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ORIGINAL RESEARCH ARTICLE

Anaerobic Germination Tolerance in Selected African Rice (*Oryza glaberrima* Steud.) Accessions: Physiology, Carbohydrate Quantification and Identification of AG1 Gene

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ABSTRACT

Anaerobic germination (AG) tolerance is one of the most important agronomic traits in rice cultivation in both rainfed and irrigated ecosystems, especially in direct-seeded rice (DSR). In this study, we assessed seven (7) rice accessions under anaerobic, anoxic and aerobic conditions. A complete Randomized Design (CRD) was used with three replications in all treatment, 30 imbibed seeds (4°C, 2 days) in 50ml vials of anoxic water and the lid was closed tightly and kept in the dark for 7 day and the coleoptile length of all seedlings was measured and recorded using meter rule. For anaerobic germination, 30 seeds of each accession were sown on a tray with 1.5cm of soil and overlaid with 0.5cm of soil. The next day, the imbibed seeds were sown under the soil (2cm depth). Trays were submerged under 10cm submergence chamber with daily adjustment. Germination was scored when coleoptiles reached≥1 mm. Survival was scored after 14 days, and control germination was performed in air. The Landraces (LR) showed a higher survival rate (72.22 ±10.72^{a}), rapid coleoptile elongation (18.22±2.11^{a}) and higher carbohydrate status (5.38±2.69^{a}) compared to the Release Accessions (RA), survival rate (26.67±12.02^{b}), P = 0.008, coleoptile elongation (5.01±2.74^{b}), *P = 0.002,* and Carbohydrate (1.05±0.14^{b}) P=0.049. Carbohydrate status was strongly positively correlated to coleoptile r=1 in both LR and RA, and strongly correlated to survival rate (LR=0.70) (RA=0.89). The molecular analysis revealed the putative presence of the AG1 gene in the landraces. Therefore, we can deduce that landraces could serve as promising resources for improving AG tolerance in rice, particularly for cultivation in flood-prone areas to enhance food security in the the face of climate change.

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Rice; Anaerobic germination, Coleoptiles, Survival rate, AG1gene

INTRODUCTION

Rice is a semi-aquatic crop and can withstand excess water but will eventually wither and dies when completely submerged in water for long (Sultana *et al.,* 2018) Tolerance to flooding during seed germination and very early seedling growth referred to as anaerobic germination (AG), is one of the most important traits necessary to ensure good seedling establishment in direct-seeded rice (DSR) in both rainfed and irrigated ecosystems (Ismail *et al.,* 2009; Kretzschmar, *et al.,* 2015). The ability of rice to germinate under flooded conditions is generally low. Most modern rice varieties either fail to germinate completely under water or fail to elongate the coleoptile and develop roots and shoots for further development under anoxic conditions (long period of oxygen deprivation), resulting in partial to complete crop failure (Magneschi and Perata, 2009; Narsai *et al.,* 2015; Ghosal *et al.,* 2019; Yadav *et al.,*2024). However, genetic variation exists among rice varieties for anaerobic germination (AG), with some landraces being able to germinate under water. One of the most spectacular adaptive growth features of germinating rice seeds to tolerate oxygen deficiency (anoxic) in flooded soils is the accelerated growth of the coleoptile. Several landraces with anaerobic germination (AG) tolerance have been identified (Angaji *et al.,* 2010). Anaerobic conditions affect glycolysis, promoting ethanol and lactate production through the ethanolic fermentation pathway, and the toxicity of ethanol can diffuse out of the cell, resulting in plant death (Chirkova and Yemelyanov, 2018). Varietal differences in terms of anaerobic germination and submergence tolerance have been shown to exist by several workers, especially wild species from the *Oryza* genus, which commonly grow in constantly or seasonally wet habitats, and thus, submergence tolerance could also be found in other species (Vaughan, 1994; Gumi and

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Aliero, 2018; Gumi *et al.,* 2018). Globally and in Nigeria, several studies in the literature reveal research on anaerobic germination. However, there is no known work, or very little, on anaerobic germination (AG) in the north-west of Nigeria, especially regarding the integration of carbohydrate quantification. The choice of landraces was largely due to their constant cultivation by local farmers in the northwestern states, which are often affected by flooding. We also hypothesize that the landraces are anaerobic germination-tolerant than the released accessions. Therefore, this research focused on anaerobic germination, its physiology, carbohydrate quantification, and the identification of the AG1 gene, although without a corresponding gene expression analysis, which aims to boost rice production in the face of global climate change.

