AATCC Review

21 January 2024: Received
05 February 2024: Revised
17 February 2024: Accepted
04 March 2024: Available Online

www.aatcc.peerjournals.net

Research Article | Open Access

Establishment of a highly efficient and reliable protocol for seed germination and callus formation from cultured rice seeds

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ABSTRACT

In the present investigation, dehusked rice seeds were tissue cultured on seven MS basal media with the supplementation of divergent concentrations and conjunction of kinetin and 2,4-dichlorophenoxy acetic acid to avail in vitro seed germination and callus formation. It was noticed that medium M1 (MS + 2.50 mg/L 2, 4-D) revealed the higher formation of callus but the lowest seed germination in rice varieties. Among auxins, 2,4-Dichlorophenoxy acetic acid is detected to be the utmost potent auxin for in vitro callus formation in rice. Moreover, the higher concentration of 2,4-dichlorophenoxy acetic acid in the remaining six media, namely, M2, M3, M4, M5, M6, and M7 enhanced the formation of callus and decreased germination of rice seed. Additionally, the highest kinetin concentration enhanced the germination of seed and decreased the formation of callus in rice. Thus, the formation of the callus can accelerate the rice improvement through somaclonal variation. Furthermore, in vitro-formed calluess, shoot buds, and roots can be employed as rudimentary explants for successful genetic transformation. Therefore, a highly efficient and reliable protocol was established for in vitro germination of seeds and the formation of callus from cultured rice seeds. This protocol will be very reproducible for acquiring in vitro germination of seed and formation of callus based on varied biotic and abiotic stress tolerance mechanisms in rice.

Keywords: *callus, 2,4-D, in vitro, protocol, rice, seed germination, somaclonal variation*

Introduction

Rice is the prime food supplier of cereal crops later than maize and wheat globally. For half of the world's population, this cereal is the central food and calorie source [1] [2]. Its embryo and pericarp accommodate 70-80% starch, 7% protein, and 1.5% oils. It has the highest pure protein in comparison to maize and wheat. In comparison to other cereals, it also constitutes excess carbohydrates and calories per hectare [3]. Plant tissue culture technique contributes a chance to create a wide range of genetic variation among rice varieties which may be further utilized for the improvement of cereal crops especially when rice plantlets are regenerated from *in vitro* built-up calli. Thus, *in vitro* callus formation becomes a requisite element for making use of plant tissue culture in rice improvement via somaclonal variation [4]. In addition, dehusked rice seed culture is a potent plant tissue culture strategy for the formation of callus and somaclonal variation accomplishment. Besides, medium is the major ingredient for getting the desired results of any plant tissue culture work and hence, convenient medium selection is the prime footstep to do any plant tissue culture experiment. Furthermore, auxins are primal phytohormones to stimulate cell-division and result in the formation of callus from cultured rice seeds. Among auxins, 2, 4-D examined the extremely fateful phytohormones for *in vitro* formation of callus. The interconnection of auxin with cytokinin is also well significant element for judging the type of organogenesis and callus formation from the cultured rice seeds. Thus, the establishment of a reliable protocol well efficient for *in vitro* germination of seed and the formation of calli from cultured seeds of rice is a fateful approach for cereal crop improvement.

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DOI: https://doi.org/10.58321/AATCCReview.2024.12.01.245
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Materials and Methods

This investigation was performed at Deptt. of AB & MB, FBS & H, Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar. Seeds of six rice varieties (listed in Table 1) were tissue cultured on selected culture media. A highly efficient and reliable protocol was established for seed germination and callus formation from cultured rice seed varieties. It has the following steps;

1. Explants selection

Dehusked rice seeds were taken as explant for seed germination and callus formation (Fig. 1).

Fig. 1 Rice explant

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2. Media selection, media composition, and media preparation

Murashige and Skoog (MS) medium [5] with some modifications is generally used just as basal medium for *in vitro* germination of seeds as well as the formation of calli in rice varieties, because it is the most favorable medium uniquely for the formation of callus and regeneration of plantlets. MS medium is so broadly granted medium for doing tissue culture of rice varieties. MS basal medium consists of inorganic salt as well as organic components. This inorganic salt contains major as well as minor elements including the source of iron. Furthermore, the organic components contain nutrients such as amino acids, and vitamins as well as sucrose as a carbon source. Like a gelling agent, agar is applied. Sterilized dehusked rice seeds were mainly cultured on MS media with the supplementation of divergent conjunctions as well as concentrations of kinetin and 2,4-D for *in vitro* germination of seed and formation of callus from cultured seeds of rice varieties. It was found that 2,4-D is extremely potent auxin among all phytohormones for the formation of callus. Even so, initially lots of media were prepared by using a differing concentrations in phytohormones, but finally, 7 media (M1, M2, M3, M4, M5, M6, and M7) were selected for acquiring *in vitro* germination of seed and formation of callus and 4 media (M8, M9, M10, and M11) were selected for getting regeneration in rice varieties (Table 2).

