

## Comparative Analyses of Regeneration Potentiality of Eight Indigenous Aromatic Indica rice (*Oryza sativa* L.) Varieties

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**Abstract**— The development of an efficient *in vitro* regeneration system for economically-important aromatic rice cultivars is of urgent need for undergoing crop improvement through genetic modification. In the present work, comparative analysis of *in vitro* callus induction and regeneration efficiency of eight indigenous aromatic rice cultivars, viz., Kalonunia (KN), Gobindobhog (GB), Pusa Basmati (PB), Tulaipanji (TP), Radhunipagal (RP), IET 21261 (IET), Gandheswari (GN) and Radhatilak (RT) was carried out. The matured seeds were used as explants on Murashige and Skoog (MS) medium supplemented with 3 mg L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid and 30 g L<sup>-1</sup> sucrose for callus induction, while embryogenic calli were transferred to MS medium supplemented with combination of hormones, 1 mg L<sup>-1</sup> naphthalene acetic acid, 2 mg L<sup>-1</sup> 6-benzylaminopurine and 4 mg L<sup>-1</sup> kinetin for plant regeneration. The present study showed that all the varieties exhibited a high frequency of callus induction, ranging from 63.19% to 98.33%. The plant regeneration potential ranged from 93.43% to 33% depending on the genotypes examined. The maximum plant regeneration (%) was noted in KN (93.43%) followed by GB (90.83%), TP (89.34%), RP (89%), IET (75.92%), GN (74.66%) and RT (71%), whereas the minimum regeneration potential was observed in PB (33%). The regeneration potential was highly correlated with callus browning and callus production. The *in vitro* regenerated plants showed successful rooting and were well acclimatized under the conditions in growth chamber. Our observation highlighted a significant effect of genotype on callus induction ability, embryogenic response and plant regeneration capacity, suggesting the varietal difference in regeneration efficiency among the selected aromatic rice varieties.

**Keywords**— Aromatic rice, *Oryza sativa*, Indica rice, Regeneration, Callus induction, Plant growth regulators, Tissue culture, Matured seed explant

### I. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops and the major source of calories for half of the world population; hence, genetic improvement of economically important rice cultivars, aimed at achieving higher rice productivity, has gained impetus in recent era of climate change and agricultural loss. India, among the rice-growing countries, accounts for nearly one-fourth (22%) of the rice produced in the world, with the first place occupied by China. Aroma and fragrance add value to the quality of rice, making it more demanding in Asia as well as Southeast Asia; further, it has recently gained popularity in the Middle East and Western markets [1, 2].

In India, aromatic rice is normally cultivated in Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Uttarakhand and some parts of West Bengal and Kerala. The most popular aromatic rice in India is Basmati, which has a medium texture with a slender shape and long grain and less

chalkiness [3]. Though a high-volume commodity, a class of aromatic, superfine premium rice has evolved its own market niches, making India one of the leading exporters of aromatic rice in the international market. In spite of high demand of aromatic rice in the international market, there are some constraints for the farmers to cultivate aromatic rice varieties, such as poor agronomic trait and reduced yield, dearth of disease resistant and salt-tolerant variety, lack of high yielding scented rice variety and proper cultural management. Because of its superior traits, Basmati rice gets a high price in international markets for its distinct aroma, long grain and soft and flaky texture. In addition, the country also produces hundreds of indigenous short-grained aromatic cultivars and landraces grown in distinct pockets of different states. West Bengal, being the 'rice bowl' of India is especially famous for its wide diversity of indigenous aromatic rice varieties such as Gobindobhog, Pusa Basmati, Tulaipanji, Radhunipagal, Gandheswari, Radhatilak, etc. These aromatic rice varieties also possess exemplary quality traits like aroma, fluffiness and taste. Gobindobhog in

particular is a highly popular aromatic rice variety, cultivated in some districts of West Bengal, and is highly prized for its magnificent aroma and soft white texture; however, the variety is extremely salt-susceptible and even requires seed priming strategy to overcome the adverse effects of salt stress [4, 5]. Hence, there is an urgent need for an efficient regeneration system for aromatic rice cultivars in order to undergo crop improvement through genetic modification or hybrid production.