MATERIALS AND METHODS

Study Area (Experimental Site)

This research work was carried out in the Plant Physiology Laboratory and the Biological Garden of Usmanu Danfodiyo University, Sokoto, located within latitude 13°.12'47.82667"N and longitude 5°.12'12.367640"E and an altitude of 302m above sea level. Sokoto State is characterized by the Sudan savanna agro-ecological zone with 570-750mm rainfall and temperature of 15 °C – 45 °C from November to March, respectively in Nigeria. The inhabitants are predominantly Hausa-Fulani and are mostly farmers, traders and artisans. The common agricultural crops grown are rice, millet, maize, guinea corn, onion and tomatoes; Biochemical (Carbohydrate quantification) analysis was carried out in the Biochemistry laboratory of UDUS.

Germplasm Collection Environmental Characterization

Rice seeds of seven accessions were collected directly from the local farmers, which include *Kamrum, Bvofort, Fanjim and Danruwa,* while the released accessions were obtained from the Federal Ministry of Agriculture and Rural Development (FMARD), which include FARO 44, FARO 66 and FARO 67 were evaluated for the physiological, Biochemical and molecular basis of anaerobic germination. Dormancy breaking in a hot-air oven at 60 ℃ for 72 h over 3 days was conducted (Shiratsuchi *et al*., 2017). A complete Randomised Design (CRD) was used across all experimental conditions, with three replications per condition. However, the study was limited by the inability to secure some internationally recognized genotypes, such as FR13 and Swarna sub I, as controls at the time the research was conducted.

Nursery Preparation

The nursery bed was prepared with garden soil, where seeds were sown separately in holes, five (5) seeds per hole, and were watered morning and evening for a a viability test for fourteen (14) days.

Percentage Germination Test

Thirty (30) seeds of each rice accessions was divided into 3 replicates of 10 seeds and placed in a waterproof tray (Petri dishes) laden with a saturated absorbent material (Whatmann filter paper). For every 10 days, the absorbent material (Whatman filter paper) was ensured to remain moist (wet), and the number of seed germinated was recorded on a a daily basis. The percentage germination was determined using the formula below.

$$Germination\ rate = \frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ sown} \times 100\% \quad \text{.....(1)}$$

Evaluation of Anoxic Germination

30 imbibed seeds (4 °C, 2 days) in 50 ml vials of anoxic water with a tightly closed lid. The seeds were kept in the dark for 7 day and the coleoptile lengths of all seedlings were measured and recorded using meter rule. (Kuya, 2019).

Seed Vigor Index

This was calculated by determining the germination percentage and seedling length of the same seed lot. 30 seeds in 3 replications were planted in the germination tray for a germination test. To evaluate the number of normal seedlings at the time of final count, the seedling length of 5 plants was randomly selected and measured. The seed vigor index was calculated as shown below.

$$SVI = \text{Germination Percentage (\%)} \times \text{Seedling Length (mm)} \quad \text{..(2)}$$

Survival Rate Evaluation of Anaerobic Germination

30 seeds of each accession and parental control were sown on a tray with 1.5 cm soil and overlaid with 0.5 cm soil. The next day, the imbibed seeds would be buried under the soil (2 cm depth) by tweezers. Trays were submerged under 5-7cm of water measured from the soil surface, with daily adjustments to ensure the level doesn't fall below 5 cm. Water depth was maintained throughout the experiment. Germination was scored based on ≥1 mm coleoptiles emergence. Survival was scored 14 days after planting, and control germination was performed in air under aerobic conditions (Alam *et al*., 2020). The number of seedlings that emerged from the water surface at 14 days was counted, and the percentage out of 30 seeds was defined as the survival rate (Kuya, 2019; Mlaki *et al.,* 2019).

