Optimization of in vitro culture media for improvement in yield of Navara ancient Indian medicinal rice

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Abstract

Medicinally important ancient Navara rice (GI Kerala, India 2007) is a very short duration (60–70 days) variety with a yield of only 0.5 tonnes/hectare costing ~Rs. 400/kg. It is used for indigenous treatment for chronic diseases by local and oral consumption. In this study, scutellum-derived calli were generated from mature Navara seeds and these were inoculated on different CIM-1 to CIM-5 media supplemented with 2.5 mg/l 2,4-dichlorophenoxyacetic acid. Regeneration of calli on different regeneration media RI, RII and RIII media were performed. Regeneration of 30-day-old calli on RI media showed 30%, for RII it showed no regeneration and on RIII media only 12% regeneration was obtained. The addition of glutamine and proline showed a 30–40% improvement in somatic embryogenesis. The 74–88% callus induction frequency was obtained on CIM-1 to CIM-5. The fresh weight (mg) of 30-day-old calli is CIM-2 < CIM-3 < CIM-4 < CIM-1 << CIM-5 and corresponding size shows CIM-2–CIM-3 < CIM-5 < CIM-1 < CIM-4. A negative correlation between the callus fresh weight and the regeneration efficiency was observed. In CIM-5, 20–25 days 3.4-fold increase and 25–30 days a 1.7-fold increase in fresh weight of calli is noted. The 20-day-old calli transfer to RI media shows 80% regeneration frequency and 6–7 plantlets/callus, which are twofold higher as compared with 30-day-old calli. The somatic embryogenesis and its regeneration on synthetic media provide an alternative for biotechnological intervention for yield improvement, in turn cost reduction for Navara rice.

Keywords Navara · Callus · Regeneration · Plant tissue culture

Introduction

The *Oryza sativa* ssp. *indica* variety Navara (GI Kerala, India 2007) is very early maturing (60–70 days) with medicinal properties (Rao et al. 2010; Shalini et al. 2012), provides the opportunity for sustainable agriculture. The ethnopharmacology of Navara includes ‘Navarakizhi’ treatment which is the immune-enhancing rejuvenation therapy, beneficial in muscular wasting, paralysis, rheumatism, back pain, high blood pressure and cholesterol, general weakness and certain skin problem (<http://www.dhanwantriayurved.com>). Also, the rice powder, root, bran and gruel of Navara used in the

treatment of psoriasis, osteoporosis cirrhosis of liver, mouth ulcer, urinary tract infection and piles treatment (<http://www.navara.in>). The antibacterial, anti-inflammatory and antioxidant activity of Navara grain and bran were reported (Rao et al. 2010; Shalini et al. 2012).

The biotechnological interventions by genetic transformation using miRNA technology would improve the yield without affecting the medicinal properties. For instance, the overexpression of miR397 shows an increase in grain size, 13.4% increase in 1000-grain weight and 20% increase in grain number on per main panicle leading to increase in yield by 25% (on field trial) in *O. sativa* ssp. *japonica* Zhonghua 11 variety (Zhang et al. 2013). The first step in the value addition requires the generation of somatic embryos (SEs). The development of SEs involves a tracheal system separation from a single cell of maternal tissue by the bipolar development of the shoot apical meristem/root apical meristem, which is similar to zygotic embryogenesis (Omar et al. 2016). The carbohydrate metabolisms, followed by energy, lipid and nucleotide metabolism pathway are significantly enriched during the somatic embryogenesis (Zhang et al.

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2017). The somatic embryogenesis has two phases: restructuring from somatic cells to embryogenic cells and regeneration of embryogenic cells in *in vitro* conditions (Yang and Zhang 2010). The SEs is an ultimate reflection of cell totipotency as well as an excellent model system for early stages of embryogenesis study (Nowak et al. 2015; Zimmerman 1993). It is a well established system for studying the crop improvement due to the well known illustration of cell totipotency, the dedifferentiation and redifferentiation through restructuring to generate embryogenic cells and to the regeneration of new plants (Yang and Zhang 2010). Generation of SEs from suitable explant and its regeneration are the first step towards genetic improvement and incorporation of the favourable trait. The pre-transformation phases for the genotype have vast effects of other factors on the plant's adaptation to *in vitro* experiments such as types of explants, age of explants, seasonal variation, oxygen gradient, light intensity, temperature and ploidy level can effect and may be changes in the genotype feedback in different experiments (Aggarwal et al. 2012).

In this study, we used different basal media (CIM-1 to CIM-5) supplemented with 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Different additives like proline, glutamine, tryptophan and casein hydrolysate were optimized for better somatic embryogenesis, and suitable media and additives for somatic embryogenesis were selected. The somatic embryogenesis was monitored for calli size and fresh weight at various time intervals. This is the first report wherein the age of the calli plays an important role in efficient regeneration and plantlet formation for Navara.

Materials and methods

Chemicals and plastic ware

The reagents were obtained from Himedia (India) and used for media preparation. The chemicals phytagel and phytohormones were purchased from Sigma-Aldrich (USA). The 90 mm disposable polystyrene Petri dishes (Tarsons, India) were used in this study.

Plant material and sterilization

The Navara mature seeds were obtained from KVK (Krishi Vigyan Kendra) Ernakulam, India. The seeds were pre-treated with 6% salt solution and these were then washed 5–10 times with water and air dried, before using them for culture. The seeds were then manually dehusked and subjected to surface sterilization as given in the schematic diagram Fig. 1.

