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Genotype-media Interactions in Aromatic Rice (*Oryza sativa* L.) Landraces of India, relation with hypoxic Germination

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ABSTRACT: An experiment to optimize media for callus induction frequency (CIF) and shoot regeneration efficiency (SRE) of ten aromatic rice genotypes including nine local landraces and one released variety of Odisha using mature seed embryos as explants in five strengths of 2,4-dichlorophenoxy acetic acid (2,4-D) (1.0mgl^{-1}, 1.5mgl^{-1}, 2.0mgl^{-1}, 2.5mgl^{-1}, 3.0mgl^{-1}) and two concentrations of 6- benzylaminopurine (BAP) (1.0 mg^{-1} and 2.0 mgl^{-1}) in combination with α- naphthalene acetic acid (NAA) and kinetin (Kn) in Murashige & Skoog (MS) medium was performed. MS medium with 2mgl^{-1} of 2,4-D, 0.5mgl^{-1} of NAA, 0.1mgl^{-1} of Kn, 0.25mgl^{-1} of BAP and half strength MS medium with 2mgl^{-1} BAP, 0.25 mgl^{-1} Kn, 0.1mgl^{-1} NAA was found most efficient for CIF and SRE respectively. Tested genotypes showed varied responses towards CIF, SRE and callus morphology in the same medium. The response of genotypes to callus induction and shoot regeneration was supported by their behavior towards hypoxic germination and subsequent seedling development.

Keywords: Aromatic rice, callus induction, regeneration, MS medium, hypoxic.

INTRODUCTION

Aromatic rice is a special group of rice having pleasant aroma or flavour and excellent taste. Accumulation of the compound 2 acetyl-1 pyrroline (2AP), due to truncation and loss of function of Betaine Aldehyde Dehydrogenase (BADH2) enzyme, results in aroma of these genotypes (Bradbury *et al.,* 2005; Chen *et al*., 2008). World famous basmati rice is synonymous to aromatic rice. Besides basmati, there is also non-basmati type aromatic rice belonging to *aus* sub-population under *indica* sub-species of Asian cultivated rice (*Oryza sativa* L.) (Garris *et al.,* 2005; Civan *et al.,* 2019). *Aus* group aromatic rice are short and medium grain flavoured rice possessing excellent aroma, taste and many rare alleles for stress tolerance (Casartelli *et al.*, 2018). These aromatic rice occupy only around 1% of the total rice grown area in Odisha, mainly due to their low yielding capacity (Das and Khanda, 2020). Genetic improvement of these aromatic rice is hindered due to their cross incompatibility with other rice groups as well as due to undesirable linkage in the resultant recombinants (Desai *et al*., 2021). A well-organised research programme involving *in vitro* culture and conventional breeding methods for genetic enhancement of yield, while maintaining their aroma and taste is required (Gosal and Kang, 2012).

The *indica* sub-species is considered to be less responsive and recalcitrant to tissue culture compared to *japonica* lines (Hasan *et al.,* 2021). Genotypes within the *indica* sub-species show different response to *in vitro* culture (Ali *et al.,* 2021). As the conditions suitable for regenerating certain rice genotypes have been ineffective to regenerate other genotypes and the *in vitro* techniques are specific for a species and even specific for genotypes within a species (Niizeki and Oono, 1968), callus induction frequency (CIF) of a genotype in the culture medium is considered as the basic information required for selecting the genotype

for any research based on *in vitro* culture (Carsono *et al.*, 2021).

Concentration of auxins, cytokinins, their combinations and study of genotype-media interaction to find appropriate media composition is important for enhancing *in vitro* response of a particular genotype in rice (Liang *et al.*, 2021). 2,4-D is the most suitable auxin for callus induction of rice but its optimum concentration varies with the explant source and genotype. No callus formation occurs in the absence of 2,4-D , while MS medium with 2.0 mgl^{-1} to 3.0 mgl^{-1} 2,4-D (depending on genotype) is best for callus induction through seeds in *indica* species (Shobhana *et al.*, 2018). Shoot regeneration in *indica* rice from calli of mature embryos is possible in MS (Murashige and Skoog, 1962) medium with 0.5 mgl^{-1} - 2.0 mgl^{-1} BAP depending on the genotypes (Hnatuszko-Konka *et al*., 2021). Auxins especially NAA (2.0 mgl^{-1}) are applied to stimulate root initiation from tissue cultured shoots in MS medium (Rout *et al.*, 2016).

