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Quantifying enzyme activities under anaerobic germination in traditional rice landraces to identify donors for direct seeded rice cultivation

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ABSTRACT

Climate change has increased environmental risks globally having an adverse effect on agriculture productivity. Among the abiotic stresses, anaerobic germination stress has been identified as a major stress for seed emergence, plant growth and food production. By understanding the manipulation of germination, antioxidant and fermentation enzymes, adaptations to anaerobic conditions can be improved. The ability of rice to emerge under oxygen deprivation is a determinant of anaerobic germination tolerance, critical for successful direct seeding. There is an urge to identify novel rice genotypes associated with better germination and higher enzymatic activities under anaerobic conditions in order to improve seedling establishment. In the present study, twenty-two rice genotypes were characterized for their anaerobic germination potential by assessing the activities of α- amylase, antioxidant enzymes viz., catalase and peroxidase, and fermentative enzyme viz., alcohol dehydrogenase and pyruvate decarboxylase under anoxic stress. α-amylase, catalase, peroxidase, alcohol dehydrogenase and pyruvate decarboxylase activities showed a significant positive association with seed germination under anaerobic conditions. Higher expression of five enzymatic activities confirms anaerobic germination stress tolerance in rice genotypes. This study identified four tolerant genotypes namely Karuppukavuni, Kalanamak, CBMAS 14065, and Kodavilayan, and two moderately tolerant genotypes namely TKM13 and Anna R4 based on principal component analysis and correlation analysis.

Keywords: *Rice landraces, α-amylase, Catalase, Peroxidase, Alcohol dehydrogenase, Pyruvate decarboxylase, Anaerobic germination, Direct-seeded rice cultivation.*

Introduction

Rice is the second largest cereal crop in the world and the worlds total rice production comes from Asia with a productivity of 510 million tons [1]. Shortage of water and labor input makes rice production more expensive, less profitable, and unsustainable [2]. In India now a day's farmers are slowly adapting direct seeded rice (DSR) technology by broadcasting dry seeds [3]. In Tamil Nadu especially Cauvery delta zones, nearly 3- 4 lakh acres of land are cultivated under DSR technology [4].

Germination is an active process that requires greater energy to sustain growth [5]. During germination α-amylase available in the endosperm is the sole supplier of energy [6]. Soil flooding during the germination stage restricts oxygen supply to the germinating seeds and therefore induces alcoholic fermentation. Alcohol dehydrogenase (ADH) is an important enzyme that increases under low oxygen level to sustain germination and growth [7]. Pyruvate decarboxylase (PDC), alcohol dehydrogenase, catalase and peroxidase are thought to be essential for the sustained production of ATP under oxygen-limiting conditions [8].

Existing rice varieties were not notably developed for direct-seeded ecosystems. Earlier rice genotypes under direct-seeded conditions showed yield decline. In Tamil Nadu under dry direct seeded conditions landraces like Kallurundaikar, Sivapuchithiraikar, and Kuruvaikalanjiyam showed maximum grain yield in Paramakudi [9]. Till date, no landraces are reported to show tolerance under wet direct-seeded conditions. Induction of enzyme for ethanolic fermentation under anaerobic response enhanced rice genotypes to survive clearly under anoxia. Exposing plants to conditions that induce enzyme response greatly improves anoxic stress tolerance in many genotypes.

Under anoxic conditions, there is an urge to study the role of germination enzymes and other antioxidant and fermentation enzymes underlying anaerobic germination (AG) tolerance. Hence, this study was aimed with the following objectives (i) To screen the rice genotypes for anaerobic germination tolerance based on germination percentage and enzyme activities. (ii) To evaluate the contribution of different enzymes to anaerobic germination tolerance in rice germplasm by PCA and correlation. (iii) To identify the anaerobic germination tolerant rice genotypes to be used for DSR cultivation.

Materials and methods

Plant Material and Growth Conditions

Rice genotypes (22 no's) were sourced from Tamil Nadu

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Agricultural University (TNAU), Coimbatore, and the experiment was conducted at the Department of Crop Physiology, TNAU during March 2022. Thirty mature seeds were placed at the oven for 5 days at 50 °C to break the dormancy. Further, they are sterilized with 0.2% $HgCl_2$ for 5 min and washed thrice with distilled water. Soil was mixed with NPK. Seeds were sown in rows inside the flooding tank. One tank was maintained as control (the thin film of water was maintained) and another as anaerobic germination (15cm of water level was maintained until 21 DAS). Two independent, replicated experiments were performed for each rice genotype.

