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Research Article

Optimization of *in vitro* culture system in tropical *Japonica* and *Indica* rice (*Oryza sativa* L.) genotypes

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Abstract
The plant growth regulator (PGR) effects on callus induction frequency (CIF) and plantlet regeneration were studied on four rice genotypes. Mature seeds were used as explant for callus induction in different combinations of 2, 4-D, Kn and NAA using MS and N6 medium. The maximum CIF recorded was 86.0% (Azucena) and 83.3% (Palwan) in N6 media augmented with 2,4-D, Kn and NAA at 2.5, 0.5 and 1.0 mg/L, respectively. In genotypes CB174R and CB87R, 66.7% and 71.6% CIF were obtained in N6 media supplemented with 2,4-D, Kn and NAA at 2.0, 0.25 and 0.5 mg/L, respectively. In MS medium a total plantlet regeneration of 70% was observed in Palwan and 10% in CB174R augmented with NAA (0.5 mg/L) + BAP (1.0 mg/L) + Kn (0.5 mg/L) and NAA (0.5, 1.0 mg/L) + BAP (0.5, 1.0 mg/L) + Kn (0.5, 2.0 mg/L), respectively. The efficiency of plantlet regeneration depended on genotype, media constituents and PGR.

Keywords: Rice, Embryo culture, callus induction, regeneration, callogenesis, rhizogenesis

INTRODUCTION

Rice (*Oryza sativa* L.) is the second most cultivated crop around the world and serves as a staple food for over 2.7 billion people. With increasing world population, demand for food production is increasing and developing countries are struggling to achieve food security. There are several genetic and environmental factors affecting the productivity and quality of rice (Azmi *et al*., 2012; Bzour *et al*., 2018; Zulkarnain *et al*., 2013). To sustain with the projected future rice productivity of 852 million metric tonnes in 2030 from 756 million metric tonnes (2021) (Noor *et al.,* 2022), current crop improvement techniques have to be improved and upgraded in order to bridge this productivity gap. Combining traditional breeding approaches with biotechnological tools like tissue culture and genetic engineering will address the gap between the consumption and productivity (Bajaj and Mohanty, 2005, Tai 2002). New genetic variants with high productivity, superior quality, resistance to biotic and abiotic stresses can be obtained by combining conventional and *in vitro* culture methods. They can significantly accelerate the genetic gains, ultimately increasing production and productivity (Mostafiz and Wagiran, 2018).

Using *in vitro* method, various varieties have been improved in different crops and rice is highly amenable to *in vitro* manipulation. In any crop, the critical phase in adapting the biotechnological tools for yield improvement is the availability of protocol for generating the *in vitro*

plants. The success of the biotechnological tools depends on the callus formation and further regeneration from the calli. Under various growth stimulating factors, explants initiate callus as an undifferentiated cell. Then eventual regeneration of plant is needed further. The factors like genotype of the plant, type of explant used, composition of basal medium, concentration of plant growth regulators, culture environment and growth stages of explants decide the success of the *in vitro* culture (Abiri *et al*., 2017). Past studies have shown the presence of recalcitrance in *indica* varieties, which affects the callusing and regeneration ability in *indica* types in comparison to *japonica* genotypes. In rice, most of the *indica* genotypes have been reported to be less receptive to genetic alterations through *in vitro* methods than *japonica* lines, due to their poor callusing and low regenerating potential (Mostafiz and Wagiran, 2018).

For somatic embryogenesis, MS media is generally employed. However, N6 media is specifically used for *japonica* rice varieties. Several studies have reported that the composition of basal nutrients in the media will have an influence on the *in vitro* culture, callus induction and regeneration potential (Ge *et al*., 2006; Abiri *et al*., 2017). Previous callus induction studies in rice revealed the potential of mature embryo for callogenesis compared to other explants (Lee *et al*., 2003, Carsono and Yoshida, 2006). In general, the development of protocols is limited to genotype and not widely adapted. So, there is demand for improving each cultivar with callus induction and regeneration protocols (Mostafiz and Wagiran, 2018 and Tripathy, 2021).

Thus, in order to develop genotype specific protocol for callus induction and regeneration, an attempt was made in the present study to optimize the concentration of plant growth regulators for callus induction and plant regeneration in two tropical *japonica* genotypes *viz*., Palwan, Azucena and two *indica* genotypes *viz*., CB174R, CB87R. The developed protocol can be utilized in future for genetic modification and crop improvement.

MATERIALS AND METHODS

To standardize the callus induction and regeneration, mature healthy seeds from two tropical *japonica* rice genotypes *viz*., Palwan and Azucena and two *indica* genotypes CB174R and CB87R were used. The dehulled mature seeds were initially surface sterilized using 70% ethanol (v/v) for two minutes followed by immersion with 0.1% mercuric chloride for five minutes. Afterwards, the seeds were immersed in 2% sodium hypochlorite with a drop of Tween-20 for 10 minutes. Finally, the seeds were rinsed several times with sterile distilled water to remove the adhered chemical residues. The excess water was drained and surface sterilized seeds were dried using sterile tissue paper before inoculation.

The explants sterilized were cultured on MS basal medium (Murashige and Skoog, 1962) and also in N6 basal medium (Chu, 1975) with B5 vitamins (Gamborg *et al*., 1968) augumented with different concentrations of growth regulators for callus induction. The plant growth regulators used were 2,4-dichloro phenoxy acetic acid (2,4-D- 2.0 and 2.5 mg/L), Kinetin (Kn- 0.25 and 0.5 mg/L) and Naphthalene acetic acid (NAA- 0.5 and 1.0 mg/L). Agar at a concentration of 8g/L and sucrose at 30g/L were added to the medium.

The pH was adjusted to 5.8 and autoclaved (121℃ at 15psi or 103.4kPa for 20 min). In both the media, for each treatment, 20 seeds were inoculated in petri plates and replicated thrice. The inoculated cultures were incubated in dark at 25±1℃ under aseptic conditions. After 10 days, callus growth was observed in the scutellum portion of the seeds. Days taken for callus initiation was recorded and Callus Induction Frequency (CIF%) after three weeks of inoculation was worked out.