Attempts to improve rice yield through conventional breeding program have met with very limited success. At present, *in vitro* technique of plant propagation from cell or tissue explants is an important and essential component of biotechnology, which is required for the genetic manipulation of crops. Efficient regeneration through *in vitro* micropropagation is very essential for the successful utilization of biotechnology in crop improvement of rice [6]. The identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration through *in vitro* system is a vital step in rice genetic improvement program [7, 8]. The use of matured seed embryos has a distinct advantage over other explants as starting material for *in vitro* rice regeneration [2, 9]. Recent advancement in biotechnology, such as transformation has enhanced the introgression of new genes from different sources to the cultivated species [10].

In spite of the low frequency of plant regeneration from callus, matured rice embryo is considered as one of the best explants for genetic transformation because of its availability throughout the year and easy handling as an explant compared to other tissues [11, 12]. Lesser information is available on the *in vitro* regeneration procedure using matured embryos, particularly in indica rice than japonica type. In addition, a number of economically valuable indica varieties are recalcitrant to *in vitro* manipulation due to their poor callus production and regeneration ability [13]. Hence, it is essential to strategize an efficient protocol using matured embryos of indica rice for establishing a highly reproducible regeneration system that will enable rice transformation [6]. The dehusked form of the matured seed of the aromatic rice cultivar acts as a highly suitable explant for the formation of callus, amenable to multiple shoot formation and could be used for genetic transformation studies. However, an effective and robust tissue culture protocol system in aromatic rice varieties is relatively limited and less established. The selection of embryogenic callus is also critical before any regeneration study is initiated. Therefore, the purpose of the present investigation was to initiate high quality embryogenic calli from matured seeds, using the optimal concentration of 2, 4 dichlorophenoxyacetic acid (2,4-D), followed by establishing an easy regeneration frequency (%) was calculated as below [15]. The calli were transferred to the fresh medium and the percentage of

protocol for eight indigenous aromatic rice cultivars, viz., Kalonunia (KN), Gobindobhog (GB), Pusa Basmati (PB), Tulaipanji (TP), Radhunipagal (RP), IET 21261, Gandheswari (GN) and Radhatilak (RT). The differential potentiality of the aforementioned aromatic rice varieties for callus induction and regeneration using matured dehusked seeds was analyzed with the optimal concentration of plant growth regulators.

## II. MATERIALS AND METHODS

### 2.1. Plant materials

The seeds of the eight aromatic rice varieties, viz., KN, GB, PB, TP, RP, IET, GN and RT were obtained from Chinsurah Rice Research Station, Hooghly, West Bengal, India. The matured seeds were used for the initiation of embryogenic calli and plant regeneration. The manually dehusked seeds of the aromatic rice cultivars were surface sterilized with 70% (v/v) ethanol for two minutes, followed by immersion in 0.1% (w/v) HgCl<sub>2</sub> for a time period of 15 minutes and shaken to mix well. The seeds were rinsed 8-10 times thoroughly using sterile distilled water, blotted dry on sterile Whatman paper (3 mm), prior to subjecting them for callus induction.

### 2.2. *In vitro* germination

The sterilized dehusked seeds of the eight varieties were separately inoculated for *in vitro* germination in Murashige and Skoog (MS) media [14] supplemented with 3% (w/v) sucrose and solidified with agar (8 g L<sup>-1</sup>). The seeds were then incubated at 24 ± 1°C in dark for four days. Each Petri plate consisted of 10-12 seeds and ten plates were maintained for each variety. The experiment was replicated thrice. Seed germination was observed after four days in the form of radicle emergence, and the germination percentage was expressed as: Embryo germination percentage (%) = (Number of embryo germinated / Total number of seeds inoculated) × 100.

