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Differential Reponses to Indirect Organogenesis in Rice Cultivars

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Abstract- An investigation was made on the initiation, maintenance and regeneration of callus from mature seeds as explants of eight rice cultivars, viz., Khitish, IR 64, IR 36, IR 72, PNR 546, Swarna, Taraori Basmati and Gobindabhog. Mature seeds were used as explants. MS medium supplemented with 2mgL-1 of 2, 4-D was found suitable for inducing high amount of embryogeniccalli in the studied genotypes. In this hormonal concentration, maximum 95% callus was induced in IR 64, followed by 91.6% in PNR 546. Maximum root - shoot induction (67%) was also observed in the same media component in IR 64. IR 64 was found most suitable for *in vitro* culture among these genotypes used. Plants regenerated *in vitro* were successfully established in soil. However among the genotypes studied IR 64 was rated as good and PNR 546 moderate in respect to regeneration capability. Media 5 (2, 4 D and BAP 2mg/l) and 6 (NAA and BAP2.50 and .050 mg/l) were selected for callusing. However media 5 showed better regeneration potential.

Index Terms- Callus induction, ideal media, genotypic variation, regeneration ability

Rice is a monocotyledonous cereal crop plant responsible for feed supplement for half the people in the world and as such varietal improvements of rice for resistance against insects, pathogens and other stresses including nutritional quality are necessary. Genetic transformation is a powerful tool for improving crops. The ability of the targeted plant cell to regenerate into plantlet and subsequently develop into a mature plant is a prerequisite for genetic transformation. *In vitro* culture is an important component of any genetic transformation protocol because it provides sources of materials that can be used as recipient of introduced foreign genes. The starting material, the explant, could originate from different plant parts. Explants from different species demonstrate varying callusing ability. In the case of rice, it is generally known that *japonica* type readily forms callus while *indica*type is more recalcitrant. The resulting calli may follow different pathways of development—they may remain as is, or regenerate shoots, roots, or entire plantlets. For genetic transformation studies, however, plantlets from calli are desired. This can happen only if the calli are embryogenic, i.e., the calli are capable of forming embryos.

I. INTRODUCTION

Induction of callus is one of the major step for genetic transformation and somaclonal variation. Successful callus culture of rice was first achieved by [1] cultured the nodes of young seedlings on Hellers medium with vitamins and 2, 4-D (2ppm) and later [2] successfully re-differentiated rice callus tissue from seeds into intact plantlet. Several laboratories have also described regeneration of plants from various rice explants [3-5]. The study of *in vitro* tissue culture of rice was intended to screen different rice varieties for their ability to produce embryogeniccalli and regenerate into whole plant [6,7]. Genotype differences in both callus induction and organ differentiation and subsequent callus growth and plant regeneration potential have not been fully understood. Although immature embryos and mature seeds are commonly used for callus induction, the use of mature seeds has the advantage as they can be obtained at anytime throughout the year regardless of growing season, [8]. The aim of this research was to study the callus and regeneration variation in eight rice cultivars in West Bengal.

Explants from different species demonstrate varying callusing ability. In the case of rice, it is generally known that *japonica* type readily forms callus while *indica*type is more recalcitrant. The resulting calli may follow different pathways of development—they may remain as is, or regenerate shoots, roots, or entire plantlets. For genetic transformation studies, however, plantlets from calli are desired. This can happen only if the calli are embryogenic, i.e., the calli are capable of forming embryos

The long term goal of this research is to generate salt tolerant rice by genetic transformation or somaclonal variation. Salt affected areas have grown to more than 100,000 ha in the last decade. To improve rice production, the use of salt tolerant varieties is sought as an option. As a first step, this paper aims to determine the tissue culturability of genotypes of rice— Khitish, IR 64, IR 36, IR 72, PNR 546, Swarna, Taraori Basmati and Gobindabhog. The quality of callus, the embryogenic response and regeneration potential are said to be influenced by genotype. Previous studies on *Saccharumsp*[9-10], *Sorghum bicolor* [11], and *Triticum*[12] demonstrated this. Similar findings were also shown in *Oryza sativa* [6,13]. Thus, results of this study will identify the genotype that could serve in subsequent study and also identify a media that will serve as a broad term.

The regeneration protocol developed from dehusked and sterilized seeds of rice variety with different ranges of 2,4-D (1-4 mg L-1) and combinations of 6-Benzyl Aminopurine (BAP) (2-4 mg L-1) and α-Naphthalene Acetic Acid (NAA) (1 mg L-1) can be used for somatic embryo formation and plantlets regeneration respectively. The embryogeniccalli obtained from 2 mg L-1 2,4-D concentration performed better than the other three used concentrations of 2,4-D. The regeneration response of mature ice embryos from eight varieties (Oryza sativa varindica) was separately measured in terms of regeneration percentage and total

number of regenerated plantlets obtained when cultured on standard MS media, the response was classed as high , medium or poor.