Callus Induction Frequency (%) =

$$\frac{\text{No. of seed explants responded for callus induction}}{\text{Total number of seed explants inoculated}} \times 100$$

Then the calli obtained from the scutellum region were excised and transferred to a fresh medium of same basal salts and growth regulators for further proliferation after 15 days of inoculation in case of two tropical *japonica* genotypes and 18 days in case of two *indica* genotypes. The parameters recorded during proliferation were colour, nature and texture of callus, fresh weight of callus at 15th (CW15) and 30th day after subculture (CW30).

To standardize the plant growth regulator combination for regeneration, the proliferated calli from four rice genotypes were placed in a media constituted with different concentrations and combinations of Kn (0.5, 1.0, 1.5 and 2.0 mg/L), BAP (0.5 and 1.0 mg/L) and NAA (0.5 and 1.0 mg/L). Four-week-old callus clumps of each cultivar were transferred to each treatment and replicated thrice. The transferred calli were incubated in a growth room at 25±1℃ with a photoperiod of 16/8 h (light/dark). The regeneration response was recorded based on the manifestation of the green spot on calli. Greening percentage and browning percentage (necrotic calli) were recorded. Individual shoots developing from the calli were separated from callus and allowed for root development. The separated shoots were transferred to half strength MS salts without hormones for root development at 25±1°C under light.

Green plantlet differentiation rate (%) =

$$\frac{\text{No. of green plantlets differentiated}}{\text{Total no. of calli inoculated in regeneration medium}} \times 100$$

Presence of significant differences between treatments

and genotypes were assessed using Three-way ANOVA followed by a post-hoc test of Duncan's Multiple Range Test (DMRT) for mean comparisons at $p \leq 0.05$. All the analyses were carried out in R-Statistical Package (v1.0.2) (R Core Team, 2018). The base function *aov()* was used for ANOVA computations and the function "*duncan.test*" R package *"agricolae"* (de Mendiburu, 2019) was used for DMRT computations. To find groupings or patterns within treatments, media and genotypes, Principal Component Analysis was implemented in R package *"factoextra"* (Kassambara and Mundt, 2017) was used.

RESULTS AND DISCUSSION

The aim of the present study was to identify the optimum concentration and combination of plant growth regulators (2,4-D, Kn and NAA) for callus induction and plantlet regeneration in selected genotypes; evaluation and comparison of the *in vitro* efficiency of N6 and MS basal salts supplemented with 2,4-D, Kn and NAA.

The experiment was first designed to identify the suitable basal salts in combination with optimum plant growth regulators for callus induction in selected rice genotypes. The callus induction frequency of selected genotypes in MS and N6 media are presented in **Table 1**. In N6 media, the callus induction frequency ranged from 15.0% to 86.6%, whereas in MS media, it ranged between 15.0% and 80.0%. Among the two types of media, the highest callus induction frequency of 86.6% (Azucena - T6 treatment), followed by 83.3% (Palwan-T6 treatment), 71.6% (CB87R- T3 treatment) and 66.7% (CB174R-T3 treatment) was observed in N6 media. The lowest callus induction frequency was noted in CB174R (15.0%) followed by CB87R (18.3%) Azucena (31.6%) and Palwan (45.0%) in MS media. Thus, N6 media supplemented with specific combinations of plant growth regulators showed higher callus induction frequency than MS media.

In general, N6 media showed increased callus induction in all four selected rice genotypes. The findings obtained were similar with the results of Paramasivam *et al*. (2010) in MR 219 rice cultivar of *indica* genotype. Saleem *et al*. (2004) reported high callus induction frequency of 83.3% in super basmathi rice in N6 media when compared with MS media. The superiority of MS media over N6 for callus induction was contradictorily reported by Ming *et al*. (2019) in Malaysian rice cultivar MR 220-CL2. Similar results were reported by Barman *et al*. (2016) on ten BRRI Dhan rice varieties in MS medium when compared with N6 medium. This variation may be due to differences in basal nutrient composition of N6 and MS medium. The salt composition of N6 media would have favoured the explant to differentiate into callus due to its higher amount of nitrogen source (Roy and Mandal, 2005; Kaushal, 2014).

In both MS and N6 basal media, the prominent calli were seen from the mature embryo 7-12 days after inoculation, when incubated under dark condition **Fig. 3A**. The calli were obtained from the scutellum region of zygotic embryos. The tropical *japonica* genotypes, Palwan and Azucena showed initiation of callus after 7-10 days of inoculation, whereas *indica* genotypes CB174R and CB87R responded around 10-12 days after inoculation. Paramasivam *et al*. (2010); Rameshkumar *et al*. (2019); Manoharan *et al*. (2020); Tripathy (2021); Dhamotharan and Saraswathi (2021) previously reported scutellum derived calli from dehusked mature rice. Paramasivam *et al*. (2010), Kumar and Ajinder (2013) and Kumari *et al*. (2016) reported callus initiation 7-9 days after inoculation from mature seeds, which supports our findings.

The CIF of different growth regulator combinations of 2,4-D, Kn and NAA ranged from 45.0% to 83.3% in Palwan and 31.6% to 86.6% in Azucena. In CB174R, the CIF varied from 15.0% to 66.7% and in CB87R the frequency varied from 18.3% to 71.6% among the twelve treatments evaluated. The CIF of different plant growth regulators in MS and N6 media in the four genotypes are given in **Table 1**. Mean comparison boxplot of selected genotypes represented in **Fig.1**. Different treatments showed significant differences for CIF, CW15 and CW30 in **Table 2**. The callus induction frequency was high in Azucena (86.0%) followed by Palwan (83.3%), CB87R (71.6%) and was low in CB174R (66.7%). Among the four genotypes, the tropical *japonica* genotype Azucena showed better potential for callus induction. However, the *indica* genotype CB174R exhibited low callus induction, when compared with other genotypes studied. The responsiveness of genotypes for callus induction frequency, callus weight at 15^{th} and 30^{th} day after subculture are presented in **Table 1**, **Fig. 3B.**