Measurement of germination percentage and α amylase activity

Seeds were allowed to germinate under submerged conditions. A number of seeds with the emergence of coleoptile and radicle were counted and expressed as % of total number of seeds germinated with respect to total number of seeds sown.

To estimate α amylase activity, 0.5 g of seed sample were homogenized in 1.8 ml of cold 0.02 M sodium phosphate buffer and centrifuged at 20,000 rpm for 20 min. 0.1 ml of enzyme extract and 1 ml 0.067 % starch solution were added. By addition of one ml of iodine hydrochloric acid solution, the reaction was stopped after 10 min of incubation at 25°C. Change in color was measured at 620 nm. The activity was calculated and expressed in mg maltose per min [10].

Assay for catalase and peroxidase activity

To determine the catalase activity, grind the coleoptile (0.1g) with 0.1M phosphate buffer, pH 7.0 in a prechilled mortar and pestle. Centrifuge at 15,000 for 30min at 4°C. Use the supernatant as an enzyme source. Pipette out 3ml of phosphate buffer, 2ml of H_2O_2, and 1ml of enzyme extract at 20°C for 1min. After 1min stop the reaction by adding 10ml of 0.7N H_2SO_4. Titrate the reaction mixture against 0.01N $KMNO_4$ to find out the residual H_2O_2 until a faint purple color persists for at least 15sec [11].

For peroxidase assay, homogenize the sample in ice-cold 0.1M phosphate buffer, pH 6.0 (1:10, w/v). Centrifuge the homogenate at 16,000g for 20 min at 4°C and use the supernatant as an enzyme source. Pipette out 1ml of O-dianisidine, 0.5ml of H_2O_2, 1 ml of phosphate buffer, and 2.4ml of distilled water into the test tube. Incubate at 30°C and start the reaction by adding 0.2ml of the enzyme. After 5min, stop the reaction by adding 1ml of 2N H_2SO_4. Read the absorbance at 430nm [12].

Determination of ADH and PDC enzyme activity

ADH and PDC enzyme activities in extracts were measured spectrophotometrically as described by [8] and [13] respectively. The extraction and assay of the enzymes were done using rice coleoptiles after 7 days of germination. The sample was centrifuged at 15, 000g for 20 min at 4°C. The assay medium of ADH contains 50 mM TRIS-HCl (pH 7.5), 62.5 mM $MgCl_2$, 3 mM NADH, 100 mM Acetaldehyde and 0.2 ml enzyme extract. The total volume of the assay medium was 3 ml. The absorbance was taken immediately after the addition of NADH and after 3 min of reaction at 340 nm.

For PDC, extracts were incubated at 25°C for 30 min. 100µl of the extract was placed in a spectrophotometer cuvette containing final concentrations of 57.5 mol/ m^3 MES Ph 6.0, 1.14 mol/ m^3 MgCl2, 0.5 mol/ m^3 TPP, 50 mol/ m^3 oxamate, 0.3 mg/ml ADH and 0.21 mol/ m^3 NADH. OD at 340 nm continuously, pyruvate was added to the final concentration of 7 mol/m^3. PDC enzyme activity was estimated from the maximal slope of the decline in absorbance over time after the addition of the substrate from which the rate of decline before the addition of the substrate was subtracted. The enzyme activities were expressed as µmol/g FW/min.

Experimental design and statistical analysis

The experiment was set up in a completely randomized design with two replications. Data were summarized followed by mean, standard errors, and Analysis of variance (ANOVA) was used to identify significant differences between the treatments. Statistical analysis was performed using SPSS (Statistical Analysis System, version 23.0) and Microsoft Excel. Principal component analysis (PCA) and correlation were performed by GRAPES 1.0.0 software (General R-shiny based Analysis Platform Empowered by Statistics).

