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VG Shobhana
Ph. D., Plant Breeding and Genetics, School of Agricultural Sciences, Kalasalingam Academy of Research and Education, Krishnankoil - 626126, Virudhunagar District, Tamil Nadu, India

R Nirmal Kumar
Indian Institute of Food processing Technology, Thanjavur, Tamil Nadu, India

A Karthikeyan
Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

K Ashokkumar
Division of Crop Improvement, Cardamom Research Station, Kerala Agricultural University, Pampadumpara, Idikki, Kerala, India

A Sheeba
Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

P Vivekanandan
Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Correspondence
VG Shobhana
Ph. D., Plant Breeding and Genetics, School of Agricultural Sciences, Kalasalingam Academy of Research and Education, Krishnankoil, Virudhunagar District, Tamil Nadu, India

Evaluation of new plant type (NPT) lines of rice (*Oryza sativa* L.) for callus induction and *in vitro* plant regeneration

VG Shobhana, R Nirmal Kumar, A Karthikeyan, K Ashokkumar, A Sheeba and P Vivekanandan

Abstract
An investigation was made to evaluate the callus induction and regeneration capabilities of nine new plant type (tropical *japonica*) lines compared to a high yielding *indica* rice variety, MDU 5, as control. The results of the experiment inferred that the combination of growth regulators containing MS + CH (1gl^{-1}) + Kinetin (0.5 ml^{-1}) + 2,4-D (2 mgl^{-1}) was outstanding in promoting early callus induction, high callus induction percentage and fresh weight of callus. In the case of regeneration, the combination of hormones, namely, MS + IAA (3 mgl^{-1}) + BA (3 mgl^{-1}) + kinetin (1 mgl^{-1}) was found to be the best in regenerating maximum number of plantlets. The new plant type line of rice, IR 75282-10-3-3-2, was identified for its better ability in callus induction and calluses with increased fresh weight and also the capacity to regenerate more number of plantlets. The findings of this experiment will be of much use in any future study, aiming to exploit the tissue culturing potentials of new plant type lines of rice.

Keywords: Rice, new plant type lines, *indica* lines, callus induction and regeneration

1. Introduction
Rice is a functional commodity and primary food source for more than half of the world population. A focus on 'the complex rice-based ecosystem' influences issues of global concern, such as food security, poverty alleviation, preservation of cultural heritage and sustainable development (IRRI, 2004) [24]. To augment the yield potential of rice following the hybrid rice revolution, a new plant type was conceptualized in the International Rice Research Institute. The ideotype method of breeding put forth the idea of redesigning the entire architecture of the plant, by reducing the number of tillers, by increasing the number of grains per panicle and by increasing the stiffness of the stem. Several breeding lines with these modified plant characteristics have been developed and most of them have out yielded the majority of high-yielders by 15-20%. They are popularly known as the New Plant Type (NPT) or the Super Rice in 1989. The NPT lines had 3-4 productive tillers, with 200-250 grains per panicle; a plant height of 90-100 cm; thick and sturdy stems; thick dark green and erect leaves; vigorous root system and 100-130 days duration with increased harvest index (Khush and Virk 2000) [28]. The establishment of a successful regeneration protocol in NPT lines will facilitate to explore and expand the degree of possibilities in somaclonal variation, gene cloning and other genetic transformation studies. Rice tissue culture keeps evolving day by day, but even with standard protocols, it is still a motivating area of research since it is genotype-dependent (Pazuki and Sohani 2013 and Yaqoob *et al.*, 2016) [44, 51]. The present study was principally undertaken up to standardize the callus induction and regeneration procedures in new plant type rice lines in comparison along with a local control, MDU 5, a very popular cosmopolitan variety in southern Tamil Nadu.

Success in the tissue culture studies of rice has been reported from a number of laboratories around the world. Callus induction from rice was first achieved by Fukuhashi and Yatazawa (1964) [17]. They cultured the nodes of young seedlings of rice on Heller's medium with vitamins and 2, 4- D (2 ppm). Maeda (1965) [32] succeeded in inducing callus from rice seedlings on a medium containing 2, 4- D and yeast extract. In earlier times, only a few evidences of plant regeneration in Graminae described only shoot morphogenesis (Green and Philips, 1975 and Nishimura *et al.*, 2006) [20, 43].

