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# Effect of growth regulators and Physiological Gradients on the High frequency plant regeneration from the long-term callus cultures of different germplasms of Rice (*Oryza sativa* L.)

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**Abstract**

Callus cultures of rice were initiated from mature embryos of different cultivars on LS medium containing 2 mg/L 2,4-D. Increasing concentrations of 2,4-D and 2,4-5T also increased the frequency of callus initiation in all the cultivars tested. Of different cultivars, Tellahamsa was found to be superior for callus initiation. Genotypic differences for plant regeneration were also observed. Cultivar Tellahamsa showed the highest (65-75%) frequency of plant differentiation followed by DGWG, Yerragaluvadlu, Surekha, Basmati-370, Bala, Chakko amubi, Jaya and IR-8. Callus cultures of rice cultivar Bala grown on a shoot-forming medium (LS + 1 mg/L IAA + 4 mg/L KN + 2% sucrose) were exposed to gibberellic acid and abscisic acid for varying lengths of time and at different periods during culture. Gibberellic acid totally suppressed the organogenesis in callus cultures of rice. The results suggest that if the tissue accumulated sufficient gibberellic acid prior to the initiation of meristemoids and shoot primordia, repression of shoot formation occurred. This repression was not reversed by increasing the levels of IAA and KN in the medium, but abscisic acid could partially overcome the gibberellic acid repression of shoot formation in rice callus. It has been observed in rice that shoots usually emerge from the basal portions of callus. This observation suggested that perhaps physiological gradients of materials were operative during the organ initiation process. To test this hypothesis, starch content and the enzyme activity of malate dehydrogenase in upper and lower portions of shoot-forming and non-shoot-forming callus were determined. Starch began to accumulate in both upper and lower portions of the shoot forming tissues within 4 days of culture. The rate of accumulation however, was faster and more in the lower portion of the callus leading to a peak of accumulation on day 8 in culture, i.e., prior to shoot formation. Non-shoot-forming callus cultures accumulated little starch during the same period of culture. Malate dehydrogenase (MDH) activity was examined in order to know the overall rate of respiration. In the upper segment of shoot-forming callus, the activity of MDH was very high by day 4 but declined continuously thereafter. The rate of activity of the enzyme was significantly higher beyond four days in culture in case of the lower portion of the shoot forming callus. Enzyme activity was lower in the non-shoot-forming portions (both upper and lower) of the callus. The higher rate of enzyme activity for the upper portion of the tissue could be attributed to increased oxygen availability. Thus, the evidence for the idea that concentrations of gradients or physiological gradients of substances into the callus tissue may be the operative factors promoting organ initiation *in vitro* is presented.

**Keywords:** Callus cultures, *Oryza sativa,* matured embryo, organogenesis, plant regeneration.

## INTRODUCTION

Rice (*Oryza sativa* L.) is the main staple food for more than half of the world population and has also become a model monocot system for genetic and functional genomics studies. In recent years, considerable efforts are being directed toward the improvement of important agronomic traits of rice through biotechnological techniques. Remarkable attention also is being paid to the functions of rice genes controlling various traits accompanying the large release of rice genomic sequences. Asian cultivated rice (*Oryza sativa* L.) consists of two major groups, which are known by the subspecies names *indica* (*Oryza sativa* ssp. *indica*) and *japonica* (*Oryza sativa* ssp. *japonica*). The *indica* subspecies is the most widely cultivated form of rice produced worldwide.

It has been known that the potential for callus induction and regeneration in rice tissue culture depends on a number of factors, such as the genotype of the donor plant, the type and physiological status of the explant, the composition and concentration of the basal salt, and the organic components and plant growth regulators in the culture medium. Various tissue culture techniques are being applied for varietal development of cereal crops including rice in different countries [1]. The success of *in vitro* culture depends mainly on the growth conditions of the source material [2, 3], medium composition and culture conditions [4]. For the first time, the successful callus culture of rice was cultured from the nodes of young seedlings on Heller's medium with vitamins and 2 mg/L 2, 4-D [5]. The nutritional requirements for growth and differentiation in rice callus tissues were determined [6- 11]. Plant regeneration in rice has been achieved in callus cultures derived from embryos and ovaries [12], endosperm [13] internodes [14], roots [15, 16], immature embryos ([7, 18] and anthers [19, 20], matured dehusked rice seeds [21]. After examining

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66 *indica* and *japonica* cultivars, [22] it was found that the culturability of these varieties was distinctly different. Compared with *japonica* rice, *indica* rice was less responsive to callus induction as well as regeneration [16, 22- 24]. The strong influence of genotypes on callus induction and plant regeneration was studied extensively [25-31]. Rice cultivars respond differently to culture techniques and the genotype is a critical factor in tissue culture. As yet, genotype differences both in callus growth and plant regeneration potential, their relationship, and histological processes leading to callus induction and organ differentiation, have not been fully understood. Accordingly, the present work on rice was undertaken to find the genotypic and explants differences for callus induction and proliferation and subsequent plant regeneration with a high frequency. In the present study, we investigated the impact of the growth regulators, physiological gradients, energy and osmotic agents on the high frequency plant regeneration from matured embryo derived long term callus cultures of rice genotypes.