Table 1 Six selected rice varieties applied in the existing experiment

S.No.	Rice variety	Origin
i.	Pokkali	UAS, Dharwad
ii.	CSR-30	CSSRI, Karnal
iii.	Pusa Basmati-1	IARI, New Delhi
iv.	Rajendra Bhagwati	RAU, Pusa
v.	IR-29	IRRI, Philippines
vi.	Pusa Sugandh-2	IARI, New Delhi

Table 2 Constituents of selected media for acquiring in vitro germination of seed, formation of callus, and plantlets regeneration from cultured seeds of rice varieties

S.No.	Media name	Composition of seed germination and callus formation media
i.	M1	MS + 2.50 mg/L 2,4-D
ii.	M2	MS + 2.50 mg/L 2,4-D + 0.50 mg/L KIN
iii.	M3	MS + 2.50 mg/L 2,4-D + 1.00 mg/L KIN
iv.	M4	MS + 2.00 mg/L 2,4-D + 0.50 mg/L KIN
v.	M5	MS + 2.00 mg/L 2,4-D + 1.00 mg/L KIN
vi.	M6	MS + 1.50 mg/L 2,4-D + 0.50 mg/L KIN
vii.	M7	MS + 1.50 mg/L 2,4-D + 1.00 mg/L KIN
Sl. No.	**Media name**	**Composition of regeneration media**
viii.	M8	MS + 1.50 mg/L NAA + 1.00 mg/L KIN
ix.	M9	MS + 1.50 mg/L NAA + 1.50 mg/L KIN
x.	M10	MS + 1.00 mg/L NAA + 1.00 mg/L KIN
xi.	M11	MS + 1.00 mg/L NAA + 1.50 mg/L KIN

Stock solutions preparation

In the present study, stock solutions of different components like major, minor, iron as well as vitamins were separately prepared. For this purpose, first of all, dissolved required amounts of different agents in sterile distilled water. In the object of stock solutions of major salts preparation (Table 3), calcium chloride was added at the end. Accordingly, distinct stock solutions of auxins namely, α-Naphthaline acetic acid, Indole-3 acetic acid, and 2, 4-Dichlorophenoxy acetic acid as well as cytokinins namely, 6-Furfuryl amino purine (KIN) and 6-Benzyle amino purine (BAP) were also made. For making auxin stock solution firstly, the requisite amount of constituent was dissolved in 2-3 ml ethanol and subsequently in sterile distilled water to build the desired volume. Similarly, for making cytokinin stock solution firstly, requisite amount of constituent was dissolved in 5-10 ml solutions of NaOH (N/10) subsequently sterile distilled water was added to build final volumes (Table 4). After that stock solution was kept in the refrigerator at 4°C.

Table 3 MS medium components and their stock solutions

S. No.	Stocks	Constituents	Quantity in stock solution (mgs)	Quantity in stock solution (gms)	Dissolved in distilled water and made	Conc.	The quantity used/lit
	Inorganic						
1.	Major element	NH_4NO_3 (Ammonium nitrate)	16500	16.5 gram	500 ml	20X	50 ml
		KNO_3 (Potassium nitrate)	19000	19.0 gram			
		$CaCl_2.2H_2O$ (Calcium chloride)	4400	4.4 gram			
		$MgSO_4.7H_2O$ (Magnesium sulphate)	3700	3.7 gram			
		$KH_2 PO_4$ (Potassium dihyro-orthophosphate)	1700	1.7 gram			
2.	Minor element	H_3BO_3 (Boric acid)	620	0.62 gram	500 ml	200X	5 ml
		$MnSO_4H_2O$ (Manganese sulphate)	2230	2.23 gram			
		$ZnSO_4.7H_2O$ (Zinc sulfate)	860	0.86 gram			
		KI (Potassium iodide)	83	0.083 gram			
		Na_2MoO_4 (Sodium molybdate)	25	0.025 gram			
		$CuSO_4.5H_2O$ (Cupric sulphate)	2.5	0.0025 gram			
		$CoCl_26H_2O$ (Cobalt chloride)	2.5	0.0025 gram			

