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Research Article

Designing and validation of a rapid and reliable protocol for screening anaerobic germination tolerance in rice

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Abstract
Anaerobic germination is one of the important traits for direct seeded rice cultivation. Identification of tolerant rice genotypes is needed for improving early vigour under anaerobic germination. This study was aimed to identify a rapid and reliable protocol to identify the tolerant rice genotypes for anaerobic germination. Four screening methods *viz.,* protrays, plastic trays, plastic tumbler and mud tumbler methods were developed and 22 rice genotypes were subjected to anoxic stress. Mud tumbler method was identified as the best screening protocol. Mud tumblers are eco friendly and also mimic the pot culture experiments. Principal component and cluster analysis revealed, Karuppukavuni and Kalanamak as tolerant genotypes. They recorded higher coloeptile traits and showed enhanced germination under early flooding. TKM13 and Anna R4 were identified as moderately tolerant genotypes under anaerobic germination. Higher genotypic coefficient of variance, phenotypic coefficient of variance coupled with high heritability was noticed in shoot length, crude protein and proline indicating scope for enhancing anaerobic germination tolerance.

Keywords: Rice, Anaerobic germination, Coleoptile traits, Screening methods, Genetic analysis.

INTRODUCTION

Rice (*Oryza sativa* L.) is an important food crop for half of the world's population (Roy and Sharma 2014). Rice is normally grown by transplanting, which requires more labour and water (Rauf *et al*., 2019). Hence, transplanted rice cultivation under current scenario is less profitable as, these resources are becoming increasingly scarce. To overcome this problem direct seeded rice (DSR) is the best cultivation method. Currently, direct seeded system is widely adopted by farmers. For DSR cultivation, rice varieties that can tolerate anaerobic stress during early seedling stage are a pre-requisite. Anaerobic germination (AG) stress is critical abiotic stress that cannot be predicted in rice cultivation (Lee *et al.,* 2023). This stress often occurs in Southeast Asia, South Asia, and Africa (Collard *et al*., 2013).

Coleoptile elongation and germination percentage are the two most basic and essential traits known to be associated with AG tolerance (Miro *et al.,* 2017). Early vigour is an important factor when breeding DSR varieties (Sandhu *et al.,* 2021). Understanding the mechanism underlying anaerobic germination tolerance in rice and coleoptile responses in AG helps to promote the development of rice for direct seeding technology (Su *et al.,* 2021). Seed quality traits like amylose content and crude protein are regarded as the most important factor as it determines rice eating and cooking quality (Zhang *et al.,* 2019).

The major bottleneck in identifying tolerant genotypes for anaerobic germination is the availability of rapid and reproducible screening protocols. Test tubes were used to evaluate the germination rate and seedling growth in rice under submerged condition (Lee *et al.,* 2023). Rice genotypes for anaerobic germination tolerance were screened in plastic trays based on alcoholic fermentation enzymes expression (Miro *et al.,* 2017). Since, plastics are non-biodegradable; an alternate methodology for anaerobic germination tolerance in rice is required. India has a wealth of rice germplasms and landraces to be screened for anaerobic germination tolerance. This study was aimed to develop a novel method and screen the available rice germplasm for anaerobic germination tolerance. Hence, the objectives of this study were (i) To identify a rapid and reliable screening protocol to identify anaerobic germination tolerance in rice. (ii) To dissect the genetic diversity of coleoptile traits and correlate with AG tolerance. (iii) To elucidate information under AG condition on genetic variability, heritability and genetic advance in traditional rice genotypes. (iv) To identify AG tolerant rice genotypes using the best screening method.

MATERIALS AND METHODS

Plant Materials: Nijavara, Thavalakannan, Adukkan, Mapillai samba, Vellimuthu, Kunjoanju, Kothamalli samba, Manvilayan, Karuppukavuni, Nootripathu, Kalanamak. Kodavilayan, Kallurundai samba, Aanaikomban, Norungan , Varappu kudainchan, Rasagadam, Kullakar, CBMAS 14065, CO53, Anna R 4 and TKM 13 were used for this study.

Experiment 1: Development and validation of rapid and reliable protocol for anaerobic germination : Four methods were designed as given below, to evaluate 22 rice genotypes comprising of 18 landraces and 4 varieties for AG tolerance during March 2022. This experiment was conducted in the laboratory of Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore. In all the methods, soil was mixed with 0.145 g of N, 0.036 g of P and 0.044 g of K per kg of soil.