Result and Discussion

Germination percentage and α amylase activity under AG condition

Seed germination is an essential criterion during AG conditions. Generally, germination percentage declined under anoxic stress compared to control. Control condition showed 100% germination was recorded in Thavalakannan, Mapillai samba, Vellimuthu, Kothamalli samba, Karuppukavuni, Kodavilayan, CBMAS 14065 and Kalanamak (Fig. 1a). Among the rice landraces, highest germination percentage was observed in Karuppukavuni (100%) followed by CBMAS 14065 and Kalanamak (97.5%), Kodavilayan (95%) under anoxic condition. Tolerant genotypes exhibit rapid and uniform germination under anoxia conditions [14]. TKM13 and Anna R4 had germination percentages of 82.5% and 82.5% respectively under AG (Fig. 1a). Germination rate was directly related to anaerobic seedling establishment under wet DSR [14].

The activity of α amylase increased significantly in all rice genotypes under AG. In four tolerant rice genotypes namely Karuppukavuni (9.81mg maltose/min), Kodavilayan (7.04mg maltose/min), CBMAS 14065 (6.31mg maltose/min), and Kalanamak (9.69mg maltose/min), the activity of α amylase increased significantly. TKM13 (3.90mg maltose/min) and Anna R4 (4.65mg maltose/min) had lower amylase activity under anoxic stress compared to the control (Fig. 1b). Expression of α amylase was greater in tolerant genotypes than susceptible genotypes under AG [15]. Alpha amylase and germination percentage were positively correlated under anaerobic germination (Fig. 1b).

Catalase and peroxidase enzyme activities

Under anoxic stress, catalase and peroxidase enzyme activities tend to increase compared to controlled conditions in rice genotypes. The catalase values ranged from 10.77 to 29.99 moles of H_2O_2/min/g in rice genotypes. Higher catalase activity of 31.06, 30.60, 26.15, and 31.86 were recorded in Karuppukavuni, Kalanamak, CBMAS 14065, and Kodavilayan respectively during anaerobic germination (Table 1). TKM13 and Anna R4 showed higher catalase values of 25.68 and 23.04 respectively. Catalase activity significantly increases with increasing stress exposure times [16].

Peroxidase values ranged from 4.48 to 31.62 in unstressed plants. Peroxidase enzyme activity showed significantly higher values in Karuppukavuni (37.03), Kalanamak (38.68), CBMAS 14065 (30.28), and Kodavilayan (23.60) genotypes compared to

TKM13 (16.75) and Anna R4 (16.17) under AG conditions (Table 1). Higher catalase and peroxidase activity might be the reason for AG survivability leading to Karuppukavuni, Kalanamak, CBMAS 14065 and Kodavilayan genotypes becoming tolerant to anaerobic germination. Contradictory results of decreased antioxidant enzymes *viz.,* catalase and peroxidase activities were observed in fully submerged rice plants [17].

ADH and PDC enzyme activities

The activity of ADH was higher under stress compared to control. Among the different ethanolic fermentative enzymes, ADH is the most important enzyme, which was upregulated under oxygen deficiency [18]. Under normal conditions, values of ADH activity ranged from 1.20 to 13.20 µmol/g FW/min. Maximum activity of ADH was noticed in Karuppukavuni (18.00), Kalanamak (18.00). Minimum ADH was recorded in CO53 (4.80) and Rasagadam (5.20) during AG (Table 2). TKM13 and Anna R4 stressed plants showed ADH values of 7.20 and 6.40 respectively. Similar results were observed in rice genotypes under anaerobic germination [15].

A similar trend was also recorded in PDC enzyme activity. But the expression of PDC activity was lesser compared to ADH in all rice genotypes. Lesser expression of PDC activity promotes the accumulation of ADH protein [19]. PDC activity was slightly increased under AG than control. During AG condition, Karuppukavuni, Kalanamak, CBMAS 14065, and Kodavilayan had their PDC values of 5.00, 4.52, 4.36, and 4.60. TKM13 and Anna R4 had PDC activity of 4.00 and 3.60 respectively (Table 2). PDC activity was positively correlated with germination percentage and all other enzyme activities (Fig. 2b). The enzymatic activity of PDC is higher in cultivars tolerant to anoxia compared to sensitive cultivars [20].