### 2.3. Callus induction medium

For callus induction, the germinated embryos were cut and inoculated on Petri plate on callus induction medium (CIM) and incubated at 24 ± 1°C in dark for 14 days. The scutellum-derived embryogenic calli were next transferred to fresh CIM for two week under the same condition; the non-embryogenic calli (compact, non-friable calli that develop root like structures) were discarded. CIM was prepared using basal MS medium containing all vitamins [14] supplemented with 30 g L<sup>-1</sup> sucrose, 3 mg L<sup>-1</sup> 2,4-D, gelled with 8 g L<sup>-1</sup> agar, and pH adjusted to 5.8. The data were recorded after four week of culture initiation. The callus induction browning in the embryogenic callus was calculated. Callus induction frequency (%) = [(Total number of cultured seed

with callus — Total number of initiated callus) / Total number of cultured seed] × 100.

#### 2.4. Plant regeneration

After four week of maintenance, the embryonic calli were transferred to MS regeneration medium (RM) supplemented with 1 mg L<sup>-1</sup> naphthalene acetic acid (NAA), 2 mg L<sup>-1</sup> 6-benzylaminopurine (BAP), 4 mg L<sup>-1</sup> kinetin, 30 g L<sup>-1</sup> sucrose, 8 g L<sup>-1</sup> agar, and pH adjusted to 5.8. The culture was maintained in growth chamber at 24°C under 16 h day/8 h night photoperiodic cycle, with a light intensity of 2000 lux using fluorescent tubes. The frequency of plant regeneration was calculated as below [15]. Regeneration frequency (%) = [Number of regenerated calli / Number of calli maintained] × 100.

#### 2.5. Statistical analysis

All statistical analyses were performed using the R statistical program. The analyses were performed by one-way analysis of variance (ANOVA) for each cultivar and the P value was obtained. Three independent biological replicates for each trait of all the genotypes were used for this analysis. The Tukey's honestly significant difference (HSD) test was conducted to identify the mean values that were significantly different from each other at P < 0.05 level.

### III. RESULTS AND DISCUSSION

Tissue culture technique has been proposed as a novel strategy in plant breeding program that allows the development of genetic variability. This technique involves standardizing a culture condition in which de-differentiated cells or tissues could be established to allow their proliferation and subsequent regeneration. However, an effective and robust tissue culture protocol system in indigenous aromatic rice varieties is relatively limited and less established. In the present study, *in vitro* techniques for callus induction and plant regeneration have been compared using matured seeds as explants from eight indigenous aromatic rice varieties of West Bengal, India, to facilitate the regeneration protocol which can be used to recover germplasm.

#### 3.1. Percentage of embryo germination

Rice improvement through *in vitro* approaches or hybridization requires an efficient regeneration protocol especially for indica rice varieties which, among cereals, are less amenable to culture. Seed dormancy, embryo abortion and failure of complete plant production are the results of interspecific barriers of hybridization program [16]. In addition, matured seeds of some traditional rice varieties may not always germinate in culture media due to their recalcitrant nature or due to seed dormancy. Hence, it is

important to develop an efficient protocol for breaking seed dormancy and plant regeneration from matured seed cultures in rice. Here, we carried out embryo germination test using dehusked matured seeds of eight aromatic rice genotypes, viz., KN, GB, PB, TP, RP, IET, GN and RT on germination medium (Figure 1a, b). In MS medium supplemented with 3% (w/v) sucrose, the seed germination was initiated within five to nine days. The fastest germination time was recorded for KN, RP, GN and RT followed by GB and TP; while the maximum time necessary for germination was noted for PB and IET (Figure 1a). In addition, the embryo germination percentage was also recorded. The highest embryo germination percentage was noted for GB (96.7%) and RP (96.3%) followed by TP (93%), GN (85.3%), RT (83.6%), KN (60.4%), IET (44.7%) and the lowest in PB (34.3%) (Figure 1b). A significant (p < 0.001) negative correlation was observed between embryo germination time and germination percentage (Figure 5a). The results of embryo germination percentage and germination time indicated that among the eight aromatic rice cultivars, PB and IET showed low response to MS culture media (3% sucrose) possibly because of prevailing dormancy in the seed. Previous studies have also used MS medium with agar as a solidifying agent [17–20] and sugar [6] for germination of rice seeds under *in vitro* conditions.