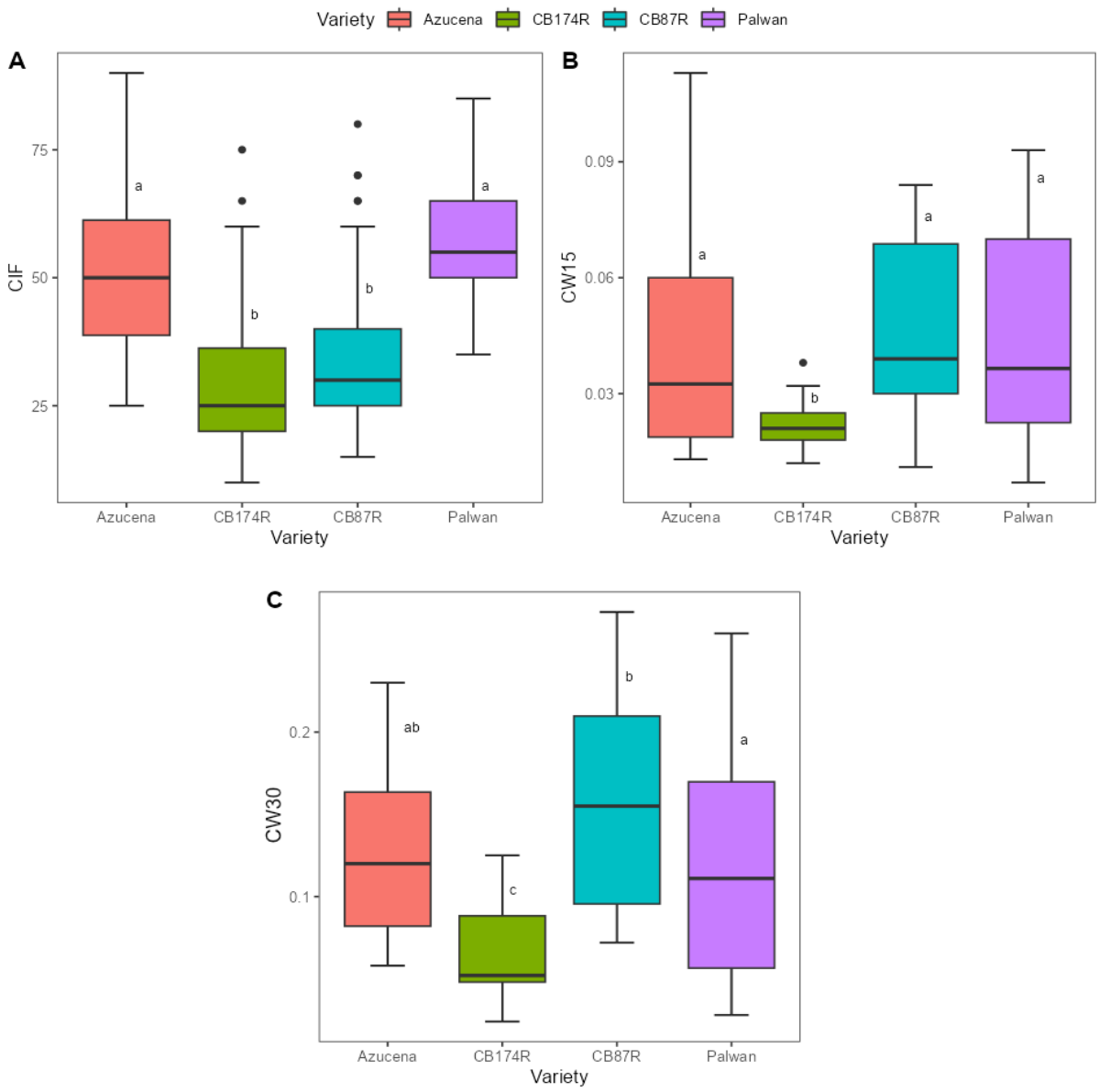
The present study showed the maximum callus induction frequency of 83.3% and 86.6% on N6 medium containing 2,4-D (2.5 mg/L) + Kn (0.5 mg/L) + NAA (1.0 mg/L) in Palwan and Azucena genotypes. The callus obtained in Palwan was embryogenic, compact and creamy yellow in colour, whereas in Azucena, the callus was friable and watery. Similarly, the N6 medium supplemented with 2,4-D (2.0 mg/L) + Kn (0.25 mg/L) + NAA (0.5 mg/L) induced 66.7% callus induction in CB174R and 71.6% callus induction in CB87R. The callus obtained was creamy yellow in colour. Texture of calli varied as compact in CB174R and friable in CB87R.

In all four genotypes, the callus induction frequency was low, when supplemented with 2,4-D and Kn. Addition of optimum NAA to the media further increased the callus induction frequency. In Palwan genotype, the addition of NAA at 1.0 mg/L exhibited the callus induction frequency of 83.3% in N6 basal medium. Likewise in Azucena, the callus induction frequency was increased to 86.6% after adding NAA at 1.0 mg/L concentration along with 2,4-D and Kn. In case of genotypes, CB174R and CB87R,

Table 1. Callus induction of selected tropical *japonica* and *indica* genotypes on different plant growth regulator concentrations