PCA and correlation analysis

The PCA under AG stress condition indicated that among six PCs (principal components) initial two PCs contributed up to 76% variation with eigenvalue more than one. The PC1 contributed higher variation due to traits survival rate (0.718), α amylase (0.759), catalase (0.679), peroxidase (0.881), ADH (0.710), and PDC (0.621) with higher positive factor loading values. All the traits were closely associated with each other (Fig. 2a). All the enzyme activities were positively correlated with germination percentage under AG conditions (Fig. 2b). Similar results were supported by [20]. PCA and correlation results showed that, Karuppukavuni, Kalanamak, CBMAS 14065 and Kodavilayan to be tolerant genotypes. TKM13 and Anna R4 were grouped as moderately tolerant genotypes.

Conclusion

Alcoholic fermentation pathways were strongly activated in rice during anaerobic germination as reflected in the induction of key enzymes, including pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH). Higher α amylase, catalase, and peroxidase activity helped to improve energy supply and survivability under anoxic stress. Anaerobic germination tolerance in rice is associated with higher germination enzymes, antioxidant enzymes, and alcoholic enzymatic activities. Finally, Karuppukavuni, Kalanamak, CBMAS 14065, and Kodavilayan were identified as tolerant genotypes as donors for direct seeded rice cultivation to be used. TKM13 and Anna R4 were identified as moderately tolerant genotypes.

Future scope of the study: Advancing the knowledge on enzymatic activities during anaerobic stress could lead to identifying the tolerant genotypes for anaerobic germination stress. This will ultimately help in breeding resilient rice landraces that are more resilient and adapted to direct-seeded cultivation.

Conflict of interest: The authors have declared that no conflict of interest exist

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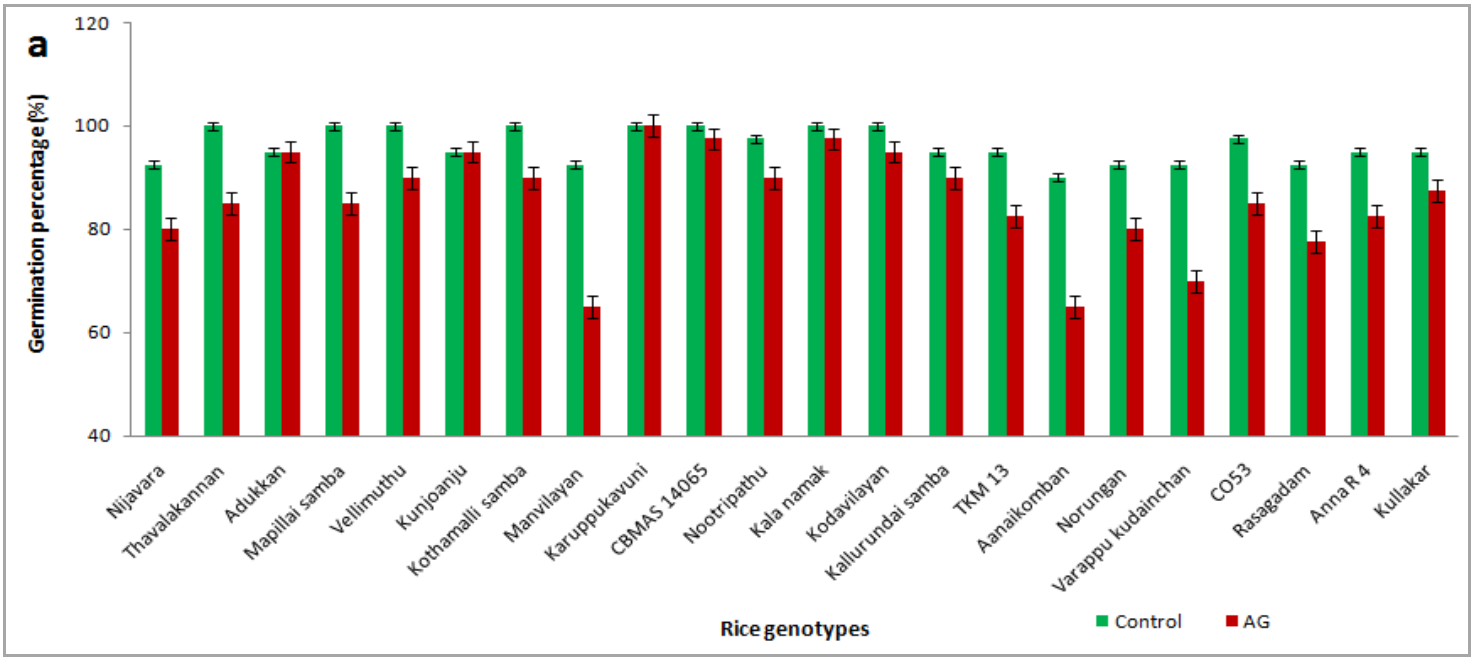