Generation of scutellum-derived callus

The sterilized seeds were placed on a Petri dish containing CIM-1 to CIM-5 (Table 1). The constant 2.5 mg/l 2,4-D concentration was used in all type of CIM. Media used for the investigation includes CIM-1 containing MS basal (Murashige and Skoog 1962), CIM-2 containing B5 basal (Gamborg et al. 1968), CIM-3 containing L3 basal (Lin and Qifa 2005), CIM-4 containing N6 basal (Chu 1975) and CIM-5 containing DI basal (Lin and Qifa 2005). In addition, the proline (500 mg/l), glutamine (500 mg/l) (Lin and Qifa 2005; Pawar et al. 2015), tryptophan (100 mg/l) (Chowdhry et al. 1993) and casein hydrolysate (100 mg/l) (Anshika and Shukla 2015) in different combinations were added to CIM-1 for optimization of suitable combination. The pH of the media was adjusted to 5.8 before autoclaving and 25 ml sterilized media was poured in sterilized Petri dish. For callus development, seeds were incubated at 27 ± 2 °C as given in the schematic diagram Fig. 1.

Regeneration and plantlet formation of the somatic embryos

The 30-day-old healthy calli were transferred to three different regeneration media RI, RII and RIII (Shoeb et al. 2001; Sivakumar et al. 2010) (Table 1). Also, the 20 days, 25 days and 30-day-old calli were transferred to RI regeneration media. The regeneration plates were kept for 16 h light and 8 h dark conditions at 25 ± 2 °C with 80% relative humidity (RH). After shoot initiation, the regenerating calli were transferred to rooting media for plantlet formation (Fig. 1). The single rooting media were used in these studies, wherein the basal salts of CIM-1 were used (Table 1).

Software and statistical analysis used in the investigation

The mean and standard errors were calculated from three independent experiments. The pictures of the calli were taken by the Sony digital camera (16 megapixels) and the microscopic picture by Nikon SMZ-1000 (Kalve et al. 2014).

Measure™ 2.0 software

The diameters of calli were measured using Measure™ 2.0 software (<http://www.cthing.com/Measure.asp>). Only the healthy calli were considered, brown calli were accounted, but these were not included in the different sizes reported herein. On CIM-1 to CIM-5, the 15 days data were obtained after measuring 116, 133, 99, 129 and 54 number of calli,

Fig. 1 The schematic diagram of the procedure used for seed sterilization, callus induction, regeneration and plantlet formation of *Oryza sativa* var. Navara

respectively. The data for 30 days were obtained by measuring 63, 97, 61, 83 and 44 number of calli raised on CIM-1 to CIM-5, respectively.

The Unscramble X software

The correlation between calli fresh weight, size, CIF (callus induction frequency), browning, white/yellow/cream colour (WYC) and green spot frequency (GSF) was analysed using “The Unscramble X”, a tool for multiple variant analyses (Sinha et al. 2019) where the PC-1 (Y-axis) and PC-2 (X-axis) show the level of confidence by which we can say data are reliable. If it is more than 80% (cumulative), then it is more reliable and reproducible.

The formula for calculations for CIF

$$\text{CIF (\%)} = \frac{\text{Number of seeds exhibiting callus formation}}{\text{Number of seeds cultured}} \times 100$$

The formula for callus browning

$$\text{Browning (\%)} = \frac{\text{Number of callus brown}}{\text{Total number of callus}} \times 100$$

The formula for white/yellow/cream (WYC) colour callus

$$\text{WYC (\%)} = \frac{\text{Callus with white/yellow/cream colour}}{\text{Number of seeds cultured}} \times 100$$

The formula for green spot frequency (GSF)

$$\text{GSF (\%)} = \frac{\text{Number of callus exhibiting green spots}}{\text{Total callus transferred on regeneration media}} \times 100$$

The formula for regeneration frequency (RF)

$$\text{RF (\%)} = \frac{\text{Number of callus regenerated}}{\text{Total callus transferred on regeneration media}} \times 100$$

Table 1 Media composition (mg/l) for callus induction, regeneration and root development

Composition	CIM-1 ^a	CIM-2 ^b	CIM-3 ^c	CIM-4 ^d	CIM-5 ^c	RI ^e	RII ^f	RIII	Rooting ^e
Macronutrient components									
KNO ₃	1900	2500	1900	2830	2900	1900	1900	1900	950
NH ₄ NO ₃	1650	—	1700	—	—	1650	1650	1650	825
(NH ₄) ₂ SO ₄	—	134	—	463	450	—	—	—	—
KH ₂ PO ₄	170	150	170	400	400	170	170	170	85
MgSO ₄ ·7H ₂ O	370	250	370	185	185	370	370	370	185
CaCl ₂ ·2H ₂ O	440	150	400	166	170	440	440	440	220
Micronutrient components									
FeSO ₄ ·7H ₂ O	27.9	27	41	27.9	55.9	27.9	27.9	27.9	13.95
Na ₂ EDTA	37.3	37	55.9	37.3	74.5	37.3	37.3	37.3	18.65
MnSO ₄ ·4H ₂ O	22.3	10	100	4.4	100	22.3	22.3	22.3	11.15
ZnSO ₄ ·7H ₂ O	8.6	2	20	1.5	20	8.6	8.6	8.6	4.3
H ₃ BO ₃	6.2	3	30	1.6	30	6.2	6.2	6.2	3.1
KI	0.83	0.75	7.5	0.8	7.5	0.83	0.83	0.83	0.41
CuSO ₄ ·5H ₂ O	0.03	0.03	0.25	—	0.25	0.03	0.03	0.03	0.01
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	2.5	—	2.5	0.25	0.25	0.25	0.12
CoCl ₂ ·2H ₂ O	0.03	0.03	0.25	—	0.25	0.03	0.03	0.03	0.01
Organic components									
Glycine	2	—	2	2	2	2	2	2	1
Thiamine HCL	0.4	10	1	1	1	0.4	0.4	0.4	0.2
Pyridoxine HCL	0.5	1	1	0.5	1	0.5	0.5	0.5	0.25
Nicotinic acid	0.5	1	1	0.5	1	0.5	0.5	0.5	0.25
Inositol	100	100	100	100	100	100	100	100	50
Additional components									
Kinetin	—	—	—	—	—	—	—	1	—
BAP	—	—	—	—	—	3	0.5	—	—
NAA	—	—	—	—	—	1	—	—	—
Casein hydrolysate	—	—	—	—	—	300	—	—	—
L-Glutamine	500	500	500	500	500	—	—	—	—
Proline	500	500	500	500	500	2.5	—	—	—
Maltose	—	—	—	—	—	3%	—	—	—
Sucrose	3%	3%	3%	3%	3%	—	3%	3%	2%
2,4-D	2.5	2.5	2.5	2.5	2.5	—	—	—	—
Agar	—	—	—	—	—	—	0.70%	0.70%	—
Phytigel	0.25%	0.25%	0.25%	0.25%	0.25%	0.40%	—	—	0.40%