Method 1 (Protray method): Protrays 7 x 14 holes along the length and breadth respectively were filled with soil. The seeds of 22 rice genotype were taken, and two seeds/hole were sown. Control protrays were watered normally, while in stress the protrays along with the seeds were dipped in 15 cm of water maintained in plastic tubs (**Plate 1**).

Method 2 (Plastic tray method): All the 22 rice genotypes seeds were sown directly in plastic tub trays filled with 6 cm of clay soil and irrigated under control. For stress, the seeds were sown in plastic tub trays of 6 cm of soil and flooded upto 15 cm of water above the soil (**Plate 1**).

Method 3 (Plastic tumbler method): Plastic tumblers were taken for screening anaerobic germination. One set of plastic tumblers were kept as control and another set kept as anaerobic germination condition. Seeds were sown directly in plastic tumblers filled with 3 cm of clay soil and irrigated under control. For stress the plastic tumblers along with the seeds were filled with 15 cm of water covered with aluminum foil (**Plate 1**).

Method 4 (Mud tumbler method): Seeds were sown directly in mud tumblers filled with 3 cm of clay soil and irrigated under control. For stress the mud tumblers along with the seeds were dipped in 15 cm of water maintained in plastic tubs (**Plate 1**).Observations on germination percentage and coleoptile length were taken on 7th day after stress.

Experiment 2: Evaluation of coleoptile traits for anaerobic germination tolerance: The identified best screening method was used to grow 22 rice genotypes. Coleoptile traits of the emerged seeds were placed inside the scanner of root image analysis system. Coleoptile length (CL), coleoptile surface area (CSA), coleoptile volume (CV), and coleoptile diameter (CD) were measured (WinRHIZO REGENT INSTRUMENTS Inc., Québec, Canada).

Measurements on seedling and seed quality traits: Shoot and root length were measured by scale. Amylose content was estimated by the method of Juliano (1971). Crude protein was quantified as per procedure of Magomya *et al.* (2014). Proline quantification was measured as per the procedure of Carillo and Gibon (2011).

Statistical analysis: Experiment I was set up as two factorial design with two replications and Experiment II was designed as completely randomized design. Data were analysed as mean, standard, summary statistics. Genetic parameters were performed by TNAUSTAT-statistical package. Analysis of variance (ANOVA) was used to identify significant differences between the treatments. Statistical analysis, principal component analysis (PCA) and correlation were performed by GRAPES 1.0.0 software (General R-shiny based Analysis Platform Empowered by Statistics).Cluster analysis was performed by R studio version 4.3.0.

RESULTS AND DISCUSSION

Experiment 1: Development and validation of rapid and reliable protocol for anaerobic germination : Screening methods were compared by using germination percentage as a trait to identify the best screening method for AG. Generally, germination percentage decreased under anaerobic germination conditions compared to control. In control condition, higher mean values of germination were recorded in mud tumbler (96.25%) and protrays (90.45%) methods, while plastic trays (84.40%) and plastic tumbler (83.75%) recorded lesser mean values of germination (**Fig.1**). Under AG stress higher percentage of germination mean was observed in mud tumbler (86.59%) followed by protray method (77.27%). Higher germination rate was the basic trait for selecting the suitable method of AG tolerance in rice. Higher genetic variability for survivability was recorded to select the best