S.No.	Genotype	Media used	Plant growth regulators (mg/l)				CIF (%)	Callus weight 15 days after subculture (mg)	Callus weight 30 days after subculture (mg)	Proliferation rate and Colour of callus	Texture of callus
			Trt. No.	2,4-D	Kn	NAA					
1	Palwan	N6	T1	2.0	0.25	-	55.0	0.06	0.25	Very High,Cy	Cp, D
2			T2	2.5	0.5	-	50.0	0.08	0.23	Very High,Cy	Cp, D
3			T3	2.0	0.25	0.5	60.0	0.04	0.13	High,Cy	Cp, D
4			T4	2.5	0.5	0.5	65.0	0.09	0.13	Moderate,Cy	Cp, W
5			T5	2.0	0.25	1.0	45.0	0.08	0.16	High,Cy	Cp, D
6			T6	2.5	0.5	1.0	83.3	0.06	0.19	Very High,Cy	Cp, W
7		MS	T7	2.0	0.25	-	55.0	0.01	0.03	Very Low,Cy	Fr, W
8			T8	2.5	0.5	-	63.3	0.02	0.09	Moderate,Cy	Fr, D
9			T9	2.0	0.25	0.5	60.0	0.03	0.08	Moderate,Cy	Fr, W
10			T10	2.5	0.5	0.5	51.6	0.01	0.06	Moderate,Cy	Fr, D
11			T11	2.0	0.25	1.0	50.0	0.03	0.03	Very Low,Cy	Fr, W
12			T12	2.5	0.5	1.0	80.0	0.02	0.04	Very Low,Cy	Cp, W
13	Azucena	N6	T1	2.0	0.25	-	44.6	0.06	0.13	Moderate,Cy	Cp, D
14			T2	2.5	0.5	-	41.6	0.05	0.11	Moderate,Cy	Fr, W
15			T3	2.0	0.25	0.5	55.0	0.10	0.21	Very High,Cy	Cp, D
16			T4	2.5	0.5	0.5	35.0	0.07	0.10	Low, Cy	Cp, D
17			T5	2.0	0.25	1.0	73.3	0.03	0.19	Very High,Cy	Cp, D
18			T6	2.5	0.5	1.0	86.6	0.06	0.22	Very High,Cy	Cp, D
19		MS	T7	2.0	0.25	-	31.6	0.01	0.08	Moderate,Cy	Fr, W
20			T8	2.5	0.5	-	50.0	0.01	0.13	Very High,Cy	Fr, W
21			T9	2.0	0.25	0.5	31.6	0.02	0.08	Moderate,Cy	Fr, W
22			T10	2.5	0.5	0.5	66.6	0.02	0.15	Very High,Cy	Fr, W
23			T11	2.0	0.25	1.0	60.0	0.02	0.06	Low, Cy	Fr, W
24			T12	2.5	0.5	1.0	43.3	0.02	0.07	Low, Cy	Fr, W
25	CB174R	N6	T1	2.0	0.25	-	21.6	0.02	0.10	High,W	Cp, W
26			T2	2.5	0.5	-	15.0	0.02	0.12	High,W	Fr, D
27			T3	2.0	0.25	0.5	66.7	0.03	0.05	Very Low,W	Cp, D
28			T4	2.5	0.5	0.5	40.0	0.03	0.06	Very Low,W	Fr, D
29			T5	2.0	0.25	1.0	16.7	0.03	0.05	Very Low,W	Cp, D
30			T6	2.5	0.5	1.0	15.0	0.01	0.05	Low, W	Cp, D
31		MS	T7	2.0	0.25	-	15.0	0.02	0.09	Moderate,W	Cp, W
32			T8	2.5	0.5	-	20.0	0.01	0.03	Very Low,W	Fr, W
33			T9	2.0	0.25	0.5	36.7	0.02	0.05	Low, W	Fr, W
34			T10	2.5	0.5	0.5	28.3	0.02	0.05	Very Low,W	Fr, W
35			T11	2.0	0.25	1.0	15.0	0.02	0.05	Low, W	Fr, W
36			T12	2.5	0.5	1.0	25.0	0.02	0.09	Moderate,W	Fr, W
37	CB87R	N6	T1	2.0	0.25	-	26.6	0.01	0.22	Very High, By	Cp, W
38			T2	2.5	0.5	-	20.0	0.07	0.27	Very High, By	Cp, W
39			T3	2.0	0.25	0.5	71.6	0.06	0.24	Very High, By	Fr, W
40			T4	2.5	0.5	0.5	25.0	0.08	0.17	Moderate, By	Cp, D
41			T5	2.0	0.25	1.0	20.0	0.07	0.17	Very High, By	Cp, D
42			T6	2.5	0.5	1.0	30.0	0.07	0.14	Moderate, By	Fr, D
43		MS	T7	2.0	0.25	-	40.0	0.04	0.08	Low, By	Fr, W
44			T8	2.5	0.5	-	20.0	0.04	0.09	Low, By	Cp, D
45			T9	2.0	0.25	0.5	18.3	0.03	0.20	Very High, By	Fr, W
46			T10	2.5	0.5	0.5	30.0	0.03	0.12	Very High, By	Fr, W
47			T11	2.0	0.25	1.0	40.0	0.03	0.10	High, By	Fr, W
48			T12	2.5	0.5	1.0	65.0	0.03	0.08	Low, By	Cp, D

CIF (%) – Callus Induction Frequency, Cy– Creamy Yellow, Fr – Friable, Cp – compact, D – Dry, W – Wet



CIF (%) – Callus induction frequency, CW15- Callus weight 15 days after subculture, CW30-Callus weight 30 days after subculture

Fig. 1. Mean comparison boxplot of selected japonica and indica genotypes for CIF, CW15 and CW30

the callusing efficiency increased to 66.7% and 71.6%, respectively at the NAA concentration of 0.5 mg/L supplemented in N6 basal medium with optimum amount of 2,4-D (2.0 mg/L) and Kn (0.25 mg/L).

The callogenesis mainly depends on exogenous supply of auxin and its concentration. The addition of 2,4-D to the media promotes embryogenic callus formation. In higher concentrations of 2,4-D, the calli decreases its regeneration potential and causes browning (Cepeda and Giraldo, 2014). Addition of 2,4-D in the media causes hypermethylation of DNA and keeps the cell in mitotic stage. Tariq *et al*. (2008) found 2.5 mg/L of 2,4-D in N6 basal medium induced callus on three basmati rice varieties. Abiri *et al*. (2017) reported the 2,4-D range of 2 to 3 mg/L induced callus in *indica* genotypes. In MR 219 *indica* rice variety, the highest callus induction of 75% was obtained with 2 mg/L of 2,4-D. The differences in callus induction percentage with 2,4-D (2.0 mg/L and 2.5 mg/L) was found to be due to the genotype of the selected rice (Mostafiz and Wagiran, 2018). Barman *et al*. (2016) reported 2,4-D at 2 mg/L induced callus in BRRI Dhan