## MATERIALS AND METHODS

### Rice Materials, Surface Sterilization, Callus induction, Subculture and Differentiation

Seeds of different varieties of rice (*Oryza sativa* L. Sub-species *indica*) such as Tellahamsa, Surekha, Dee-Gee-Woo-Gen (DGWG), Yerra galuvodlu, Bala, Jaya, IR-8, Basmati-370 and Chakko amubi were obtained from Central Rice Research Institute, Cuttack and Directorate of Rice Research, Hyderabad. The dehusked seeds were sterilized sequentially with 70% ethanol for 2 min immediately followed by immersion in a solution of 0.1% $HgCl_2$ for 15 min and then rinsed five times with sterilized water. The seeds were finally placed on sterilized filter paper to blot excessive water for 5 min before inoculation on to the callus induction medium. Matured embryos of the above cultivars were isolated aseptically and 4-5 embryos were inoculated into each test-tube containing 15 ml of LS agar medium [32] fortified with different concentrations (0.5, 1.0 and 2.0 mg/L) of 2, 4-5T separately. Culture tubes were incubated in diffused light (10 µ $Em^{-2}$ $S^{-1}$) at 26 ± 2ºC. Induction of callus from the embryos of all the cultivars was observed within 4 to 5 days. Callus cultures were routinely sub cultured every 25-30 days onto LS medium supplemented with 2 mg/L 2, 4-D and 2% Sucrose. Callus tissues of rice grown on sucrose plus sorbitol were deep yellow in colour, compact and smooth. LS medium containing 1 mg/L IAA, 4 mg/L KN and 2% Sucrose was used for organogenesis. Percent frequency of plant regeneration was scored at the end of 30 days.

### Estimation of Starch

Starch content of callus tissues was measured by *Hassid* method [33], and expressed in terms of mg/gram fresh weight of tissue.

### Estimation of Malate dehydrogenase (MDH) activity

Callus cultures from different flasks were pooled together weighed (500 mg) and homogenised in a chilled mortar using 0.05 M phosphate buffer pH 7.0, 500 mg of neutral glass powder and 250 mg of polyvinylpolypyrrolidone. The tissue homogenate was centrifuged for 20 minutes at 10,000 RPM in a refrigerated centrifuge. All steps in the preparation of the enzyme extract were carried out at 0 to -4ºC. For each sample of the enzyme, two replicates were prepared and data represent average values of 4 determinations taken from two independent experiments. Enzyme malate dehydrogenase (MDH, EC 1.1.1.37) was assayed [34]. This enzyme catalyzes the reduction of oxaloacetate to L-malate in the presence of NADH.

$$\text{Oxaloacetate} + \text{NADH} + H^+ \rightleftharpoons \text{L- malate} + NAD^+$$

The assay system in a final volume of 2 ml contained 0.2 µ moles of nicotinamide adenine dinucleotide reduced (NADH), 0.5 µ moles of oxaloacetic acid (neutralized), 150 µ moles of phosphate buffer pH 7.4 and a suitable aliquot of enzyme preparation. The mixture was inverted rapidly in a quartz cuvette and the decrease in O.D as a result of oxidation of NADH was followed spectrophotometrically at 340 nm for a minute. Specific activity of the enzyme is defined as 1 nanomole of NADH oxidized per minute per mg of protein. All experiments were repeated at least once. The level of significance was found out by performing standard t-test.

## RESULTS

### Optimization of media for callus induction and proliferation

The percent frequency of callus initiation from mature embryos of different rice cultivars is represented in Table 1. Increasing concentrations of 2, 4-D and 2, 4-5T also increased the frequency of callus initiation. Both the auxins at 2 mg/L were found to be good compared to lower concentrations (0.5 and 1 mg/L). Of all the cultivars, Tellahamsa showed highest percent (100) frequency of response followed by Bala (95), Basmati-370 (92),Surekha (91),Jaya (90),Yerragaluvadlu (90), DCWG (89), IR-8 (87) and Chakko amubi (81) when 2 mg/L 2, 4-D was used. Compared to 2, 4-D, 2, 4-5T was less effective as evidenced by the callusing ability (Table 1). Induction of callus from the embryos of all the cultivars was observed within 4 to 5 days. Callus culture of Bala growing on sucrose is shown in Figure 1A.

Table 1. Effect of growth regulators on callus induction from mature embryos of rice cultivars*

| LS+Growth regulator | Concentration (mg/l) | % Frequency of callus initiation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Tellahamsa | DGWG | Yerragalu -adlu | Surekha | Basmathi -370 | Bala | Jaya | IR-8 | Chakko amubi |
| 2,4-D | 0.5 | 22 | 19 | 18 | 25 | 21 | 22 | 19 | 20 | 21 |
| 2,4-D | 1.0 | 80 | 81 | 80 | 79 | 81 | 85 | 78 | 79 | 70 |
| 2,4-D | 2.0 | 100 | 89 | 90 | 91 | 92 | 95 | 90 | 87 | 81 |
| 2,4-5T | 0.5 | 10 | 9 | 8 | 10 | 9 | 12 | 13 | 14 | 16 |
| 2,4-5T | 1.0 | 30 | 26 | 25 | 29 | 25 | 34 | 28 | 29 | 24 |
| 2,4-5T | 2.0 | 48 | 39 | 51 | 44 | 47 | 52 | 43 | 39 | 37 |

* Data represents an average of 25-30 replicates per treatment scored at the end of 25 days.

