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Plant Regeneration *in vitro* by Organogenesis on Callus Induced from Mature Embryos of Three Rice Varieties (*Oryza sativa* L. ssp. *indica*)

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ABSTRACT. *Callus induction and regeneration in vitro of three rice varieties (Oryza sativa L. ssp indica), Bg 94-1, Moraberekon and Bg 300 were examined. Phenotypic variations of regenerated and parent plants were compared and the method of plant regeneration was confirmed by histology. Callusing was induced by culturing the mature embryos of the three rice varieties in a liquid medium of distilled water containing 13.2 mg/L 2, 4-D and 2% sucrose for 7 days. Calli were proliferated for three weeks on solidified MS medium containing NAA and BAP at 1 mg/L each and 2% sucrose. Regeneration of calli was induced by culture solidified MS medium containing 100 mg/L tryptophan and 3% sucrose. The proliferation and regeneration media were solidified using 3 g/L gelrite. Regeneration was further stimulated by callus dehydration using silica gel and culture for 3 days on the latter medium solidified by 1% agarose. Callus induction frequencies of Bg 94-1, Moraberekon and Bg 300 were 61%, 67.5% and 55% respectively. The shoot bud induction frequencies of these varieties were 6.5%, 7.5% and 7.5% respectively. Regeneration frequencies of Bg 94-1 and Moraberekon were 100% and 43% respectively. Bg 300 calli did not regenerate. The comparison of regenerated and parent plants at a phenotypic level on maximum tiller number, maximum height and seed weight, indicated that the regenerated plants of Bg 94-1 were similar to parent plants, while Moraberekon showed variations, compared to parent plants. Histological studies revealed that regeneration occurred via indirect organogenesis from calli initiated from the rice scutellum.*

INTRODUCTION

Progress in plant genetics and biotechnology is highly dependent on the use of *in vitro* techniques; hence the establishment of effective *in vitro* plant regeneration systems enabling a rapid production of fertile, genetically stable plants is important to biotechnologists. High frequency callus induction and efficient plant regeneration are pre-requisites for the use of biotechnological approaches for rice improvement such as genetic transformation and improvement through somaclonal variation.

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Shoot meristems and embryo like structures are often observed in callus cultures, which eventually develop into whole plants. A basic regulatory mechanism underlying organized development involves a balance between auxins and cytokinins. At present, successful aseptic culture and improvement of the culture media and methods permit organ formation in callus cultures from many different plant species. However, it is suggested that various growth regulators must be applied to cells in the correct quantity and sequence under optimal conditions.

Various studies have been carried out in the field of callus induction and regeneration in rice (*Oryza sativa* L.). Khanna and Raina (1998) showed that regeneration and shoot bud induction frequencies were influenced by the genotype, callus induction medium, regeneration medium as well as the interaction between the two media and the interaction between the genotype and the two media. Fillipov *et al.* (2006) studied the effects of genotype, different concentrations and exposure time to Dicamba, 2, 4. D and 2,4,5-T (2,4,5- trichlorophenoxyacetic acid) and combinations of other auxins with Dicamba, on somatic embryogenesis and plant regeneration from mature embryos of spring and winter genotypes of wheat. The study showed a significant effect of the above factors on embryogenic callus formation and shoot induction with 0.5 mg/L indole-3-acetic acid (IAA) and 12.0 mg/L Dicamba in the callus induction medium giving maximum results.

Abe and Futsuhara (1986) have carried out a study on sixty rice varieties of *japonica, javanica* and *indica* for their capacity for callus growth and regeneration. The results have shown that variabilities exist in callus growth and plant regeneration potentials among the varieties tested. It was also observed that varietal variation exists within a subspecies and that callus growth potential was not correlated with regeneration potential.

In a study carried out by Lutts *et al*. (1999), the effects of absisic acid (ABA), polyethelene glycol (PEG), proline, tryptophan and IAA on rice callus regeneration were studied at various doses of NaCl on two *japonica* and two *indica* varieties of rice. It was shown that the amino acid tryptophan stimulated regeneration and increased subsequent survival rates of regenerated plantlets in all cultivars at all NaCl doses. ABA had a decreasing effect on regeneration, while the amino acid proline did not have a marked effect. IAA reduced shoot regeneration and increased root regeneration. Yang *et al*. (1999) showed that the addition of proline at a concentration of 8.69 mmol to a pre-culture medium prior to culture in regeneration medium increased the growth rate of the callus masses, and increased plant regeneration. Addition of 6- benzylaminopurine (BAP) at 12.49 mg/L in the pre-culture medium also had an enhancing effect.