Table 2. Mean comparison of selected rice genotypes for CIF, CW15 and CW30 based on DMRT

	Groups	CIF	CW15	CW30
Media	MS	40.49^{b}	0.02^{b}	0.08^{b}
	N6	47.36^{a}	0.06^{a}	0.16^{a}
Treatments	T1-MS	35.42^{c}	0.02^{c}	0.07^{de}
	T1-N6	40.83^{bc}	0.04^{bc}	0.18^{ae}
	T2-MS	37.08^{cc}	0.02^{cc}	0.08^{de}
	T2-N6	38.75^{cc}	0.06^{ac}	0.18^{ae}
	T3-MS	34.17^{cc}	0.03^{cc}	0.10^{de}
	T3-N6	63.33^{ac}	0.06^{ac}	0.16^{abe}
	T4-MS	44.17^{bc}	0.02^{cc}	0.09^{dee}
	T4-N6	41.25^{bc}	0.07^{ac}	0.11^{bcd}
	T5-MS	41.25^{bc}	0.02^{cc}	0.06^{ecd}
	T5-N6	43.75^{bc}	0.06^{ac}	0.14^{abc}
	T6-MS	50.83^{abc}	0.02^{cc}	0.07^{dec}
	T6-N6	56.25^{abc}	0.05^{ab}	0.15^{abc}
Variety	Azucena	51.39^{a}	0.04^{a}	0.13^{b}
	CB174R	30.56^{b}	0.02^{b}	0.07^{c}
	CB87R	35.56^{b}	0.05^{a}	0.16^{a}
	Palwan	58.19^{a}	0.05^{a}	0.12^{b}

Significant differences are noted with different alphabets
CIF (%) – Callus induction frequency, CW15- Callus weight 15 days after subculture, CW30-Callus weight 30 days after subculture

rice varieties. In *japonica* rice varieties Yan *et al*. (2010) obtained high callus induction of 72% with 2,4-D (2 mg/L), Kn (0.5 mg/L) and NAA (1.0 mg/L) in M8 medium based (Mei *et al*., 1988). Friable callus with creamy yellow in colour was obtained. Our results on callus colour and texture were similar with the findings of Kumar and Ajinder (2013) and Abiri *et al*. (2017).

Azizi *et al*. (2015) also found reduction in callus induction percentage when 2,4-D and NAA were increased above 3 mg/L and 1 mg/L, respectively. In *indica* rice genotypes, Kadhimi *et al*. (2016); Kalhori (2017) and Abiri *et al*. (2017) reported callus induction at 2.0 mg/L of 2,4-D. Similarly, in the study with SR4 rice genotype, reported high callus induction of 53.33% with 2.5 mg/L of 2,4-D and 3.5 mg/L of NAA (Noor *et al*., 2022). Our findings agree with the report of Azizi *et al*. (2015) in which the combination of 2,4-D and NAA increased callus induction upto 90%. To overcome the genotypic influence, each variety should be standardized with different plant growth regulator combinations. Mostafiz and Wagiran (2018) found combinations of auxin (NAA+2,4-D) better than using single auxin source (2,4-D or NAA). Lower concentrations of 2,4-D with NAA stimulated the callus induction and increased the regenerative potential (Cepeda and Giraldo, 2014). The NAA concentration in the media increased the embryogenic frequency. To overcome the genotypic influence, diverse hormonal combinations are needed (Mohddin *et al*., 2016). The combination of 2,4-D and Kn was reported to improve embryogenic proliferation and cell division (Rameshkumar *et al*., 2019).

While comparing the callus induction frequency of selected genotypes, Azucena showed better potential for callus induction compared to other genotypes. The *indica* genotypes, CB174R and CB87R, showed less response. The results obtained are similar to the statement of Rameshkumar *et al*. (2019), who reported less amenable nature of *indica* rice genotypes for *in vitro* culture compared to *japonica* cultivars.

The mean weights of callus in different concentrations of plant growth regulators in MS and N6 media were compared and depicted in **Table 3**. The mean weight of callus on 15 days after subculture on different treatments was 0.04 mg (Azucena), 0.02 mg (CB174R), 0.05 mg (CB87R) and 0.05 mg (Palwan). The proliferation rate of initiated calli after 30 days of subculture was high in N6 media, when compared to MS media (**Table 3**). The proliferation rate after 30 days of sub culture was high in CB87R (0.16 mg) followed by Azucena (0.13 mg), Palwan (0.12 mg) and was low in CB174R (0.07 mg) (**Table 3**). Among the treatments, the proliferation rate was high in N6 media supplemented with 2,4-D (2.5 mg/l) and Kn (0.5 mg/L) (**Table 1**). Sidek *et al*. (2022) calculated fresh weight of callus and reported callus weight as a measure of callus growth and the weight of callus depended on the concentration of plant growth regulators.

Principle component analysis was performed using Callus Induction Frequency (CIF), callus weight on 15th day after subculture (CW15) and callus weight on 30th day after subculture (CW30) as variables (**Fig. 2A**). The PC1

contributed about 59.3% to the total variance with the higher contribution of CW30 and PC2 contributed about 28.9% with higher contribution towards CIF among the traits. The responses were grouped based on different partitions like the type of medium (MS and N6), sub-species (*indica* and tropical *japonica*), genotypes (Palwan, Azucena, CB174R and CB87R) and different treatments and are presented in **Fig. 2**.

PCA biplot showed considerable grouping based on basal media used. In basal media based grouping (**Fig. 2B**), horizontal separation was observed. Hence, it can be said that basal media had major influence on callus weight and progressive development of callus. However, grouping based on sub species showed that the clustering of responses in the vertical axis (PC2) was majorly contributed by CIF (**Fig. 2C**). Thus, it portrayed in our study that, sub species had a greater influence in the case of callus induction, which is in correspondence with previous studies on *indica* and tropical *japonica* rice. Grouping of genotypes (**Fig. 2D**) showed the higher influence of sub species based groups on *in vitro* response. No considerable grouping or patterns were observed based on treatments (**Fig. 2E**) in our study, which showed that treatments or concentration of growth regulators have to be optimized for each variety.