But in recent decades, extensive evidences are available for plant regeneration, which was previously considered to be a rare phenomenon in all major species of cereals.

Seed cultures have been used to compare to capacity for callus induction and plant regeneration, because these are comparatively easy to establish (Abe and Fuksuhara, 1986) [2]. Among the several explants used *viz*., seed, leaf, node, internode, root, leaf sheath, anthers, embryo, immature embryo and immature inflorescence, the seeds gave the best response. Calli produced from the seed scutellum is the best explants in rice and exhibited high totipotency, whereas those derived from other explants were highly recalcitrant Cho *et al.*,., 2004; Khaleda and Al-Forkan 2006) [12, 27].

Yamada *et al.* (1976) [49] reported techniques for callus induction, cell culture, re-differentiation and regeneration of cereals. Cereal callus is of atleast two types (Heyser *et al.*, 1983; Nabors *et at.*, 1983) [22, 41] so called, embryogenic callus has a small white knobby appearance and is composed of small, isodiametric cells. Non-embryogenic callus is yellow to translucent, wet and rough to crystalline in appearance and is composed of larger, elongated cells. In relation to callus cultures numerous factors influencing callus induction have been published by Maeda (1965) [32], Yamada *et al.* (1967) [49]. The genotype used and the composition of the nutrient media are the major factors in rice tissue culture (Mikami and Kinoshita 1988; Gul *et al.*, 2000; Al-Forkan *et al.*, 2005) [39, 21, 3].

A combination of growth hormones *viz*., 2, 4-D and kinetin for improved callus induction frequency and NAA, kinetin and Benzyl aminopurine (BAP) for higher plant regeneration was reported by several workers. White and compact embryogenic calli can regenerate into a complete plant when transferred to a suitable regeneration medium. MS regeneration medium supplemented with different concentrations of auxin and cytokinin is superior in plant regeneration Manimekalai and Sree Rangaswamy, 1988 [37]; Mandal and Bandyopadhyay, 1997 [34]; Bishnoi *et al.*, 2000[7]; Azria and Bhalla, 2004) [6]. Chu *et al.* (1976) [13] observed equal frequency of plant regeneration in the media supplemented with or without growth regulators. Chaleff and Stolarz (1982) [10] claimed as high as 70 per cent response in the modified MS medium supplemented with NAA and kinetin for both callusing and regeneration. Azria and Bhalla (2004) [6] have studied the influence of BAP + NAA in regeneration of the callus. Aparnamaiti and Mandal (1998) [5] opined that higher concentrations of carbon source (9% and 12%) had inhibitory role in plant regeneration. The size of the callus and its age play a crucial role in plant regeneration.

2. Materials and Methods

The plant materials consist of nine New Plant Type (NPT) lines, namely, IR 71700-247-1-1-2, IR 72158-11-5-2-3, IR 72165-63-2-3-3, IR 72981-92-1-1-2-2, IR 72985-65-3-1, IR 73896-51-2-1-3, IR 73907-53-3-2-2, IR 73935-51-1-3-1 and IR 75282-10-3-3-2. One high yielding cosmopolitan variety of rice, MDU 5, was used as control. The details of the genotypes used are given in the Table 1. The *in vitro* study was carried out at the tissue culture laboratory, Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai. The nine new plant type germplasms were evaluated for their callus induction and regeneration abilities with the control line, MDU 5. Matured seeds were used as explants under four altered concentrations in the MS media. The nutrient media, MS (Murashige and Skoog, 1962) [40], was used for callus induction and regeneration. The composition of media used was given in Table 2.

