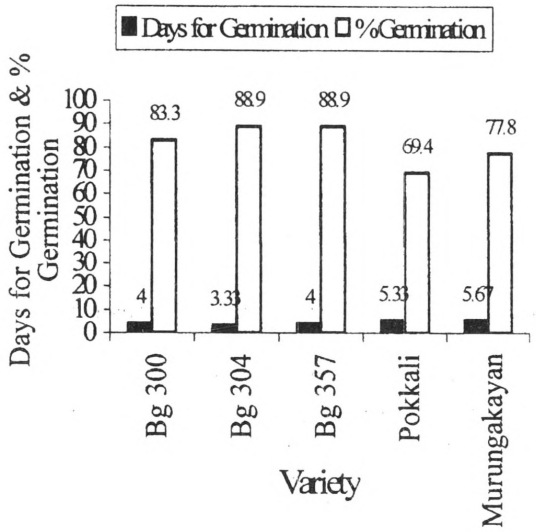


Figure 2. Days taken for germination and the percentage of germination in different genotypes

After 10 days in culture 88.9% seed germination was observed in Bg 304 and Bg 357, while Bg 300 showed 83.3% and relatively lower values were shown by Pokkali (69.4%) and Murungakayan (77.8%) (Figure 2).

Callus Culture Initiation

In all five genotypes, callus induction was observed within 3 to 5 days of the 2,4-D treatment.

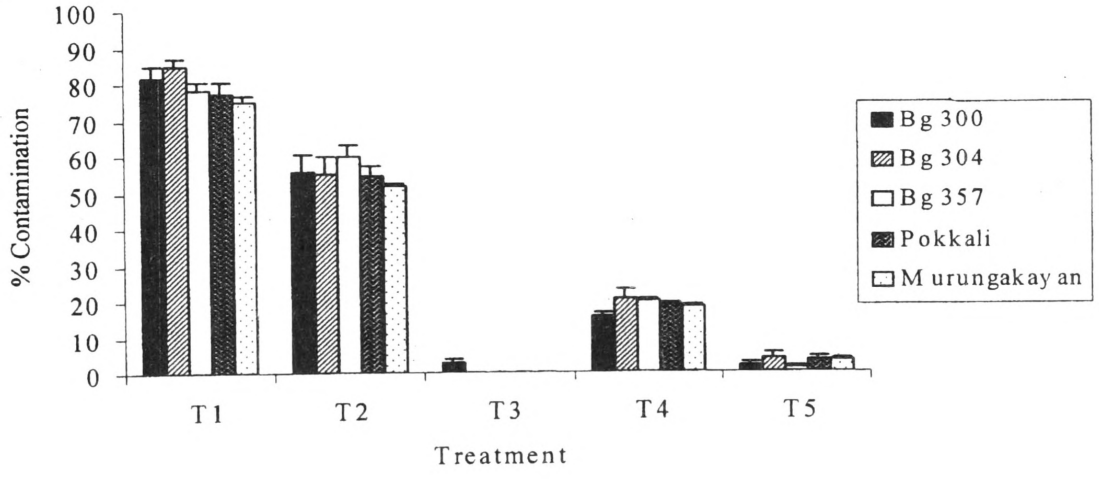


Figure 1. Percentage contamination of rice caryopses cultures of five genotypes in different treatments

The callus appeared like a thin layer around the coleoptile. The tissue produced was slow growing and no significant improvement in callus size was observed in subsequent transfer to hormone free MS for 1 week. Furthermore, it was observed that treating with 2,4-D for more than 3 days was inhibitory for regeneration. Such caryopses cultures turned brown and later degenerated. It was observed that liquid media was more effective than agar media for both callus induction and plantlet regeneration.

The callus colour, compared using a colour chart, revealed a slight variation among five genotypes (Table 3).