The calli obtained from N6 media supplemented with 2,4-D (2.0 and 2.5 mg/L) + Kn (0.25 and 0.5 mg/L) + NAA (0.5 and 1.0 mg/L) was transferred to regeneration media. The regeneration media used had different concentrations of NAA, BAP and Kn. The green spots were seen in calli after 14 days of transfer to regeneration media and subsequently after 14 days, shoots were induced from the green spots of calli (**Fig. 3C&3D**).

The regeneration response of calli on different plant growth regulators is presented in **Table 3**. The total plantlet regeneration of 70.0% was recorded in MS media containing NAA (0.5 mg/L) + BAP (1.0 mg/L) + Kn (0.5 mg/L) in Palwan (Treatment-RM5) (**Fig. 3G**). The total plantlet regeneration rate of 10.0% was seen in CB174R when MS media was supplemented with NAA (0.5 and 1.0 mg/L) + BAP (0.5 and 1.0 mg/L) + Kn (0.5 and 2.0 mg/L) (Treatment-RM4 (**Fig. 3H**), RM7). For rejuvenation of calli, the auxin and cytokinin concentrations should be in equilibrium. The genotypes, Azucena and CB87R, showed green spots and did not regenerate into plantlet and become necrotic (**Fig. 3E**).

The regeneration rates observed in other treatments of the genotype Palwan were 30.0 % with 40.0% greening rate (RM1), 40.0% from 60.0% (RM2), 20.0% from 30.0% (RM3), 30.0% from 40.0% (RM4), 50.0% from 70.0% (RM6 & RM7). In Palwan, multiple shoot induction was seen in RM4 treatment (MS + NAA (0.5 mg/L), BAP (0.5 mg/L), Kn (2.0 mg/L)) (**Fig. 3F**). Likewise, in CB174R the regeneration rate of 10.0 % was seen in RM4 and RM7 treatments. Among the two treatments, the multiple shoot induction was observed in RM4 treatment (MS+ NAA (0.5 mg/L) + BAP (0.5 mg/L) + Kn (2.0 mg/L)) with greening rate of 20.0%. In RM1 and RM2 the browning rate was higher compared to greening rate. The *indica* genotype, CB87R, produced green spots in MS regeneration medium with NAA, BAP at 0.5 mg/L and Kn at 1.0 mg/L (Treatment-RM2). Similarly, the genotype, Azucena, produced the highest greening in MS medium supplemented with NAA, BAP at 0.5 mg/L and Kn at 2.0 mg/L concentration (Treatment-RM4). The genotypes, Azucena and CB87R, did not produce plantlets as the calli were friable and watery in nature, with very poor in embryogenic potential.

Cytokinins are needed for regulating shoot growth and to promote cell expansion (Azizi *et al*., 2015). In the study by Azizi *et al*. (2015) 0.5 mg/L BAP, 1.5 mg/L Kn along with 0.5 mg/L NAA induced 100% regeneration in Panderas, Malaysian cultivar. Noor *et al*. (2022) suggested increased concentration of cytokinin and decreased concentration of auxin induced 100% regeneration frequency in SR4 rice line. The use of kinetin in regeneration media, is suggested to differentiate mature chloroplasts from protoplastids and help in protein synthesis and vascular differentiation. The role of NAA is to strengthen the generated shoots (Mostafiz and Wagiran, 2018). Yan *et al*. (2010) observed 0.5, 1.0 mg/L of BAP combined with auxin source responded for regeneration in *japonica* and *indica* rices. For induction of micro tillering in zinc rich rice Chittimuthyalu, Tripathy (2021) experimented the combination of two cytokinin sources BAP and kinetin. For stimulating callus to shoot differentiation and regeneration, the cytokinin BAP (2.0 mg/L) was used (Sakthivel *et al*., 2021). Libin *et al*. (2012) reported NAA and Kn concentration at 1.0 mg/L induced plant regeneration in biris rice. The combination of BAP with lesser amount of auxin was found to produce multiple shoots (Rameshkumar *et al*., 2019). In TRY 2 and TRY 3 rice varieties shoots were induced in MS media containing 0.5mg/L of NAA and 2.0 mg/L of BAP (Dhamotharan and Saraswathi, 2021).

For regeneration, in particular for the shoot growth and proliferation, cytokinins were found to play a vital role. Lee *et al*. (2003) reported the combination of cytokinin BAP and Kn increased plantlet regeneration in *japonica* rice lines in the presence of optimum NAA concentration. The shoot regeneration was increased in lower concentration of kinetin compared with BAP. NAA in combination with BAP stimulated the shoot regeneration in rice cultivars (Kumar and Ajinder, 2013). Compared to the earlier works, Azizi *et al*. (2015) observed more regeneration from mature embryos of *Japonica* lines than *Indica* lines. When BAP was added to the media, the cells differentiated and formed buds. Mohddin *et al*. (2016) reported 100% regeneration in 0.5 mg/L of NAA, BAP, thidiazuron with 1.5mg/L of kn in Panderas Malaysian cultivar. The lower regeneration frequencies were observed in media with cytokinin alone