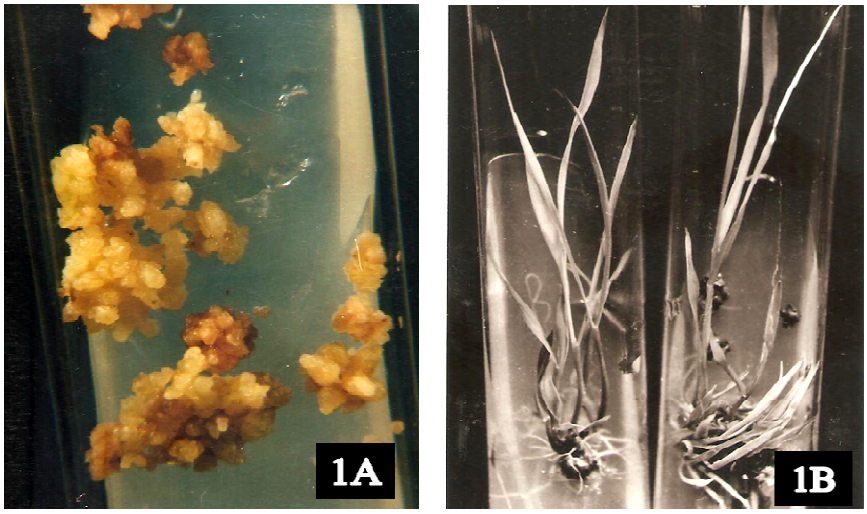


Fig 1. Callus cultures of Bala grown on LS + 2 mg/L 2, 4-D + 2% Sucrose (Fig. 1A), and Plantlet regeneration from callus cultures of Bala is shown in Figure 1B (LS + 1 mg/L IAA + 4 mg/L KN + 2% Sucrose).

Genotypic differences for plant regeneration from mature embryo derived callus cultures of rice was carried out using 50 day old callus and represented in the Figure 2. Of different genotypes tested, Tellahamsa exhibited the highest frequency (65-75%) of plantlet formation followed by DCWG (45-50%), Yerragaluvadlu (40-45%), Surekha (40-45%), Basmati-370 (35-40%), Bala (20-25%), Chakko amubi (20-25%), Jaya (20-22%) and IR-8 (20-22%). Thus, the percent frequency of differentiation was least in callus cultures derived from the cultivars Jaya and IR-8. Omission of 2, 4-D from the culture medium failed to give any shoots but rhizogenesis was observed in about 10-15 days in all the cultures. However, addition of 1 mg/L IAA and 4 mg/L KN to the culture medium resulted in the development of roots in 85-96% of cultures though formation of shoots with roots was less frequent. Roots appeared in 8-11 days and their growth in all the cultivars was prolific. Shoots appeared any day between 16 to 25 from several sites on the callus and 2% sucrose was found optimum for shoot organogenesis (Fig. 1B). In 3-6% of cultures shoot organogenesis occurred in callus tissues that did not produce roots. Albino plantlets were also observed in 10-20% of the differentiated cultures. The percent frequency of plantlets represented in the figure 2 includes albino plants.

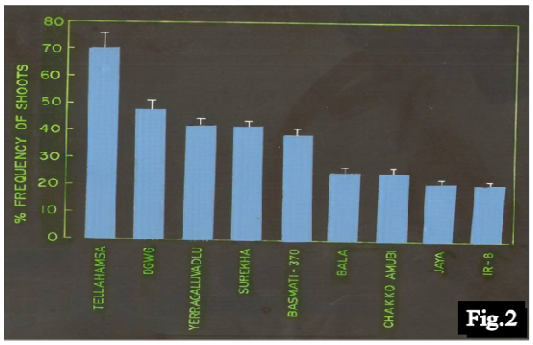


Fig 2. Genotypic differences for plant regeneration in rice (LS + 1 mg/L IAA + 4 mg/L KN + 2% Sucrose).