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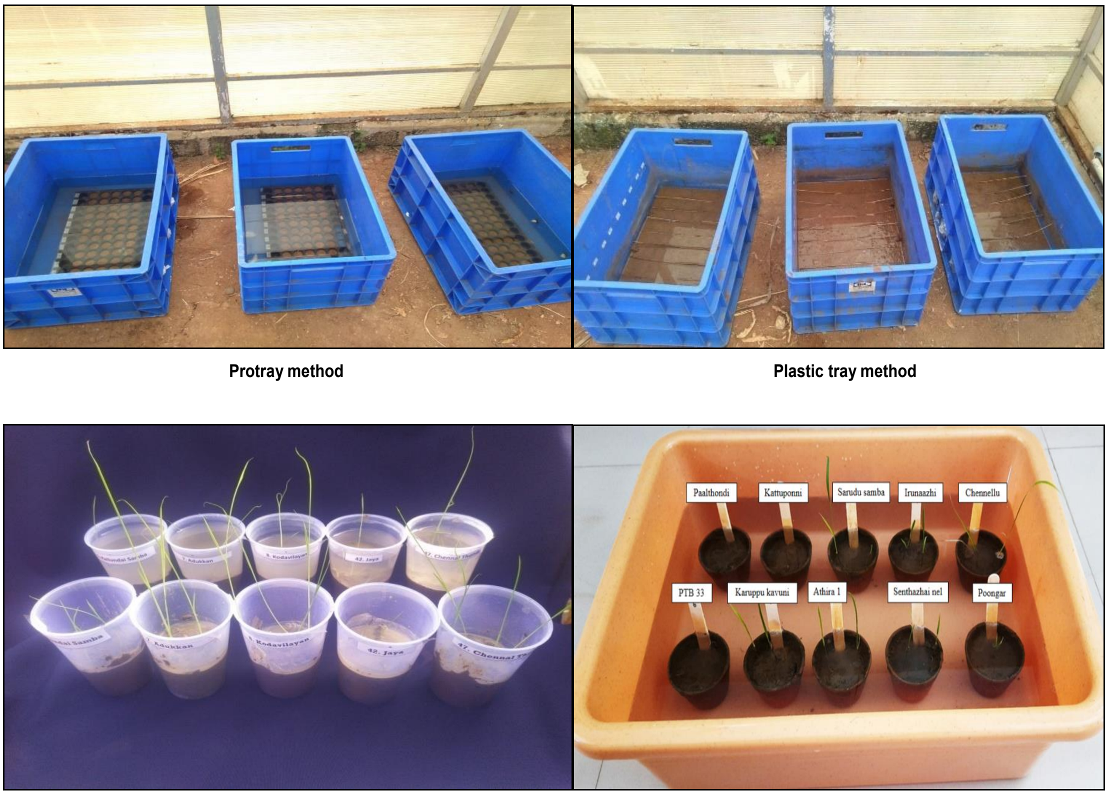


Protray method

Plastic tray method

Plastic tumbler method

Mud tumbler method

Plate 1. Methods used to screen for anaerobic germination tolerance

method for anaerobic germination (Ghosal *et al.,* 2020). Plastic tumbler and protray methods had lower mean values of germination of 72.84% and 71.59% respectively under stress condition (**Fig.1**).ANOVA was used to test the suitability of the screening methods. Treatments, screening methods and interaction had p-values less than 0.05 indicating statistical significance (**Table 1**).Our results revealed that, higher percent of rice genotypes were germinated in mud tumbler method. Hence, it was identified as a rapid and reliable protocol for screening rice genotypes for anaerobic germination tolerance. Mud tumblers are eco friendly methods and also mimic the pot culture experiment. Similarly, different screening methods were used to evaluate the rice germplasm for tolerance to low temperature (Sharma *et al.,* 2021).

Experiment 2: Evaluation of coleoptile traits for anaerobic germination tolerance: The 22 rice genotypes were grown in mud tumbler method and subjected to early flooding to identify tolerant genotypes for AG. On an average, there was a decrease in coleoptile traits under anaerobic germination compared to control condition (**Table 2**). Coleoptile length is the standard feature used to screen for AG in rice germplams (Miro *et al.,* 2017). Highest values of coleoptile length (5.92), coleoptile surface area (0.96), and coloeptile diameter (0.89) and coleoptile volume (11.78) were recorded in Kalanamak followed by Karuppukavuni (CL-5.59, CSA-0.98, CD-0.89, CV-11.77) under stress conditions (**Fig. 2a**). On the other hand, TKM 13 and Anna R4 recorded lesser coloeptile length of 4.77 and 4.65 and coloeptile volume of 4.42 and 4.21 respectively under AG (**Fig. 2a**). A decrease in coloeptile surface area and coloeptile diameter were observed in TKM 13 and Anna R4 compared to Karupukavuni and Kalanamak (**Fig. 2b**).