II. MATERIAL AND METHODS

The experimental materials (Khitish, IR 64, IR 36, IR 72, PNR 546, Swarna, Taraori Basmati and Gobindabhog) were collected and mature rice grain embryos were used as the sources of primary explant. Mature seeds were used as explant. Mature seeds of the studied cultivars were dehusked carefully so that the embryo remains intact. In explant selection diseases and unhealthy seeds were avoided.

The dehusked seeds were surface sterilized with 70% ethanol for 10seconds with tween 80 and after sterilization, the explant were washed 3-4 times with distilled water and left for 24 hrs. Then it was treated with 0.1% $Hgcl_2$ for 30 minutes, washed 3-4 times with sterile water and placed on sterilized filter paper to remove excess water before inoculating them on to callus inducing or germinating medium. The whole operation was carried under Laminar air flow chamber. However it was also found that seeds plated after 36 hrsimbibation period gave better results. For determining the most suitable media composition 15 seeds of each variety was plated in 6 replications and two repeats and the totals from those were taken as the actual counts for the percentage.

A. Incubation and Callus Induction

All the inoculated culture tubes and flasks were incubated in a growth chamber room providing a special culture environment. The tubes were placed on the shelves of a culture rack in the growth chamber. The culture flasks and tubes kept in the dark at 25 - 26^0C throughout the incubation period. The cultures were checked time to time for monitoring the response. The primary callus induced from explants was sub-cultured for further proliferation and developing embryogenic nature. The frequency of explants forming embryogenic callus, degree of callus growth, colour and texture were recorded after 5 - 6 weeks of culture. After 5-7 days of callus initiation, the primary tiny calli of explants were separated aseptically from the source of explants, so that no contact of parental tissue remained and set them again on the same medium for proliferation of calli without root-shoot differentiation.

The quantitative measurement of callus growth was estimated in terms of percentage of callus and degree of callus growth. The different types were distinguished based on their external features. The number of calli under each type was counted after 21 days and expressed as a percentage of the total.

B. Plant regeneration

Embryogeniccalli, after two subcultures, were transferred to MS regeneration medium [14]containing MS inorganic salts, Calli were exposed to 16 h of light and 8 h of dark period daily. Every month, calli were transferred in fresh medium until regenerates appeared. Regenerated plantlets with underdeveloped rooting were grown in MS medium [14] at half strength concentration. The resulting regenerates were eventually removed from agar, grown in water for one to three days under culture conditions and then in cups filled with sterile soil in a cool place for a week. The number of calli that regenerated plants at the end of a three-month period was counted and expressed as a percentage of the total number of calli that were induced to regenerate.

The number of plantlets regenerated at the end of a three month period was also counted. Those that regenerated by organogenesis were counted separately from those that regenerated by somatic embryogenesis.

C. Plant Regeneration

After desired passage time the developing embryogeniccalli were selected and tested for their regeneration ability using different kinds and concentrations of cytokinin and auxins. The cultures were incubated at 26^0-28^0C under white light for 16/8 hours light/dark condition. After 4-5 weeks differentiation of shoots and roots were observed. The number of calli producing plantlets and the total number of plants were counted for each treatment. Shoots having insufficient root system was not suitable to transfer into soil. So when the shoots attained a size of 3-5 cm they were aseptically separated from each other. After that they were transferred to half strength of MS salts and vitamins, supplemented with 2% sucrose without growth regulators for root proliferation.

D. Transplantation

The regenerated plants with well developed sufficient root system were ready for transferred to soil. When the plantlets remained in culture they were brought out of the controlled environment of growth room and were kept in the room temperature for 2-3 days to bring then in the contact of normal temperature. The plantlets were then rescued very carefully from the culture tubes. Agar attached to the root was washed gently under running tap water. Immediately after that they were transplanted to small pots containing sterilized ground soil, sands in the ratio of 1:2:1.Half MS strength liquid was added to this. The pots with plantlets were kept in shade place and necessary cultured management was undertaken for good growth and development of the plant. After 7 days, the pots with plants were transferred to direct sunlight and after 15-25 days plantlets were finally transferred in new pots.

III. RESULTS AND DISCUSSIONS

Explants from different species demonstrate varying callusing ability. In the case of rice, it is generally known that *japonica* type readily forms callus while *indica*type is more recalcitrant. The resulting calli may follow different pathways of development—they may remain as is, or regenerate shoots, roots, or entire plantlets. For genetic transformation studies, however, plantlets from calli are desired. This can happen only if the calli are embryogenic, i.e., the calli are capable of forming embryos.