Table 3. Callus colour of five genotypes

Genotype	Callus colour
Bg 300	Light yellow
Bg 304	White
Bg 357	Yellow
Pokkali	Cream
Murungakayan	Cream

The callus induction frequency varied between 16.7% and 93% in five genotypes (Figure 3). In the statistical analysis, a chi-square test was performed (Pr=0.05) and accordingly the effect of different concentrations of 2,4-D was significant (Pr<0.0001). However, a defined pattern of callus formation could not be identified at varied 2,4-D concentrations among five different genotypes. In certain genotypes an increment in the percentage of caryopses developing calli was observed when increasing the auxin concentration. In Bg 300 and Pokkali, over 90% of cultures had developed calli at the highest level of 2,4-D (80 µM). In other genotypes highest rate of callusing was achieved at 20 and 40 µM levels and it slightly decreased at 80 µM level.

Most of the calli (>80%) were <1mm in length, measured at the broadest point of the callus. They mostly possessed the ability to regenerate into complete plantlets within 15 to 20 days. Around 5% of cultures developed calli >1mm in size, which did not possess the ability to regenerate. They were viable for 2-3 weeks in the culture and later turned brown and degenerated. This was frequently observed at higher concentrations of 2,4-D (*i.e.* 80 µM).

Shoot Development and Rooting

All the genotypes responded to TDZ treatment and developed shoot buds. Shoot development frequency varied between 8.3% and 50% (Table 4).

Data were analyzed using the CATMOD procedure (Pr=0.05). The effect of 2,4-D as well as TDZ was found to be significant (Pr>Chisq = 0.0013 and 0.0018 respectively). But the interaction effect of 2,4-D x TDZ was not significant. Statistical analysis revealed that the genotype has a significant effect on the shoot development frequency ((Pr>Chisq, <0.0001). Shoot development frequency of Bg 300 was found to be significant when compared to other four genotypes (Table 5).

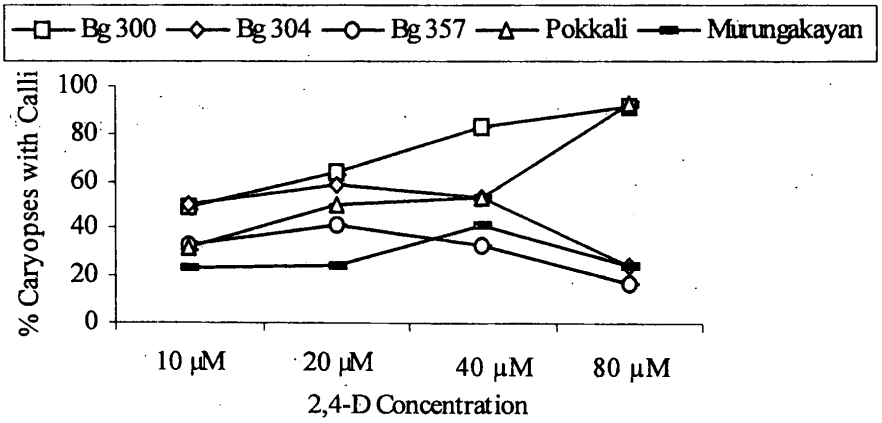


Figure 3. Rice caryopses culture, frequency of culture developing calli at different concentrations of 2,4-D

Table 4. Shoot development frequency at different concentrations of 2,4-D and TDZ

Genotype	Percentage of cultures regenerated into plantlets							
	2,4-D 10 µM TDZ		2,4-D 20 µM TDZ		2,4-D 40 µM TDZ		2,4-D 80 µM TDZ	
	5 µM	10 µM	5 µM	10 µM	5 µM	10 µM	5 µM	10 µM
Bg 300	33.3	50	25	36	19.4	24.9	13.8	19.4
Bg 304	8.3	16.6	11	22.2	0	0	0	0
Bg 357	8.3	16.6	19.4	36	0	11	0	0
Pokkali	13.8	16.6	8.3	13.8	0	0	0	0
Murungakayan	13.8	25	22.2	47.2	0	8.3	0	0

Bg 300 was the most responsive genotype to tissue culture followed by Murungakayan, while Bg 304 was the least responsive genotype. Among the five genotypes, only Bg 300 had responded in all 4 levels of 2,4-D. Higher levels of 2,4-D were found to be inhibitory for other genotypes. Among the 2,4-D levels applied, 10 and 20 µM levels were found to have a significant effect on shoot development (Table 5). None of the genotypes except Bg 300, had responded at 80 µM level and just few had responded at 40 µM level. However, the regeneration ability increased significantly by doubling the TDZ concentration (10 µM) (Table 4 and 5). Mostly the regeneration ability had increased by 2 folds at TDZ 10 µM level. Treating rice caryopses for more than 4 days with TDZ was found to be inhibitory for their further development.