For plant breeders increase in PCA will make more accessible to discover the number of plants and it would be helpful in identifying tolerant genotypes. PCA of coloeptile traits for all the 22 rice genotypes evaluated under AG condition were found to exhibit extreme ranges. Karupukavuni, Kalanamak, CBMAS 14065 and Kodavilayan were positively correlated and closely associated with coloeptile traits, whereas TKM 13 and Anna R4 were not closely associated with coloeptile traits (**Fig. 3a**). The findings are in line with Mohanapriya *et al.* (2022), who reported, Karuppukavuni as a tolerant landrace for anaerobic germination. To explain variation among rice genotypes PC1 and PC2 principal components and associated traits were the most reliable (Beena *et al.,* 2021). Cluster analysis also revealed similar results as coloeptile traits. Karupukavuni, Kalanamak,

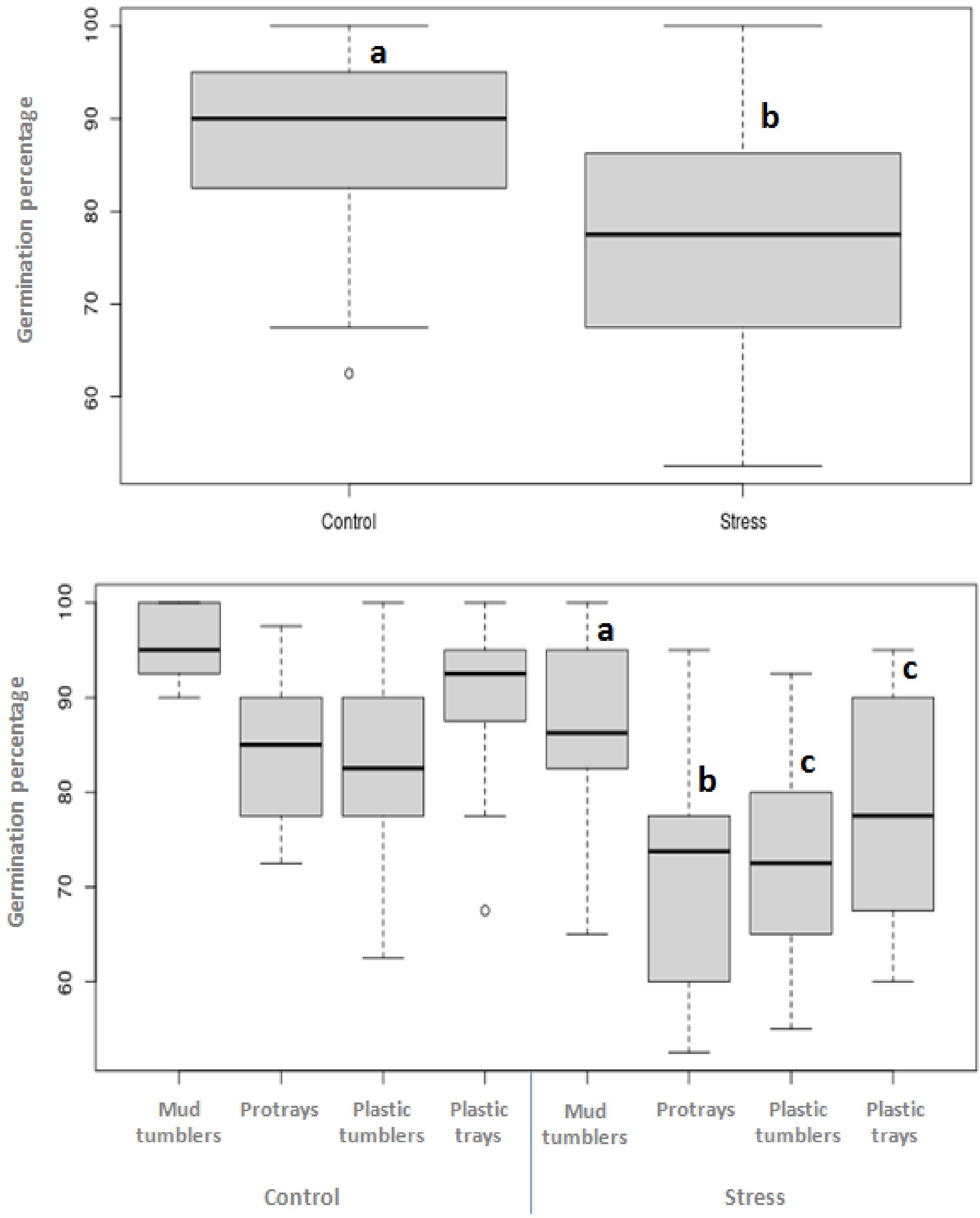


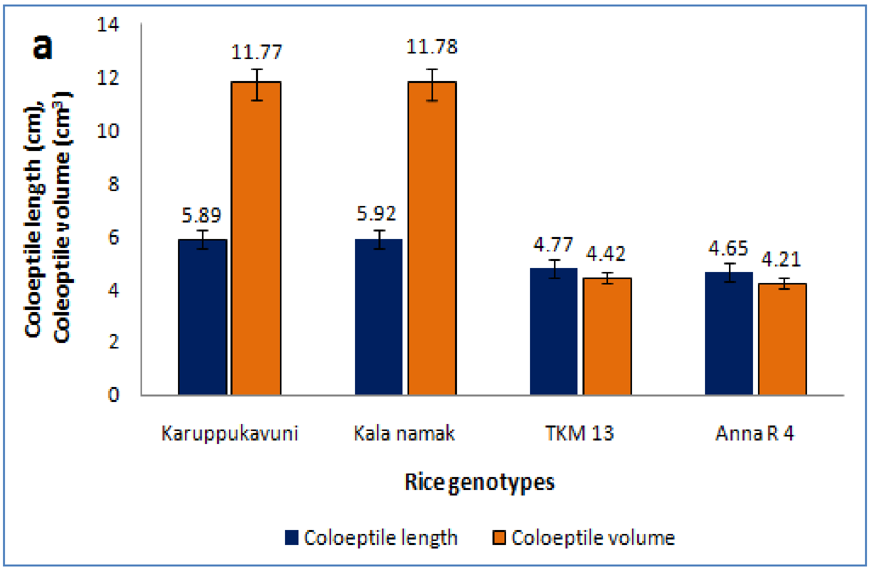
Fig. 1. Box plot showing the genetic variability for anaerobic germination tolerance using different screening protocol