#### 3.2. Callus induction and percentage of browning

Murashige and Skoog medium is considered as the most suitable culture medium for callus induction of rice cultivars [21, 22]. Previous reports have also shown high callus induction on MS medium supplemented with 2,4-D [23]. In the present work, a comparative study was carried out with several indigenous aromatic rice cultivars showing the best callus induction potential (Figure 2). The germinated embryos of eight aromatic rice cultivars were inoculated on MS media supplemented with 3 mg L<sup>-1</sup> 2,4-D for callus induction. Our results showed that callus induction occurred between 8-14 days after inoculation with the cultivars GB, TP and RP, requiring minimum time for callus induction, followed by KN, GN and RT. The maximum time for callus induction was noted for IET followed by PB which was significantly different from that for GB, TP and RP (Figure 3a). The varietal difference with regard to callus induction time was further supported by our ANOVA study which showed significant (p < 0.001) effect of rice genotypes on callus induction time. A significant (p < 0.001) negative correlation between callus induction time and percentage of callus induction was also noteworthy (Figure 5b). Almost all the inoculated seeds developed scutellar calli within 14 days of inoculation on CIM; however, non-embryogenic calli were discarded and only embryogenic calli were subcultured further on CIM. The callus induction frequencies ranged from 63.19% to 98.33% depending on the genotypes examined. The maximum (98.33%) callus induction

frequency was observed in RP which was not significantly different from GB (97.67%), TP (93.29%) and GN (89.97%), but significantly different from KN (74.84%), PB (64.75%) and IET (63.19%) (Figure 3b). This result clearly indicated the varietal effect on the callus induction, which was further supported by ANOVA, revealing a significant ( $p < 0.001$ ) effect of genotype on callus induction frequency (Table 1).

Previous reports also showed the effect of rice genotypes on the variability of callus induction potential [24, 25]. The role of 2,4-D in promoting somatic embryogenesis, dedifferentiation and cell division in rice has been reported earlier [26]. The phytohormone also modulates the endogenous indole acetic acid (IAA) level in explant tissues [27]. These genotypic variations are probably

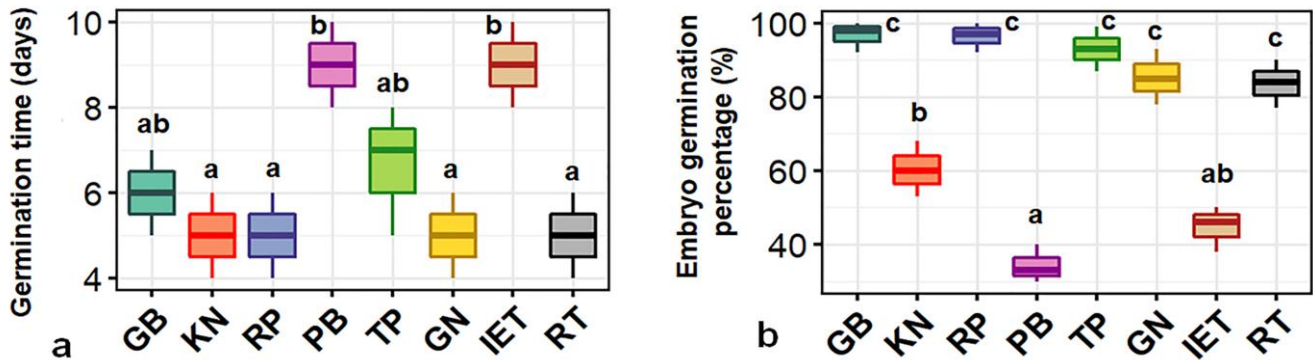


Figure 1. Embryo germination time (a) and germination percentage (b) from matured seeds of eight aromatic rice cultivars, viz., Gobindohog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipani (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT). For all box plots, central bold line indicates the median; boxes delineate 1st and 3rd quartiles, while the lower and upper lines refer to minimum and maximum values respectively. Three biological replicates ( $n = 3$ ) were used for analysis. Similar letters indicate no significant difference ( $P < 0.05$ ) using Tukey's HSD test.