Several studies were done on the effect of dehydration stress on regeneration frequency of plants from callus. A study by Desai *et al*. (2004) on sugarcane indicated that increase in time exposure to silica desiccation from 1 to 7 hrs significantly increased plant regeneration frequencies. Another study by Abeyratne *et al.* (2004) on *indica* rice varieties demonstrated an enhanced effect of regeneration when callus was desiccated for 24 hrs.

Histology studies were carried out by previous authors to determine the method of regeneration from callus tissue. Abe and Futsuhara (1985) reported somatic embryogenesis on rice callus induced from root segments.

The current study was carried out to induce callusing and regeneration from Sri Lankan rice varieties to ultimately enable the introduction of new traits using genetic transformation on the resulting callus phase. This procedure was developed as the currently available protocols fail to give consistent response on local varieties.

Therefore, the objective of the study was to induce callusing and regenerate plants from the mature seeds of three *indica* rice varieties, Moraberekon, Bg 94-1 and Bg 300 using plant growth regulators, callus dehydration and tryptophan. It also aimed at a preliminary comparison between regenerated plants and parental plants on maximum tiller number, maximum plant height and seed weight and to investigate the method of plant regeneration from callus cells using histology studies.

MATERIALS AND METHODS

Mature seeds of Moraberekon, Bg 94-1 and Bg 300 obtained from the Plant Genetic Resources Center, Gannoruwa, Sri Lanka were used as the explants in this study.

Surface sterilization

The seeds were dehusked, washed in Teepol water and rinsed with tap water. They were sterilized using 5.25% (v/v) sodium hypochlorite containing 2 - 3 drops of Tween 20 for 20 min and 95% (v/v) ethanol for 1 min and rinsed thrice in sterile distilled water.

Callus induction

The sterilized seeds were cultured in a liquid callus induction medium of distilled water containing 13.2 mg/L 2, 4 D, 0.15 mg/L BAP and 2% (w/v) sucrose for a period of 7 days. Cultures were aerated using a rotary shaker.

Callus proliferation

Subsequently, the germinated seedlings were aseptically dissected to remove the shoots, roots and endosperm. The dissected embryos were cultured on a callus proliferation medium of MS (Murashige and Skoog, 1962) supplemented with NAA and BAP, each at 1 mg/L, 2% (w/v) sucrose and solidified using 3 g/L gelrite. Embryos were subcultured to fresh medium every 5 days for a period of 3 weeks.

Plant regeneration

The embryogenic calli were dehydrated for 1 hr using 2.5 g of sterile, dehydrated silica gel per 5 calli and transferred to a regeneration medium of ½ MS supplemented with naphthaleneacetic acid (NAA) and BAP, each at 1 mg/L, 0.4 mg/L thiamine, 100 mg/L tryptophan, 3% (w/v) sucrose and solidified using 3 g/L gelrite. The calli were cultured for a period of 2 weeks. Afterwards, they were further dehydrated for 3 days by culturing in the above regeneration medium solidified using 1% agarose, and transferred to fresh regeneration medium solidified by 3 g/L gelrite for 8 weeks.

The pH of all culture media were adjusted to 5.8 and autoclaved at 121^{o}C and 1.05 kg/cm^{2} for 20 min. All cultures of callus induction, proliferation and regeneration were maintained at conditions of 25^{o}C and 16 hrs photoperiod.

Plant growth and acclimatization

The regenerating calli were transferred to hormone-free MS medium for shoot and root growth. The plants obtained were acclimatized under the above culture conditions in liquid MS medium, with the sucrose concentration decreasing every 7 days from 3% to 0%. Subsequently, the plants were maintained in tap water for 3 - 4 days inside a growth chamber at 25^{o}C and 16 hrs photoperiod and finally potted and transferred to the greenhouse.

Experimental design

Each experimental unit consisted of 8 culture vessels (baby food jars) for each variety, containing 5 explants (seeds) cultured in 20 mL of culture medium. Therefore, the data for each unit was a composite of all 10 vessels. The varieties were considered as the treatment, while all culture conditions were identical. Since the units were arranged in a completely randomized design, observations were recorded for each unit.