Table 1. ANOVA for germination percentage in different screening methods under control and AG stress conditions

	DF	Sum of Squares	Mean sum of squares	F-value	Pr(>F)
Factor A (Treatments)	1	10985.740	10985.740	96.671	0.000
Factor B (Methods)	3	9371.436	3123.812	27.488	0.000
A X B	3	1026.447	342.149	3.011	0.032
Error	168	19091.625	113.641		

Table 2. Range, mean, standard deviation, coefficient of variation of coleoptile traits under control and AG in mud tumbler method (values in parenthesis shows AG data)

Variables	Minimum	Maximum	Mean	SE. mean	S.D	Coef.var
Coleoptile length (cm)	5.170 (2.600)	6.860 (5.920)	5.810 (4.418)	0.088 (0.227)	0.415 (1.064)	0.071 (0.241)
Coleoptile surface area (cm^2)	0.630 (0.210)	6.940 (0.980)	1.445 (0.637)	0.266 (0.051)	1.250 0.241	0.865 0.378
Coleoptile diameter (mm)	0.620 (0.240)	0.970 (0.890)	0.805 (0.575)	0.021 (0.041)	0.100 (0.194)	0.125 (0.337)
Coleoptile volume (cm^3)	9.250 (2.220)	14.790 (11.780)	11.998 (6.640)	0.330 (0.665)	1.547 (3.120)	0.129 (0.470)