due to the differential accumulation of phytohormones, depending upon the spatial and temporal distribution, and their physiological and developmental stages [28]. The critical concentration of 2,4-D for callus induction and embryogenic callus formation from matured seeds of cereal crops is between  $10^{-5}$  to  $10^{-7}$  M [29]. The possible reason for requiring different concentrations of 2,4-D for callus proliferation may be due to the variation of genetic makeup in the endogenous levels of phytohormones which influence the callus induction and proliferation [27]. One of the inevitable components of tissue culture and transformation processes is subculturing, and callus growth and browning becomes more evident with the increased passages of subculture. The main obstacle for transgenic manipulation of rice by callus culture from matured seed is callus browning which prevents normal regeneration process due to the death of brown calli in indica rice variety. We measured the percentage of callus browning for the eight aromatic rice cultivars. Overall, the highest browning occurred in PB (71.55%) followed by TP (44.25%) which was significantly different from GB (11.30%) and RT (12.25%) (Figure 3c). The GB and RT showed minimum callus browning in MS medium supplemented with  $3 \text{ mg L}^{-1}$  2,4-D. This result clearly indicated the genetic variability of rice cultivars with regard to callus browning which corresponds with the earlier reports [30, 31]. The data from ANOVA supported our

observation, showing significant ( $p < 0.001$ ) effect of genotype on callus browning. We also noted a significant ( $p < 0.01$ ) negative correlation between callus induction and percentage of browning (Figure 5c), pointing to the fact that the decrease of callus induction was related to the increased rate of callus browning. An earlier study also reported that a decrease in the rate of callus growth is occasionally related to the appearance of a brown coloured area [32]. The callus browning or regeneration of albino plants depend on the low concentration of 2,4-D [33] and high temperature [8]. However, in our study,  $3 \text{ mg L}^{-1}$  2,4-D and  $24^\circ\text{C}$  were used for callus induction, which are supposed to be the optimum conditions for callus induction [34]. The relationship of peroxidase (POD) and polyphenol oxidase (PPO) activities with callus browning has been shown earlier [35]. During cell damage, the cytoplasmic and vacuolar contents are mixed and phenolic compounds can readily become oxidized by air, PODs or PPOs. The catalytic activity of the enzyme may be inhibited by oxidized phenolic compounds which cause darkening of the culture medium and subsequent lethal browning of explants [36]. The unsuitable culture conditions are reported to lead to callus browning, viz., inappropriate  $\text{NH}_4^+$  concentrations [37], light level [38], temperature [8], thiamine [39], and auxin [40]. The genetic linkage analysis indicated that one single locus was responsible for rice callus browning and that mapped to chromosome 1 [41].





Figure 2. Callus induction and regeneration of matured embryos of eight aromatic rice cultivars, viz., Gobindobhog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipanji (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT); (a) embryo germination, (b) callus induction, (c) shoot bud initiation, (d) shoot induction and elongation of multiple shoots, (e) regenerated plant.