Callus induction media (CIM-1 to CIM-5) and regeneration media (RI to RIII)

^aMS (Murashige and Skoog 1962)

^bB5 (Gamborg et al. 1968)

^cL3 and DI (Lin and Qifa 2005)

^dN6 (Chu 1975)

^eSivakumar et al. (2010)

^fShoeb et al. (2001)

Results

Effect of the additional components to synthetic media in somatic embryogenesis and its regeneration

The media without any additional component CIM-1B

(negative control) show 20% higher CIF as compared to those obtained in the presence of all components in media CIM-1A (positive control) (Fig. 2). In the presence of single component, glutamine (CIM-1C) and casein hydrolysate (CIM-1F) show higher CIF as compared with the presence of proline and tryptophan. In the presence of two components, proline and glutamine (CIM-1G) show 57% CIF,

Fig. 2 Influence of additives with media (CIM-1) in callus induction frequency of Navara (15 days). The callus induction frequency (CIF) was determined on media containing A (Glutamine, Proline, Tryptophan and Casein hydrolysate), B (No additive), C (Glutamine), D (Proline), E (Tryptophan), F (Casein hydrolysate), G (Glutamine, Proline), H (Glutamine, Tryptophan), I (Glutamine, Casein hydro-

lysate), J (Proline Tryptophan), K (Proline, Casein hydrolysate), L (Tryptophan, Casein hydrolysate), M (Glutamine, Proline, Tryptophan), N (Proline, Tryptophan, Casein hydrolysate), O (Glutamine, Proline, Casein hydrolysate) and P (Glutamine, Tryptophan, Casein hydrolysate). Asterisk represents the significant $p < 0.001 = ***$, $p < 0.01 = **$ and $p < 0.05 = *$

which is significantly ($p < 0.01$) higher when compared to negative control. In the presence of three components, glutamine, tryptophan and casein hydrolysate (CIM-1P) showed better percentage of somatic embryogenesis. The presence of glutamine and proline in CIM-1 was effective in improvement of CIF when compared with other combinations (CIM-1A-F, I and M-P) (Fig. 2). To identify the additional supplement provided in CIM could play a role in regeneration, the 20-day-old calli obtained from the media CIM-1 (A to P) were transferred to RI regeneration media (Fig. 3). The media such as CIM-1B, CIM-1G, CIM-1N and CIM-1P showed 100, 80, 80 and 100% RF, respectively (Table 2). The higher 6–7 plantlet formation per callus was obtained for CIM-1G, CIM-1N and CIM-1P as compared to other combination (Table 2). These results suggest that supplementation of CIM influences CIF and RF in the *O. sativa* Navara genotype. The combination of proline and glutamine

showed higher CIF (57%) and RF (80%) with 6–7 plantlet formation per callus. The combination selected was added to CIM-1 to CIM-5 (Table 2).

Somatic embryogenesis in different callus induction media

The swelling in the Navara scutellar region is seen only after 3 days post-inoculation on CIM-1 to CIM-5. The CIF obtained on CIM-1 to CIM-5 used in this study showed 87, 86, 80, 88 and 74%, respectively (Table 3). The white/cream/yellow (WYC) colour calli referred to as healthy or embryogenic callus are 58–75%. After 15 days post-inoculation, these calli show 1.6–6.3% browning on CIM-1 to CIM-5 (Fig. 4, Supplementary Fig. 1, 2, 3, 4 and Table 3).