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3.	Iron sources	$Na_2EDTA.7H_2O$ (EDTA-disodium salt)	373	0.373 gram	250 ml	40X	25ml
		$FeSO_4.7H_2O$ (Ferrous sulfate)	278	0.278 gram			
	Organic						
4.	Amino acid & vitamin	$C_2H_5NO_2$ (Glycine)	100	0.1 gram	250 ml	200X	5 ml
		$C_6H_{12}O_6$ (Inositol)	5,000	5.0 gram			
		$C_6H_5NO_2$ (Nicotinic acid)	25	0.025 gram			
		$C_8H_{11}NO_3HCl$ (Pyridoxine hydrochloride)	25	0.025 gram			
		$C_{12}H_{17}ClN_4O_5.HCl$ (Thiamine hydrochloride)	5	0.005 gram			
5.	Carbon Source	$C_{12}H_{22}O_{11}$ (Sucrose)	30,000	30 gram			
6.	Gelating Agent	Agar	8000	8 gram			

Table 4 Stock solutions of distinct phytohormones preparation

S. No.	Phytohormones	Quantity (mg)	Quantity (gm)	Initially dissolved in	Made up of distilled water	Strength
1.	Indole-3 acetic acid (IAA)	25	0.025	2-3 ml ethanol	250 ml	1mg/10ml
2.	2,4-Dichlorophenoxy acetic acid (2,4-D)	25	0.025	2-3 ml ethanol	250 ml	1mg/10ml
3.	α-Naphthaline acetic acid (NAA)	25	0.025	2-3 ml ethanol	250 ml	1mg/10ml
4.	6-Benzyle amino purine (BAP)	25	0.025	5-10 ml slightly heated solutions of NaOH (N/10)	250 ml	1mg/10ml
5.	6-Furfuryl amino purine (KIN)	25	0.025	5-10 ml slightly heated solutions of NaOH (N/10)	250 ml	1mg/10ml

One litre medium preparation

Firstly a volumetric flask of one litre was taken for preparing one litre medium. After that the needful amount of divergent stock solutions such as major, minor, iron, amino acid, as well as vitamins were taken into this flask. Subsequently, stock solutions of phytohormones were added as per their requirements and then 30 grams of sucrose was added into it. By adding distilled water, sucrose was dissolved gradually. And finally, volume was made slightly less than one litre, by adding distilled water. The medium pH was tested using a pH meter and it was adjusted to 5.8 by drop-wise adding diluted NaOH or diluted HCl as required. After adjusting its pH 5.8 then made final volume one litre of medium using distilled water. Then, took one litre of conical flask and poured this medium into it and in the last 8 gram agar was added as gelling agent. Finally, this prepared one-litre medium containing a conical flask was heated for mixing agar correctly for 15-20 mins or until the medium looked transparent. Approximately, 15-20 ml medium was poured into every tube culture (size: 18mmX150mm/ 25X150). Each one tube was plugged using non-absorbent plugs of cotton and then capped with aluminium foil.

Sterilization of prepared medium

The prepared medium was sterilized in an autoclave. The capped culture bottles containing homogenized medium were put in perforated baskets. The date and precise medium number were marked on each basket. Thereafter, the basket containing culture bottles was kept inside autoclave for 20 mins at 121°C and at 15 psi (pounds per square inch) or 1.0kg/cm^2 pressure. At room temperature, culture tubes/bottles were brought, and then cooled after autoclaving. The medium was left for a day to set and inoculation was done the next day, or kept in a refrigerator for later use.

3. Explants collection and preparation

Seeds soaking and explants surface sterilization

First of all, before starting to surface sterilization, the seeds of rice were soaked in water overnight. The next-day, washed soaked rice seeds for 15-20 mins in running tap water afterwards treated with 70% ethanol for 30 sec afterwards it was washed with sterile distilled water. Then lastly, seeds were treated for 10-15 minutes with 0.1% $HgCl_2$. Under laminar air flow, seeds were 4 times rinsed using distilled water.

4. Inoculations

Sterilization under laminar air-flow (LAF)

Before use, all inner sides of LAF such as the working chamber; containing the floor are pertinently wiped with 70% ethanol with the help of cotton. After that inoculating instruments namely, needles, sterilized petridishes, forceps, scalpels, media, and spirit lamps were put under the chamber. Then the chamber is closed and then switched on UV lamp for 30 mins to sterilize chamber.