Reduced growth and necrosis in some rice genotypes during callus culture is mainly due to the accumulation of ethylene and to some extent carbon dioxide in the culture medium. This causes depletion of oxygen in the nutrient medium as ethylene is found to restrict callus growth (Stephen *et al*., 1993). The genotypes which show high callus induction frequency when inoculated in the culture medium are able to tolerate high concentrations of ethylene. The response towards tissue culture is genotype dependent, same in the case of genotypes which possess certain QTLs associated with tolerance to low oxygen stress and can germinate under high concentrations of ethylene (Ismail *et al*., 2009; Barik *et al*., 2019).

Genotype response to *in vitro* callus induction and subsequent plant regeneration is the prerequisite for employing any research program involving tissue culture (Silva *et al*., 2015). In this context our study was focused to relate the genotype - media interaction towards callus induction and shoot regeneration and led to optimization of media for CIF and SRE in responsive genotypes. The association between the capacity of a genotype to germinate under anaerobic (hypoxic) conditions and subsequent seedling development with its CIF and SRE respectively, was also found positive.

MATERIALS AND METHODS

The research work was conducted in the tissue culture laboratory, Crop Improvement Division, ICAR-National Rice Research Institute, Cuttack, India during July 2018 to November 2019. Seeds for the experiment were provided by Regional Research and Technology Transfer Station (RRTTS), Bhawanipatna (OUAT), Kalahandi, Odisha (Table 1).

Table 1: Characteristic features of the aromatic rice genotypes used in the experiment.

Sr. No.	Genotype	Category	Grain type	Aroma level	Plant height (cm)	Duration (Days)	Avg. yield per plant (g)
1.	Basumati	Landrace	Short medium	Strong	152	138	32.1
2.	Kalikati	Landrace	Short bold	Medium	161	134	34.30
3.	Parijat	Landrace	Short bold	Medium	138	134	24.83
4.	Kanakachampa	Landrace	Short medium	Mild	144	132	25.16
5.	Jeerakasal	Landrace	Medium	Medium	142	134	27.0
6.	Karpurajeera	Landrace	Short bold	Medium	141	134	24.2
7.	Parbatjeera	Landrace	Short bold	Medium	156	138	16.33
8.	Gangabali	Landrace	Short medium	Strong	159	146	30.73
9.	Dhanaprasad	Landrace	Short medium	Mild	145	133	36.33
10.	CR Dhan-907	Released variety	Medium slender	Mild	94	145	56.06

Explants. Manually dehusked, surface sterilized, (using recommended bactericides and fungicides-ethanol, NaOCl and $HgCl_2$) mature seeds of aromatic rice were used as explants in the present experiment.

Culture Media and inoculation. MS media with five concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}), 0.5 mgl^{-1} NAA, 0.25 mgl^{-1} BAP and 0.1 mgl^{-1} Kn along with 100 mgl^{-1} myo-inositol, 3% (w/v) sucrose (pH 5.8) and 8 gl^{-1} agar were formulated for callus induction (Table 2). Autoclaved at 15*psi* and 121°C for 20 minutes, 50ml of it was dispensed into each sterilized disposable 100mm×15mm petri plate. Surface sterilized seeds were inoculated horizontally (12-15 in each petri plate) on to the media under laminar flow unit and incubated at 25±2°C in dark conditions. Each treatment with 100 seeds was repeated three times. Data on callus induction frequency was taken after 4 weeks of inoculation.

Half strength MS medium with two concentrations of BAP (1 mgl^{-1}, 2 mgl^{-1}), 0.1 mgl^{-1} NAA and 0.25 mgl^{-1} Kn, 100 mgl^{-1} myo-inositol, 3% w/v sucrose (pH 5.8) and 0.8% w/v agar was used for shoot regeneration. 25 ml of molten media was poured into culture tubes (25 x 150 mm), plugged with non- absorbent cotton, autoclaved, removed and cooled by slanting at an angle of 45° to increase the surface area of medium for

subsequent callus inoculation. 2-4 calli of size 2mm diameter were inoculated in each culture tube, incubated at 25 ± 2°C with a photoperiod of 12 hrs at 2000 lux light intensity, each treatment with three replications. Data on shoot regeneration efficiency was taken after 4-6 weeks depending on the genotype response. MS medium supplemented with NAA (2.0 mgl^{-1}), Kn (0.5 mgl^{-1}) and 50 gl^{-1} sucrose with 7 gl^{-1} agar-agar was used for root initiation from 1-2 cm long green plantlets in 25 × 150 mm size culture tubes having one shoot per tube and incubated at 25± 2°C. Observations for root initiation was taken after 10-15 days of inoculation.

