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Research Note

Evaluation of anaerobic germination and submergence tolerance in rice (*Oryza sativa* L.) suitable for direct seeded condition

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Abstract
The leading target domain in lowland areas is improving the tolerance for anaerobic germination and submergence, especially in direct-seeded rice (DSR). The present study identified elite genotypes with tolerance for anaerobic germination and submergence among 25 diverse rice genotypes. This study adopted four experimental conditions (three in the lab and one in the field): anaerobic germination in water, anaerobic germination with soil and water, submergence condition and direct seeded rice in the field. The recorded traits showed significant variation under all four stresses. *Chitiraikar* performed well in anaerobic germination and DSR experiments. CO55 registered the highest trait value of seedling height and early vegetative vigour in the anaerobic experiment with water. *Aanaikomban, Karunguruvai, Karuppukavuni* and *Chitiraikar* outperformed other genotypes in most of the above experimental conditions. Thus, choosing lines based on germination percentage, early seedling vigour, elongation index and seedling survival rate can increase tolerance for anaerobic germination and submergence conditions in the lab and DSR method. Among all the experiments conducted, anaerobic germination with soil and water and the DSR method was found to be reliable for screening a large population.

Keywords: rice, anaerobic germination, submergence tolerance, direct seeded rice.

Rice (*Oryza sativa* L.) is one of the most important cereal food crops and the main food source for more than one-third of the world's population. Over 90 per cent of the world's rice is produced and consumed in Asia (Bandumula, 2018), where a tremendous ecological diversity exists due to the various climatic conditions, soil, cultural practices, and human selection. Rice occupies an area of about 46.38 million hectares with a production of 130.29 million tonnes and productivity of 2809 kg/ha during 2021-22 (4th advance estimates, E&S Division, DA&FW, 2021). Abiotic stresses like water deficit, absence of anaerobic respiration, submergence, salinity, and deficiencies of P and Zn greatly affect rice production worldwide. There is a 50% reduction in average yields of all major crops due to abiotic stresses and it is the main cause of crop failure worldwide (Varshney and Tuberosa, 2013).

Direct-seeded rice (DSR) has emerged as a feasible alternative to address water and labour shortages (Sun *et al.,* 2015). The term direct seeding of rice refers to the method of establishing rice plants by sowing seeds directly into the field. There are three methods of rice direct seeding: dry seeding (sowing dry seeds into dry soil), wet seeding (sowing pre-germinated seeds on wet puddled soil), and water seeding (sowing seeds into standing water) (Xu *et al.,* 2019). Because of its lower planting cost, DSR is more easily adopted by rice growers whose goal is to maximize economic returns. At the beginning of the 21st century, DSR occupied 21% of Asia's total rice planting area (Xu *et al.,* 2019). In recent years, DSR cultivation has been increasingly adopted by farmers in many traditional transplanted rice (TPR) regions (Sun *et al.,* 2015).

DSR is affected by anaerobic stress during the seed germination and seedling stage. Due to excessive flooding in the field, the seeds sown will experience anxiety and suffer from a lack of oxygen. Anaerobic germination-tolerant rice genotypes showed seed germination and the emergence of seedlings above the surface of water (Chamara *et al.,* 2018). Improvement of rice genotypes for tolerance against anaerobic germination and submergence during the seedling stage is very important for direct seeded rice production (Septiningsih *et al.,* 2013). Flash flooding is a serious, naturally occurring problem for rice production in the rain-fed lowlands. Fifty per cent of the rice growing area in this ecosystem is affected by flash flooding at various stages of growth. Higher-yielding modern rice varieties die within a week of complete submergence, making them unsuitable alternatives to traditional rice landraces. Complete submergence of the rice crop for 10-15 days during flash flooding is a severe constraint to rice production in areas of high-rainfall lowland ecosystems.