Explant inoculations

Under sterilized laminar air flow, explants inoculation was carried out. Firstly, both hands were wiped with 70% ethanol, and then instruments such as scalpels and forceps were dipped into 70% ethanol after that flamed on burner under laminar air flow hood. During aseptic inoculation work this procedure was carried out again and again. Further, during the inoculation period tube light and air flow/HEPA filter

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(high-efficiency particulate) must be switched on. After it, surface sterilized rice seeds were sharply inoculated into the medium by forceps over the sprit lamp flame.

5. Incubation

In this step, the bottles/tubes of inoculated culture were kept into a culture room under a controlled environment state. In other sense, temperature and relative humidity (RH) was maintained at 25°C±2°C and 70 to 80% respectively. The continuous light approx. 2 kilo lux intensity was provided via a fluorescent tube

6. Subculture

Every 5-6 weeks, established cultures were regularly transferred by subdividing shoot clusters using scalpel blade. For sub-culturing, the outer dead tissue from the base of the explant was removed. The developed multiple shoots were splited into 2-4 groups after that inoculated to individual culture bottles.

7. Acclimatization

Regenerated rice shoots having sufficient no. and length of roots were extracted from the culture vessels. The root was washed entirely but slowly in running tap water to extract traces of media and finally was transferred in a pot having sterilized sands and FYM (farm yard manure) in the 1:1 ratio. The pots were initially placed under high humid conditions and progressively acclimatized to minimized humidity for their acclimatization and hardening in acclimatization chamber.

8. Observations, photography, and statistical analysis

At regular intervals, the rice varieties responses were observed concerning establishment frequencies of aseptic cultures, formation of callus, and differentiation of shoots as well as roots. Further, observations were done for the nature of callus, the colour of callus as well as no. of differentiated shoots per culture. As per the table given (Table 5), the callus growth as well as existed shoots or differentiated root and shoot were observed.

Table 5 Observation parameter for growth responses and formation of callus

Sign adopted	Description	Growth measurements		
		Calli (Diameter in cm)	Shoots (Height in cm)	Roots (Length in cm)
-	No growth	0.0	0.0	0.0
+	Low growth	<1.0	<1.0	<1.0
++	Medium growth	1.0-1.5	1.0-1.5	1.0-1.5
+++	Good growth	1.5-2.5	1.5-2.5	1.5-2.5
++++	Excellent growth	>2.5	>2.5	>2.5

9. Photography

Photographs of responses were captured using 16.1 Mega Pixels, Sony Cyber-shot, 5X, OZD camera, (Model No. DSC-W570).

10. Statistical Analysis

With three replications, a plant tissue culture study was carried out in a factorial CRD (completely randomized design). All data were analyzed by ANOVA (analysis of variance) using OP Stat (Kumari et al. 2015; Kumari et al. 2017; Kumari et al. 2020). The means were compared by DMRT (Duncan's multiple range test) to detect the difference at a 5% (P<0.05) level. The result was expressed as mean ± SE. One-way ANOVA was made for comparing mean values.

Results

Establishment of cultured seeds of six rice varieties

Establishment of any cultured explants depends upon surface sterilization. In the present investigation, establishment of cultured dehusked rice seed was examined with reference to effect of media as well as effect of varieties which were further depicted in Table 6 and graphically depicted in Figs. 2 and 3.

On different media among six rice varieties, cultured rice seeds exhibited no any variations for responses of establishment. The frequency of establishment was at par among the six rice varieties when rice seeds were cultured on three media namely, M1, M2 and M3. Further, overall mean establishment frequency of all varieties was revealed the maximal in M3 (89.990 percent) medium followed by medium M2 (89.160 percent) and medium M1 (88.050 percent) respectively (Fig. 3). When evaluating three media M1, M2, and M3, the overall mean establishment frequency was revealed the maximal in variety Pokkali (91.100 percent) followed by varieties Pusa Basmati-1 (89.990 %), Rajendra Bhagwati (89.440 %), IR-29 (88.880 %), CSR-30 (88.330 %), Pusa Sugandh-2 (86.660 %) respectively (Table 6 and Fig. 2).

Table 6. Establishment percentage of cultured rice seeds on three media

Media	varieties									
	Pokkali	CSR-30	Pusa Basmati-1	Rajendra Bhagwati	IR-29	Pusa Sugandh-2	Mean	CD	CV	SE(m)
M1 medium	88.33 ± 1.660	85 ± 5.770	88.33 ± 4.410	91.66 ± 3.330	90 ± 2.880	85 ± 2.880	88.050	0.000	7.330	3.720
M2 medium	91.66 ± 1.660	90 ± 2.880	93.33 ± 1.660	85.00 ± 5.000	86.66 ± 3.330	88.33 ± 1.660	89.160	0.000	5.760	2.960
M3 medium	93.33 ± 1.660	90 ± 5.000	88.33 ± 4.410	91.66 ± 1.660	90.00 ± 5.000	86.66 ± 1.660	89.990	0.000	6.920	3.600
Mean	91.100	88.330	89.990	89.440	88.880	86.660				
CD	0.000	0.000	0.000	0.000	0.000	0.000				
CV	3.160	9.240	7.170	6.970	7.500	4.300				
SE (m)	1.660	4.710	3.720	3.600	3.840	2.150				