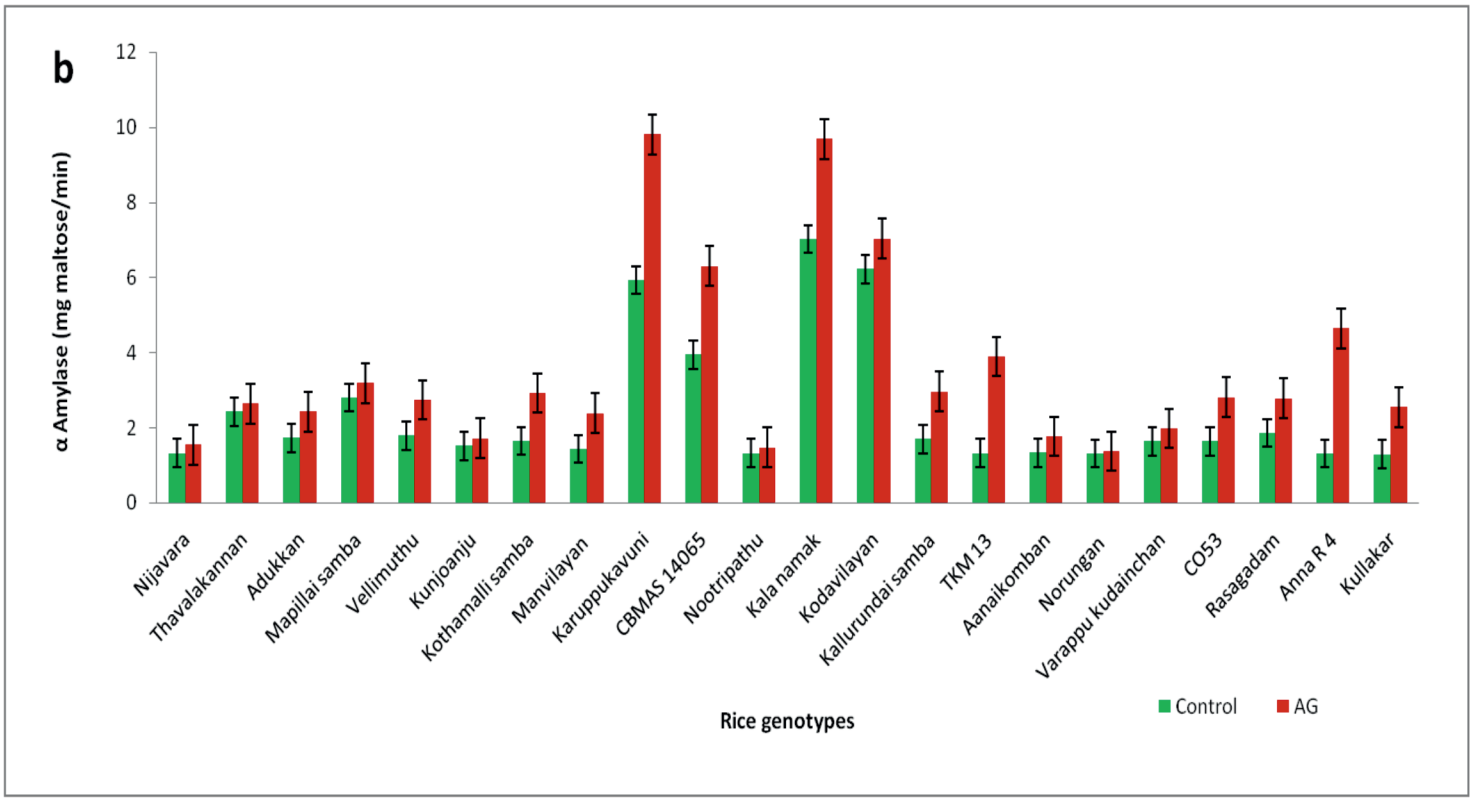

Fig.1: Genetic variation for germination percentage (a) and alpha amylase (b) in rice genotypes subjected anaerobic stress