Submergence is the condition where the rice seedling completely submerges in water for several days. After salinity and drought, submergence and anaerobic germination are important abiotic stresses influencing rice production. Nearly 22 million hectares of rainfed lowland areas in South and South-East Asia are affected due to flooding, out of 6.2 million hectares of rice lands in India (Azarin *et al*., 2017; Dar *et al*., 2017). Out of 22 million hectares, 15 million hectares of rainfed lowland are affected by short-term flash flooding (Singh *et al*., 2017), and economic loss is estimated to be One billion US dollars. The flood-prone ecosystem comprises about 7% of the global rice area and produces 4% of world rice (Yang *et al*., 2017). Rice is remarkably well adapted to submergence conditions and it can germinate in the complete absence of oxygen. Anaerobic germination includes a lengthening of the coleoptile, that, analogous to the escape strategy, aims to make aerial contact but considerable variation exists among rice genotypes in coleoptile extension during anoxia (Loreti *et al.,* 2016). Respiration is arrested in 7- to 14-day-old seedlings, and the seeds are unable to germinate underwater. Flash flooding results in complete submergence of rice crops and the uptake of O_2 for respiration and CO_2 for photosynthesis is greatly impeded. Matured rice seedlings which are above 14 days after transplant or direct seeded are affected due to this phenomenon. Rice adopts two strategies to overcome the submergence stress: submergence tolerance and escape mechanism (Luo *et al*., 2011). Four QTLs for anaerobic germination were recently identified from an Indian cultivar, BJ1 (Ghosal *et al*. 2020). Submergence tolerance is expressed by rice varieties adapted to flash floods where a rapid increase in water level causes partial to complete submergence for up to two weeks. On the contrary, certain plants that can survive in waterlogged areas for extended periods use escape mechanisms through their rapid shoot elongation capability. This study aims to understand the response of various genotypes to different anaerobic stress conditions and submergence, to identify elite breeding material suitable for DSR conditions.

Twenty-five different rice genotypes (**Table 1**) collected from all over Tamil Nadu were selected and used as the experimental materials. Three different experiments to simulate submergence namely, anaerobic germination with water (Exp 1), anaerobic germination with soil + water (Exp 2), and submergence tolerance (Exp 3) were conducted in the Genetics and Plant Breeding laboratory of Karunya Institute of Technology and Sciences. DSR experiment (Exp 4) was conducted in the South Agricultural Farm of Karunya Institute of Technology and Sciences during *Kharif* 2023.

Experiment 1: Anaerobic germination experiment only with water

Ten seeds of all 25 genotypes were directly immersed in plastic cups of 11 x 9 x 5.6 cm size filled with water up to 10 cm *[***Fig. 1***]*. It was replicated thrice in Completely Randomized Design (CRD). Observations of germination percentage, seedling height and early seedling vigour were recorded.

Experiment 2: Anaerobic germination experiment with soil and water

The procedure adopted by Vinitha *et al.* (2023) was followed for this study. Sowing was done in the soil filled in plastic cups of 11 x 9 x 5.6 cm size *[***Fig. 1***]*. Water was then filled, and the water level was maintained up to 5 cm. The study was replicated thrice in a Completely Randomized Design (CRD). Observations on germination percentage, germination rate, seedling height and early seedling vigour were recorded.

Experiment 3: Submergence tolerance experiment

Ten seeds of all 25 genotypes were directly immersed in plastic cups of 11 x 9 x 5.6 cm size filled with soil and water. The soil was filled up to 3/4th of the volume of the cup. Each cup was irrigated regularly. Submergence was imposed after 14 days of aerobic growth conditions. Plastic buckets (45 x 45 cm) were used as a submergence medium. Water was filled for 30 cm and each cup was