Data were collected on callus and green shoot bud induction, regeneration and number of plants obtained. They are expressed as percentage response against total explants treated (frequencies) for each variety.

$$\text{Callusing frequency} = \frac{\text{Number of explants with callus} \times 100}{\text{Number of explants cultured}}$$

$$\text{Shoot bud induction frequency} = \frac{\text{Number of calli with shoot buds} \times 100}{\text{Number of calli cultured}}$$

$$\text{Regeneration frequency} = \frac{\text{Number of shoot bud induced calli producing plants} \times 100}{\text{Number of shoot bud induced calli cultured}}$$

Preliminary comparison of parent plants and regenerated plants

The acclimatized plants were raised in the greenhouse to maturity and seeds were harvested. Five parent plants from each variety were raised from mature seeds in parallel under the same conditions. Data were collected on tillering, maximum plant height (from base of shoot to base of flag leaf) at physiological maturity and 1000 seed weight, and compared to determine the variation between tissue culture regenerated and seed derived parent plants.

Histology

Calli from the variety Moraberekon showing greening and shoot induction were used for sectioning (A separate replicate was cultured for callus sectioning). They were dehydrated in an ethanol series of 50% - 100% over a period of 8 hrs and left overnight in 100% ethanol. Subsequently, the specimen were infiltrated with xylene in a graded series of ethanol -xylene from 75% - 0% ethanol (from 25% - 100% xylene) over a period of 8 hrs and left overnight in pure xylene.

The calli were infiltrated under a temperature of 60^{o}C with paraffin wax (melting point 58 - 60^{o}C) by adding wax shavings gradually until the xylene was saturated with wax. Half of the wax solution was poured out and replaced by new melted wax every 2 hrs. Three such changes were done. Finally two complete changes of pure wax were made and the callus was embedded. The wax blocks containing the calli were sectioned using a rotary microtome to obtain sections of 20 μm thicknesses. The sections were mounted on slides, air-dried for 1 week, stained using saffranine and covered using cover slips mounted on Canada balsm. The sections were examined under an Olympus® BH-2 series microscope.

RESULTS AND DISCUSSION

Callus induction

The scutellum of the embryos of all varieties produced prominent calli containing a nodular but watery surface, under the influence of 2, 4 D in callus induction medium. Callus induction was carried out in a distilled water based liquid medium to increase the efficiency of exposure of the explants to the hormones. MS basal medium components were not added as the embryo is attached to the endosperm at callus induction, from which it would derive all nutrients required. Calli were proliferated on solid MS proliferation medium under the influence of NAA and BAP in equal concentrations to produce a large light yellow callus with a dry nodular surface (Plate 1A). According to Rueb *et al*. (1994) and Desai *et al*. (2004), the appearance of calli was embryogenic.

The three varieties used in the study, Moraberekon, Bg 94-1 and Bg 300 gave comparable callus induction frequencies of 61%, 67.5% and 55% respectively, (Figure 2).

The time period for maximum callusing was 21 - 31 days from culture initiation. Similar results were reported by Abeyratne *et al*. (2004), where data on callus induction and growth were recorded after one month in culture. The study by Khanna and Raina (1998) recorded callus induction and growth after 3 weeks in culture and another study by Yang *et al*. (1999) obtained callusing in one month. Therefore, the duration to callus induction in the studied varieties is in accordance with these previous results.

Green shoot bud induction

The varieties Moraberekon, Bg 94 - 1 and Bg 300 gave green shoot bud induction frequencies of 6.5, 7.5 and 7.5%, respectively (Figure 1). The calli produced green shoot buds 26 - 40 days after transfer to regeneration medium (Plate 1B).

Plant regeneration

The shoot bud induced calli of varieties Bg 94-1 and Moraberekon regenerated to produce plants (Plate 1C). Regeneration frequencies of the above were 43% and 100%, respectively. Shoot bud induced calli of Bg 300 failed to regenerate. The results indicate a higher ability of induced shoot buds to regenerate into plants, and a higher distinction on regeneration frequencies between varieties when compared with callusing and shoot bud induction frequencies (Figure 2). Similar results were shown by Abe and Futsuhara (1986), where sixty rice varieties were tested to a single procedure for callus induction and regeneration. Their study indicated that callus growth and regeneration in rice is highly variety dependent and that the callus growth potential was not correlated to the regeneration potential. Therefore, it can be suggested that a genetic basis exists for regeneration of plantlets from calli.