**Influence of gibberellic acid and abscisic acid on differentiation of shoots in callus cultures of rice**

Fifty day old callus cultures of rice cultivar Bala were transferred onto LS medium containing 2 mg/L 2, 4-D, 2% sucrose and 3% mannitol or 3% sorbitol. Cultures on this medium were sub cultured for every 25-30 days. After two subcultures, the callus growing on 3% sorbitol was used for this experiment. The effect of gibberellic acid and abscisic acid on shoot formation is represented in Table 2. When no hormones were added into the medium no shoot formation was observed as noted in the previous experiment. However, some green spots and root differentiation was noticed with 90% frequency. In presence of 1 mg/L IAA, 4mg/L KN and 2% sucrose, shoot regeneration was observed with 47% frequency. Also, 8 to 9 shoots per callus mass were produced on this medium. Incorporation of 0.9% mannitol into the above regenerating medium not only enhanced the frequency of shoot regeneration to 85%, but also increased the number of shoots formed per callus mass significantly (11 to 14). Enhancement in the IAA (2 mg/L) and KN (6 mg/L) concentrations only decreased the percent frequency (36%) as well as the number of shoots formed per callus considerably. However, addition of 0.9% mannitol to 2% sucrose increased the frequency of response from 36 to 53%. But, gibberellic acid when used alone at 10mg/L level, shoot differentiation was totally

suppressed (Table 2). Likewise, 4 mg/L ABA also inhibited organogenesis. However, when gibberellic acid (10 mg/L) or abscisic acid (4mg/L) were added along with IAA (1mg/L) and KN (4 mg/L), very low i.e., 2 to 7% frequency of response was observed. Shoots formed per callus piece were only 1 to 2 on these media. Incubation of callus tissues in a medium containing a combination of 1 or 2 mg/L IAA, 4 or 6 mg/L KN, 10 mg/L $GA_3$ and 4 mg/L ABA led to the production of shoots with slightly higher percent frequency (9 to 17%) of response (Table 2).

Table 2. Effect of gibberellic acid and abscisic acid on regeneration of shoots in callus cultures of rice cultivar Bala*

| LS+ Hormonal Conc. (mg/l) | Sucrose (%) | Mannitol (%) | % Frequency of shoot regeneration | No. of shoots formed per callus mass |
|---|---|---|---|---|
| No Hormones | 2 | - | Green spots (No. of shoots) | Nil (only roots) |
| 1 IAA + 4 KN | 2 | - | 47 (± 5)** | 8-9 |
| 1 IAA + 4 KN | 2 | 0.9 | 85 (± 6) | 11-14 |
| 2 IAA + 6 KN | 2 | - | 36 (± 3) | 4-5 |
| 2 IAA + 6 KN | 2 | 0.9 | 53 (± 4) | 7-8 |
| 10 $GA_3$ | 2 | 0.9 | Nil | Nil |
| 4 ABA | 2 | 0.9 | Nil | Nil |
| 1 IAA + 4 KN + 10 $GA_3$ | 2 | 0.9 | 6 (± 1) | 1-2 |
| 2 IAA + 6 KN + 10 $GA_3$ | 2 | 0.9 | 2 (± 1) | 1-2 |
| 1 IAA + 4 KN + 4 ABA | 2 | 0.9 | 7 (± 2) | 1-2 |
| 2 IAA + 6 KN + 4ABA | 2 | 0.9 | 2 (± 1) | 1-2 |
| 1 IAA + 4 KN + 10 $GA_3$ + 4 ABA | 2 | 0.9 | 17 (± 2) | 2-3 |
| 2 IAA + 4 KN + 10 $GA_3$ + 4 ABA | 2 | 0.9 | 9 (± 1) | 1-2 |

* Data were scored at the end of 30 days from 25 replicates; *Figures in parenthesis represent standard error.

In the first series of experiments, callus cultures of Bala were grown on the shoot forming medium (LS + 1 mg/L IAA + 4 mg/L KN + 2% sucrose) with or without $GA_3$ (10 mg/L) for varying lengths of time. Growing the tissues on a $GA_3$ - containing medium for as little as one day and then transferring to a normal shoot-forming medium led to an appreciable reduction in the percent frequency (50%) of shoot regeneration. Complete repression of shoot regeneration took place after two days on $GA_3$ containing medium (Fig. 3). If the tissue was grown on a non-$GA_3$ medium at first, and then transferred permanently to a $GA_3$-containing medium any time during the first10 days of culture, essentially no shoot formation was observed (Fig. 3). If the transfer of the tissues was made after day 12 in culture, the percent frequency of shoot regeneration increased but still was markedly less (19-30%) than the control. Incubation of callus in $GA_3$ for one hour at any time during culture effectively reduced the percent frequency of shoot regeneration (Fig. 4). On day 0, when the callus incubated in $GA_3$, there was no reduction in the regeneration frequency. But on day 1, there was a slight reduction. $GA_3$ treatment was most effective between days 6 and 9, where less than 10% frequency of regeneration was noticed. $GA_3$ treatment from day 15 onwards did not have a pronounced repressive effect on shoot formation (Fig. 4).

When the tissues were grown on a shoot-forming medium and incubated at day 8 (preceding shoot differentiation) in distilled water, $GA_3$ (10 mg/L), ABA (4 mg/L) and a mixture of $GA_3$ plus ABA at the concentrations mentioned above for one hour, the following results were obtained (Fig. 5). The controls, including incubation in sterile water, produced about 50% frequency of shoot differentiation. Number of shoots per callus mass in these two treatments ranged 7 to 8. But, incubating the tissues in 10 mg/L $GA_3$ (one hour) significantly reduced the percent frequency of response to 9 and the number of shoots per callus mass to 2. Incubating the tissues on the other hand in 4 mg/L ABA resulted in a shoot regeneration frequency of 38%. However, incubation of callus in a mixture of $GA_3$ and ABA led to the shoot regeneration frequency of 25%, i.e., better than the corresponding treatment in $GA_3$ alone (Fig. 5).