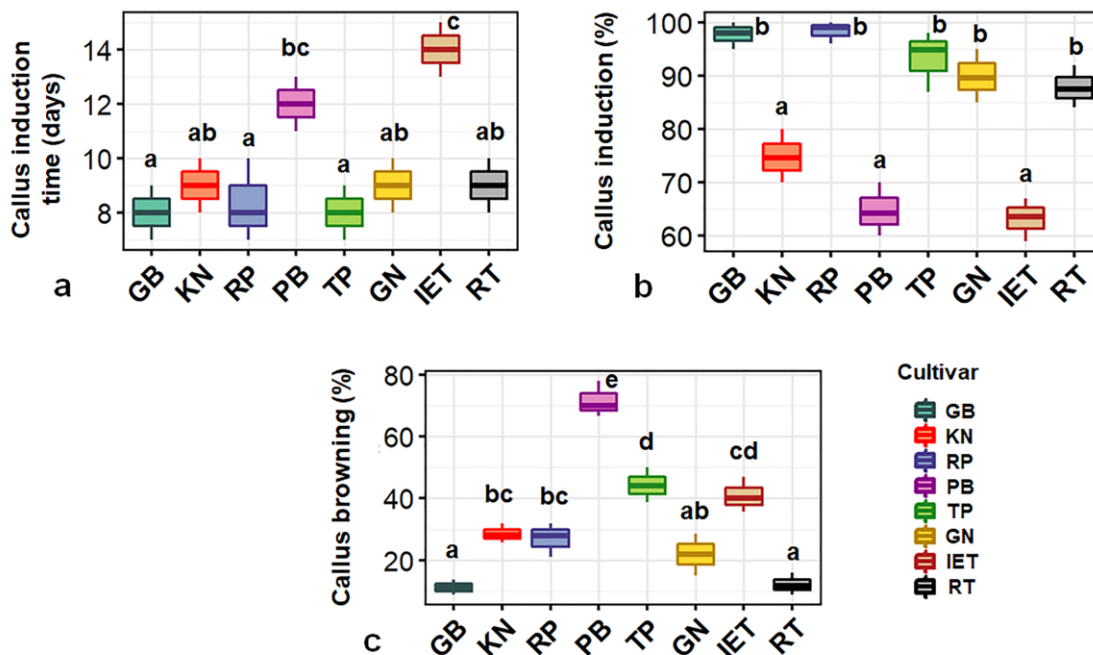


Figure 3. Callus induction time (a), percentage of callus induction (b) and percentage of callus browning (c) of eight aromatic rice cultivars, viz., Gobindobhog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipanji (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT). For all box plots, central bold line indicates the median; boxes delineate 1st and 3rd quartiles, while the lower and upper lines refer to minimum and maximum values respectively. Three biological replicates (n = 3) were used for analysis. Similar letters indicate no significant difference (P < 0.05) using Tukey’s HSD test.

Table 1. ANOVA table for different trait, related to callus induction and regeneration of eight aromatic rice cultivars, viz., Gobindobhog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipanji (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT). P-values of one-way ANOVA analyzing the effects of genotypes (G) are shown. The asterisks represent significant effects of G on the callus induction and regeneration frequency.

Trait	Genotype (G)
Embryo germination time	0.0003236 ***
Embryo germination percentage	1.053e-09 ***
Callus induction time	2.491e-05 ***
Callus induction percentage	2.795e-08 ***
Callus browning	1.723e-09 ***
Regeneration frequency	2.677e-08 ***

Significance level: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

### 3.3. Plant regeneration

The main objective of the present study was to compare the regeneration potential of eight indigenous aromatic rice cultivars, since the regeneration step is important and necessary to derive plantlets for crop improvement program [42]. Many high yielding, elite rice cultivars show low regeneration potential; however, regeneration capacity of plant varies, depending on their genetic background [43] and is considered as a major bottleneck for crop improvement via introgression of desirable genes in rice genome through transgenic approach [44,45].

In the current study, high quality embryogenic calli were transferred to the regeneration medium (RM) in order to investigate their potential for plant regeneration. The plant regeneration potential ranged from 93.43% to 33% depending on the genotypes examined. The maximum plant regeneration capability was noted in KN (93.43%) followed by GB (90.83%), TP (89.34%), RP (89%), IET (75.92%), GN (74.66%) and RT (71%). The minimum regeneration potential was observed in PB (33%) which was significantly different from other aromatic rice cultivars (Figure 4).