The stock solutions for the media were prepared using double distilled water, kept in sterile glass stoppered bottles and stored in a refrigerator at five degree Celsius. The stock formulations and the quantity of stocks used per litre of the respective media were given in Table 3. Auxins *viz*., 2,4-D and IAA (25 mg) were dissolved in few drops of ethanol, slightly heated and gradually the volume was made upto 100 ml with double distilled water. Twenty five mg of Cytokinin *viz*., Kinetin (6-furfuryl amino purine) was dissolved in few drops of 0.5N NaOH, slightly heated and gradually diluted to 100 ml with distilled water. Four ml of stock solution is equivalent to one mg of growth hormones. A quantity of 3.725g of Na_2EDTA and 2.785g of $FeSO4.7H_20$ were dissolved separately in 50ml of distilled water. Na_2EDTA solution was boiled and then added to warm solution of $FeSO_4.7H_20$. For preparing one litre of solid medium, the required quantity of stock solutions and high purity sucrose (BDH-Analar R) were dissolved in double distilled water. The pH was adjusted to the requirement with 0.1N HCL or 0.1 NaOH and then 0.8 per cent melted agar was added and the volume was made up to one litre. The medium was shaken well for uniform mixing of the constituents and distributed to about 10 to 15 ml quantities into 150 x 125 ml test tubes and plugged with non-absorbent cotton. The cotton-plugged test tubes were autoclaved at 1.01 $kgcm^{-2}$ pressures at 121ºc for 20 minutes. The medium was allowed to cool at room temperature and stored at ten-degree Celsius.

Table 1: Details of parents taken up for study

S. No.	Parent	Designation of Parent	Parentage	Origin
1	IR 71700-247-1-1-2	L_1	IR66159-164-5-3-5 / IR64	IRRI, Philippines.
2	IR 72158-11-5-2-3	L_2	BG90-2 / 1R67962-84-2-2-2	IRRI, Philippines
3	IR 72165-63-2-3-3	L_3	IR44962-161-2-4-4 / IR 68022-3-2-2	IRRI, Philippines
4	IR 72981-92-1-1-2-2	L_4	IR71605-1-1-1-3-2 / IR 66738-118-1-2	IRRI, Philippines
5	IR 72985-65-3-1	L_5	PSBRC2 / IR 67962-84-2-2	IRRI, Philippines
6	IR 73896-51-2-1-3	L_6	BG90-2 / IR67962-84-2-2	IRRI, Philippines
7	IR 73907-53-3-2-2	L_7	IR43 / IR 68011-15-1-1	IRRI, Philippines
8	IR 73935-51-1-3-1	L_8	IR65629-157-3-2-3-2-1 / IR69132-17-2-2-2	IRRI, Philippines
9	IR 75282-10-3-3-2	L_9	IR67966-84-2-3-2 / IR68450-36-3-2-2-3	IRRI, Philippines
10	ADT 45	T_1	IR 50 / ADT 37	Tamil Nadu Rice Research Institute, Aduthurai
11	ASD 16	T_2	ADT 31 / CO 39	Rice Research Station, Ambasamudram
12	IR 72	T_3	IR 19661-9-2-3 / IR 15795-199-3-3	IRRI, Philippines
13	MDU 5	T_4	IR 9129-209-2-2-2-1 *O. glaberrima* / Pokkali	Agricultural College and Research Institute, Madurai

Note: IRRI, International Rice Research Institute

Table 2 Composition of tissue culture medium – MS

Constituents	MS (mg l^{-1})
Macro Elements	
$NH_4 NO_3$	1650.00
$(NH_4)_2 SO_4$	-------
KNO_3	1900.00
$MgSO_4 TH_2O$	370.00
KH_2PO_4	170.00
$CaCl_2 2.H_2O$	14.00
Micro Elements	
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7.H_2O$	8.60
H_3BO_3	6.20
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	--
KI	0.83
Iron Source	
Na_2 EDTA	37.25
$FeSO_4.7H_2O$	27.85
Organic Supplements	
Myoinositol	100.00
Thiamine HCL	0.1
Nicotinic Acid	0.5
Pyridoxine HCL	0.5
Glycine	2.0
Carbon Source	
Sucrose	30000.00
Agar	8000.00
pH	5.8

Table 3: Preparation of stock solutions for MS media

S. No	Ingredients	W/V Concentration (mg)	Volume of Stock Solution prepared (ml)	Volume of Solution taken per litre of medium (ml)
1	**Macro elements**			
	NH_4NO_3	16500		
	KNO_3	19000		
	$MgSO_4.7H_2O$	3700	500	50
	$CaCl_2.2\ H_2O$	4400		
	KH_2PO_4	1700		
2	**Micro elements**			
	$MnSO_4.4H_2O$	2230		
	$ZnSO_4.7H_2O$	860		
	H_3BO_3	620	500	5
	$Na_2MoO_4.2H_2O$	25		
	$CuSO4.5H_2O$	2.5		
3	**KI**	166	200	1
4	**Iron Sources**			
	Na_2 EDTA	3725		
	$FeSO4.7H_2O$	2785	100	1
5	**Organic Supplements**			
	Myoinositol	1000	100	10
	Thiamine HCL	10		
	Nicotinic Acid	50		
	Pyridoxine HCL	50	250	2.5
	Glycine	200	100	1