**Physiological gradients and shoot initiation in callus cultures of rice**

Callus cultures of Bala growing in LS medium supplemented with 2 mg/L 2, 4-D, 2% sucrose and 3% sorbitol was used for this experimentation. The emergence of shoots from the basal or lower portion of the callus can be seen clearly in all rice varieties. This observation suggested that perhaps physiological gradients were operative during the organ initiation process. To test this hypothesis we examined the starch content and the enzyme activity of malate dehydrogenase (MDH) in upper and lower portions of shoot-forming and non-shoot-forming callus, and inverted the callus tissues at different times during culture. For regeneration, LS medium containing 1 mg/L IAA, 4 mg/L KN and 2% sucrose were used.

The starch content of the upper and lower pieces of non-shoot-forming (NSF) and shoot-forming (SF) callus tissues as expressed in mg/g fresh weight is shown in the figure 7. Starch began to accumulate in both upper and lower portions of the shoot forming tissues within 4 days of culture. However, the rate of accumulation was faster and more in the lower portion of the callus leading to a peak of accumulation on day 8, i.e., preceding shoot differentiation. This peak occurred by day 12 in the upper portion of the SF callus. The NSF callus tissues accumulated little starch during the culture period. Also, no marked differences were observed in starch content of the upper and lower portions of the NSF callus. Thus, there were clear differences between the SF and NSF callus cultures of rice (Fig. 6).

Malate dehydrogenase (MDH) is the last enzyme in the Krebs cycle converting malate to oxaloacetate. The activities of this enzyme represent the overall rate of respiration. In order to find out the rate of respiration in the SF and NSF tissues, the activity of MDH was determined. The activities of enzymes MDH in the upper and lower segments of shoot-forming and non-shoot-forming tissues are shown in the Figure 7. In both SF and NSF tissues, there was an initial rise in the activity of the enzyme MDH, which is probably

partially a consequence of wounding. In the upper segment of SF callus, the activity peaked by day 4 but declined continuously thereafter. On the other hand, in the lower portions of SF callus, the rate of activity of the enzyme was significantly higher beyond 4 days in culture (Fig. 7). This higher respiratory rate as measured by the enzyme activity was maintained throughout the remainder of the culture period. There was very little difference in the enzyme activity between the upper and lower portions of the NSF callus tissue during culture. The higher rate for the upper portion of the tissue could be attributed to increased oxygen availability. It should be pointed out that most of the new callus proliferation occurs on the upper portion of this tissue.

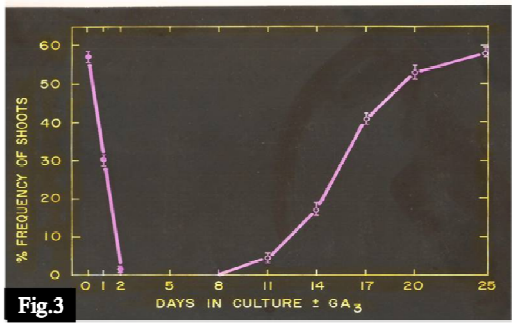


Fig 3. Percent frequency of shoots formed on a shoot forming medium ± $GA_3$. (●) Tissue transferred from + $GA_3$ (10 mg/L) medium to - $GA_3$ medium on day incubated by point on figure (o). Tissue transferred from - $GA_3$ medium to + $GA_3$ medium.

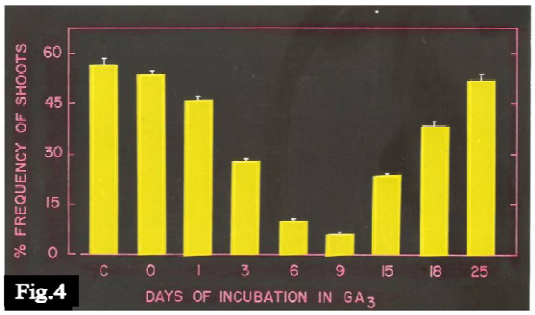


Fig 4. Percent frequency of shoots formed on a shoot forming medium (MS + 1 mg/L IAA + 4 mg/L KN). Incubation of tissue for one hour in a medium containing $GA_3$ on day shown and replanted. C-Control, no incubation.

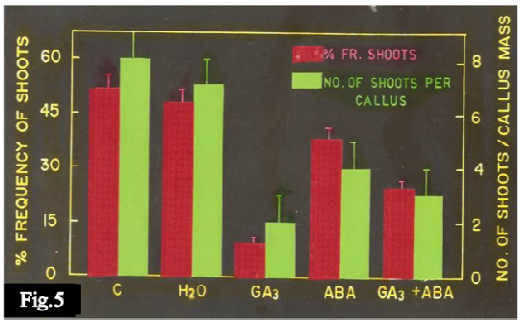


Fig 5. Percent frequency of shoots formed on a shoot forming medium. Tissues were incubated on day 8 in water, $GA_3$ (10 mg/L, ABA (4 mg/L) or a mixture of $GA_3$ (10 mg/L) + ABA (4 mg/L) at above concentrations for one hour and replanted. C – Control, no incubation.