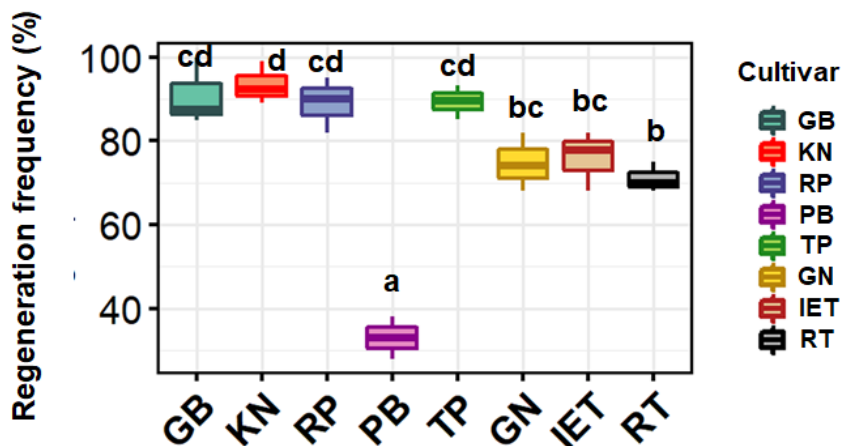


Figure 4. Regeneration frequency of eight aromatic rice cultivars, viz., Gobindobhog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipanji (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT). For all box plots, central bold line indicates the median; boxes delineate 1st and 3rd quartiles, while the lower and upper lines refer to minimum and maximum values respectively. Three biological replicates (n = 3) were used for analysis. Similar letters indicate no significant difference (P < 0.05) using Tukey’s HSD test.

A significant (p < 0.001) negative correlation was observed between callus browning and plant regeneration capacity (Figure 5d), while positive correlation (p < 0.01) between percentage of callus induction and plant regeneration capacity (Figure 5e), indicating callus induction capacity as an ideal index for the ability of the callus to generate plantlets during micropropagation. The aromatic rice cultivars which showed high callus induction percentage during the first week of culture were more probable to regenerate plantlets after several weeks of culture. An earlier study also reported high positive correlation between the embryogenic calli production and capacity for plant regeneration [46]. Upon transfer of the calli to RM and maintaining there for two weeks, greening effect was observed for all the varieties. After three weeks, the green

spots developed multiple shoots, followed by *in vitro* plantlet formation, and then developing into healthy plants (Figure 2). Naphthalene acetic acid was used in the RM according to Iqbal et al. [24]. Earlier study reported that weak plantlets were developed in the absence of NAA [47] and also that higher amounts of BAP and kinetin may stimulate initial cell division, playing an important role in somatic embryogenesis and plantlet regeneration [34, 48]. In our study, a combination of 1 mg L<sup>-1</sup> NAA, 2 mg L<sup>-1</sup> BAP and 4 mg L<sup>-1</sup> kinetin contributed to the highest plantlet regeneration in all the indigenous aromatic rice cultivars. The role of kinetin in improving somatic embryogenesis by influencing mitosis, cytokinesis, total protein synthesis, lignin biosynthesis and vascular differentiation is well known from earlier reports [49].