2.1. Callus induction medium

The callus induction medium used was MS supplemented with different concentrations of 2, 4-D and Casein hydrolysate. The treatments are as follows: a). MS+CH (1g lt^{-1}) + Kinetin (0.5mg lt^{-1}) + 2, 4-D (2mg lt^{-1}); (b). MS+CH (1g lt^{-1}) + Kinetin (0.5mg lt^{-1}) + 2, 4-D (1mg lt^{-1}); (c). MS +Kinetin (0.5mg lt^{-1}) + 2, 4-D (2mg lt^{-1}); (d). MS +Kinetin (0.5mg lt^{-1}) + 2, 4-D (1mg lt^{-1}). The dehusked seeds were surface sterilized using 70 per cent ethanol for two to three seconds followed by 0.1 per cent mercuric chloride for eight to ten minutes and washed two to three times with sterile double distilled water. The seeds were carefully inoculated in test tubes having callus induction media at a density of 2 to 3 per tube (Figure 1a). The cultures with explant were inculcated in dark at 25±2 ºC. All these operations were carried out under aseptic conditions in culture room. The explants were inoculated at the rate of 2 to 3 per tube in each treatment with three replications. The total number of explant in a tube that produced the calli was observed. After ten days, the callus induction percentage and the fresh weight of callus were taken up (Figure 1b and 1c). Number of days taken for the explant to produce callus was recorded in each genotype. The ratio of number of seeds producing callus to the number of seeds inoculated were calculated and expressed in percentage. The fresh weight of callus was taken after 15 days of inoculation and expressed in gram.

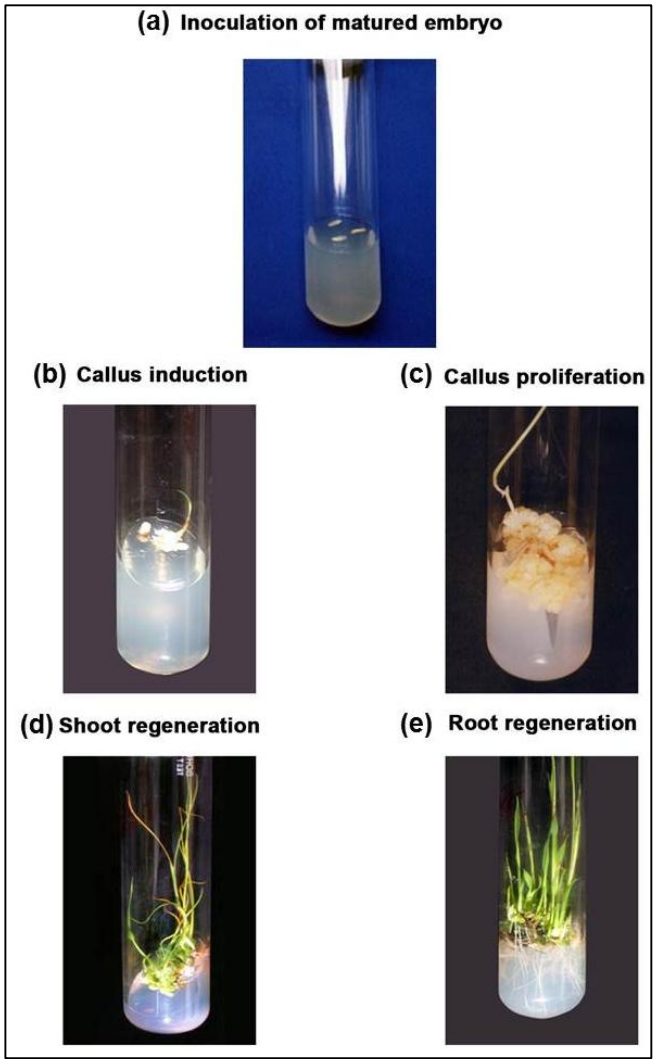

Fig 1: (a) Inoculation of matured embryo in culture medium; (b) Callus induction; (c) Callus proliferation; (d) Shoot regeneration; (e) Root regeneration