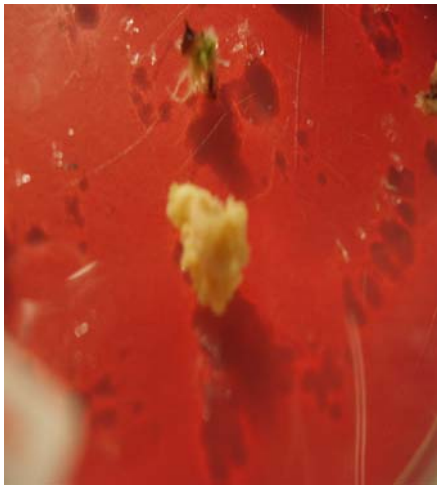
Regeneration was obtained under the influence of dehydration and tryptophan. Influence of dehydration on regeneration was also shown by Abeyratne *et al*. (2004), where calli were desiccated for 1 - 2 days resulting in an optimal desiccation period of 1 day and by Desai *et al*. (2004), where calli were desiccated for 1 - 7 hrs in sugarcane resulting in a increased frequency with increased desiccation.

In addition to silica desiccation, the embryogenic calli were maintained for 3 days in 1% agarose supplemented regeneration medium. Therefore, the current study used two dehydration treatments on the calli, *i.e.* desiccation using silica gel to reduce water content of calli and culture in regeneration medium solidified by 1% agarose to limit water uptake. Both methods result in making the calli drier and more embryogenic. This condition may also improve the oxygen supply to the embryogenic cells and enhance plant regeneration.

A previous study by Jain *et al*. (1996) indicated that an increase in agarose concentration on regeneration medium from 0.5% to 1.0% increased the frequency of shoot formation to 86%. In the current study, the calli were removed from agarose-solidified medium after shoot induction, and transferred to the previous regeneration medium to avoid severe dehydration and death.

Tryptophan was used in the regeneration of plants from calli in previous studies. A study carried out by Lutts *et al*. (1999) increased regeneration by use of tryptophan in combination with NaCl in the regeneration medium on two *indica* and two *japonica* varieties. The authors suggest that tryptophan, as a precursor of IAA, acts by altering endogenous levels of this hormone.

Bg 94-1 produced shoots after 27 days of culture on regeneration medium. Moraberekon gave shoots from calli containing shoot buds after 30 days of culture on regeneration medium. Previous studies on other rice varieties of both *indica* and *japonica* have shown different results. Khanna and Raina (1998) obtained regeneration of calli 5 weeks after transfer to regeneration medium. Abeyratne *et al*. (2004) recorded calli greening frequency after 4 weeks on regeneration medium. The above studies were on *indica* varieties. The current study indicated that the time period for regeneration was 3 - 4 weeks on regeneration medium, which was lower than recorded in the above studies.



(A)

(B)

(C)

(D)

Plate 1. Callus induction, shoot bud induction and regeneration from mature seeds of variety Bg 94-1.

Note: (A) embyogenic callus showing a dry, nodular surface (1.5x); (B) callus showing green shoot buds (1x); (C) shoot and root regeneration from callus (1.5x); (D) regenerated plant grown to maturity.

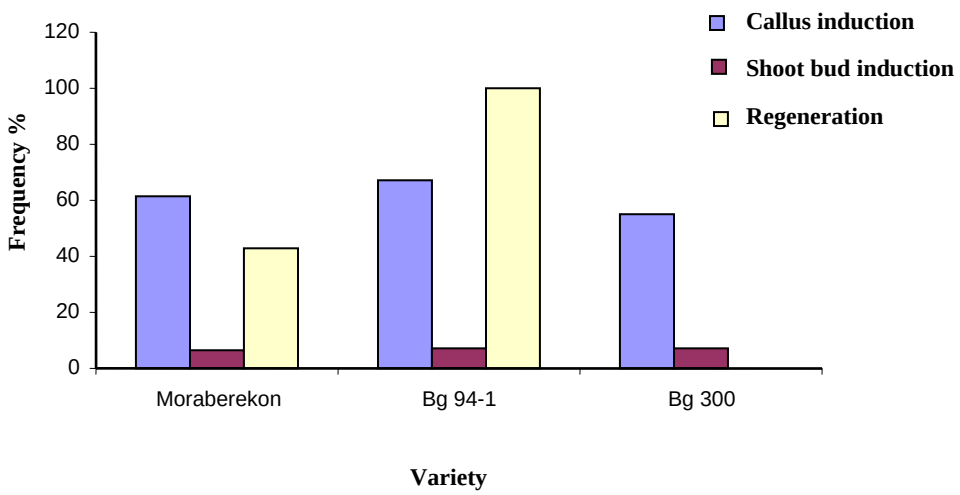


Figure 1. Callus induction, shoot bud induction and regeneration frequencies of Moraberekon, Bg 94-1 and Bg 300.