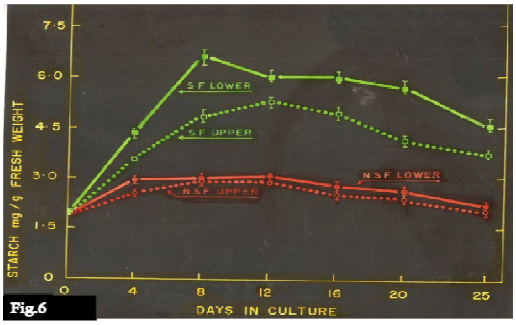


Fig 6. Starch content (mg per gram fresh weight of callus) in the shoot-forming (SF) lower (-■-), shoot-forming (SF) upper (--□--), non-shoot forming (NSF) lower (-●-) and non-shoot forming (NSF) upper (-○-) portions of rice callus (cultivar Bala).

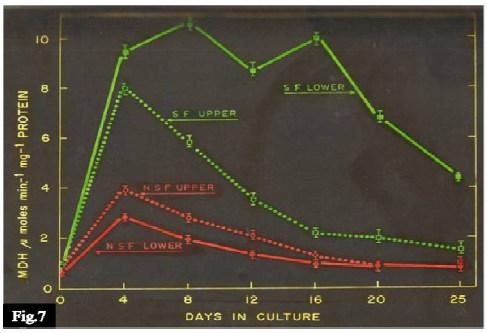


Fig 7. Activity of malate dehydrogenase (MDH) in the shoot-forming (SF) lower (-■-), shoot-forming (SF) upper (--□--), non-shoot forming (NSF) lower (-●-) and non-shoot forming (NSF) upper (-○-) portions of rice callus (cultivar Bala).

The results obtained in the inversion studies are very interesting. The appearance of shoots in the tissues inverted after 4 days in culture was similar to control, i.e., shoots were differentiated from the lower portions of the callus. When the tissue was inverted after 8 days in culture, the number of shoots formed were drastically reduced (3 to 4) compared to the control (8 to 9) and they emerged from the new as well as the old basal portions of the callus. Most of the shoots, however, emerged from the new basal portion. When the tissue was inverted after 12 days in culture, two patterns of development occurred. In some of the tissues, a few well developed shoots were observed. These emerged from the new upper i.e., the old lower portion of the callus. In this type of development essentially no shoots were formed on the new basal portion of the callus. In the second type of development a few shoots (1 to 2) were formed. These were not as developed as the other type and emerged from both upper and lower portions of the callus. They were also fewer in number (1 to 2) and less developed than those which appeared on tissue inverted after 8 days in culture. Inversion of tissues after 16 days in culture did not produce shoots on the new lower portion of the callus. All shoots (7 to 8) appeared from the new upper i.e., the old lower portion of the callus.

## DISCUSSION

In this study, we concentrated on initiation and establishment of callus cultures of rice, the examination of growth responses as influenced by growth regulators, regeneration of plants from callus cultures with a high frequency, physiological gradients and its role during organogenesis in rice.

### Callus initiation and organ differentiation in rice

Callus was induced from mature embryos of different cultivars of rice on LS medium containing different concentrations of 2, 4-D and 2, 4-5T. Callus initiation was found to be better in 2, 4-D containing medium than 2, 4-5T. Genotypic differences for callus induction were also observed. Cultivar Tellahamsa seemed to be superior when compared to other cultivars. Earlier, specific minimum 2, 4-D concentrations in the medium were found to be necessary for induction of callus from different explants of rice. While it was 0.5 mg/l for roots and embryos, it was 1 mg/l for immature inflorescences [35]. It was found earlier that 2, 4-D was necessary for callus induction in rice [36]. Most researchers working on rice have used only 2, 4-D for induction, proliferation and maintenance of callus either for tissue culture or for transformation experiments [37-40]. A number of studies on rice tissue culture have been conducted with special reference to the effect of exogenous phytohormones (11, 41-43]. These studies involved a limited number of genotypes, though in some of them differences in callus formation or plant regeneration potential among rice genotypes were reported [8, 24, 44]. Seed cultures have been used to compare the capacity for callus growth and plant regeneration because they are comparatively easy to establish. Genotypic differences for plant regeneration from mature embryo derived callus cultures of rice were observed in the present investigation (Fig. 2). While Tellahamsa exhibited highest frequency (67 to 75%) of response, Jaya and IR-8 showed the lowest (20-22%) frequency of plantlet regeneration.