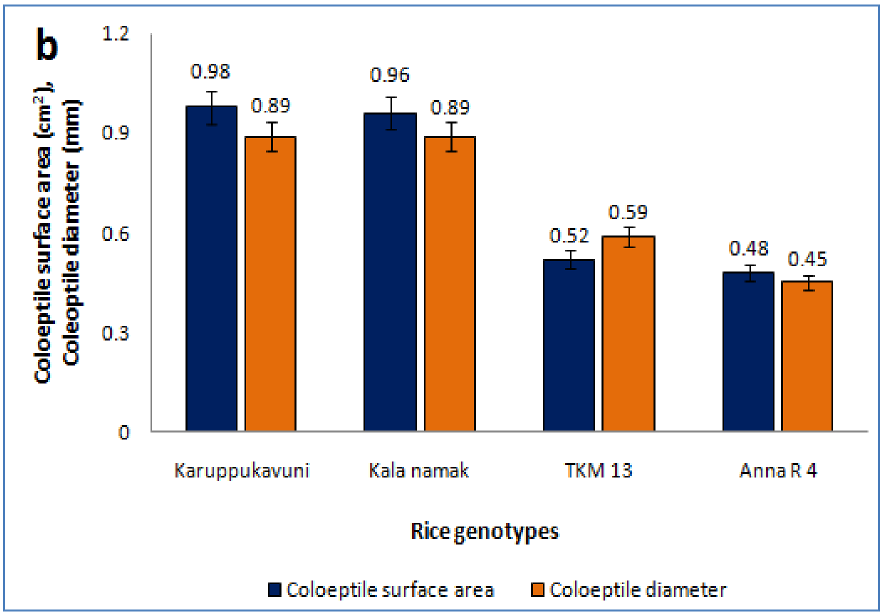


Fig. 2. Genetic variation of coleoptile traits in rice genotypes under AG stress (a) coleoptile length and coleoptile volume (b) coleoptile surface area and coleoptile diameter