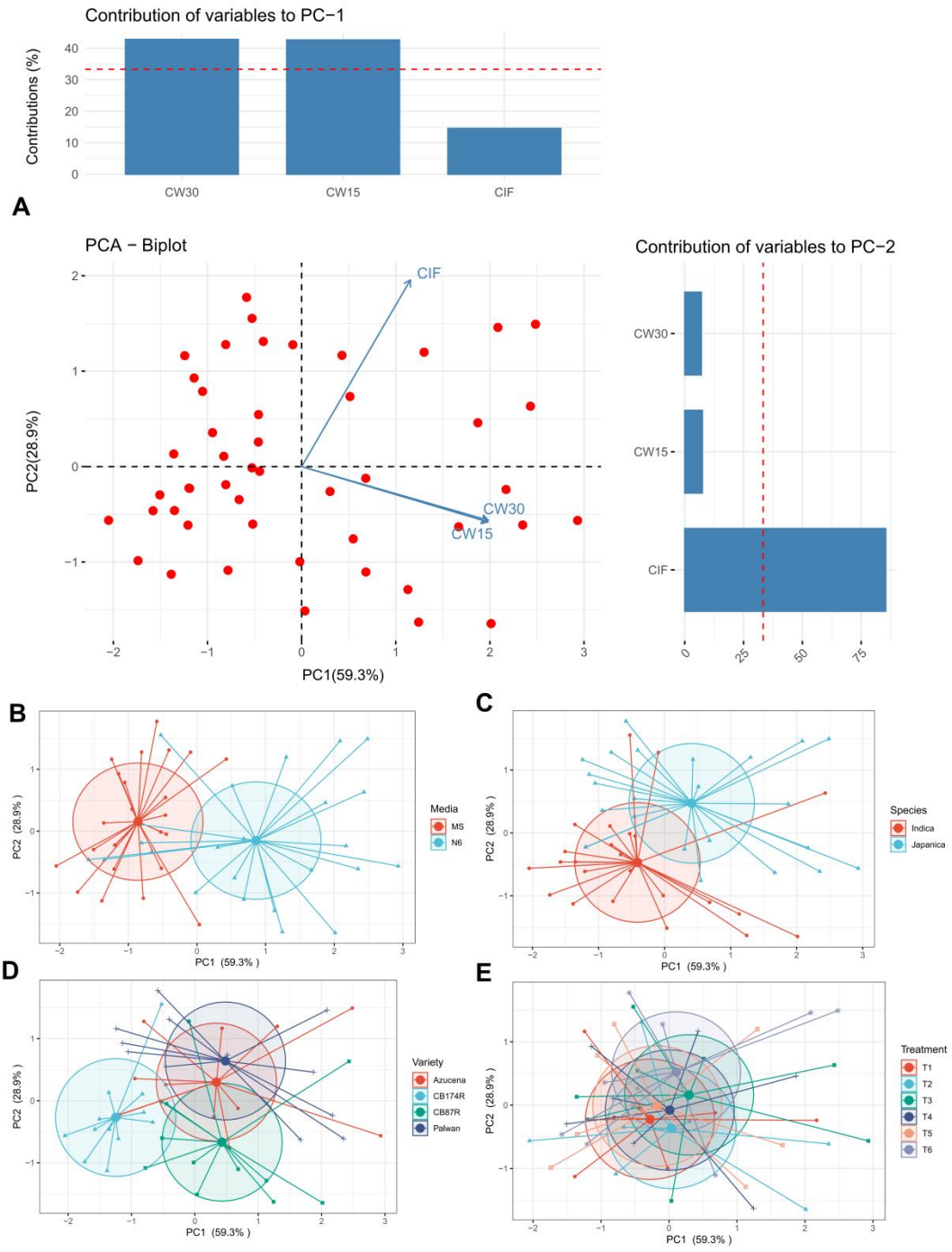


Fig. 2. A-Two dimensional PCA biplot of PC1 (59.3%) and PC2 (28.9%). B- Grouping based on media, C- Grouping based on sub-species, D- Grouping based on genotype, E- Grouping based on treatments

without NAA. Hence, the balanced ratio of cytokinin and auxin induced cell cycle and morphogenic potential of growth. The auxin and cytokinin were found to interact and promote caulogenesis (Mostafiz and Wagiran, 2018). Barman *et al*. (2016) reported that Kn (2.0mg/L) was found effective for regeneration with NAA (2.0 mg/L), when compared to BAP in *indica* rice genotypes. Similar results were found by Abiri *et al*. (2017) in MR 219. Combination of NAA and kn induced shoots from calli, and the optimum NAA and BAP concentration increased the number of

Table 3. Response of tropical *japonica* and *indica* rice calli on different regeneration media

S. No	Genotype	Trt. No.	MS + Plant Growth Regulator concentration (mg/L)			Greening Percentage (%)	Browning Percentage (%)	Per cent plantlet regeneration from green calli	Percentage of total plantlet regeneration from all calli	Regeneration response
			NAA	BAP	Kn					
1	Palwan	RM1	0.5	0.5	0.5	40.0	30.0	75.0	30.0	Caulogenesis
2		RM2	0.5	0.5	1.0	60.0	40.0	66.7	40.0	Caulogenesis & Rhizogenesis
3		RM3	0.5	0.5	1.5	30.0	70.0	66.7	20.0	Rhizogenesis
4		RM4	0.5	0.5	2.0	40.0	50.0	75.0	30.0	Caulogenesis & Rhizogenesis
5		RM5	0.5	1.0	0.5	70.0	30.0	100.0	70.0	Caulogenesis & Rhizogenesis
6		RM6	0.5	1.0	1.0	70.0	30.0	71.4	50.0	Caulogenesis
7		RM7	1.0	1.0	0.5	70.0	30.0	71.4	50.0	Caulogenesis Rhizogenesis
8	Azucena	RM1	0.5	0.5	0.5	20.0	80.0	-	-	Caulogenesis
9		RM2	0.5	0.5	1.0	30.0	70.0	-	-	Caulogenesis Rhizogenesis
10		RM3	0.5	0.5	1.5	20.0	80.0	-	-	Caulogenesis
11		RM4	0.5	0.5	2.0	40.0	60.0	-	-	-
12		RM5	0.5	1.0	0.5	30.0	70.0	-	-	Caulogenesis
13		RM6	0.5	1.0	1.0	20.0	80.0	-	-	-
14		RM7	1.0	1.0	0.5	20.0	80.0	-	-	Caulogenesis
15	CB174R	RM1	0.5	0.5	0.5	-	100.0	-	-	-
16		RM2	0.5	0.5	1.0	-	100.0	-	-	-
17		RM3	0.5	0.5	1.5	20.0	80.0	-	-	-
18		RM4	0.5	0.5	2.0	20.0	80.0	50.0	10.0	Caulogenesis & Rhizogenesis
19		RM5	0.5	1.0	0.5	20.0	80.0	50.0	-	Caulogenesis & Rhizogenesis
20		RM6	0.5	1.0	1.0	10.0	90.0	-	-	Caulogenesis & Rhizogenesis
21		RM7	1.0	1.0	0.5	20.0	80.0	50.0	10.0	Caulogenesis & Rhizogenesis
22	CB87R	RM1	0.5	0.5	0.5	-	100.0	-	-	-
23		RM2	0.5	0.5	1.0	33.3	66.7	-	-	Caulogenesis & Rhizogenesis
24		RM3	0.5	0.5	1.5	-	100.0	-	-	-
25		RM4	0.5	0.5	2.0	-	100.0	-	-	-
26		RM5	0.5	1.0	0.5	-	100.0	-	-	-
27		RM6	0.5	1.0	1.0	-	100.0	-	-	-
28		RM7	1.0	1.0	0.5	-	100.0	-	-	-