Value is indicated as mean ± SE

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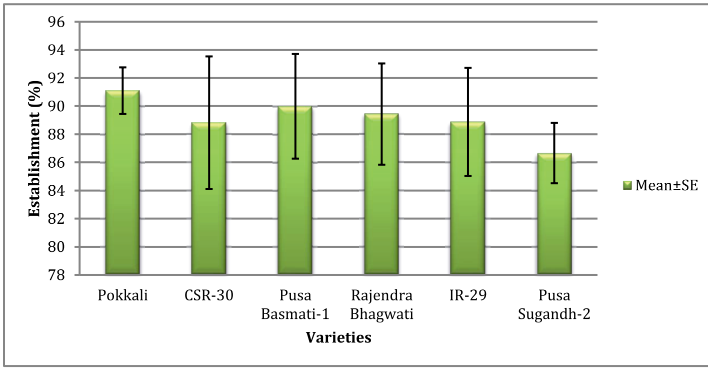

Fig. 2 Effect of varieties on establishment percentage of cultured rice seed

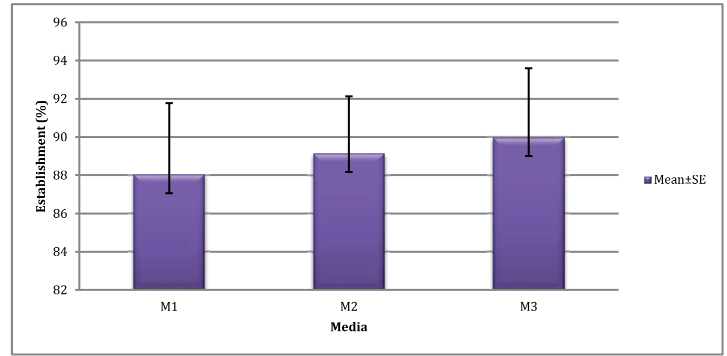

Fig. 3 Effect of media on establishment percentage of cultured rice seeds

Germination from cultured seeds of six rice varieties under *in vitro*

Rice seeds started germination after four days of inoculation. It was observed that maximum rice cultures showed either the formation of shoot or root formation. But, a few rice cultures exhibited the formation of both shoots as well as roots.

The germination % of cultured rice seeds of six varieties on the three callusing media namely, M1, M2, and M3 was at par. Accordingly, among all varieties, the effect of these 3 media on germination percentage was at par. Even so, medium M3 (94.010 percent) revealed the best germination percentage among all varieties followed by M2 (92.220 percent) and M1 (91.500 percent) media (Fig. 5). Additionally, in the effect of varieties, the percentage of germination amongst six varieties was at par. Furthermore, the overall best germination percentage was found in variety CSR-30 (93.590 percent) followed by varieties Pusa Basmati-1 (92.670 %), Pokkali (92.610 %), IR-29 (92.410 %), Pusa Sugandh-2 (92.280 %), Rajendra Bhagwati (91.910 %) respectively (Table 7 and Fig. 4).

Table 7. Germination percentage of cultured rice seeds on three media

Media	Varieties									
	Pokkali	CSR-30	Pusa Basmati-1	Rajendra Bhagwati	IR-29	Pusa Sugandh-2	Mean	CD	CV	SE(m)
M1 medium	92.78 ± 1.950	92.32 ± 2.410	90.61 ± 2.060	90.83 ± 1.950	90.23 ± 4.490	92.26 ± 2.010	91.500	0.000	5.000	2.640
M2 medium	92.68 ± 1.900	94.42 ± 0.170	92.78 ± 1.950	90.39 ± 2.160	90.62 ± 1.740	92.46 ± 2.120	92.220	0.000	3.420	1.810
M3 medium	92.37 ± 2.070	94.05 ± 0.400	94.63 ± 3.040	94.52 ± 0.200	96.39 ± 1.800	92.13 ± 1.960	94.010	0.000	3.430	1.860
Mean	92.610	93.590	92.670	91.910	92.410	92.280				
CD	0.000	0.000	0.000	0.000	0.000	0.000				
CV	3.690	2.610	4.490	3.170	5.570	3.810				
SE(m)	1.970	1.410	2.400	1.680	2.970	2.030				