Table 2: Different combinations of plant growth regulators used for callus induction, shoot and root regeneration.

Sr. No.	Media Code	Plant growth regulators (mgl^{-1})				Medium
		2,4-D	BAP	NAA	Kn	
1	M1	1.0	0.25	0.5	0.1	Callus induction
2	M2	1.5	0.25	0.5	0.1	
3	M3	2.0	0.25	0.5	0.1	
4	M4	2.5	0.25	0.5	0.1	
5	M5	3.0	0.25	0.5	0.1	
6	RM1	-	1.0	0.1	0.25	Shoot regeneration
7	RM2	-	2.0	0.1	0.25	
8	R1	-	-	2.0	0.5	Root regeneration

Acclimatization and hardening of plantlets. Plantlets, 20 days after root initiation, were separated from the culture medium, washed thoroughly under tap water and incubated by dipping in 1% Hoagland's solution for 48 hours in 25 ± 2°C temperature before planting into pots which were kept in green house for 7 days before transferring to net house.

Hypoxic germination. Germination percentage of 100 seeds of each genotype, dipped in 12 cm long test tube (10-12 in each test tube) filled with 15 ml of water (10 cm standing water) and kept in standing position for 10 days without disturbing was calculated.

Seedling development after withdrawal of oxygen stress

After 20 days, standing water from all the test tubes with seeds was drained, seeds were rinsed for 2-3 times with tap water and placed in the petri plates containing thin layer of water. Observations for root and shoot growth was taken on 3rd and 7th day after plating and seedling development of the genotypes was calculated. Following calculations were done for each genotype:

$$\text{Callus Induction Frequency (\%)} = \frac{\text{Number of seeds produced calli}}{\text{Number of seeds inoculated}} \times 100$$

$$\text{Shoot regeneration efficiency (\%)} = \frac{\text{Number of calli produced shoots}}{\text{Number of calli cultured}} \times 100$$

$$\text{Root regeneration efficiency (\%)} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot cultured}} \times 100$$

$$\text{Germination \%} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds soaked}} \times 100$$

$$\text{Seedling development (\%)} = \frac{\text{Number of seedlings developed}}{\text{Number of oxygen stressed seeds}} \times 100$$

Statistical analysis. Research methods arranged in a completely randomized design with three independent replications per treatment for each genotype adopting standard analysis of variance techniques (ANOVA). Means of each treatment were separated by Duncan's Multiple Range Test (DMR), least significant difference (LSD, $p<0.05$) using MSTATC. Comparison of the traits was realized using Excel sheet.

RESULTS AND DISCUSSION

From the genotype- media interaction resulting in callus induction and shoot regeneration the following inferences was drawn.

Effect of different strengths of 2, 4 D on callus induction of the genotypes. Different concentrations of 2,4-D had a considerable influence on callus induction in different genotypes and it was observed that callus induction for all the media tested was statistically significant ($p<0.05$). MS medium with 2.0 mgl^{-1} 2,4-D, 0.5 mgl^{-1} NAA and 0.25 mgl^{-1} BAP and 0.1 mgl^{-1} Kn was found to induce maximum calli in most of the landraces. Landrace Kalikati was found most responsive to callus induction (72.3%) followed by Basumati (66.6%) and Dhanaprasad (61.0 %).CIF was found to be maximum at 2.5 mgl^{-1} concentration of 2,4-D in case of the released aromatic rice variety CR Dhan 907.

Least CIF was found in the landrace Gangabali (19.6%), in all the concentrations (Table 3). CIF was low in lower concentrations of 2,4-D (1.0 mgl^{-1} and 1.5 mgl^{-1}) in all the genotypes which increased initially with increase in the concentration of 2,4-D but decreased at higher concentrations which was earlier reported by Din *et al.*, (2016). Response of genotype towards callus induction was found to be significantly different from each other in same medium. As CIF is genotype dependent character, each genotype showed different response towards the same medium as was also found by Mostafiz and Wagiran, (2018).