The calli raised on CIM-1 for 15 days, showed the semi-compact, irregular shape and were yellow in colour

Fig. 3 Influence of additives with media (CIM-1) in regeneration efficiency of calli of Navara after 20 days of transfer on RI media (Bars = 5 mm). Where A (Glutamine, Proline, Tryptophan and Casein hydrolysate), B (No additive), C (Glutamine), D (Proline), E (Tryptophan), F (Casein hydrolysate), G (Glutamine, Proline), H (Glutamine,

Tryptophan), I (Glutamine, Casein hydrolysate), J (Proline Tryptophan), K (Proline, Casein hydrolysate), L (Tryptophan, Casein hydrolysate), M (Glutamine, Proline, Tryptophan), N (Proline, Tryptophan, Casein hydrolysate), O (Glutamine, Proline, Casein hydrolysate) and P (Glutamine, Tryptophan, Casein hydrolysate)

(Supplementary Figure 1a). On the 30th day, the outer surface of the callus showed white globular and inner side appeared friable type and pale yellow in colour (Supplementary Figure 1b). The CIM-2, on the other hand, showed cream colour, compact type after 15 days post-inoculation (Supplementary Figure 2a). These calli 30 days post-inoculation turned to yellow colour became friable and became irregular in shape (Supplementary Figure 2b). The calli raised on CIM-3 (Fig. 4a) and CIM-5 (Supplementary Figure 3a) were light yellow colour, semi-compact and also irregular in shape after 15 days post-inoculation. The 30-day-post-inoculation calli were deep yellow and showed an uneven distribution of dividing cells (Fig. 4b and Supplementary Figure 3b). Very few > 2.5% of the calli developed necrotic areas originating from the tip of the root leading to the browning of whole callus (Fig. 4a, b and Table 3). However, the calli obtained on CIM-4 showed light yellow colour and were compact in structure (Supplementary

Fig. 4a). These calli were on 30 days post-inoculation and showed semi-compact mass with distinct air pocket formation in the calli and with no change in colour (Supplementary Figure 4b).

The fresh weight of calli obtained after 10 days, 15 days, 20 days, 25 days and 30 days post-inoculation on CIM-1 to CIM-5 is given in Table 4. The increasing order of fresh weight of calli is CIM-5 < CIM-3 < CIM-4 < CIM-2 < CIM-1 for 15 days post-inoculation. Also, a change in trend for 30 days post-inoculation shows CIM-2 < CIM-3 < CIM-4 < CIM-1 < CIM-5 (Supplementary Fig. 5). This result suggests the concentration of media salts along with time post-inoculation influences on the fresh weight of calli.

Callus size

The diameter (mm) of calli obtained on 15 days on CIM-1 to CIM-5 was 6.2 ± 1.0 , 6 ± 0.9 , 5.3 ± 0.6 , 4.8 ± 0.7 and

Table 2 Influence of additives with media (CIM-1) in regeneration efficiency of Navara

Callus induction media	Regeneration frequency (%)	Plantlet/calli
CIM-1A (proline, glutamine, tryptophan, casein hydrolysate)	20	3
CIM-1B	100	2–4
CIM-1C (glutamine)	40	2
CIM-1D (proline)	50	2–3
CIM-1E (tryptophan)	0	–
CIM-1F (casein hydrolysate)	66	2
CIM-1G (glutamine, proline)	80	6–7
CIM-1H (glutamine, tryptophan)	0	–
CIM-1I (glutamine, casein hydrolysate)	20	2
CIM-1J (proline, tryptophan)	0	–
CIM-1K (proline, casein hydrolysate)	22	1
CIM-1L (casein hydrolysate, tryptophan)	0	–
CIM-1M (proline, glutamine, tryptophan)	50	2
CIM-1N (proline, tryptophan, casein hydrolysate)	80	6–7
CIM-1O (proline, glutamine, casein hydrolysate)	33	1
CIM-1P (glutamine, tryptophan, casein hydrolysate)	100	6–7

The different additives glutamine, proline, tryptophan and casein hydrolysate used either alone or in combinations in CIM-1 media supplemented with 2.5 mg/l 2,4-D, 3% sucrose and 0.25% phytigel

Table 3 Comparison of callus induction frequency, browning and green spots frequency of Navara

Media	Callus induction frequency (%; CIF)	Callus white/yellow/cream (%; WYC)	Callus browning (%; CBW)	Green spot frequency (%; GSF)
CIM-1	87.2 ± 1.4	58.2 ± 0.6	2.7 ± 0.3	46.0 ± 6.7
CIM-2	86.7 ± 1.7 ^{ns}	74.9 ± 1.1 ^{**}	1.6 ± 0.1 ^{ns}	53.0 ± 6.7 ^{ns}
CIM-3	80.7 ± 2.6 ^{**}	71.5 ± 1.0 ^{ns}	2.1 ± 0.2 ^{ns}	60.0 ± 0.0 ^{ns}
CIM-4	88.5 ± 1.9 ^{ns}	72.0 ± 0.7 ^{ns}	6.3 ± 0.4 ^{ns}	46.0 ± 6.7 ^{ns}
CIM-5	74.0 ± 4.0 ^{**}	68.0 ± 1.0 ^{**}	1.6 ± 0.2 ^{ns}	26.0 ± 6.7 ^{ns}

The callus induction frequency and browning of calli raised on CIM-1, CIM-2, CIM-3, CIM-4 and CIM-5. The regeneration was evaluated on RI media. Data represent mean ± SE. ns = non significant, Asterisk (*) $p < 0.05$ and (**) $p < 0.02$ with respect to CIM-1 media value

5.1 ± 0.9, respectively (Fig. 5a). After 30 days post-inoculation, this diameter on CIM-1 to CIM-5 is 9.5 ± 1.1, 8.2 ± 1.1, 8.5 ± 1.1, 11.2 ± 1.7 and 8.8 ± 1.3, respectively (Fig. 5b). The measurement of the diameter indicates maximum growth of calli in CIM-4 where 1.7 times the diameter corresponding to calli raised on CIM-2 is noted for 30 days old calli. The distribution of the size of healthy calli generated on different CIM-1 to CIM-5 shows CIM-3 with minimal size variations, and a maximum diameter of 5.9 mm, 9.6 mm and minimum diameter of 4.7 mm, 7.4 mm were obtained after 15 days and 30 days post-inoculation, respectively (Fig. 5). After 30 days, the calli with the distribution of size ranging from 8.4 mm to 10.6 mm for CIM-1, and 7.1 mm to 9.3 mm for CIM-2 show minimal variation in size.