Table 5. Probability values obtained by the statistical analysis

Contrast	Pr>Chisq
Bg 300 Vs Bg 304	0.0002
Bg 300 Vs Bg 357	0.0025
Bg 300 Vs Pokkali	<0.0001
Bg 300 Vs Murungakayan	0.0313
2,4-D 10 µM Vs 20 µM	0.3031
2,4-D 10 µM Vs 40 µM	0.0101
2,4-D 10 µM Vs 80 µM	0.0244
TDZ 5 µM Vs 10 µM	0.0018

Shoot development occurred within 15 to 20 days of culturing and it was a very rapid process. More than 20 shoot buds developed around the coleoptile and the coleoptile was longer than the other shoot buds. Dissecting of callus and subculturing it on hormone free MS was not found to be effective in retrieving the regeneration ability.

A clump of roots developed within one week of subculturing on hormone free MS medium. They further developed during the acclimatization process. Plantlets with well-developed roots were phenotypically normal and similar to the parent plants.

Plantlet Establishment in Soil

Five weeks old plantlets were transferred to the greenhouse and more than 90 % of them developed successfully in all genotypes. No phenotypic variation was observed. Pre-mature mortality of plantlets was associated with severe dehydration. It could be successfully overcome by covering the plantlets with polythene cover having sufficient number of small holes for air circulation.

Panicle formation was observed in more than 90% of well-developed plants in 2-3 months.

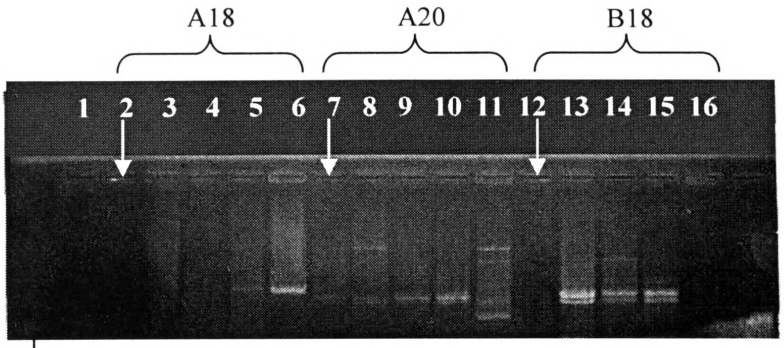
Confirmation of the Genetic Basis of the Tissue Cultured Plants

Following the gel electrophoresis, the DNA concentration of each sample was estimated as 40 ng/µl (Plate 1). Therefore, approximately 2.5 µl genomic DNA was used for the amplification reactions.

The PCR amplified products were visualized by agarose gel electrophoresis. Among the Bg 300 callus-derived plants tested, the plant no. 4 (Lane 11) has produced a polymorphic banding pattern with respect to the random primer A20 (Plate 2).

1- Bg 300 seed raised parent
2,3,4 and 5 - Four different callus-derived Bg 300 plantlets

Plate 1. Quality and quantity assessment of extracted DNA using gel electrophoresis



- Bg 300 seed raised parent

Other four lanes – Callus-derived Bg 300 plants

Plate 2. Gel photograph obtained after the PCR

DISCUSSION

Contamination is a major drawback in establishing *in vitro* culture of rice. In somatic tissue culture, seeds are excellent explant material because the vigorous surface sterilization results in minimal contamination of the resultant callus culture (Abeyratne *et al.*, 2004). In this study a survival percentage of over 95% of rice caryopses could be achieved by either increasing the chlorox concentration (*i.e.*5%) or by extending the agitation period in lower concentrations of chlorox (30 min). However, rice caryopses were found to be highly vulnerable to phenolic oxidation and browning at higher concentrations of chlorox, limiting the *in vitro* establishment of cultures. It has been recorded that the use of mercuric chloride ($HgCl_2$) at lower concentrations (<1%) was also effective in controlling bacterial contamination in rice (Abeyratne *et al.,* 2004 and Gairi and Rashid, 2004). However, the use of bleach as a surface sterilant is common because of its low toxicity and high performance in controlling contamination.