Table 1: Early Response of different hormones and varieties in callus production(%)

	Media 1	Media 2	Media3	Media 4	Media 5	Media 6	total
Khitish	42	Plant regenerate	Plant regenerate	40	55	65	33.7

IR64	77.5	85	89.5	90	95	89.5	87.75
IR 36	42	Plant regenerate	85	85	55	72.7	56.62
IR72	85	92.5	95	78.5	80	80.7	85.28
PNR 546	89.9	85	95	84.5	91.6	76.8	87.13
Swarna	45	35	45	40	40	40	40.83
Taraori Basmati	65	75	75	80	85	85	77.5
Gobindabhog	94	plant regenerate	Plant regenerate	74.7	86	65	53.28
total	67.55	46.56	60.56	71.58	73.45	71.8	65.25

Media 1 – 2,4 D :BAP = 5:1 , Media 2 2,4 D :BAP = 4:1
Media 3 2,4 D :BAP = 3:1 , Media 4 2,4 D :BAP = 2:1
Media 5 2,4 D :BAP = 1:1, Media 6 NAA :BAP= 5:1

Nature of calli, their growth rate, texture and colour varied on number of factors e.g. variety or cultivar, and media composition. The mature seeds derived calli were creamy white to creamy in MS medium. The nature of mature seeds derived calli were compact but fragile and tend to be very dry with increasing concentration of 2, 4-D. MS medium supplemented with 2, 4-D and BAP in 1:1 ratio was found most effective in callus induction in all cultivars (Table 1). It produced 55%, 95%, 55%, 80%, 91.6%, 40%, 85% and 76% callus in Khitish, IR 64, IR 36, IR 72, PNR 546, Swarna, Taraori Basmati and Gobindabhog respectively. Similar result was also reported by several workers in *Oryza sativa*,[5].