Callus regeneration

The calli (30 days old) obtained on CIM-1 were transferred to regeneration media RI, RII and RIII, and RI showed 30% RF and 1–4 plantlets per callus which are higher than RII and RIII media (Table 5). For further experiments, RI regeneration media were used. The emergence of green spots was seen on 7–10 days post-transfer to RI regeneration media for calli raised on CIM-1 (Supplementary Figure 1c), CIM-2 (Supplementary Fig. 2c), CIM-3 (Fig. 4c), CIM-4 (Supplementary Figure 4c) and CIM-5 (Supplementary Figure 3c). The calli raised in CIM-1 to CIM-5 after 30 days old transfer to RI regeneration media; the GSF after 30 days post transfer showed 46, 53, 60, 46 and 26%, respectively (Table 3, Fig. 4d, Supplementary Figures 1d, 2d, 3d and 4d). In addition to that

Fig. 4 Scutellum-derived callus on CIM-3 media. **a** After 15 days post-inoculation is seen as semi-compact, irregular shape and are light in yellow colour, **b** after 30 days post-inoculation loose compact are dark yellow colour. **c** In regeneration condition after 7 days

post transfer, green spots were seen, **d** after 30 days post transfer, the regenerating calli show browning along with greening. Bar = 30 mm in **a**, 40 mm in **b** and **d** and 1 mm in **c**

Table 4 Comparison of fresh weight (mg) of callus in different media

	Day 10	Day 15	Day 20	Day 25	Day 30
CIM-1	120.0 ± 5.8	169.0 ± 5.2**	383.3 ± 6.7**	423.3 ± 14.5**	707.0 ± 6.9**
CIM-2	90.6 ± 4.7	196.6 ± 2.7**	358.6 ± 2.0**	508.0 ± 4.4**	577.0 ± 4.4**
CIM-3	46.6 ± 3.5	124.6 ± 2.4**	331.0 ± 1.0**	570.6 ± 3.8**	614.6 ± 8.2**
CIM-4	61.6 ± 1.8	197.0 ± 4.0**	270.0 ± 11.5**	454.3 ± 3.5**	630.6 ± 6.4**
CIM-5	30.0 ± 2.9	190.6 ± 2.4**	216.6 ± 13.3**	741.6 ± 3.8**	1270.0 ± 43.6**

The changes in fresh weight (mg) of callus on CIM-1 to CIM-5 supplemented with 2.5 mg/l 2,4-D, 500 mg/l proline, 500 mg/l glutamine, 3% sucrose and 0.25% phytigel. Data represent mean ± SE of 50 callus. Asterisk (**) $p < 0.02$ with respect to 10 days post inoculation of each CIM

Fig. 5 The diameter of the calli (mm) of Navara measured by Meazure™ 2.0 software after **a** 10 days, **b** 15 days post-inoculation raised on CIM-1, CIM-2, CIM-3, CIM-4 and CIM-5 supplemented with

2.5 mg/l 2,4-D, 3% sucrose, 500 mg/l proline and 500 mg/l glutamine and incubated at 27 ± 2 °C, 80% RH, in dark

Table 5 Comparison of regeneration frequency of Navara

Regeneration media	Age of calli (days)	Regeneration frequency (%)	Plantlet/callus
RI	20	80	6–7
	25	40	3–6
	30	30	1–4
RII	30	—	—
RIII	30	12	1

The calli raised on CIM-1 with 2.5 mg/l 2,4-D, 3% sucrose, 500 mg/l proline and 500 mg/l glutamine and incubated at 27 ± 2 °C, 80% RH, in dark

the statistical analysis using “The Unscramble X” shows a negative correlation of calli fresh weight with GSF and CIF (Fig. 6). A positive correlation between CIF and GSF of Navara in different CIM (1–5) and RI media is obtained.

The influence of age of calli on regeneration efficiency was monitored by transferring calli of ages: 20 days, 25 days and 30 days to RI regeneration media (Fig. 7). It may be noted that 80, 40 and 30% RF in RI media for transferred calli of 20 days, 25 days and 30 days was noted (Table 5). The plantlet formation is twice the number 6–7 plantlets/callus when calli of 20 days were transferred as against 25 days where 3–6 plantlets/callus and 30 days where 1–4 plantlets/callus were obtained in rooting media conditions (Table 5).

Discussion

The callus development shows an initial slow rate of cell division in *O. sativa* ssp. *indica* Navara variety and no visible changes are noted till 3 days post-inoculation when

compared to our other varieties studies like CR1009, IW Ponni, BPT5204, ADT43, TKM9, ADT39, KKLR, CO(R)48 and Savitri/Ponmani (Dhamangaonkar and Shukla 2013). In this study, the scutellum-derived 75–80% calli were obtained on CIM-1 to CIM-5 where the amount of auxin was only 2.5 mg/l of 2,4-D (Table 3) as reported earlier in CR1009, IW Ponni, BPT5204, ADT43, TKM9, ADT39, KKLR, CO(R)48, Savitri/Ponmani and IR64 rice varieties (Dhamangaonkar and Shukla 2013; Karthikeyan et al. 2009; Sahoo and Tuteja 2012). We did not experiment with higher amount of auxin as the higher concentration of 2,4-D has a tendency to decrease the CIF (Verma et al. 2011).