Table 3: Response of the genotypes towards different media and oxygen stress.

Genotype	Callus Induction Frequency (CIF) (%)					Shoot Regeneration Efficiency (SRE)(%)		Anaerobic (hypoxic) germination (AG)(%)	Seedling development on 7th day (SD) (%)
	M1	M2	M3	M4	M5	RM1	RM2		
Basumati	34.6b	46.2b	66.6ab	50.0bc	47.3a	41.6c	54.0c	73.3a	26.0 d
Kalikati	42.6a	60.6a	72.3a	69.6a	50.0a	62.3ab	68.0b	80.0a	76.6 a
Parijat	20.1c	22.3d	36.0de	33.3d	22.3c	27.6d	35.3de	16.6d	6.6 ef
Kanakachampa	22.3c	39.0c	44.3cd	22.3de	14.0d	27.9d	44.6d	20.0d	60.0 b
Jeerakasal	34.6b	36.0c	53.0bc	47.3c	39.0ab	20.3de	25.0f	36.6c	43.3 c
Karpurajeera	20.3c	24.1d	33.3def	27.6d	8.0d	53.3b	62.0b	33.3c	73.3 a
Parbatjeera	19.6c	50.0b	55.6bc	52.6bc	27.6b	25.6d	32.0ef	63.3b	3.3 ef
Gangabali	2.6d	6.0e	19.6f	11.0e	8.3d	77.3a	79.3a	13.3d	80.0 a
Dhanaprasad	20.2c	22.3d	61.0ab	52.6bc	22.3c	18.6e	24.3f	63.3b	23.3 d
CRDhan-907	10.0cd	15.3b	26.0ef	61.0ab	45.6a	36.6c	43.0d	40.0c	13.3 e
LSD (0.05)	5.4	5.5	8.9	7.1	5.0	5.5	6.1	8.9	11.4

Effect of different concentrations of BAP on shoot regeneration of the genotypes. Two concentrations of BAP (1 mgl^{-1} and 2 mgl^{-1}) showed statistically significant ($p<0.05$) difference in the plant regeneration capacity of the genotypes studied (Table 3). Half MS basal medium with 2.0 mgl^{-1} BAP, 0.25 mgl^{-1}Kn and 0.1 mgl^{-1} NAA produced maximum green shoots. Landrace Gangabali (79.3%) followed by Kalikati (68.0 %) and Karpurajeera (62.0%) were found to have high SRE while Dhanaprasad (24.3%) and Jeerakasal (25.0%) had least SRE. SRE was found to increase with increasing BAP concentration from 1.0 mgl^{-1} to 2.0 mgl^{-1} as found by Biswas and Mandal (2007). Response of shoot regeneration was found to be different in all the genotypes studied as SRE in a particular medium was found to be highly genotype dependent (Lardon *et al*., 2020).

Genotypic response for CIF and SRE. Gangabali and Karpurajeera showed low CIF (19.6% and 33.3% respectively) but high SRE (79.3% and 62.2% respectively). This is because the genes responsible for callus induction are different from shoot regeneration (Nguyen *et al.*, 2020). Out of the ten genotypes studied, six (Kalikati, Basumati, Dhanaprasad, Jeerakasal, Parbatjeera and CR Dhan-907) had CIF and only four (Gangabali, Kalikati, Karpurajeera and Basumati) had SRE above 50%. All these genotypes belonging to *indica* sub-species were found to be less responsive to tissue culture as stated earlier by many rice researchers (Sundararajan *et al*., 2020).

Effect of genotype and culture medium on the callus morphology of different genotypes. Callus type and texture is the characteristic of a genotype (Lee *et al.*, 2002) (Table 4). Calli of Parijat, Dhanaprasad, Parbatjeera, Jeerakasal, Gangabali, Kanakachampa and CR Dhan-907 were creamy to creamy white while calli of Kalikati and Basumati were pale yellow in colour. Calli of Kalikati, Gangabali, Karpurajeera and Basumati were granular, compact and dry and that of Parijat, Jeerakasal, Dhanaprasad and Parbatjeera were smooth and mucilaginous in texture (Fig. 1).

Table 4: Callus morphology of tested genotypes in M3 medium.