2.2. Plant regeneration

The callus tissues derived from the explants were used for regeneration studies. The callus tissues upon reaching 2 mm in diameter were transformed to MS-Plant regeneration medium with different levels of kinetin and NAA. The treatments were as follows: (1). MS + 3.0 mgl $^{-1}$ BAP + 3.0 mgl $^{-1}$ IAA + 1.0 mgl $^{-1}$ Kinetin and (2) MS + 3.0 mgl $^{-1}$ BAP

+ 3.0 mgl $^{-1}$ IAA + 0.5 mgl $^{-1}$ Kinetin. The cultures were kept under continuous light (3000 lux intensity) at 25 $\pm$2 ºC (Figure 1d and 1e) and the percentage of total plant regeneration was worked out as follows:

$$\text{Total plant regeneration} = \frac{\text{No. of plants produced}}{\text{Total number of embryogenic calli inoculated}} \times 100$$

2.3. Statistical analysis
The effect of different treatment combinations on days to callus induction, callus induction percentage, total and green plant regeneration percentage of explant were studied by subjecting the mean values of replicated arc sine transformations into factorial completely randomized design.

3. Experimental Results
3.1. Callus Induction
The days taken for callus induction for each genotype on four media compositions are illustrated in Figure 2A. The least number of days taken for callus induction was noticed in the MDU 5 (14.09) and the maximum in IR 72158 (19.34). With regard to the treatments, C_1 (13.44 days) was found to be earlier in callus induction and C_4 (19.70days) was found to be of longer duration for inducing callus. While considering the interactions between the lines and four different media compositions, the least number of days for callus induction was found for the genotype IR 75282 treatment C_1 (11.19) and the highest number of days to callus induction was observed in IR 72158 at the treatment C_4 (22.39).

The callus induction percentage of the nine NPT lines on four media compositions was given in Table 4. The callus induction percentage ranged between 74.48% (IR 71700) and 90.11% (IR 72981) among the genotypes. The comparison among different media compositions revealed that the treatment combination C_1 had pronounced effect on callus induction (88.71%), while the treatment C_4 had the least callusing response (76.31%). Among the genotypes, IR 72981 at C_1 (95.56%) recorded the highest callus induction percent and IR 73896 at C_4 (70.41%) registered the lowest callus induction percent. Five genotypes, two treatments and 18 of genotype x treatment interactions recorded higher callus induction percentage than the grand mean (82.59%).

Fresh weight of callus varied from 0.340 (IR 73935) to 0.506 g (IR 75282) among the genotypes, 0.283 (C_4) to 0.617g (C_1) among the treatments and 0.165 (IR 72158 in the treatment C_4) to 0.745g (IR 75282 in the treatment C_1) among the genotype x interactions. Five genotypes including the control, the treatment C_1 and 20 of genotype x treatment interactions were found to have higher fresh weight than the general mean (0.430g) (Table 5).

Table 4: Callus induction percentage in the nine NPT lines and the control

Genotypes	Treatments				Mean
	C_1	C_2	C_3	C_4	
IR 71700	80.05 (63.47)	70.45 (57.07)	75.03 (60.02)	72.39 (58.30)	74.48 (59.72)
IR 72158	82.01 (64.91)	77.52 (61.70)	80.57 (63.85)	70.62 (57.18)	77.68 (61.91)
IR 72165	86.54 (68.48)	78.30 (62.24)	81.60 (64.60)	74.63 (59.75)	80.27 (63.77)
IR 72981	95.56 (77.85)	88.24 (69.94)	91.05 (72.60)	85.58 (67.68)	90.11 (72.02)
IR 72985	93.06 (74.74)	83.29 (65.87)	89.34 (70.95)	80.32 (63.67)	86.50 (68.80)
IR 73896	82.06 (64.94)	75.02 (60.02)	78.29 (62.23)	70.41 (57.04)	76.44 (61.06)
IR 73907	90.90 (72.45)	80.59 (63.86)	88.39 (70.08)	80.43 (63.75)	86.26 (68.44)
IR 73935	88.84 (70.49)	81.10 (64.23)	85.03 (67.24)	75.46 (60.30)	82.48 (65.47)
IR 75282	92.94 (74.60)	85.40 (67.54)	88.13 (69.85)	72.46 (58.35)	83.66 (66.76)
MDU 5	95.44 (77.25)	85.40 (67.54)	90.81 (72.36)	80.78 (64.00)	88.10 (70.29)
Mean	88.71 (70.92)	80.50 (64.00)	84.82 (67.38)	76.31 (61.00)	82.59 (65.82)