Table 1. List of rice genotypes used in the present study

S. No.	Genotypes	Landraces /Varieties	Place of collection	S. No.	Genotypes	Landraces /Varieties	Place of collection
1	CO54	Variety	Tamil Nadu	14	*Karunguruvai*	Landrace	SSF, Kanyakumari
2	*Sorna masuri*	Landrace	Tamil Nadu	15	*Sivappukavuni*	Landrace	Tamil Nadu
3	*Kalasar nel*	Landrace	Kottaram	16	JCL nel	Landrace	Tamil Nadu
4	*Chitiraikar*	Landrace	AC and RI, Madurai	17	CO55	Variety	Tamil Nadu
5	*Kullakar*	Landrace	Tamil Nadu	18	TPS5	Variety	Tamil Nadu
6	*Seeraga samba*	Landrace	Tamil Nadu	19	*Kichili samba*	Landrace	Tamil Nadu
7	CR1009 *Sub1*	Variety	Tamil Nadu	20	CO52	Variety	Tamil Nadu
8	*Kuzhiyadichan*	Landrace	Tamil Nadu	21	TPS3	Variety	Tamil Nadu
9	CO51	Variety	Tamil Nadu	22	CO53	Variety	Tamil Nadu
10	*Aanaikomban*	Landrace	Tamil Nadu	23	ASD16	Variety	Tamil Nadu
11	*Thanga samba*	Landrace	Thanjavur	24	*Milagu samba*	Landrace	AC and RI, Madurai
12	ADT45	Variety	Tamil Nadu	25	*Garudan Samba*	Landrace	Tamil Nadu
13	*Karuppukavuni*	Landrace	AC and RI, Madurai				

submerged in the water *[***Fig. 1***]*. The experimental set-up was replicated thrice in a completely randomized design (CRD). Observations on seedling height, and elongation index were recorded. Survival percentage and number of recovered tillers were recorded after submergence treatment.

Experiment 4: Field trial of DSR

Ten seeds of each of all the 25 genotypes were directly sown in the well-drained field. The water level was kept at a height of 5 cm until germination. Daily irrigation was done to maintain water level to a standby of 5cm and the field was enclosed by ridges. Germination percentage and seedling height were recorded on the 25th day while leaf length and width were calculated on the 45th day (**Fig. 1**). This was because the seedling stage commences within 30 days and the tillering phase will start after that. Usually, tillering starts after 30 days. After germination the field was flooded and anaerobic stress condition was induced due to submergence. The genotypes which continued to grow even after the stress were regarded as submergence-tolerant lines. The experiment was replicated twice in a randomized block design (RBD).

Methods followed to measure the phenotypic traits related to seedling growth under submergence condition: All the genotypes were germinated in the cups with three replications each containing 10 seeds per genotype. Observations on germination percentage (GP), seedling height (SH), early seedling vigour (ESV), recovered number of tillers (RNT), elongation index (EI), seedling survival rate (SSR), leaf length (LL), leaf width (LW) were recorded namely. Germination percentage was measured by calculating the number of seeds germinated to the total number of seeds sown.

$$\text{Germination percentage (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \times 100$$

Seedling height was measured in centimetres (cm). The early seedling vigour index was estimated following the method of Abdul-Baki and Anderson (1973). The vigour index was calculated by using the formula;

Vigour Index (V.I.) = Seedling germination percentage (%) x Seedling length (cm). After a submergence period of 14 days, recovered seedlings were observed and the trait, recovered number of tillers were recorded. The seedling survival rate and elongation index of the leaf were calculated using the following formula;

$$\text{Seedling survival rate} = \text{Surviving seedling} / \text{Sprouted seedling} \times 100$$

$$\text{Elongation index} = \frac{\text{Length of shoots submerged for 14 days} - \text{length at beginning of submergence}}{\text{Length of shoots (control) after 14 days} - \text{Length at beginning of submergence}}$$

Leaf length was calculated from the tip of the longest leaf down to the base of the leaf and it was measured in centimetres. Leaf width was measured at the widest portion of the leaf blade and measured in centimetres.