Genotype	Callus morphology
Basumati	Pale yellow, moderate granular, somewhat compact and dry
Kalikati	Pale yellow, moderate granular, dry and compact
Parijat	Creamy, somewhat smooth, soft and mucilaginous
Kanakachampa	Creamy, large granular, dry and compact
Jeerakasal	Creamy , Large granular, dry and compact
Karpurajeera	Creamy, moderate granular, dry and compact
Parbatjeera	Creamy, moderate granular, somewhat compact and mucilaginous
Gangabali	Creamy , small granular, compact and dry
Dhanaprasad	Creamy white, large granular, somewhat compact and mucilaginous
CRDhan-907	Creamy white, large granular, somewhat compact, dry

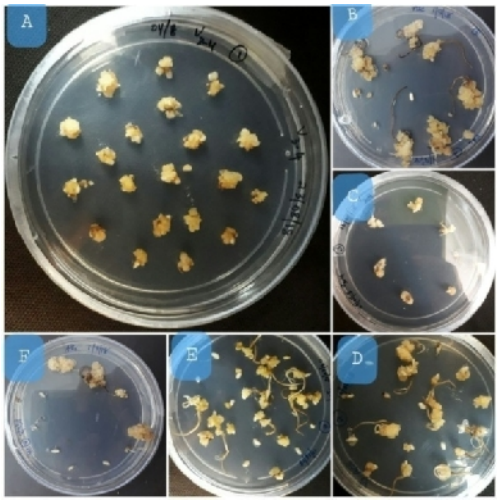


Fig. 1. Callus morphology of different genotypes A- Kalikati (Pale yellow, granular, compact), B- Kanakchampa (Creamy, large granular, compact), C- Parijat (Creamy, smooth, mucilaginous), D- Parbatjeera (Creamy, granular, mucilaginous), E- Basumati (Pale yellow, granular, compact), F- Jeerakasal (Creamy, large granular, compact).

It was found that compact, granular, dry, pale yellow to creamy colour calli (Kalikati, Gangabali), showed more plant regenerating capacity (54.0 to 79.3%) than the whitish colour calli with smooth and mucilaginous appearance (Parijat, Dhanaprasad) (24.3 to 35.2%) this relationship was earlier reported by Narciso and Hatorri (2010).

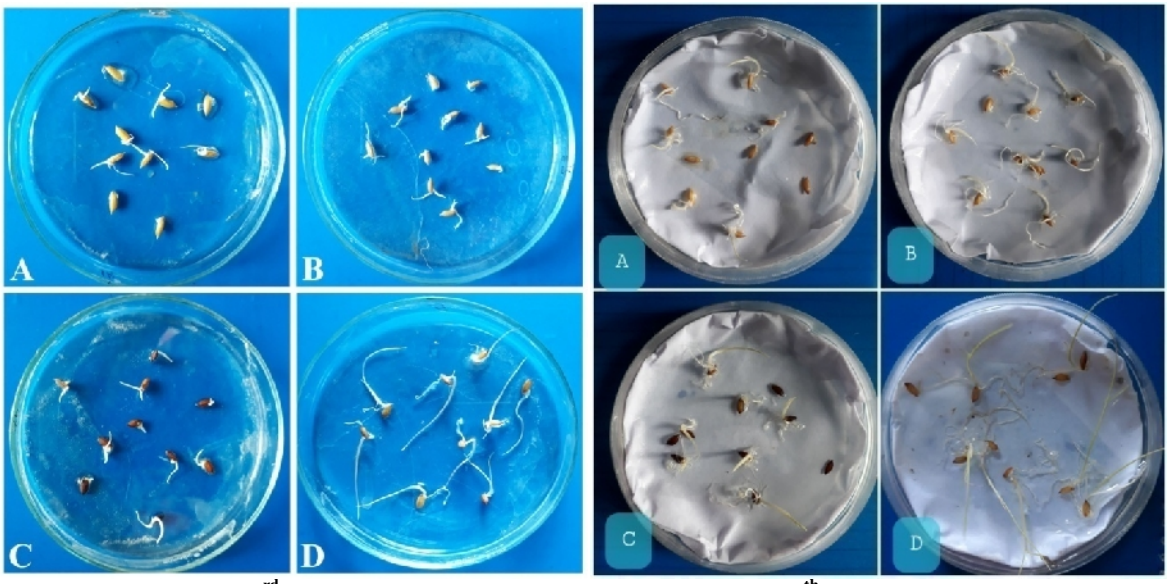
Anaerobic (hypoxic) germination capacity of genotype and its relationship with CIF. In Kalikati, Basumati, Dhanaprasad, Parbatajeera and CR Dhan-907 coleoptile elongation was observed while Gangabali, Karpurajeera, Parijat, Kanakachamapa and Jeerakasal could not germinate inside water filled test tubes (hypoxic condition) (Fig. 2).