*Transformed values in parenthesis

Table 5: Fresh weight of callus on different media compositions

Genotypes	Treatments				Mean
	C_1	C_2	C_3	C_4	
IR 71700	0.560	0.245	0.470	0.230	0.376
IR 72158	0.555	0.350	0.455	0.330	0.423
IR 72165	0.660	0.270	0.350	0.165	0.361
IR 72981	0.660	0.255	0.460	0.235	0.403
IR 72985	0.665	0.360	0.455	0.325	0.451
IR 73896	0.555	0.540	0.545	0.360	0.500
IR 73907	0.665	0.520	0.355	0.340	0.470
IR 73935	0.480	0.370	0.265	0.245	0.340
IR 75282	0.745	0.660	0.255	0.365	0.506
MDU 5	0.625	0.550	0.460	0.235	0.468
Mean	0.617	0.412	0.407	0.283	0.430

C_1	-	MS + CH (1g per litre) +Kinetin (0.5ml per litre) + 2,4-D (2mg per litre)
C_2	-	MS + CH (1g per litre) +Kinetin (0.5ml per litre) + 2,4-D (1mg per litre)
C_3	-	MS + Kinetin (0.5ml per litre) + 2,4-D (2mg per litre)
C_4	-	MS +Kinetin (0.5ml per litre) + 2,4-D (1mg per litre)

3.2. Regeneration of Plantlets
The days to plant regeneration varied between 24.23 (IR 75282) and 32.11 (IR 72158) among the genotypes and between 22.01 (IR 73935 in C_1) and 35.15 (IR 72158 in C_2) among the genotype x treatment interactions. The treatment C_1 (24.58) was earlier than the other treatment (28.64) in days to plant regeneration. A total of three genotypes only one treatment and six genotype x treatment interactions recorded earlier days to plant regeneration than their general mean (26.61) (Figure 2B).

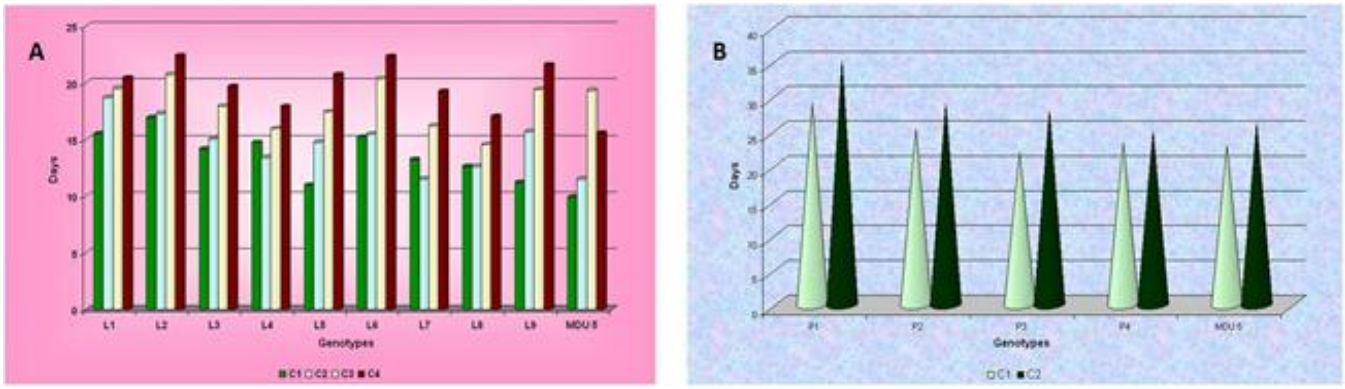

Fig 2: A), Days taken for callus induction on different media composition; B), Days to regeneration on different media composition

The percent regeneration on two different media was furnished in Table 6. The regeneration percentage varied from 75.62% (IR 72158) to 90.38% (MDU 5) among the genotypes and from 71.71% (IR 72158 in C_2) to 93.89% (IR 75282 in C_1) among the genotype x treatment interactions. The treatment C_1 (86.26%) has recorded maximum regeneration percentage.