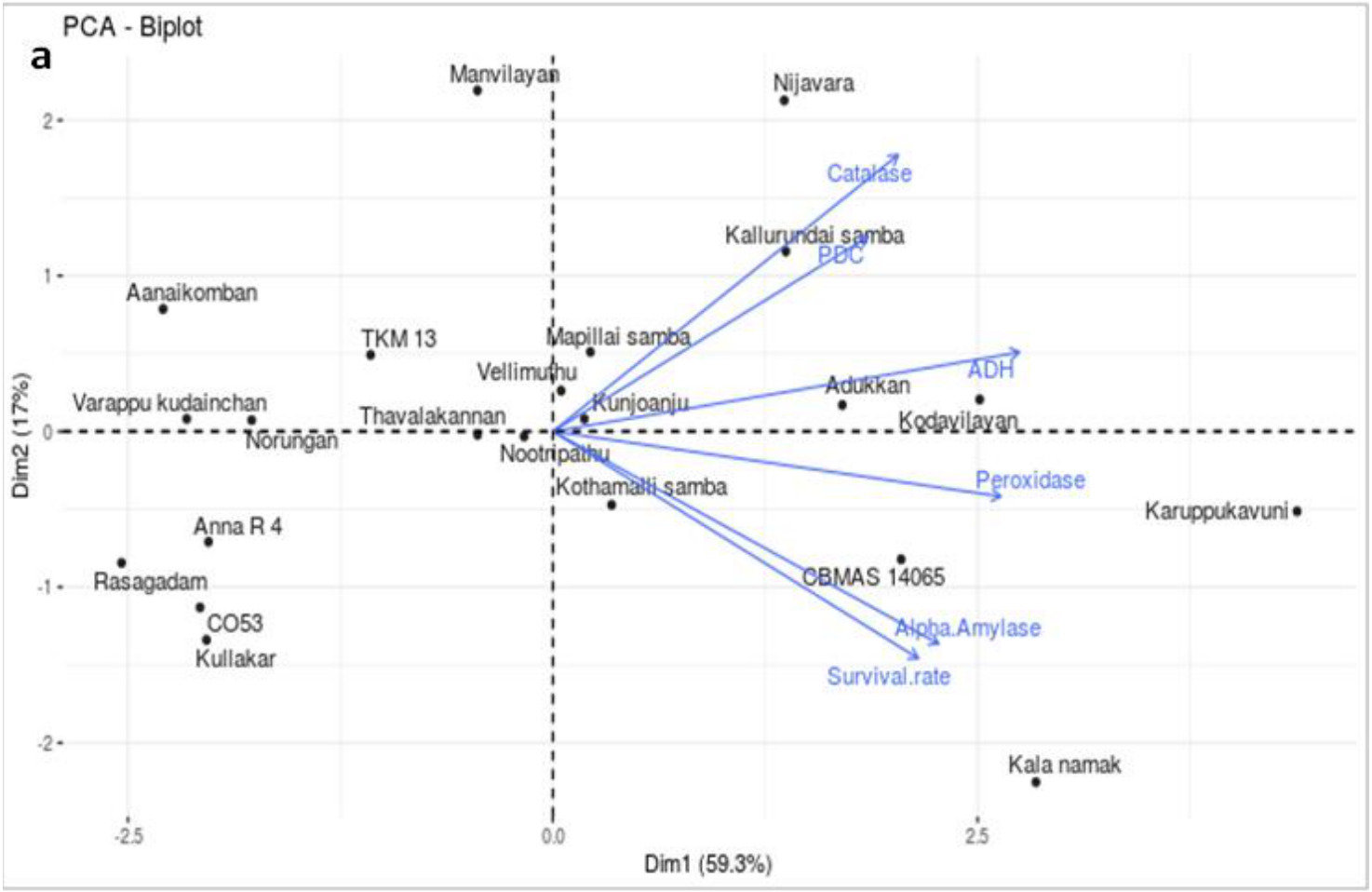

Fig.2: Genetic evaluation of anaerobic germination tolerance using enzyme activities (a) PCA analysis (b) Correlation of enzyme activities under anaerobic conditions