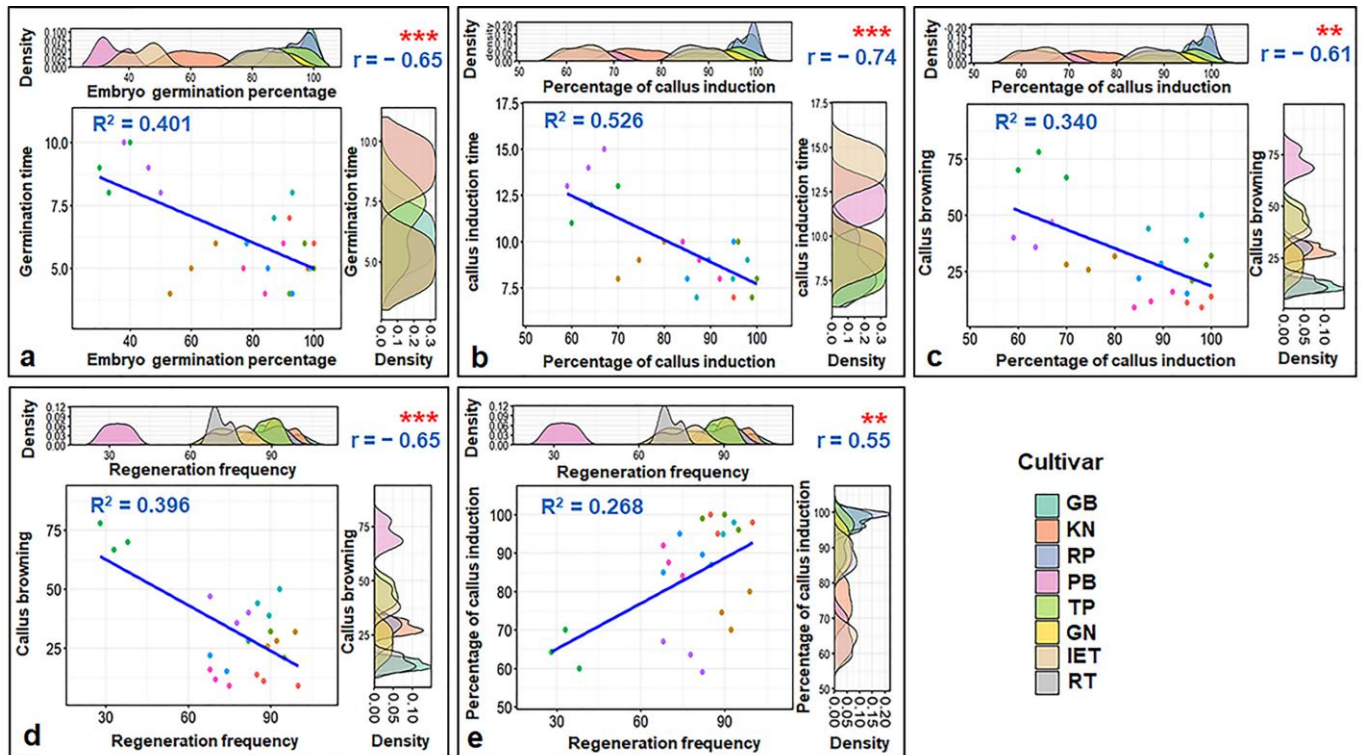


Figure 5. Correlation between embryo germination time and embryo germination percentage (a), between callus induction time and percentage of callus induction (b), between callus browning and percentage of callus induction (c), between callus browning and regeneration frequency (d), and between percentage of callus induction and regeneration frequency (e). The corresponding distribution curves of aromatic rice cultivars, viz., Gobindohog (GB), Kalonunia (KN), Radhunipagal (RP), Pusa Basmati (PB), Tulaipanji (TP), Gandheswari (GN), IET 21261 (IET) and Radhatilak (RT) are also shown for each trait. The Pearson’s correlation coefficient ( $r$ ), regression values ( $R^2$ ) and their significance level (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) are displayed on top right of each segment.

#### IV. CONCLUSION

In the present work, a comparative analysis of *in vitro* callus induction and regeneration efficiency of indigenous aromatic rice cultivars was carried out. We report here a successful high-frequency regeneration protocol from matured rice embryos of eight aromatic rice cultivars from West Bengal, India. Our result showed a high seed germination rate in MS medium supplemented with 3% (w/v) sucrose for all the varieties except PB and IET. High callus induction frequency was observed for all the rice varieties in MS medium supplemented with 3 mg L<sup>-1</sup> 2,4-D and 30 g L<sup>-1</sup> sucrose. A significant negative correlation was noted for callus induction time and callus browning with callus induction frequency. Further, our result showed the potential of plant growth hormone combination of 1 mg L<sup>-1</sup> NAA, 2 mg L<sup>-1</sup> BAP and 4 mg L<sup>-1</sup> kinetin to promote regeneration of the chosen aromatic rice varieties. The maximum regeneration potential was noted in KN followed by GB, TP, RP, IET, GN and RT, and minimum regeneration potential in PB. Further, we observed a significant negative correlation

between callus browning and plant regeneration capacity, while positive correlation between percentage of callus induction and plant regeneration capacity, indicating callus induction capacity as a good index for assessing the ability of callus to regenerate plantlets. All the eight cultivars responded, though with different extent, to *in vitro* culture especially with respect to embryogenic callus production and plant regeneration. Overall, our findings showed a significant effect of genotype on the callus induction ability, embryogenic response and plant regeneration capacity in the selected aromatic rice cultivars. The work will pave the way for future crop improvement program of economically important aromatic rice varieties through transformation of desirable genes into their genome for obtaining desirable traits.

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