The calli of Navara obtained on media using additives proline, glutamine, casein hydrolysate and tryptophan in different combination to CIM-1 showed best results for 500 mg/l proline and 500 mg/l glutamine as 57% CIF (Fig. 2) with 80% RF and 6–7 plantlets/callus formation (Table 2). Earlier studies also suggest that the presence of glutamine and proline in callus induction media influences the somatic embryogenesis, as well as regeneration efficiency (Chowdhry et al. 1993; Ge et al. 2006; Shahsavari 2011). Also, Pawar et al. (2015) found that glutamine and proline significantly increase in CIF along with the fresh weight in *indica* varieties like Jaya, Indrayani and Ambemohar. These authors suggest that the additives glutamine and proline in media could facilitate an increased cell growth. Also, these amino acids provide necessary nitrogen to the growing cells in callus. In addition, the proline and glutamine are relatively non-toxic and facilitate increase in the rate of cell growth for a longer period of incubation (Pawar et al. 2015). To facilitate development of healthy calli in Navara, 500 mg/l proline and 500 mg/l glutamine are added to CIM-1 to CIM-5.

Fig. 6 Correlation between the fresh weight of callus and regeneration efficiency using first two components from PCA approaches. **a** Classification of CIM-1 to CIM-5 on the basis of calli fresh weight,

size, CIA (callus induction ability), CBW (callus browning), WYC (white/yellow/cream colour callus) and GSF (green spot frequency) **b** correlation loading of calli fresh weight and CIA on GSF

The selection of media for this investigation broadly includes the preferred synthetic media for scutellum-derived callus, MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), L3 (Lin and Qifa 2005), N6 (Chu 1975) and D1 (Lin and Qifa 2005) (Table 1). We used these media to identify their influence on the growth of calli from the scutellum of Navara mature seeds. The scutellum produces a large number of the actively dividing cell which can convert into the embryogenic calli, therefore it is considered to be the best source of cells for in vitro culture (Wani et al. 2011). Also, mature seed is an excellent source as it could be stored for longer period and maintains homogeneous populations of explants (Ge et al. 2006).

The scutellum-derived callus of Navara rice showed efficient CIF in CIM-1 to CIM-5, and CIM-3 showed 80.7% CIF and 60% GSF (Table 3). The least GSF was obtained in CIM-5 where only 26% GSF is obtained. The relative $\text{NH}_4^+/\text{NO}_3^-$ ratios for CIM-1 to CIM-5 are 0.52, 0.08, 0.53, 0.25 and 0.24, respectively. The somatic embryogenesis and regeneration efficiency that show association with $\text{NH}_4^+/\text{NO}_3^-$ ratios are reported to provide a better result (Menke-Milczarek and Zimny 2001). The best calli which produce green spots are for CIM-3 where $\text{NH}_4^+/\text{NO}_3^-$ is 0.53. The calli of Navara obtained white or cream compact (type I) and yellow organized (type II) on CIM-1 to CIM-5 (Fig. 3 and Supplementary Figures 1–4) (Visarada et al. 2002), and it becomes friable with air pockets (Supplementary Figure 4b). The *indica* rice varieties have been divided into recalcitrant (4–7 plantlets/callus), moderately recalcitrant (9 plantlets/callus) and non-recalcitrant (16–18 plantlets/callus) (Shoeb

et al. 2001). We obtained maximum 6–7 plantlets/callus for Navara in RI media after 30 days incubation on regeneration conditions. These results suggest that Navara variety is categorized as recalcitrant rice variety.

The size of calli obtained in different media in increasing order CIM-4 < CIM-5 < CIM-3 < CIM-2 < CIM-1 for 15 days post-inoculation. On 30th days post-inoculation, the calli size are in increasing order CIM-2 < CIM-3 < CIM-5 < CIM-1 < CIM-4 (Fig. 5). Our result for Navara calli size (30 days post-inoculation) is comparable with *O. sativa* L. cv. Khao Dawk Mali 105 (3 weeks old) calli size as 9.3 mm (Chutipaijit and Sutjaritvorakul 2018). The changes in fresh weight (mg) of the calli with time on the different CIM were noted. The 10-day-old calli showed higher weight for CIM-1 and CIM-2. The CIM-1, CIM-2, CIM-4 and CIM-5 show near to same fresh weight while the calli on CIM-3 have lower weight on 15 days post-inoculation (Table 4). The difference in 30-day-old calli showed differences of more than twofold for CIM-2 and CIM-5. It may be noted that the average fresh weight of calli is higher in CIM-5 raised calli (30 days old) (Supplementary Figure 5). The regeneration efficiency was only 26%, thereby suggesting that the weight of callus of Navara is inversely correlated with regeneration efficiency (Table 3). The increase in fresh weight could be due to the presence of more water (Manimaran et al. 2013). The efficient regeneration is achieved in for calli raised on CIM-3 where the size of calli is 5.3 mm and 8.5 mm for 15 days and 30 days post-inoculation (Fig. 5). The weight of calli is 46.6 mg, 124.6 mg, 331.0 mg, 570.6 mg and 614.6 mg

Fig. 7 Response of early transfer of calli is in RI regeneration media. The different aged (20-days, 25-days and 30-days) callus transferred to RI regeneration media (Bar=2 mm). The callus raised on CIM-1

supplemented with 2.5 mg/l 2,4-D, 3% sucrose, 500 mg/l proline and 500 mg/l glutamine and incubated at 27 ± 2 °C, 80% RH, in dark

on 10 days, 15 days, 20 days, 25 days and 30 days post-inoculation, respectively (Table 4).