Statistical analyses: Analysis of variance (ANOVA) was worked out to test the difference among the genotypes in completely randomized design (CRD) for lab study and randomized block design (RBD) for field study. Comparison of mean was done by the least significant difference test when the F value showed at least $p < 0.05$ significance level. Pearson's correlation analysis between different traits was done. The parameters from the trials

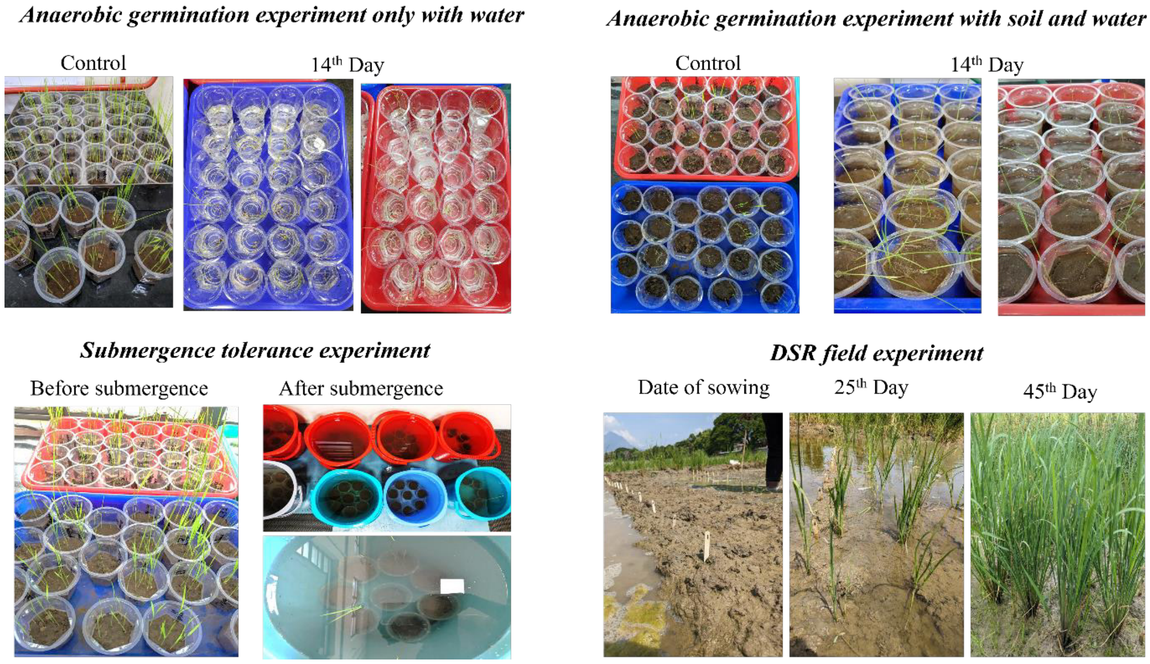

Fig. 1. Anaerobic germination and submergence screening of rice genotypes in the laboratory and DSR field condition i) Anaerobic germination experiment only with water ii) Anaerobic germination experiment with soil and water iii) Submergence tolerance experiment iv) DSR field experiment

namely germination percentage, seedling height, early seedling vigour, recovered number of tillers, elongation index and seedling survival rate are compared to the control for further study. All the statistical analyses were done with the help of the STAR tool (Statistical Tool for Agricultural Research). (http://qgb.irri.org/products)

The present study indicates the presence of considerable variation and genetic diversity for anaerobic germination and submergence tolerance among the set of rice germplasm evaluated. For direct seeding, it is critical to choose rice genotypes that can germinate well in anaerobic conditions with high seedling vigour (Mohanapriya *et al.,* 2022). Greater seedling vigour is a useful attribute in direct-seeded rice (Vu *et al*., 2016). Germination percentage is the successful trait that ensures the establishment of seedlings and subsequent seedling vigour (Miro and Ismail, 2013). Higher seedling vigour has been attributed to tolerance to anaerobic germination (Muvendhan *et al.,* 2023). Among the 25 genotypes that were germinated under anaerobic conditions, the landraces have been observed to have a high germination percentage when compared to the popular varieties. This conformed with the research outcomes of Partheeban *et al.* (2017): Vergara *et al.* (2014) also reported that the performance of landraces when compared to cultivated varieties was better under flooding conditions.