Coleoptile development	No germination
A. Dhanaprasad	F. Kanakachampa
B. Parbatjeera	G. Parijat
C. Kalikati	H. Jeerakasal
D. CR Dhan 907	I. Karpurajeera
E. Basumati	J. Gangabali

Fig. 2. Genotype response to germination under low oxygen (hypoxic) stress.

Anaerobic (hypoxic) germination capacity was found to be significantly different among the tested genotypes (Table 3). The genotypes which showed coleoptile elongation under hypoxic conditions were found to have high CIF in culture medium, while those which could not germinate under oxygen stress were also found to have low CIF (Table 3, Fig. 2 and 4). As CIF is genotype dependent it can be said that the genotypes with an ability to germinate and develop coleoptile under low oxygen and high ethylene concentrations would be more responsive to callus induction in culture medium, where seeds are subjected to similar conditions (Stephen *et al.*, 1993; Ismail *et al*., 2009).

Association of seedling development after stress with SRE: Seedling development (SD) under normal conditions after removal of oxygen stress was found to be significantly different among the genotypes tested. Gangabali, Kalikati Karpurajeera and Kanakachampa showed >50% SD (80%, 76.6%, 73.3% and 60% respectively) (Table 3, Fig. 3 and 4), corresponding to their higher SRE (79.3%, 68%, 62%, and 44.6 respectively). Parbatjeera, Parijat, CR Dhan-907 and Dhanaprasad with <30% SD (3.3%, 6.6%, 13.3% and 23.3% respectively) had low SRE (35.3%, 32%, 43% and 24.3 respectively). With few exceptions, SD and SRE were found to be positively related. As SRE is genotype dependent, it can be said that the genotypes with the ability to develop into seedlings after undergoing a stress period can be similar to regenerating shoot from stressed embryogenic callus cells. This may be due to the presence of few similar loci (QTLs) governing these characters in the genotypes as mentioned by Ghosal *et al.*, (2019); Baltazar *et al*., (2019).



3rd Day 7th Day

A. Kanakachampa B. Gangabali C. Karpurajeera D. Kalikati

Fig. 3. Seedling development (3rd and 7th day) after withdrawal of oxygen stress.

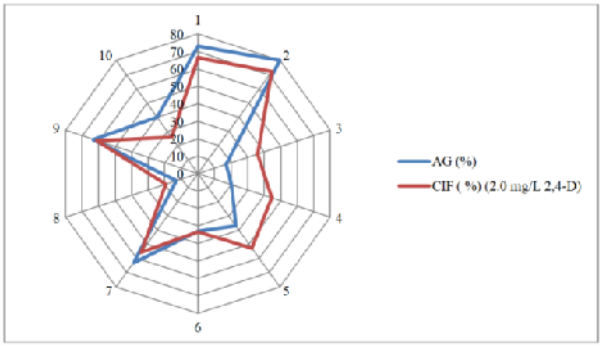


Fig. 4. Comparison of anaerobic (hypoxic) germination (AG) % with CIF (%) of the genotypes 1. Basumati, 2. Kalikati, 3. Parijat, 4. Kanakchampa, 5. Jeerakasal, 6. Karpurajeera, 7. Parbatjeera, 8. Gangabali, 9. Dhanaprasad, 10. C R Dhan 907.

CONCLUSION

Both CIF and SRE are genotype dependent. Genes responsible for callus induction are different from shoot regeneration. Each genotype has its specific media requirement for efficient CIF and SRE. The positive association of anaerobic (hypoxic) germination capacity with CIF and seedling development with SRE of the genotypes may be due to the presence of QTLs associated with these characters. This relationship can be helpful while selecting a genotype for research program involving *in vitro* techniques.

FUTURE SCOPE

As local aromatic rice cultivars under study were found to be less responsive to tissue culture, further study involving various media compositions and sugar source specific to genotype should be carried out for increasing CIF and SRE of the genotypes. Relation of hypoxic germination capacity to CIF can be evaluated in other rice cultivars for trait association.

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Conflict of Interest. None.

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