The percentage of regeneration frequency is dependent on the interaction of callus with regeneration media salts and phytohormones (Lin and Qifa 2005). The ratio of phytohormones auxin and cytokinins in regeneration media is a key factor for shoot differentiation in *indica* rice (Abiri et al. 2017). The regeneration in Navara shows a preference for 6-benzylaminopurine (BAP) than kinetin as seen earlier in *indica* rice varieties CR1009 (Anshika and Shukla 2015; Sinha et al. 2019). Also, minor concentration 0.1–0.5 mg/l of 1-naphthaleneacetic acid (NAA) along with BAP improved regeneration efficiency earlier (Lin and Qifa 2005). The 2 mg/l BAP and 0.5 mg/l NAA presence in regeneration media resulted in improvement in regeneration efficiency for *O. sativa* ssp. *indica* varieties Pawana, Jaya, Indrayani and Ambemohar (Pawar et al. 2015). For Navara, 30-day-old calli raised on CIM-1 transferred to RI media (CIM-1,

2 mg/l BAP and 1 mg/l NAA) resulted in 30% regeneration frequency (Table 5).

In this investigation, young calli were transferred to regeneration media where regeneration efficiency showed 50% improvement (Table 5). Earlier 18-day-old calli transfer to regeneration media enhanced regeneration efficiency and reduction in the time period of plantlet regeneration from 3 to 1 month was reported in *japonica* rice cultivar Nipponbare (Toki et al. 2006). Also, 6-day-old scutellum-derived calli of *indica* rice BPT 5204 show better regeneration (Manimaran et al. 2013). We found that 20-day-old calli transfer showed 80% RF which is higher when compared with 25 days and 30 days transferred calli to RI media (Fig. 7 and Table 5).

In addition to that the statistical analysis using “The Unscramble X” shows a negative correlation of calli fresh weight with GSF and CIF (Fig. 6). A positive correlation between CIF and GSF of Navara in different CIM-1

to CIM-5 and RI media is noted. The negative correlation between the GSF and calli fresh weight suggests that SEs (< 20 days) of Navara richer in totipotent cells as compared with the 25 days and 30 days post-inoculation have large diameter of callus (Fig. 7). The statistical analysis confirms young callus transfer to regeneration media for better regeneration efficiency.

This study provides evidences for the scutellum-derived callus, suitable CIM and the correlation of calli fresh weight and age of callus with regeneration efficiency for Navara. The regeneration media and suitable age of calli transfer to regeneration media are highlights of the paper. A procedure for genetic transformation for the high cost (~Rs. 400/kg), poor yield and very early maturing Navara *indica* rice is provided. The yield improvement by biotechnological interventions is feasible by scutellum-derived callus and its regeneration. The transformation could be initiated with miR397 and overexpression resulted in an increasing grain yield in rice by 25% (Zhang et al. 2013).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Abiri R et al (2017) Enhancing somatic embryogenesis of Malaysian rice cultivar MR219 using adjuvant materials in a high-efficiency protocol. *Int J Environ Sci Technol* 14:1091–1108. <https://doi.org/10.1007/s13762-016-1221-y>
- Aggarwal D, Kumar A, Sharma J, Reddy MS (2012) Factors affecting micropropagation and acclimatization of an elite clone of *Eucalyptus tereticornis* Sm. *In Vitro Cell Dev Biol Plant* 48:521–529. <https://doi.org/10.1007/s11627-012-9446-z>
- Anshika S, Shukla LI (2015) Identification of essential constituents for development of embryogenic non-recalcitrant calli from recalcitrant indica rice variety CR1 and ASD16. *GSTF e-J ICT*. https://doi.org/10.5176/2251-2489_biotech15.11
- Chowdhry CN, Tyagi AK, Maheshwari N, Maheshwari SC (1993) Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L. cv. Pusa 169). *Plant Cell Tiss Organ Cult* 32:357–361. <https://doi.org/10.1007/bf00042300>
- Chu CC (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci Sin* 18:659–668. <https://doi.org/10.1360/ya1975-18-5-659>
- Chutipaijit S, Sutjaritvorakul T (2018) Application of activated charcoal and nanocarbon to callus induction and plant regeneration in aromatic rice (*Oryza sativa* L.). *Chem Spec Bioavailab* 30:1–8. <https://doi.org/10.1080/09542299.2017.1418184>
- Dhamangaonkar SN, Shukla LI (2013) Identification of callus induction potential of nine different genotypes of *indica* rice. *J Environ Nanotechnol* 2:44–52. <https://doi.org/10.13074/jent.2013.06132013>
- Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50:151–158. [https://doi.org/10.1016/0014-4827\(68\)90403-5](https://doi.org/10.1016/0014-4827(68)90403-5)
- Ge X, Chu Z, Lin Y, Wang S (2006) A tissue culture system for different germplasms of *indica* rice. *Plant Cell Rep* 25:392–402. <https://doi.org/10.1007/s00299-005-0100-7>
- Kalve S, Fotschki J, Beeckman T, Vissenberg K, Beemster GT (2014) Three-dimensional patterns of cell division and expansion throughout the development of *Arabidopsis thaliana* leaves. *J Exp Bot* 65:6385–6397. <https://doi.org/10.1093/jxb/eru358>
- Karthikeyan A, Pandian STK, Ramesh M (2009) High frequency plant regeneration from embryogenic callus of a popular *indica* rice (*Oryza sativa* L.). *Physiol Mol Biol Plants* 15:371–375. <https://doi.org/10.1007/s12298-009-0042-6>
- Lin YJ, Qifa Z (2005) Optimising the tissue culture conditions for high efficiency transformation of *indica* rice. *Plant Cell Rep*. <https://doi.org/10.1007/s00299-004-0843-6>
- Manimaran P et al (2013) Infection of early and young callus tissues of *indica* rice BPT 5204 enhances regeneration and transformation efficiency. *Rice Sci* 20:415–426. [https://doi.org/10.1016/S1672-6308\(13\)60153-5](https://doi.org/10.1016/S1672-6308(13)60153-5)
- Menke-Milczarek I, Zimny J (2001) NH_4^+ and NO_3^- requirement for wheat somatic embryogenesis. *Acta Physiol Plant* 23:37–42. <https://doi.org/10.1007/s11738-001-0020-2>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nowak K, Wójcikowska B, Gaj MD (2015) ERF022 impacts the induction of somatic embryogenesis in *Arabidopsis* through the ethylene-related pathway. *Planta* 241:967–985. <https://doi.org/10.1007/s00425-014-2225-9>
- Omar AA, Dutt M, Gmitter FG, Grosser JW (2016) Somatic embryogenesis: still a relevant technique in citrus improvement. In: Germana MA, Lambardi M (eds) *In vitro embryogenesis in higher plants*. Springer, New York, pp 289–327. https://doi.org/10.1007/978-1-4939-3061-6_13
- Pawar B, Kale P, Bahurupé J, Jadhav A, Kale A, Pawar S (2015) Proline and glutamine improve in vitro callus induction and subsequent shooting in rice. *Rice Sci* 22:283–289. <https://doi.org/10.1016/j.rsci.2015.11.001>
- Rao AS, Reddy SG, Babu PP, Reddy AR (2010) The antioxidant and antiproliferative activities of methanolic extracts from Njavara rice bran. *BMC Complement Altern Med* 10:4. <https://doi.org/10.1186/1472-6882-10-4>
- Sahoo RK, Tuteja N (2012) Development of Agrobacterium-mediated transformation technology for mature seed-derived callus tissues of *indica* rice cultivar IR64. *GM Crops Food* 3:123–128. <https://doi.org/10.4161/gmcr.20032>
- Shahsavari E (2011) Impact of tryptophan and glutamine on the tissue culture of upland rice. *Plant Soil Environ* 57:7–10. <https://doi.org/10.17221/116/2010-pse>
- Shalini V, Bhaskar S, Kumar KS, Mohanlal S, Jayalekshmy A, Helen A (2012) Molecular mechanisms of anti-inflammatory action of the flavonoid, tricetin from Njavara rice (*Oryza sativa* L.) in human peripheral blood mononuclear cells: possible role in the