Roots were also regenerated on regeneration medium. Special rooting treatments were not required. The regenerating calli were maintained on MS hormone-free medium to facilitate shoot and root growth.

The plants obtained were acclimatized and transferred to the greenhouse (Plate 1 D). Bg 94-1 produced 11 plants of which 10 survived acclimatization. Moraberekon gave 4 plants. All survived acclimatization. The plants were grown to maturity and seeds obtained.

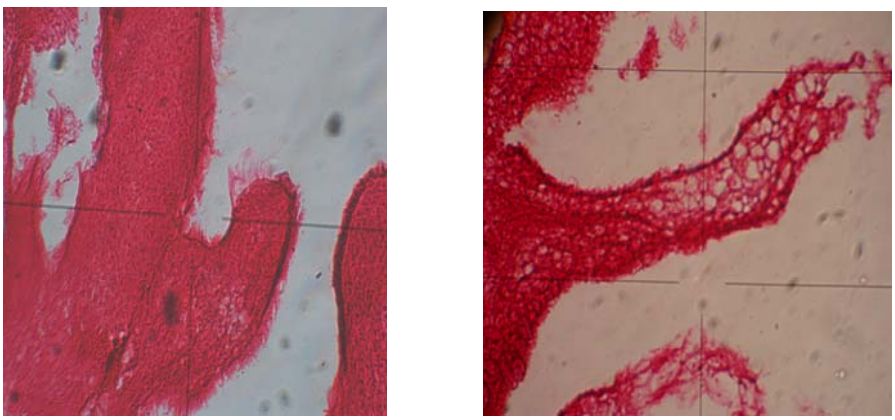
Table 1. Maximum number of tillers, maximum height and 1000 seed weight of regenerated plants and parent plants grown in parallel under greenhouse conditions.

Variety	Maximum no. of tillers	Maximum average height (cm)	1000 seed weight (g)
Bg 94-1 (regenerated plants)	5±0.84	33.0±3.85	22.29±11.10
Bg 94-1 (parent plants)	4±0.44	34.0±3.49	22.25±0.03
Moraberekon (regenerated plants)	1±0.50	55.7±14.0	28.32±14.16
Moraberekon (parent plants)	1±0.00	91.2±7.25	20.54±5.64

Preliminary comparison of regenerated and parent plants

Phenotypic comparison between callus regenerated plants and seed raised parent plants show that the regenerated plants of Bg 94-1 were very similar to parent plants. Moraberekon showed a marked decrease in height and an increase in 1000 seed weight when compared to mother plants (Table 1). These differences could be attributed to genetic changes leading to somaclonal variation, which were observed to be higher in Moraberekon compared to Bg 94-1.

Previous studies (Abeyratne *et al.,* 2004) have also shown the presence of variation in regenerated plants when compared to parent plants by means of isozyme analysis of leaf extracts.



(A) **(B)**

Plate 2. Sections from variety Moraberekon showing direct shoot organogenesis from embryogenic callus tissue.

Note: (A) - LS section through a shoot bud showing initial stage of regeneration at 400x magnification (B)- LS section through a regenerating shoot at 200x magnification.

Histology

Samples of callus tissue from the variety Moraberekon were used for microtome sectioning. The sections of regenerating callus, obtained through the points of shoot emergence, did not to show embryoids, but direct shoot and root regeneration (Plates 2 A and B). Histology studies have been carried out previously on regenerating rice calli obtained from root culture by Abe and Futsuhara (1985), showing embryoids. Sections through the embryogenic callus in this study show the nodular appearance of the callus.

CONCLUSIONS

This study shows that a variety-based variation exists in callus induction, shoot bud induction and regeneration frequencies. Reduced variation was shown among varieties

regarding callus and shoot bud induction frequencies, while a higher variation was shown among regeneration frequencies.
The regenerated plants of Bg 94-1 were essentially very similar to the parent plants, but Moraberekon regenerated plants exhibited variation on the above characters. Further studies should be carried out between plants raised by seeds harvested from regenerated plants and those raised by parent seeds to obtain a thorough comparison.

The histology studies carried out on embryogenic calli of Moraberekon showing green shoot buds confirmed the method of shoot induction as organogenesis.

The results of this study can be extended to other varieties to induce callusing and regeneration from the mature embryo. The protocol can be applied to future practical applications of rice mature embryo culture for breeding, transformation and other biotechnological applications.

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