Table 6: Total plantlet regeneration on different media compositions in four NPT lines and control

Genotypes	Treatments		Mean
	C_1	C_2	
IR 72158	79.53 (63.10)	71.71 (57.87)	75.62 (60.49)
IR 73907	80.75 (63.98)	74.27 (59.52)	77.51 (61.75)
IR 73935	84.89 (67.14)	83.91 (66.35)	84.40 (66.75)
IR 75282	93.89 (75.69)	82.20 (65.06)	84.04 (70.37)
MDU 5	92.26 (73.86)	88.51 (70.21)	90.38 (72.03)
Mean	86.26 (68.75)	80.12 (63.80)	83.19 (66.28)

*Transformed values in parenthesis

C_1	-	MS + 3 mg per litre IAA + 3 mg per litre BAP +1 mg per litre Kinetin
C_2	-	MS + 3 mg per litre IAA + 3 mg per litre BAP + 0.5 mg per litre Kinetin

4. Discussion

The traditional breeding methods have to be supplemented with plant tissue culture techniques either to achieve a specific objective, which is not possible conventionally, or end up with an increase in the efficiency. However, the attention of the scientists all over the world has been focused on these techniques to break the time consumption which is the major barrier involved in traditional methods of breeding. Several laboratories have reported tissue culture in rice using different explants such as, mature and immature seed, root, leaf and immature panicle (Deepti *et al*., 2001; Chitra *et al*., 2003 and Yamaguchi *et al.,* 2004) [15, 11, 50]. For the successful application of the tissue culture technique in crop breeding, the callus growth and plant regeneration potential must be determined (Dabul 2009; Joyia and Khan 2012; Yaqoob *et al*., 2016) [14, 25, 51].

4.1. Callus induction studies

Success in callus induction depends on the various factors like genotype, explants, media constitution and other growth environments. A number of studies on rice tissue culture have been conducted with special reference to the effect of genotypes (Abe and Sasahara, 1982 and Biswas *et al*., 2002) [1, 8] and exogenous phytohormones (Maheswaran and Sree Rangasamy, 1989 and Al-Forkan *et al.,* 2005) [33, 3].

4.2. Effects of genotypes

The varietal difference in rice tissue culture had well pronounced effect on callus induction. In the present study, the genotypes displayed difference with respect to days to callus induction, callus induction percentage and fresh weight of callus. The genotypes, IR 72981, IR 72985, IR 73907, IR 73935 and MDU 5 were earlier in callus induction. Among these, all genotypes except IR 73935 had higher callus induction percentage. The genotype, IR 75282 though late in callus induction showed high callus induction percentage. The rest of the genotypes, IR 71700, IR 72158, IR 72165 and IR 73896 failed to show either earlier callus induction or higher callusing frequency. Both lower (Siriwardana and Nabors, 1987) [47] and higher (Tsukuhara *et al*., 1996) [48] concentration of 2, 4-D have been reported for callus induction which was observed in the present study also indicating the differential response of genotypes to this auxin. This variation in callusing response attributed to varietal effect was earlier reported by Azria and Bhalla (2004) [6] and Al-Forkan *et al*., 2005) [3].

A range of variation was observed for fresh weight of callus among the genotypes. Totally, five genotypes *viz.*, IR 72985, IR 73896, IR 73907, IR 75282 and MDU 5 exhibited higher fresh weight of callus than the grand mean. While considering the three traits together namely days to callus induction, callus induction percentage and fresh weight of callus, the NPT lines IR 72985 and IR 73907 were found superior apart from the check MDU 5. The genotype IR 75282 though took longer time for callus induction had better callus induction percentage and fresh weight of callus.