Omission of 2, 4-D from LS medium failed to give whole plants in the present study, but initiated organogenesis in *japonica* rice cultivars [45]. Rice callus exhibits higher ability to form roots than shoots and addition of kinetin to the medium stimulates and prolongs the potential to initiate shoots [46]. It was noticed that, 5% plantlet formation from leaf blade induced callus tissues of *indica* cultivars [47]. Large genotypic variations in callus growth, plant regeneration potential were observed in rice. Embryogenic and non-embryogenic callus formation as well as plant regeneration is genetically determined as described in maize [48- 50], wheat [51, 52], barley and rice [15, 53]. Testing substitution lines of the common wheat variety “Chinese spring”, [54, 55] showed that particular chromosomes or chromosome arms have a strong effect on culture ability of plant tissues. Though embryogenic callus was not isolated during the present investigation, a very high percent frequency of response was noticed. Besides the effect of nuclear genes, cytoplasmic factors seem to play a role in callus production and plant regeneration ability [56]. Besides genotype, the composition of the culture medium has a strong influence on embryogenic callus formation of *indica* rice. Addition of cytokinins to the callus induction medium increased the frequency of embryoid formation and from immature barley scutelli [57]. In the present study, however, no cytokinin was used in the callus induction medium. Our investigations clearly show that genotype plays an important role during organogenesis.

### Effect of gibberellic acid and abscisic acid on differentiation of shoots in rice

Callus cultures of rice cultivar Bala grown on MS medium containing 2 mg/L 2, 4-D, 2% sucrose, and 3% sorbitol was deep yellow in colour, compact and looked like embryogenic. While 1 mg/L IAA, 4 mg/L KN, and 2% sucrose produced shoots with 47% frequency, incorporation of 0.9% mannitol into the above regeneration medium enhanced the frequency of shoot regeneration to 85%. Increase in the concentrations of IAA (2 mg/L) and KN (6 mg/L) decreased the present frequency of response (36%) as well as

the number of shoots formed per callus mass. Both gibberellic acid (10 mg/L) and abscisic acid (4 mg/L) totally suppressed the organogentic response. With the inclusion of gibberellic acid or abscisic acid to IAA (1 mg/L) and KN (4 mg/L) media, 2 to 7% frequency of response was noticed. But with a combination of 10 mg/L $GA_3$ and 4 mg/L ABA in 1 or 2 mg/L IAA plus 2 or 6 mg/L KN, the percent frequency of response was slightly increased (9 to 17%). The results also suggest that if the tissue accumulated sufficient $GA_3$ prior to the initiation of shoot primordia, repression of shoot formation occurred. This repression was not reversed, by increasing the levels of auxin and cytokinin in the medium, but ABA could partially overcome the $GA_3$ repression of shoot formation.

Gibberellins as a class of growth regulators play a very important role in plant growth and development [58]. In morphogenesis, one of their most pronounced effects was the repression of organ formation in tobacco callus under conditions which should normally lead to shoot or root formation [59, 60]. $GA_3$ repressed shoot differentiation in rice is demonstrated in the present study. This seems to be a general response to GAs and is not reversed by so-called gibberellin antagonists [61]. Gibberellins can therefore be an extremely useful tool in studying all aspects of organ formation *in vitro.* The present study showed that growing the tissue on a $GA_3$-containing medium for as short a period as 48 hours (2 days) led to total organ repression (Fig. 3). It would appear; therefore, that enough of the growth regulator was taken up into the tissues in this time to repress shoot differentiation. Transferring the tissue from a non $GA_3$ -medium to medium containing $GA_3$ showed that the inhibitory effect of $GA_3$ on shoot formation was reduced after 15 days in culture. These findings were supported by the incubation studies (Fig. 4), where again beyond 9 days in culture, the inhibitory effect was reduced. Of most significance however, was the increased effectiveness of treatment between days 6 and 9. By days 8 and 12 in culture, zones of preferential cell division, from which shoot primordia and ultimately the shoots arise, were observed. Shoot primordia then become visible on the base of the callus by 15 to 18 days in culture. Thus incubation of the tissue in $GA_3$ was most effective at the time of meristemoids and shoot primordia formation. The effectiveness of $GA_3$ at the time of meristemoids formation could be due to an increased uptake or enhancement of utilization by the metabolically active cells. Once the shoot primordia were formed, $GA_3$ appeared to be ineffective in inhibiting development. In a parallel situation, it has been shown that $GA_3$ inhibits floral or vegetative bud formation in tobacco explants but promotes development of floral primordia, which were already formed [62]. A similar finding was obtained in studies on regeneration of organs on detached *Begonia* leaves. ABA at 4 mg/L totally suppressed the shoot formation in callus cultures of rice. In the present study, ABA partially overcame the $GA_3$ repression of shoot formation. This occurred when the tissue was grown on a shoot forming medium containing IAA + KN + $GA_3$ + ABA (Table 2) or $GA_3$ or ABA or a mixture of $GA_3$ + ABA (Figs. 4 and 5). This partial reversal of $GA_3$ repression was contrary to that observed with organ regeneration in detached *Begonia* leaves, where the $GA_3$ inhibition was not reversible by ABA [63]. Interactions between ABA and $GA_3$ have been observed in lettuce seed, where ABA could inhibit $GA_3$-induced germination [64, 65]. Interactions between ABA and $GA_3$ have also been reported in organ forming callus cultures of triticale [66]. $GA_3$ is a very useful experimental tool for studying organ formation *in vitro*. The finding that shoot repression took place if the tissue was incubated in $GA_3$ at the time of initiation of organized development, therefore, extends the usefulness of this growth regulator in studies in experimental morphogenesis, irrespective of whether the approach is anatomical, physiological or biochemical.