- inflammatory signaling. *Int Immunopharmacol* 14:32–38. <https://doi.org/10.1016/j.intimp.2012.06.005>
- Shoeb F, Yadav JS, Bajaj S, Rajam MV (2001) Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism in different genotypes of *indica* rice. *Plant Sci* 160:1229–1235. [https://doi.org/10.1016/S0168-9452\(01\)00375-2](https://doi.org/10.1016/S0168-9452(01)00375-2)
- Sinha A, Solanki M, Shukla LI (2019) Evidences for differential expression of miR167d-5p, target, positional nucleotide preference, and its role in somatic and different stages of regenerating calli of *Oryza sativa*. *Plant Cell Tiss Organ Cult* 136:537–548. <https://doi.org/10.1007/s11240-018-01535-w>
- Sivakumar P, Law YS, Ho CL, Harikrishna JA (2010) High frequency plant regeneration from mature seed of elite, recalcitrant Malaysian *indica* rice (*Oryza sativa* L.) CV. MR 219. *Acta Biol Hung* 61:313–321. <https://doi.org/10.1556/abiol.61.2010.3.7>
- Toki S, Hara N, Ono K, Onodera H, Tagiri A, Oka S, Tanaka H (2006) Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *Plant J* 47:969–976. <https://doi.org/10.1111/j.1365-313x.2006.02836.x>
- Verma D, Joshi R, Shukla A, Kumar P (2011) Protocol for in vitro somatic embryogenesis and regeneration of rice (*Oryza sativa* L.). *Indian J Exp Biol* 49:958–963. <http://nopr.niscair.res.in/handle/123456789/7353>
- Visarada KBRS, Sailaja M, Sarma NP (2002) Effect of callus induction media on morphology of embryogenic calli in rice genotypes. *Biol Plant* 45:495–502. <https://doi.org/10.1023/a:1022323221513>
- Wani SH, Sanghera GS, Gosal SS (2011) An efficient and reproducible method for regeneration of whole plants from mature seeds of a high yielding *indica* rice (*Oryza sativa* L.) variety PAU 201. *N Biotechnol* 28:418–422. <https://doi.org/10.1016/j.nbt.2011.02.006>
- Yang X, Zhang X (2010) Regulation of somatic embryogenesis in higher plants. *Crit Rev Plant Sci* 29:36–57. <https://doi.org/10.1080/07352680903436291>
- Zhang YC et al (2013) Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. *Nat Biotechnol* 31:848–852. <https://doi.org/10.1038/nbt.2646>
- Zhang J, Xue B, Gai M, Song S, Jia N, Sun H (2017) Small RNA and transcriptome sequencing reveal a potential miRNA-mediated interaction network that functions during somatic embryogenesis in *Lilium pumilum* DC. *Fisch. Front Plant Sci* 8:566. <https://doi.org/10.3389/fpls.2017.00566>
- Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. *Plant Cell* 5:1411–1423. <https://doi.org/10.1105/tpc.5.10.1411>