Value is indicated as mean ± SE

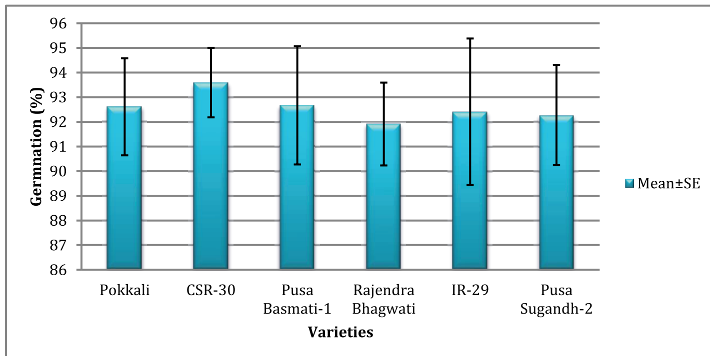

Fig. 4 Effect of varieties on germination percentage of cultured rice seeds

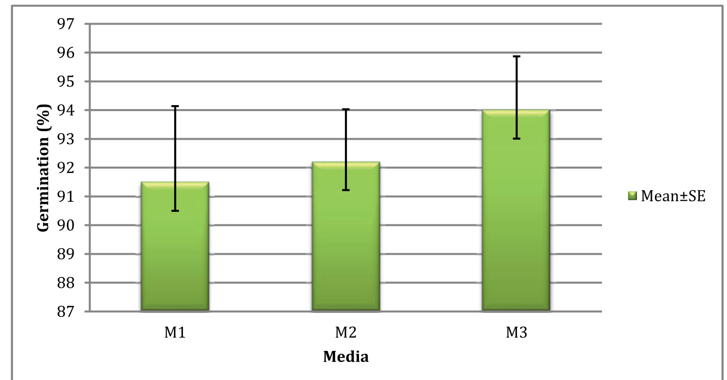

Fig. Effect of media on germination percentage of cultured seeds

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In vitro formation of callus from cultured seeds of six rice varieties

In the first week, cultured rice seeds formed callus from germ pores in few cases, while in some other cases, the base of the starting shoot swelled and finally observed formation of callus in the second week. Callus growth, color, and nature differed from one rice culture to some other rice culture. Callus growth, color, and nature also differed from one medium to some other medium. The color of the callus in the present study varied from brown to cream. Maximum calli were cream in color. The callus nature was friable but some callus were compact (Fig. 6).

The growth of callus was good (+++) to excellent (++++) in rice varieties Rajendra Bhawati, IR-29, and Pusa Sugandh-2, average (++) to good (+++) in varieties CSR-30 as well as Pusa Basmati-1 and low (+) to good (+++) in variety Pokkali.

The overall formation of callus percentage was exhibited the best on medium M1 (82.080 percent) followed by medium M2 (80.940 percent) and M3 (80.670 percent) respectively when evaluating all six varieties (Fig. 8). The percentage of formation of callus among six varieties and on 3 media M1, M2 and M3 was at par. The overall best formation of callus percentage was detected in variety IR-29 (91.720 percent) followed by varieties Pusa Sugandh-2 (91.440 percent), Rajendra Bhagwati (81.080 percent), Pusa Basmati-1 (79.080 percent), CSR-30 (73.350 percent) and Pokkali (70.700 percent) respectively examining the 3 media (depicted in Table 8 and Fig. 7). Further, M8 medium showed the maximum number of shoots and root in rice followed by media M9, M10 and M11 (Fig. 6) respectively when calluses were sub-cultured on these regeneration medium.

Table 8. Percentage of callus formation from cultured seeds on three media

Media	Varieties									
	Pokkali	CSR-30	Pusa Basmati-1	Rajendra Bhagwati	IR-29	Pusa Sugandh-2	Mean	CD	CV	SE(m)
M1 medium	73.29c ± 0 .700	75.54_b ± 3.390	77.72_b ± 0.710	83.71_b ± 2.870	88.48_a ± 3.920	93.75_a ± 3.600	82.080	9.520	6.440	3.050
M2 medium	70.75_b ± 4.090	72.25_b ± 2.820	76.60_b ± 5.190	83.17_a ± 2.710	88.55_a ± 3.490	94.33_a ± 3.210	80.940	11.490	5.890	3.680
M3 medium	68.08_c ± 4.490	72.27_c ± 2.910	82.93_b ± 0.870	76.36_c ± 1.510	98.14_a ± 1.850	86.24_b ± 1.980	80.670	7.950	5.480	2.550
Mean	70.700	73.350	79.080	81.080	91.720	91.440				
CD	0.000	0.000	0.000	0.000	0.000	0.000				
CV	9.400	7.210	6.720	5.220	6.070	5.710				
SE(m)	3.840	3.050	3.070	2.440	3.210	3.010				