**Physiological gradients and shoot initiation in callus cultures of rice**

The work presented in this experiment was designed to test the hypothesis that physiological gradients of materials into the tissue, rather than physiological isolation of cells, was operative during the initiation of organs *in vitro.* In addition, through the inversion studies, a more direct evaluation of the consequences of a diffusion gradient phenomenon in action was made. In the shoot initiation process in callus cultures of rice under the experimental conditions, zones of preferential cell division activity appeared in the tissues by 7 to 12 days in culture. In some of these zones, meristemoids and shoot primordia (12 to 14 days) and subsequently the whole shoots emerged by 16 to 25 days in culture. Associated with these organized events, the shoot forming tissues accumulated starch, which was apparently utilized in the organ initiation process (Data not shown). This is presumably a high energy requiring process, as judged by the respiratory activity of the callus tissues as was also reported in tobacco callus [20, 67, 68]. The stored starch was hypothesized to serve as a readily available reserve source of energy for the organ initiation process. Confirmation of the utilization of starch in organ development has come from the measurement of the activities of EMP pathway and Krebs cycle enzymes [69]. Determination of the starch content of upper and lower portions of the shoot-forming tissue showed that the entire tissue accumulated starch (Fig. 6). However, what was clear in this study was that the peak of starch accumulation occurred earlier in the lower half of the shoot-forming callus than the upper half. Furthermore, the decrease in accumulated starch began earlier, and was faster in the lower portion of the callus tissue forming shoots. Since the key histogenic events leading to shoot formation also took place in the lower portion of the tissue, this suggested that the starch stored in the upper part of the tissue was mobilized and utilized by the developing shoots. This significance of the respiration data is even clearer. Whereas upper and lower segments of non-shoot forming tissues have the same rate of MDH activity, the respiration rate as measured by MDH activity in the lower portion of shoot-forming tissue, in which the shoot-forming events were taking place, was much higher than in the upper portion (Fig.7). This data further strengthened the correlation between starch accumulation, respiration as measured by MDH activity and the organ initiation process. Furthermore, as a measure of the organ initiation process, they indirectly add weight to the diffusion gradient concept of organized development. More direct evidence for this concept was obtained through the inversion studies.

In non-inverted tissues the shoots mainly emerged from the basal portion of the tissue. Tissues inverted after 4 days in culture also behaved similarly, producing shoots in their new basal parts. This means that within 4 days in culture the position in which shoots would arise was not yet determined. It should be realized at this time that cell division had not yet began in the tissue. Obviously then, during this early culture period, materials (nutrients and hormones) were taken up from the medium, but presumably not enough and in the proper concentrations and ratios to bring about the areas or organized development. However, the picture began to change by 8 days in culture. Here, shoots emerged from both the old and new basal regions, although more arised from the new basal portions.

This suggested that almost enough of the substances required for organ initiation had accumulated by 8 days in culture in the original basal portion. Inversion set up new diffusion gradients leading to new zones of preferential cell division, etc., so that shoots also emerged from the new basal portion. This interpretation is further strengthened when the behavior of callus tissues inverted later in culture was examined.

With later inversion (12 days), two patterns of development were observed. In some cases the requirements for shoot formation had been achieved in the basal portion of the tissue, so that on inversion, the tissues went ahead with their normal development. Since organ size and number usually vary inversely, a few large, well developed shoots were produced. Apparently, this development prevented additional shoots from being formed in the new basal portion, possibly by mobilizing the reserve starch, which disappeared from the portion of the tissue not forming shoots. On the other hand, if the requirements for bud initiation were not present, the new diffusion gradients set up at the time of inversion led to the production of shoots in the new basal portion of the callus. A few shoots were still produced in the former basal portion of the callus, presumably because this part of the tissue ultimately achieved a favorable balance of substances. In other words, this tissue behaved similarly to that inverted at 8 days in culture. This suggests that there is a period of time in culture during which inversion can lead to shoot formation over the entire tissue, an idea which is entirely consistent with the diffusion gradient concept. For this tissue inverted at 12 days, shoots were being formed later in time. This probably accounted for their (shoots) smaller size and reduction in number, when compared to shoot-forming tissue inverted at day 8.

In conclusion, regeneration of plants from long-term cultures with a high frequency could be obtained in rice cultivars. Both gibberellic acid and abscisic acid play an important role during organogenesis in rice. Also, support for the idea that physiological gradients of substances into the tissue may be the operative factors promoting organ initiation *in vitro* has been obtained in rice. In conclusion, it seems that a complicated genetic mechanism, which may be controlled by separate groups of genes, is involved in the response to *in vitro* tissue culture. The knowledge gained from the present study has strengthened our understanding of *in vitro* callus regeneration potential of rice cultivars. This information can be used in rice transgenic experiments and other biotechnological interventions.

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