Analysis of variance: The ANOVA for each trait showed a significant variation at $p < 0.05$ for all the traits among the 25 genotypes studied (**Table 2**).

Descriptive statistics: In the anaerobic germination experiment only with water, concerning germination percentage, among all the genotypes TPS5 expressed the least germination percentage (50%), and *Chitiraikar, Aanaikomban*, JCL nel, TPS-3 and *Milagu samba* expressed the maximum germination percentage (100%). In an experiment conducted by Partheeban *et al.* (2017), CR 1009 *sub-1* showed 95% germination in an anaerobic germination experiment. Regarding seedling height, *Sivappu kavuni* showed a minimum seedling height of 16.1 cm while CO55 showed the highest seedling height of 29.2 cm. In the case of early seedling vigour *Kichili samba* had a lower trait value (1140), and CO55 had a higher trait value of 2636. Based on these parameters, CO55 could be considered tolerant to anaerobic conditions.

Comparing all the genotypes in the anaerobic germination experiment with soil and water, ASD16 expressed the lowest germination percentage (30%) and *Aanaikomban* expressed the maximum germination percentage (90%). Regarding seedling height, CO53 had the least seedling height (12 cm) and *Garudan samba* expressed the maximum seedling height (29.85 cm). CO53 had less early seedling vigour (600) and *Aanaikomban* expressed maximum early seedling vigour (2455). Comparing all genotypes *Aanaikomban* and *Garudan samba* expressed better growth than other lines.

In the third experiment (submergence tolerance), CO53 showed the least germination percentage (20%) and CO52 had the highest germination percentage (85%).

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Table 2. Analysis of Variance of 25 genotypes for anaerobic germination and submergence tolerance

Trait	AG with water		AG with water + soil		Submergence				DSR		
					Before		After				
	Genotype	Error	Genotype	Error	Genotype	Error	Genotype	Error	Genotype	Replication	Error
GP	1669.75**	159.00	2086.91**	191.00	943.83**	508.00	-	-	1328.00**	1152.00	452.00
SH	75.60**	15.88	213.33**	31.84	94.60**	30.47	259.27**	6.07	270.85**	149.95	240.78
ESV	823553.27*	144257.85	1220603.50**	190346.89	397600.16**	231843.54	-	-	2749927.16**	813067.52	713648.02
RNT	-	-	-	-	-	-	6.25**	0.62	-	-	-
EI	-	-	-	-	-	-	0.23**	0.0071	-	-	-
SSR	-	-	-	-	-	-	1808.96**	79.4	-	-	-
LL	-	-	-	-	-	-	-	-	111.99**	297.68	90.56
LW	-	-	-	-	-	-	-	-	0.08*	0	0.03