Same letter values in rows are not significantly different by using DMRT at 5%

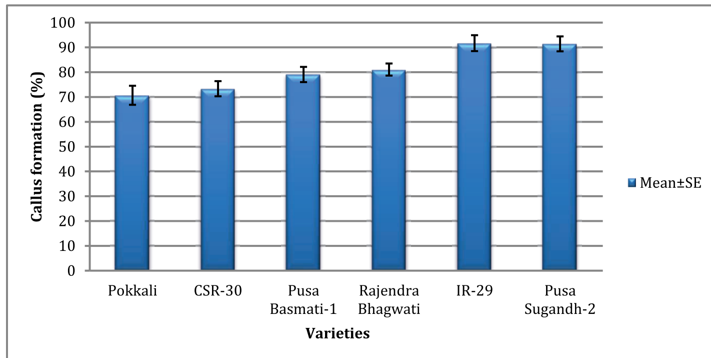


Fig. 7 Effect of varieties on the formation of callus (%) from cultured seeds

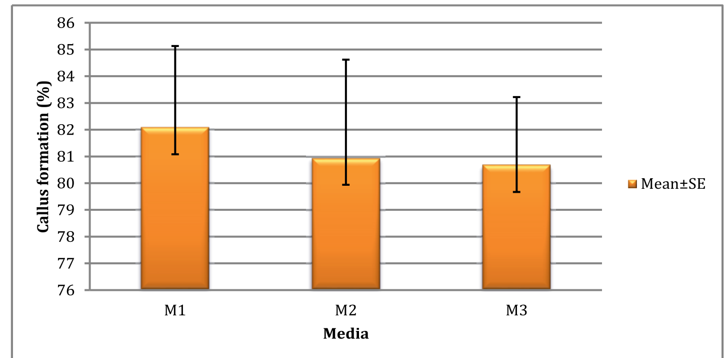
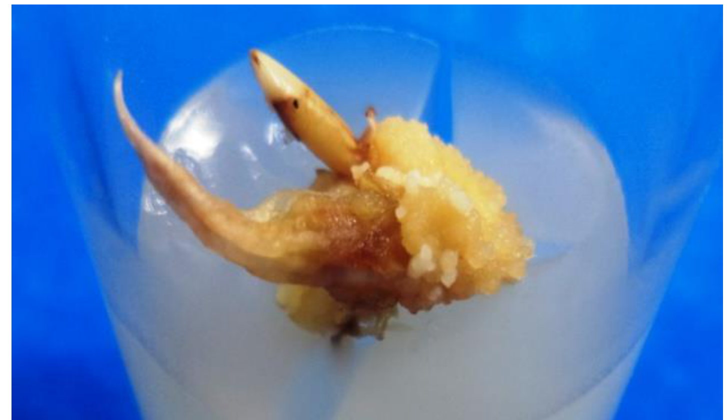


Fig. 8 Effect of media on the formation of callus percentage from cultured seeds

Pokkali (M1)



CSR-30 (M1)

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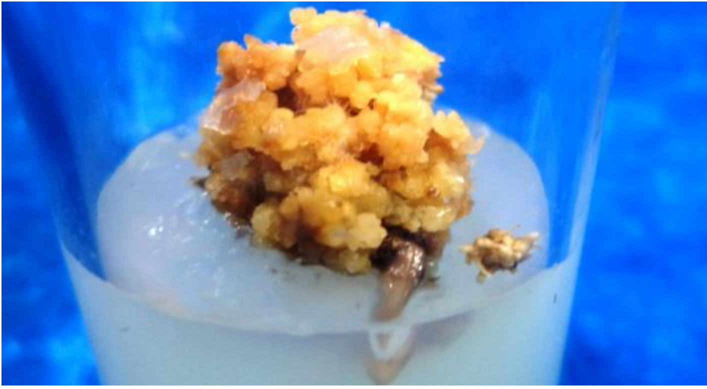
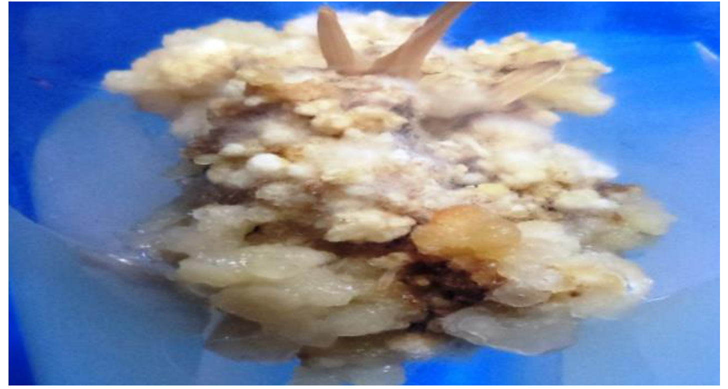