An auxin treatment is essential to induce embryogenic competence on the scutellar cells of rice caryopses (Heyser *et al.*, 1983, Abe and Futsuhara, 1986, Rueb *et al.*, 1994). The inclusion of higher concentrations of auxins in tissue culture of cereals induce callus. Of these auxins, 2,4-D was found to be the most effective at high concentrations (2-5 mg/l) (Vasil, 1995). In the present investigation also successful rates of callus induction and plantlet regeneration were achieved at 10 µM (2.2 mg/l) and 20 µM (4.4 mg/l) levels, confirming the above results.

Mostly, the calli formed at very high concentrations of 2,4-D (>5.0 mg/l) degenerate when transferred to the regeneration medium subsequent to the TDZ treatment. Similar results were obtained by Rupika, 2000. Treating caryopses with 2,4-D for more than 3 days was found to be inhibitory for regeneration. Previous studies also have produced similar results on indica rice genotypes (Gairi and Rashid, 2004).

In a liquid medium, the contact of rice caryopses with medium components is more effective than in a solid medium. Furthermore, the concentration of hormones is equal in all the places in a liquid medium. Therefore, more efficient callus induction and plantlet regeneration could be achieved when liquid medium is used.

Often the calli exceeding 1 mm in length could not regenerate into complete plantlets. At higher concentrations of 2,4-D the cell division is rapid, resulting in a big mass of callus. Though the cell division proceeds in a rapid rate, DNA synthesis will not be completed at such rates. The cells lacking enough DNA for their further development may turn brown and later degenerate.

Previously, Gairi and Rashid (2004) had described that the regeneration of somatic embryos to form shoots in rice caryopses cultures was not so frequent on cytokinin cultures, as compared to TDZ. Also they have reported specific increase in the frequency of shoot bud formation by TDZ in embryogenic callus cultures originating from caryopses of a minor millet, *Paspalum scrobiculatum.* According to their investigations, in dicot plants, TDZ substitutes an auxin-cytokinin requirement for high frequency somatic embryogenesis of *Geranium* and *Arachis.* However, TDZ plays a specific role by inducing direct shoot regeneration from caryopses cultures in rice and the reason for a better performance by TDZ remains to be investigated.

With indica rice genotypes, mature seeds as the explant material and MS as the regeneration medium, the genotype was found to be a major factor influencing callus induction and subsequent plantlet regeneration. Among the five genotypes used, Bg 300 was found to be the most responsive for tissue culture. This finding is in conformation with the results reported by Rupika (2000) and Abeyratne *et al.* (2004). This may be due to an inherent genotypic effect and the exact reason is still to be determined.

The best results in rice tissue culture so far obtained required 1 month for callus initiation and its development and 1 month for plant regeneration (Rueb *et al.,* 1994). However in this process there could be a potential for the loss of regeneration ability of embryogenic tissue. By contrast, according to the present study, a short auxin treatment of 3 days is sufficient to induce the callus and its regeneration potential could be evoked on transfer to TDZ medium and plant

regeneration accomplished within 15 days from the auxin treatment.

According to the RAPD analysis performed, the callus-derived plants of Bg 300 were genetically variable. One among the four callus-derived plants was proved to be genetically different from the parental genotype Bg 300. Similarly, Abeyaratne *et al.* (2004) also had reported presence of somaclonal variation in callus-derived plants of the genotype Bg 94-1. Plant cells grown in cultures undergo extensive genetic changes, which leads the somaclonal variation. Somaclonal variation is a general phenomenon of all plant regeneration systems that involves a callus phase. This is an attractive source of genetic variation to breeders. However, undesirable genetic changes (somaclonal variation) could be problematic when the objective is to produce true-to-type plants specially when use them as parents in a breeding programme.

Though the current study suggests the possibility of occurring somaclonal variation among the Bg 300 callus-derived plants, further studies should be carried out with representative samples using more primers in order to confirm the results obtained. Also the genetic basis of the callus-derived plants of other four varieties remains to be investigated.