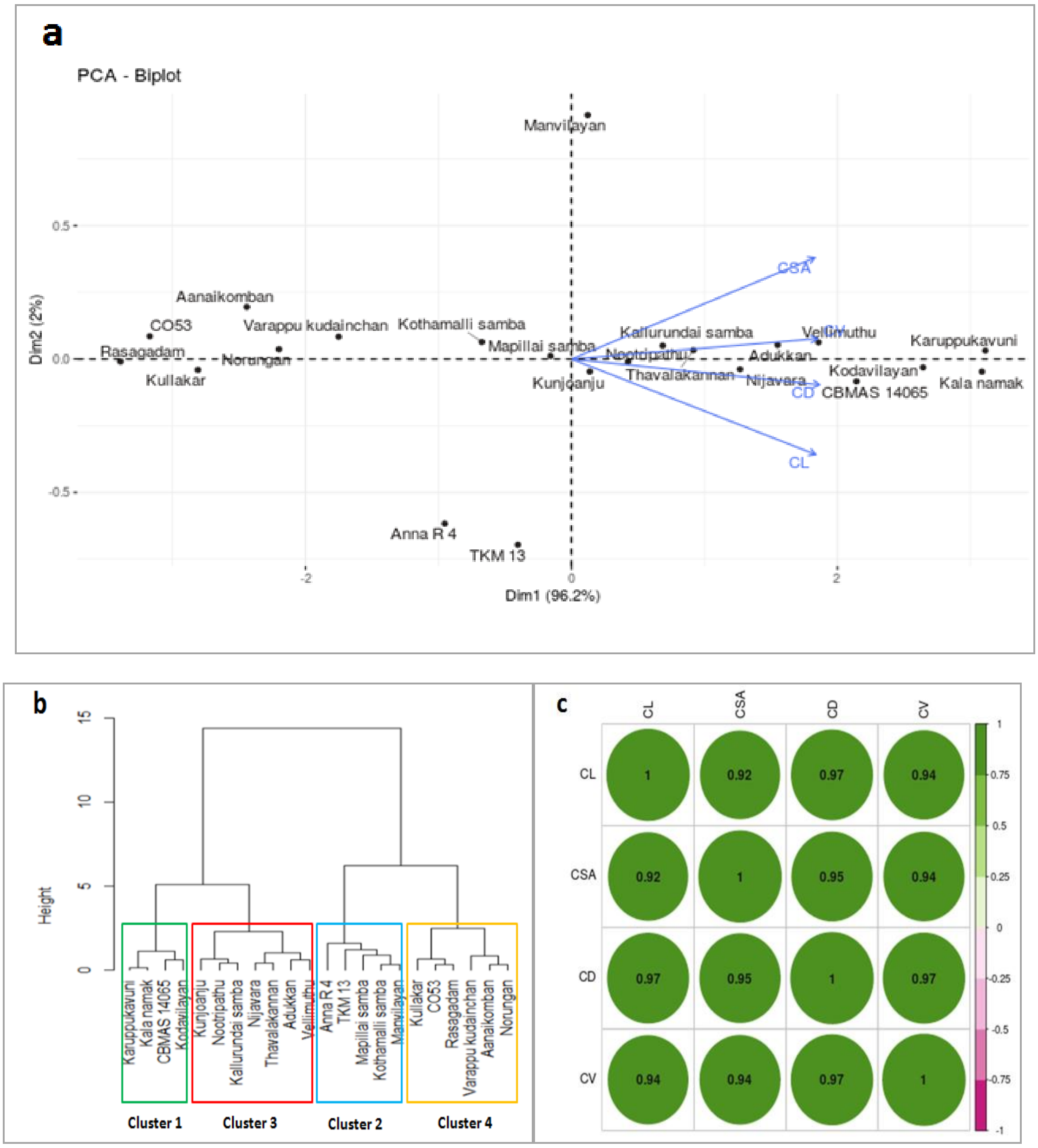


Fig. 3. (a) PCA of genotype × parameter i.e. coleoptile traits in rice genotypes (b) Clustering the rice genotypes for tolerant/ susceptibility to anaerobic germination stress (c) Correlating the coleoptile traits under stress

CBMAS 14065 and Kodavilayan were grouped in cluster 1 (Tolerant genotypes) while TKM 13 and Anna R4 were grouped in cluster 3 (Moderately tolerant genotypes) (**Fig. 3b**). Cluster 2 (Moderately susceptible genotypes) included 7 genotypes and cluster 4 (Highly susceptible genotypes) included 6 genotypes (**Fig. 3b**). Coleoptile length ranged from 5.53 to 5.92cm was grouped in cluster 1. These genotypes recorded higher ranges of coleoptile volume (10.08 to 11.78) under AG. Cluster 3 genotypes showed moderte coleoptile length (4.38 to 4.65) and coleoptile volume (4.21 to 6.29), which was higher compared to cluster 2 and cluster 4. Cluster 4 genotypes showed lowest CL and CV under AG. Coloeptile traits were used as selective criteria for identifying and grouping of rice genotypes for AG tolerance (Ghosal *et al.,* 2020). All the four coloeptile traits were correlated positively (**Fig. 3c**). These results corroborate with the findings of Miro *et al.* (2017).

Variability, heritability and genetic advance: Phenotypic coefficient of variance (PCV) was found higher than genotypic coefficient of variance (GCV) for seedling and

Table 3. Genetic parameters of rice genotypes under anaerobic germination

Traits	Variance			GCV (%)	PCV (%)	Heritability (%)	GAM (5%)
	Vg	Vp	Ve				
Shoot length	0.057	0.058	0.0008	46.176	46.487	98.661	94.483
Root length	43.466	67.208	23.741	46.092	57.313	64.675	76.359
Amylose	8.691	13.737	5.046	35.053	44.070	63.266	57.436
Crude protein	13.099	17.491	4.391	33.712	38.955	74.893	60.099
Proline	5.179	7.304	2.125	32.833	38.989	70.911	56.955

seed quality traits (**Table 3**). Difference between PCV and GCV was less for all the traits, because of less influence of environmental characters. Similar results were reported by Beena *et al.* (2021) in rice genotypes subjected to heat stress. Minimum difference of GCV and PCV were observed in shoot length, crude protein and proline contents (**Table 3**). Thus, the selection on these traits might be effective in bringing significant genetic enhancement. All the five parameters showed high heritability (>0.4) and will be useful for selection criteria. Highest heritability (0.986%) and genetic advance percent as mean (94.483%) were recorded in shoot length. Results revealed that, higher GCV, PCV coupled with high heritability was noticed for the traits *viz.,* shoot length, crude protein and proline contents. Thus, these traits might be useful as selection criterion for screening anaerobic germination tolerance.

Four different screening methods were designed to assess seed germination of rice genotypes under anaerobic germination stress. Among the methods, mud tumbler method was identified as a rapid and reliable screening protocol for AG tolerance. Rice genotypes grown in this method recorded higher germination percentages. Coleoptile traits *viz.,* coleoptile length, coleoptile surface area, coleoptile diameter and coleoptile volume were selected as the key indicators to evaluate the AG tolerance of rice. These traits were strongly correlated to anaerobic germination stress tolerance. Finally, Karuppukavuni and Kalanamak were identified as tolerant genotypes whereas TKM13 and Anna R4 were identified as moderately tolerant genotypes under AG through PCA and cluster analysis. Shoot length, crude protein and proline had higher heritability and thus, might be helpful for AG tolerance enhancement.

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