Table 1: Activities of catalase and peroxidase enzymes in traditional rice landraces under control and AG conditions

Sl. No	Genotypes	Catalase (nmoles of H_2O_2/min/g)		Peroxidase (Δ 430 nm/g/min)	
		Control	AG	Control	AG
1	Nijavara	26.64 ± 0.21	26.76 ± 0.42	26.64 ± 0.21	26.76 ± 0.42
2	Thavalakannan	22.86 ± 0.26	26.60 ± 0.20	22.86 ± 0.26	26.60 ± 0.20
3	Adukkan	28.00 ± 0.23	29.77 ± 0.42	28.00 ± 0.23	29.77 ± 0.42
4	Mapillai samba	27.41 ± 0.34	28.00 ± 0.09	27.41 ± 0.34	28.00 ± 0.09
5	Vellimuthu	27.28 ± 0.32	27.90 ± 0.36	27.28 ± 0.32	27.90 ± 0.36
6	Kunjoanju	26.76 ± 0.29	27.52 ± 0.30	26.76 ± 0.29	27.52 ± 0.30
7	Kothamalli samba	23.86 ± 0.21	25.22 ± 0.14	23.86 ± 0.21	25.22 ± 0.14
8	Manvilayan	29.00 ± 0.01	29.10 ± 0.11	29.00 ± 0.01	29.10 ± 0.11
9	Karuppukavuni	27.52 ± 0.05	31.06 ± 0.43	27.52 ± 0.05	31.06 ± 0.43
10	CBMAS 14065	23.37 ± 0.39	26.15 ± 0.01	23.37 ± 0.39	26.15 ± 0.01
11	Nootripathu	24.81 ± 0.25	26.12 ± 0.21	24.81 ± 0.25	26.12 ± 0.21
12	Kala namak	18.19 ± 0.09	30.60 ± 0.02	18.19 ± 0.09	30.60 ± 0.02
13	Kodavilayan	29.99 ± 0.47	31.86 ± 0.09	29.99 ± 0.47	31.86 ± 0.09
14	Kallurundai samba	28.16 ± 0.15	21.60 ± 0.15	28.16 ± 0.15	21.60 ± 0.15
15	TKM 13	19.28 ± 0.10	25.68 ± 0.03	19.28 ± 0.10	25.68 ± 0.03
16	Aanaikomban	18.88 ± 0.03	21.72 ± 0.04	18.88 ± 0.03	21.72 ± 0.04
17	Norungan	17.40 ± 0.22	21.54 ± 0.35	17.40 ± 0.22	21.54 ± 0.35
18	Varappu kudainchan	16.76 ± 0.05	21.24 ± 0.31	16.76 ± 0.05	21.24 ± 0.31
19	CO53	16.18 ± 0.03	18.04 ± 0.02	16.18 ± 0.03	18.04 ± 0.02
20	Rasagadam	14.95 ± 0.02	17.56 ± 0.26	14.95 ± 0.02	17.56 ± 0.26
21	Anna R 4	13.68 ± 0.03	23.04 ± 0.18	13.68 ± 0.03	23.04 ± 0.18
22	Kullakar	10.77 ± 0.11	14.88 ± 0.04	10.77 ± 0.11	14.88 ± 0.04
SE.d		1.59	1.59	1.52	1.32
CD ($p<0.05$)		5.52**	5.55**	5.28**	4.58**

Table 2: Fermentation pathway enzymes (ADH and PDC) in traditional rice landraces under control and AG conditions