Availability of an efficient regeneration system is a pre-requisite for undertaking any transformation study, which could be used for generating transgenic plants resistant to various biotic and abiotic stresses. Also there are possibilities for the production of haploids from anther/microspore culture and for the production of somatic hybrids and cybrids from isolated protoplasts of rice which are essential steps in crop improvement programmes.

CONCLUSIONS

In *in vitro* establishment of indica rice caryopses cultures, optimum surface sterilization procedure was identified as rinsing rice caryopses in liquid soap and 1% NaOCl for 30 min followed by agitation in 70% ethanol for 1 min and 2.5% NaOCl for 5 min and washing with sterilized distilled water for 3 times under aseptic conditions. Inclusion of either 10 or 20 µM 2,4-D was found to be the optimum for callus induction and subsequent plantlet regeneration, invariably dependent on genotype. TDZ at a concentration of 10 µM was very efficient in developing shoot buds. Shoot bud formation occurred within 15 to 20 days of culturing. A genotypic difference was observed in the regeneration ability and Bg 300 was found to be the most responsive genotype followed by Murungakayan. Bg 304 was the least responsive genotype. Plantlets transferred to greenhouse after the acclimatization were successfully developed irrespective of the genotype. According to the RAPD analysis, the callus-derived plants of Bg 300 were proved to be genetically variable from the original Bg 300 plants. Thus tissue culture could be used create genetic variability in rice. However, this protocol is potentially useful for applied research such as genetic transformation of embryogenic tissue.

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REFERENCES

Abe, T. and Y. Futsuhara, (1986). Genotypic variability for callus formation and plant regeneration in rice (*Oryza sativa L.). Theor. Appl. Genet.* **72**:3-10

Abeyratne, W.M., U.N. De Silva and H.M.P.S. Kumari, (2004). Callus Induction, plantlet regeneration and occurrence of somoclonal variation in somatic tissues of some Indica rice varieties. *Annals of the Sri Lanka Department of Agriculture.* **6**: 1-11.

Anon (2003). Annual Report of Central Bank, Colombo.

Anon (1999). SAS/STAT software, Institute Inc. cary, Nc27513 USA.

Bajaj, Y.P.S., (1989). Introduction and cryopreservation of somaclonal variation in wheat and rice. Review of advances in plant biotechnology 1981-88. Second international symposium of genetic manipulation in crops. Eds. A. Mujeebkazi and L.A. Sitch. 195-203.

Croughan, T.P. and Q.R. Chu, (1991). Establishment of Callus Cultures and the Regeneration of Plants. Biotechnology in Agriculture and Forestry, Ed. Y.P.S. Bajaj, Arabic press. 19 – 37.

Gairi, A. and A. Rashid, (2004). TDZ- induced somatic embryogenesis in non-responsive caryopses of rice using a short treatment with 2,4-D. *Plant Cell, Tissue and Organ Culture.* **76**: 29-33.

Heyser, J.W., T.A. Dykes, K.J. DeMott and M.W. Nabros, (1983). High frequency long term regeneration of rice from callus cultures. *Plant Science.* **65**:217-223.

Murashige, T. and F. Skoog, (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**:473-487.

Rueb, S., M. Leneman, Schilperoort and L.A.M. Hensgens, (1994). Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.). *Plant Cell Tiss. Org. Cult.* **36**: 259-264.

Rupika, L.A.S., (2000). Screening five local rice genotypes (*Oryza sativa ssp. Indica)* for callusing ability and plant regeneration. Final year project report, B.Sc. Agriculture. Department of Crop Science, Faculty of Agriculture, University of Peradeniya. 42-59.

Saharan, V., R.C. Yadav and R.Y. Neelam, (2004). High frequency plant regeneration from desiccated calli of Indica rice. *African Journal of Biotechnology.* **3**: 256-259.

Shockman, D. (2004). Gold Mine for Rice Genes. Daily News, 22 Nov. 2004.

Vasil, I.K., (1995). Cellular and molecular genetic improvement of cereals in Current issues in plant molecular and cellular biology, Ed. M.Terzi *et al.*, Kluwer Academic Publishers, Netherlands. 5-18