** Significant at 1% level *Significant at 5 % level

For seedling height, JCL *nel* expressed the lowest seedling height (5 cm) and *Aanaikomban* expressed the highest seedling height (29 cm). CO53 showed minimum early seedling vigour (117) and *Aanaikomban* showed maximum early seedling vigour (1730). Three different traits were recorded for post-submergence. *Karunguruvai* recorded the least seedling height (27.65 cm) and *Chitiraikar* expressed maximum seedling height (32.2 cm). *Aanaikomban* had a mean recovered number of tillers of 3.5 and *Kullakar* had six recovered number of tillers which was the highest. *Aanaikomban* was observed to record the least seedling survival rate (61%) and *Chitiraikar* had a high seedling survival rate (88.5%). *Aanaikomban* had the lowest elongation index (0.86%) and *Kullakar* had a high elongation index (0.97%). Among all genotypes, *Chitiraikar* had the best performance for all observed traits. In all three lab experiments, the genotype *Aanaikomban* recorded more than 90% germination. This is in line with the findings of Partheeban *et al.,* 2017. This was also reported by Vergara *et al.* (2014). Hence, the genotypes that exhibited a high level of tolerance can be utilized as contributors in programmes focused on crop improvement to anaerobic and submergence stress conditions. Seedling height in this study was observed to be directly proportional to survival percentage, as genotypes with longer shoots had higher survival rates. This study reveals that the genotypes which have the highest germination percentage and seedling height had a high vigour index. The recovered number of tillers and early seedling vigour are the important traits associated with anaerobic germination and submergence which were attributed to genetic effects. It is the ability to regrow after the effect of submergence for a particular period. Senapati *et al.* (2019) reported that seedling germination percentage did not significantly affect seedling length. This showed that establishment percentage underwater was the main hurdle rather than seedling length (Miro *et al.*, 2017). In the field experiment with the DSR condition, *Chitiraikar* showed a maximum germination percentage (100 %) and *Thanga samba* was recorded with a minimum trait value (30 %). *Karunguruvai* had the highest seedling height of 53.6 cm and JCL had the lowest seedling height (9 cm). In the case of early seedling vigour, *Chitiraikar* showed the highest trait value of 5185 and ASD16 had the lowest trait value of 780. *Karuppukavuni* showed leaf length (35 cm) and leaf width (1 cm) whereas *Thanga samba* showed the least leaf length (9.5 cm) and CO53 and *Thanga samba* showed less leaf width (0.15 cm). Comparing overall traits *Chitiraikar*, and *Karuppukavuni* performed well under submergence conditions. Regarding germination percentage, *Chitiraikar*, *Aanaikomban* and CO52 expressed maximum germination percentage in anaerobic and submergence stresses both in lab and DSR conditions. For seedling height, *Chitiraikar*, *Kullakar*, CR 1009 *sub-1*, *Aanaikomban*, *Karunguruvai* had the highest seedling height under stresses. CO55, *Aanaikomban* and *Chitiraikar* showed maximum ESV in anaerobic conditions and submerged situations (**Table 3**). The current study indicates that there is a broad genetic variation for anaerobic germination in rice.

Correlation: Correlation results (**Table 4**) of control showed that the seedling height was significantly positively correlated with germination percentage (0.10) and early seedling vigour (0.97). In the anaerobic germination experiment only with water, seedling height had less correlation with germination percentage (0.08), and early seedling vigour had a highly significant positive correlation with germination percentage (0.76) and seedling height (0.69). In the anaerobic germination experiment with soil and water, the early seedling vigour had high significant and positive correlation with germination percentage (0.94) and seedling height (0.86). Rathod *et al.* (2024) observed a high positive correlation between seedling length and seedling vigour index and between germination percentage and seedling vigour index. Doley *et al.* (2018) also reported a high positive significant correlation between seedling height and seedling vigour index. After

Table 3. Descriptive statistics of traits studied among 25 genotypes under different screening conditions

				Mean	Min	Max	Std dev	CV (%)
Anaerobic Germination	AG with water	Control	GP (%)	76.00	50.00	90.00	8.94	11.00
			SH (cm)	15.61	5.75	27.50	5.52	35.00
			ESV	839.54	75.00	1780.00	464.94	55.00
		Treatment	GP (%)	86.52	50.00	100.00	14.02	16.00
			SH (cm)	21.74	16.11	29.21	3.19	15.00
			ESV	1887.17	1140	2636.00	414.78	22.00
	AG with water + soil	Treatment	GP (%)	56.31	30.00	90.00	14.58	26.00
			SH (cm)	22.48	12.00	29.85	4.51	20.00
			ESV	1348.05	600.00	2455.00	485.44	36.00
Submergence	Submergence (before)	Treatment	GP (%)	47.39	20.00	85.00	17.69	37.00
			SH (cm)	15.16	5.00	29.00	5.56	37.00
			ESV	786.20	117.00	1730	397.49	51.00
	Submergence (After)	Treatment	SH (cm)	29.13	24.15	32.2	2.90	10.00
			RNT (nos)	4.74	3.55	6.00	0.81	17.00
			EI	0.914	0.86	0.97	0.044	5.00
			SSR (%)	76.8	61.00	88.5	11.11	14.00
DSR	Field DSR	Treatment	GP (%)	63.80	30.00	100.00	14.37	23.00
			SH (cm)	30.85	13.00	53.60	10.80	35.00
			ESV	2049.72	780.00	5185.00	973.68	48.00
			LL (cm)	21.95	9.50	35.00	6.87	31.00
			LW (cm)	0.48	0.15	1.00	0.19	40.00