Sl. No	Genotypes	ADH (µmol/g FW/min)		PDC (µmol/g FW/min)	
		Control	AG	Control	AG
1	Nijavara	7.20 ± 0.12	12.80 ± 0.11	4.32 ± 0.02	7.20 ± 0.09
2	Thavalakannan	4.00 ± 0.04	10.80 ± 0.13	2.44 ± 0.03	2.92 ± 0.03
3	Adukkan	10.00 ± 0.08	15.60 ± 0.07	3.16 ± 0.01	3.60 ± 0.03
4	Mapillai samba	6.00 ± 0.07	12.80 ± 0.16	3.60 ± 0.03	3.80 ± 0.04
5	Vellimuthu	6.80 ± 0.04	10.80 ± 0.13	2.92 ± 0.04	3.80 ± 0.03
6	Kunjoanju	8.00 ± 0.11	13.20 ± 0.09	3.00 ± 0.01	3.36 ± 0.02
7	Kothamalli samba	5.60 ± 0.04	11.20 ± 0.19	2.68 ± 0.03	3.28 ± 0.04
8	Manvilayan	4.00 ± 0.02	12.40 ± 0.17	3.80 ± 0.03	4.20 ± 0.04
9	Karuppukavuni	10.80 ± 0.06	18.00 ± 0.01	2.76 ± 0.03	5.00 ± 0.04
10	CBMAS 14065	8.40 ± 0.11	14.00 ± 0.09	3.28 ± 0.04	4.36 ± 0.04
11	Nootripathu	8.80 ± 0.03	11.20 ± 0.14	2.40 ± 0.02	3.20 ± 0.05
12	Kala namak	11.60 ± 0.14	18.00 ± 0.06	2.48 ± 0.02	4.52 ± 0.03
13	Kodavilayan	13.20 ± 0.09	16.80 ± 0.27	3.72 ± 0.01	4.60 ± 0.03
14	Kallurundai samba	10.40 ± 0.11	15.60 ± 0.06	3.60 ± 0.06	3.96 ± 0.03

15	TKM 13	1.20 ± 0.01	7.20 ± 0.01	2.48 ± 0.03	4.00 ± 0.06
16	Aanaikomban	4.40 ± 0.02	6.00 ± 0.05	3.64 ± 0.02	3.84 ± 0.04
17	Norungan	6.80 ± 0.06	7.60 ± 0.12	3.48 ± 0.05	3.56 ± 0.04
18	Varappu kudainchan	8.00 ± 0.13	10.00 ± 0.07	2.32 ± 0.03	2.48 ± 0.03
19	CO53	2.80 ± 0.03	4.80 ± 0.04	3.00 ± 0.01	3.20 ± 0.04
20	Rasagadam	2.00 ± 0.03	5.20 ± 0.04	2.36 ± 0.04	3.00 ± 0.01
21	Anna R 4	3.20 ± 0.05	6.40 ± 0.03	2.80 ± 0.02	3.60 ± 0.04
22	Kullakar	3.60 ± 0.05	7.60 ± 0.03	3.20 ± 0.02	3.40 ± 0.04
SE.d		0.44	0.07	0.14	0.02
CD (p<0.05)		0.73***	0.11***	0.23***	0.04***

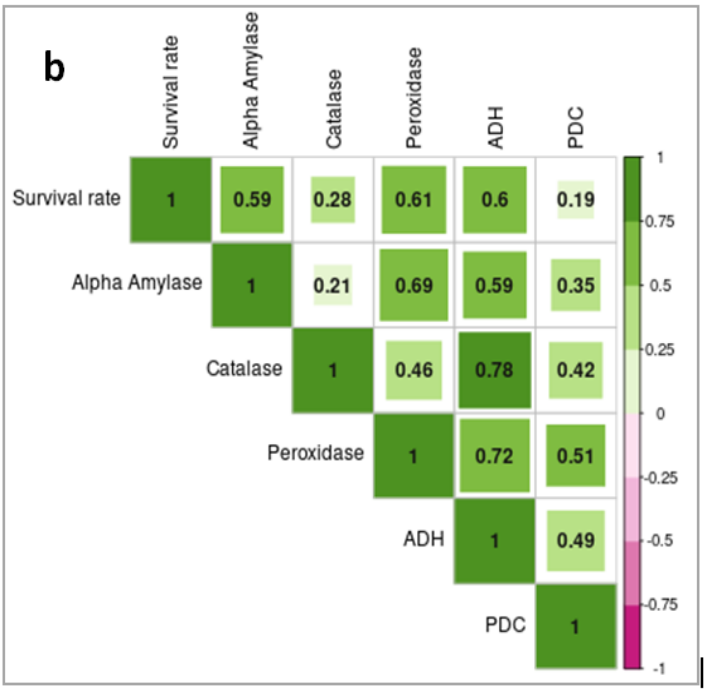

Fig.2: Genetic evaluation of anaerobic germination tolerance using enzyme activities (a) PCA analysis (b) Correlation of enzyme activities under anaerobic conditions

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