4.3. Effect of growth regulators

The most commonly used growth regulator for callus induction in cereal tissue culture is 2, 4-D (Bregitzer *et al*., 1989 and Gouranga *et al*., 2015) [9, 19]. Many cereals express embryogenic competence in the presence of 2, 4-D (George and Eapen, 1988) [18]. Heyser *et al*. (1983) [22] reported varying responses among rice varieties in producing embryogenic calli with the use of 2, 4-D. The other auxins such as NAA and IAA are available and 2, 4-D either alone or in combination with any one of the above mentioned auxins were widely used by many scientists in callus culture (Mandal and Gupta, 1995; Mandal and Bandyopadhyay, 1997) [35, 34]. Addition of cytokinins facilitated better callus induction and maintenance (Mandal *et al.*, 1998) [36]. Some of the recent studies in rice tissue culture have indicated that a combination of auxins *viz*., 2,4-D and NAA with kinetin is superior in terms of overall plant regeneration efficiency than a medium with 2,4-D or NAA alone. However, a critical level of auxin and cytokinin was found to be essential for optimum levels of

callus induction and plantlet regeneration. A combination of NAA and kinetin with CH (Laxminarayanan, 2000) [29] was studied at various concentrations to find out the optimum hormonal concentration for increased callus induction. The results revealed that the hormonal combinations of MS + CH ($1glt^{-1}$) + Kinetin ($0.5\ mglt^{-1}$) + 2, 4-D ($2\ mglt^{-1}$) was found to be superior in higher frequency of callus on MS media. The hormonal combination MS + Kinetin ($0.5\ mglt^{-1}$) + 2, 4-D ($2\ mglt^{-1}$) was the next best in callus induction. The treatment MS + CH ($1glt^{-1}$) + Kinetin ($0.5\ mglt^{-1}$) + 2, 4-D ($2\ mglt^{-1}$) was also observed to be superior in inducing callus earlier with high fresh weight. Hence, the growth regulator combination of MS + CH ($1glt^{-1}$) + Kinetin ($0.5\ mglt^{-1}$) + 2, 4-D ($2\ mglt^{-1}$) was the best for obtaining quicker callus induction, higher callus induction percentage and fresh weight of callus. The results are in accordance with that of Lee *et al.*, 2002 [30]; Azria and Bhalla (2004) [6]; Karthikeyan *et al.*, 2009 [26] and Li-na *et al.*, 2010 [31].

4.4. Regeneration studies

4.4.1. Effect of genotypes

The days to plant regeneration and regeneration percentage were studied in the nine NPT lines and MDU 5. Out of ten genotypes, only five responded for regeneration. There occurred a wide variation for these traits among the genotypes. The genotypes, IR 75282, IR 73935 and the check, MDU 5 showed minimum days for regeneration and higher regeneration percentage while the remaining two genotypes *viz.*, IR 73907 and IR 72165 took longer days for plant regeneration and lesser regeneration percentage. Such as a variation for days to plant regeneration and regeneration percentage among the genotypes was earlier reported by Ranjan *et al*. (1998) [45]; Aparnamaiti and Mandal, 1998 [5] and Hoque *et al.*, 2004 [23]. The study indicated that the genotypes IR 75282 and IR 73935 with the check, MDU 5, were the best for plant regeneration. It may be concluded that among the genotypes evaluated IR 75282-10-3-3-2 was the best suited to tissue culture studies since it excelled others in callus induction percentage, fresh weight of callus besides regeneration potential.

4.4.2. Effect of growth regulators

The concentration of growth hormones in the regeneration medium decides the regeneration of plantlets. The ratio of kinetin to auxin had a major effect on differentiation of green plants (Anonymous, 1975) [4]. Mascarenhas (1981) [38] also suggested that the delicate balance between auxin and cytokinins, is the most important factor in getting desirable results in regeneration. Hence, two combinations of kinetin and IAA were tried for plant regeneration in the present study. The results revealed that the hormone combination MS + 3 $mglt^{-1}$ IAA + 3 $mglt^{-1}$ BAP +1 $mglt^{-1}$ Kinetin was highly efficient in producing higher frequency of plant regeneration and therefore it is adjudged as the best for obtaining higher frequency of plantlet regeneration.

5. Conclusion

This *in vitro* study has generated a reliable protocol in rice for generating high frequency regeneration from mature seeds. This report will be of high utility in inducing high frequency callus which is the key footstep for the faster multiplication of plants through biotechnological techniques. These findings could also be involved in genetic transformation research for the development of cultivars serving tolerance to biotic and abiotic stresses and bio-fortified cereals.

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