Pusa Basmati-1(M1)



Rajendra Bhagwati (M1)

IR-29 (M1)

Pusa Sugandh-2 (M1)

Rajendra Bhagwati (M8)

Rajendra Bhagwati (M9)

Fig. 6 Formation of callus from cultured seed of six rice varieties on M1 medium (MS + 2.50 mg/L 2,4-D) and sub-cultured callus formed from cultured seeds of variety Rajendra Bhagwati on regeneration media M8 and M9 showing shoot and root differentiation

Discussion

Dehusked rice seeds are recognized as favorable explants for *in vitro* seed germination and callus formation [6] [7] [8] [3] [9]. Thus, plant tissue culture techniques may be applied to study abiotic and biotic stress tolerance mechanisms in cereal plant accompanied rice [4]. It was found that more somaclonal variations as well as genetic variabilities occurred when cereal crops are directly regenerated via the callus stage [3]. In the present study, *in vitro* germination of seed and formation of callus percentage in doing tissue culture of rice varieties are impacted by numerous factors like source of dehusked rice seeds, the composition of media, genotype, and climate. The divergent conjunction of cytokinin as well as auxin along with the efficacy of basal components showed a prerequisite task for organogenesis and callogenesis from the cultured tissues [10] [11] [12]. Further, the M1 medium contained only 2,4-D and revealed the best formation of callus in rice. Remaining, six media namely, M2, M3, M4, M5, M6, and M7 had the highest 2,4-D (1.50- 2.50 mg/L) concentrations with the lowest KIN (0.50-1.00 mg/L) concentrations and hence these media were very appropriate for formation of callus due to presence of higher concentrations of 2,4-D because it is the most potent inducer of callus. It was concluded that the highest 2,4-D concentrations present in the medium enhanced the formation of callus and reduced germination of seed while the highest kinetin concentrations enhanced the germination of seed and reduced the formation of callus. Divergent combinations of phytohormones in MS basal media showed an effective role in rice plant regeneration [12] [13]. Thus, in the present research an efficient protocol was established to study callogenesis as

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well as organogenesis via caulogenesis and rhizogenesis in rice cereal crops. The availability of an efficient *in vitro* seed germination and callus formation protocol is a key prerequisite for doing well regeneration and transformation steps in any cereal crop. According to Azizi et al. [14], genetic improvement of rice varieties to make better desired characteristics which needs to have a highly reliable and well-organized *in vitro* seed culture strategy. The *in vitro* seed culture protocol provides source of materials that work as recipients of acquainted exogenous DNA [15].

Conclusion

Certain significant plant tissue culture approaches such as fusion and culture of protoplast, culture of leaf and root, culture of anther, unripe embryo culture as well as ripe seed culture are significant in rice cereal to generate extra genetic variability and finally leads to make better rice crop. The established protocol will be well-becoming for germination of seed and formation of callus *in vitro* in rice cereal for acquiring biotic and abiotic stress tolerance mechanism, genetic variability as well as somaclonal variation. Additionally, formed callus, shoot and root buds in rice crops may also be employed as prime explant for successful genetic transformation experiments.

Future scope of the study: The established protocol will be utilized to study biotic and abiotic stress tolerance mechanism, genetic variability as well as somaclonal variation in any cereal crop for their improvement.

Conflict of Interest: No conflict of interest amongst the authors.

Funding: No any funds or grants were received during this research work.

Acknowledgments: All authors thankfully acknowledge the of Deptt. of Plant Breeding & Genetics, DRPCAU, Pusa, India to give the seeds of rice varieties.

Authors' contribution: Conceptualization of research (RK, VKS, PK, HK); Designing of the experiments (RK, VKS, PK, HK); Analysis of data and interpretation (RK, VKS, PK, HK); Preparation of manuscript (RK, PK); Writing-Original draft preparation (RK, PK); Supervision (VKS, HK, AK); Writing-Review & Editing (RK, PK, HK, AK).

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