Table 4. Correlation table for traits of 25 genotypes under anaerobic and submergence stress

		GP	**SH**		**ESV**	
Control	GP	1				
	SH	0.10**	1			
	ESV	0.34**	0.97**		1	
		GP	**SH**		**ESV**	
Anaerobic germination with water	GP	1				
	SH	0.08**	1			
	ESV	0.76**	0.69**		1	
		GP	**SH**		**ESV**	
Anaerobic germination with soil and water	GP	1				
	SH	0.69**	1			
	ESV	0.94**	0.86*		1	
		GP	**SH**		**ESV**	
Before submergence	GP	1				
	SH	0.54**	1			
	ESV	0.80**	0.85**		1	
		SH	**RNT**		**EI**	**SSR**
After submergence	SH	1				
	RNT	-0.12*	1			
	EI	0.11*	0.70*		1	
	SSR	0.43*	0.52*		0.75*	1
		GP	**SH**	**ESV**	**LL**	**LW**
DSR	GP	1.00				
	SH	0.41*	1.00			
	ESV	0.71*	0.88**	1.00		
	LL	0.46**	0.83**	0.74**	1.00	
	LW	0.40*	0.70**	0.61**	0.85**	1.00

* P < 0.05, significance at 5 % ** P < 0.01, significance at 1%

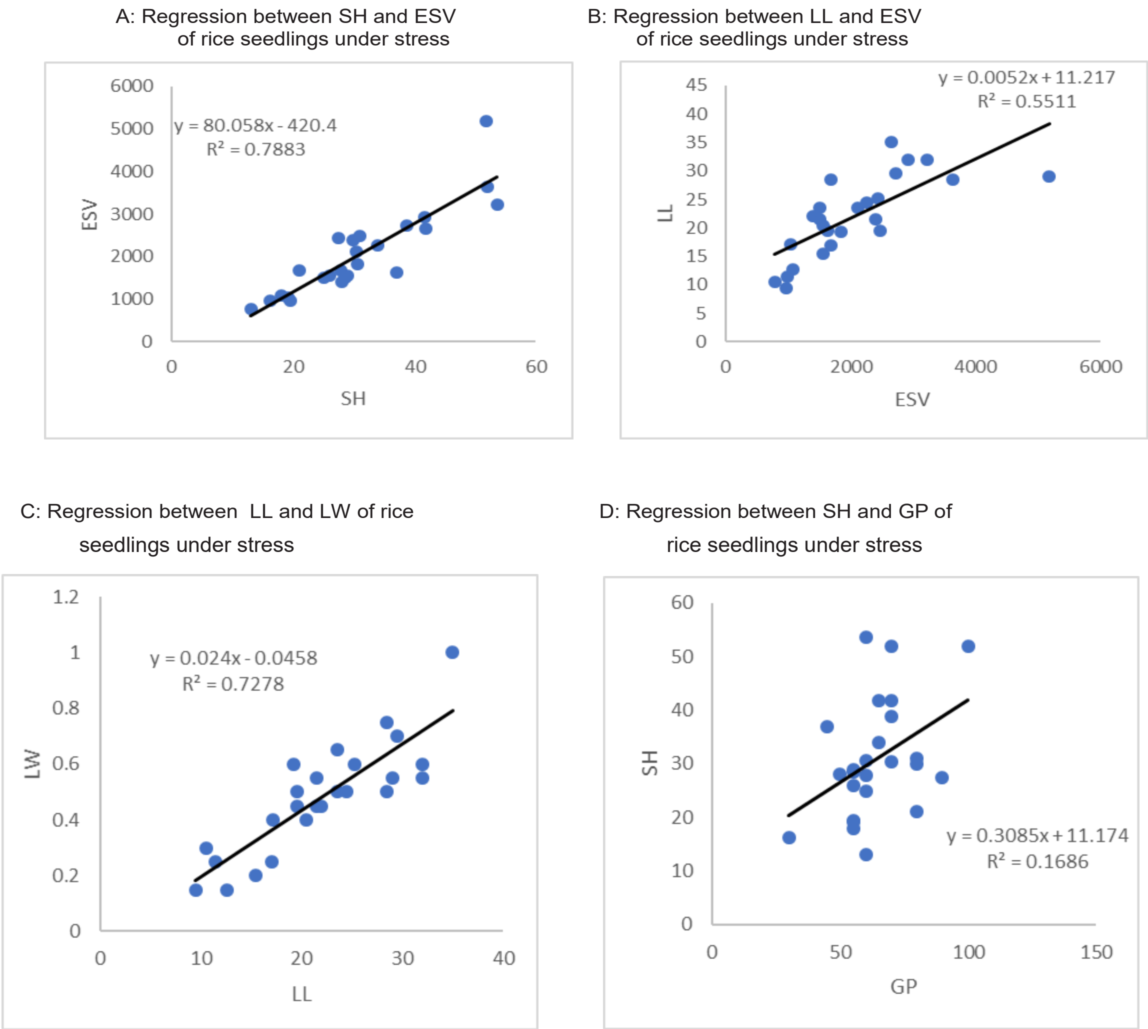

Fig. 2. Scatterplot graphs of different traits of all 25 genotypes under anaerobic and submergence stress conditions: A) Regression between SH and ESV of rice seedlings under stress. B) Regression between LL and ESV of rice seedlings under stress. C) Regression between LL and LW of rice seedlings under stress. D) Regression between SH and GP of rice seedlings under stress.

inducing submergence, the findings showed that the recovered number of tillers had a negative correlation with seedling height (-0.12). The elongation index was found to have less direct effect (0.11) on seedling height and high positive correlation with the recovered number of tillers (0.70). The seedling survival rate was found to have less relation with seedling height (0.43) and positive correlation with the recovered number of tillers (0.52) and elongation index (0.75). In the DSR experiment, the early seedling vigour had a significant positive correlation with germination percentage (0.71) and seedling height (0.88), which fulfils the major criteria of direct seeded rice (high seed vigour and high early vigour) (Xu *et al.,* 2023). The scatterplot [**Fig .2** *(A)* and **Fig.2** *(D)*] explains the relation between seedling height and early seedling vigour with germination percentage. The leaf length significantly correlated with seedling height (0.83) (**Table 4**). Leaf width had a significant positive correlation with leaf length (0.85).

To conclude, a more adaptable and affordable method using cups and buckets to screen the rice genotypes for anaerobic and submergence stress tolerance was designed in this study. This study revealed that genotypes *viz*., CO55, *Karunguruvai, Aanaikomban, Kullakar*, CR 1009 *sub-1* and *Chitiraikar* can be used as gene donors for tolerance to anaerobic stresses which contain the trait to germinate against anaerobic as well as submergence conditions. Among all the traits observed, the seedling vigour index and recovered number of tillers show high association with seedling survival percentage. These traits could be given due importance in breeding works for improved anaerobic germination and submergence tolerance under stress conditions.

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