shoots and regeneration frequency (Noor *et al*., 2022). While comparing all the genotypes, tropical *japonica* genotype (Palwan) expressed higher regeneration than other three genotypes.

In conclusion, the optimum concentration of plant growth regulators enhanced the callus induction and regeneration. In our experiment the callus induction frequency was high in N6 medium supplemented with optimum concentration of plant growth regulators in both Palwan and Azucena (N6 + 2,4-D (2.5 mg/L) + Kn (0.5 mg/L) + NAA (1.0 mg/L)). In CB174R and CB87R higher percentage of calli was induced in N6 with 2,4-D (2.0 mg/L) + Kn (0.25 mg/L) + NAA (0.5 mg/L)). The Plant growth regulator combination in MS with NAA (0.5 mg/L) + BAP (1.0 mg/L) + Kn (0.5 mg/L) and MS + NAA (0.5, 1.0 mg/L) + BAP

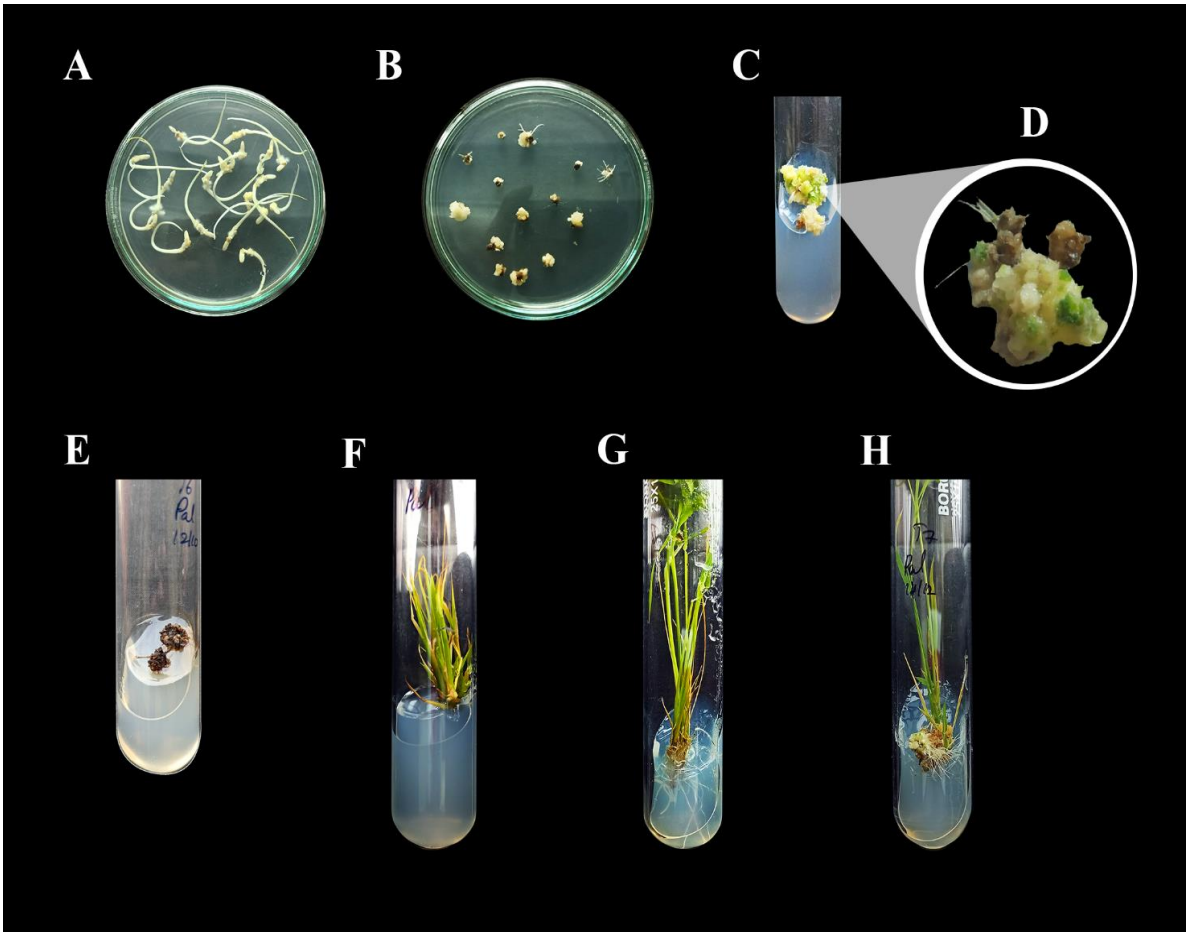


Fig. 3. A) Callus induction from seed explants B) Calli after 15 days of subculture C& D) Greening of calli after 14 days in regeneration medium E) Necrotic calli F) Multiple shoots induction in Palwan (Treatment – RM5) G) Regenerated plantlet after 45 days in regeneration medium (CB174R) H) Regenerated plantlet after 45 days in regeneration medium (Palwan)

(0.5, 1.0 mg/L) + Kn (0.5, 2.0 mg/L) induced regeneration in Palwan and CB174R. Azucena and CB87R induced greening, but regeneration could not be obtained. Further optimization of BAP and Kn concentration in regeneration media enhanced the plantlet regeneration from Palwan and CB174R. Among the four rice genotypes, Palwan had the highest potential for *in vitro* modifications.

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