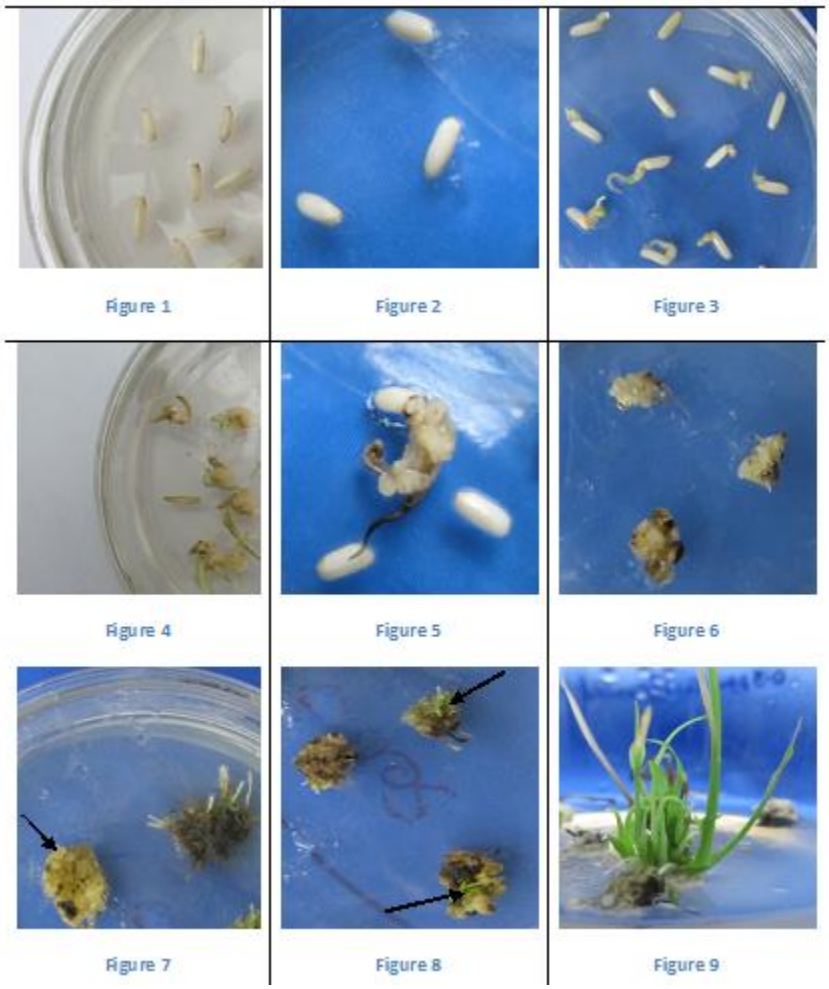

Figure 1 Figure 2 Figure 3

Figure 4 Figure 5 Figure 6

Figure 7 Figure 8 Figure 9

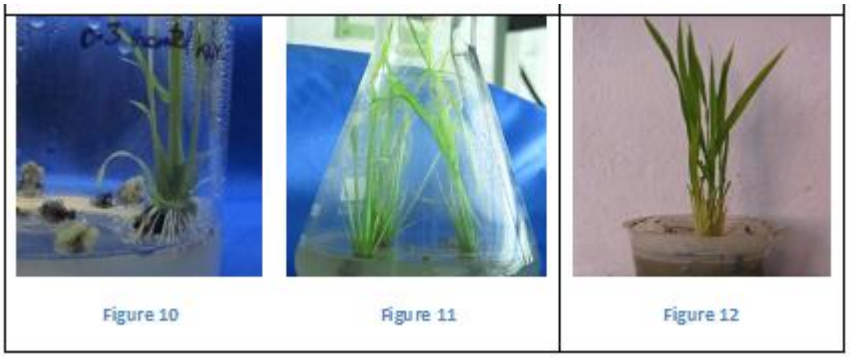

Figure1.*In vitro* responses of mature seeds of rice plated for 25 (fig. **5**) and 45 (fig. **9**) days.
1-2 Seeds plated **3-4** Germination of seed with shoot; **5** Initiation of callus **6** transfer to callus maintain media **7** Glossy, creamy white, nodular callus pointed by arrow and black necrotic callus(undesirable) **8** Appearance of green shoots pointed by arrow **9** Complete shoot initiation in regeneration media **10** Roots development **11** plantlet ready for hardening **12** transferred to pot

Callusing ability of the rice genotypes

As shown in Table 2, the seeds exhibited responses to tissue culture such as germination and callus formation; others demonstrated no response.

Table 2 Studies on average size, nature of the callus obtained in the different varieties in Media 5

variety	No of seeds plated	Germin-ated	Type of callus							No respons e
			total	nodular	Watery compa ct	Very hard	Necrotic		With differentia ted roots	
							Within 30 days of plating	After 30 days follo-wing sub-culturing		
Khitish	400	137 (34.3)	219 (54.8)	114 (28.5)	0	0	59 (14.8)	0	46 (**11.5**)	44 (10.9)
IR64	385	10 (2.5)	366 (95.2)	0	25 (6.4)	0	80 (20.7)	**	261 (**67.7**)	9 (2.3)
IR 36	396	52 (12.8)	218 (55.2)	0	62 (15.7)	24 (6)	48 (12.2)	45 (11.3)	39 (**10.0**)	126 (32.0)
IR72	415	47 (11.3)	334 (80.4)	0	164 (39.8)	0	41 (10)	80 (19.2)	49 (**12**)	34 (8.3)
PNR 546	385	21 (5.4)	353 (91.6)	59 (15.4)	0	0	133 (34.6)	**	161 (**41.6**)	11 (3.0)
Swarna	395	21 (5.7)	159 (40.3)	0	157 (40)	0	0	2 (0.3)	0	220 (56.0)
Taraori Basmati	400	31 (7.9)	341 85.2)	0	121 (30.2)	10 (2.5)	180 (45)	0	30 (**7.5**)	28 (6.9)
Gobind bhog	415	4 (1.0)	356 (86)	0	0	28 (6.7)	177 (42.6)	120 (28.9)	31 (**7.4**)	54 (13)

Note: Given numbers are actual counts from the total followed by percentages in parentheses
** notsubcultured

However the results indicate the reasons behind the popularity of IR 64 as potent material in all tissue culture or transgenic studies. It was also found that highest callus induction was observed in IR 64 varieties as earlier reported [15]. Lowest range of callus regeneration induction (41.67% - 50%) was observed in higher (6.0 mgL-1) concentration of 2, 4-D in this study. By increasing the concentration of 2,4D the response was quick but the quality of callus and the embryogenic efficiency was lowered. The results also revealed that the concentration of 2,4-D, types of genotype, had great variability for early induction and high production of callus. Effect of genotype and explant age was also reported to be important factor in callus induction in indicarice[6,13]. From proliferative callus, embryogeniccalli could be stimulated with the right type and concentration of growth regulators. This was shown in Bermuda grass [16] and in banana [17]. In some cases, however, callus medium serves both for callus induction and stimulation of embryogenic response.

This was demonstrated in the present study on rice and was previously found in sorghum [13], sugarcane [9,10] and peach palm [18].The ability to form embryogeniccalli is genotype-dependent.

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