Carbohydrate Quantification

The Carbohydrate was first hydrolyzed into a simple sugar using dilute hydrochloric acid (HCl). 100mg of rice coleoptiles-shoot oven-dried tissue was placed in a boiling tube and hydrolyzed in a boiling water bath for 3 hours with 5 mL of 2.5 N HCl, then cooled to room temperature. 1 mL of liquid sodium carbonate was added until effervescence ceased. The volume was adjusted to

100 mL and centrifuged at 630rpm. The supernatant was collected, and 0.5- and 1-ml aliquots of each genotype were used for sugar content measurements following the method of Ismail *et al.* (2009), as adopted and modified.

Conversion Formul

$$mg/g\ DW = \frac{Concentration\ (mg/ml)}{DW\ per\ ml\ extract\ (g/ml)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Since we used 100mg dry weight and 5ml solvent for each sample, we have to convert the dry weight from milligrams to grams: 100mg = 100/1000 g = 0.1g.

Dry weight per 1ml extract contains $= \frac{0.1g}{5ml} = 0.02g/ml$ DW

Genomic DNA Extraction

Genomic DNA from the selected rice accessions was isolated using the pre-standardized protocol of Borges *et al.* (2012). The harvested fresh green leaves of the plant were weighed (~100mg) and immediately used for DNA isolation using CTAB mini prep protocols. Leaf tissues were ground into a fine powder after freezing in liquid nitrogen in a pre-chilled mortar. The fine powdered tissues were then transferred to a tube of pre-warmed CTAB buffer (2.0% CTAB {w/v}); 0.1 M TrisCl, PH 8; 0.02M of EDTA, PH 8; 1.4 M NaCl) and the mixture was incubated at 65 ^{0}C for 20 minutes. The supernatant was collected after centrifugation, and an an equal volume of Chloroform: Isoamyl alcohol (24:1) was mixed. After centrifugation, the aqueous phase was collected, mixed with an equal volume of isopropanol, and incubated for 20 min at -20 °C. Centrifugation was done to pellet down the DNA. Pellet was washed with 70% (v/v) ethanol, air-dried, and dissolved in nuclease-free water. The sample was treated with RNase enzyme at 37 ^{0}C and subsequently purified by the phenol-chloroform method (Sambrook and Russelle, 2001). For DNA purification, RNase was used to incubate the dissolved DNA sample at 37 ^{0}C for 1 hour, then phenol: isoamyl alcohol (PIA) was added, and the mixture was centrifuged at 8,000rpm for 8 minutes. Subsequently, the sample was decanted and dried in laminar flow for 20-30 minutes. The concentration and quality of the purified DNA were checked using a NanoDrop spectrophotometer (Thermo Scientific, USA) by measuring the 260/280 and 260/230 ratios, and by 1% (w/v) agarose gel electrophoresis as reported by Goswami *et al.* (2015).

Primer Design

The nucleotide sequences of *the OglAG1* gene of *O. glaberrima* were retrieved from the Plant Ensemble database, and primers were designed manually. The thermodynamic properties of the oligos were checked using Vector NTI software for hairpin, primer dimer, optimal Tm values, and % GC content (Vector NTI Advance version 11.5.3, March, 2013).

Fwd- 5'-ATGGCGAAGGCGAGCGTGG 3'

Rev- 5'- TCCCACTTGATGGTCGGC 3'

Amplicon size - 870 bp

Amplification of the AG1 gene

The Polymerase chain reaction (PCR) of the AG1 gene in the selected accessions was performed using *OglAG*Igene-specific primer pairs (GSP_AG1_fwd & GSP_AG1_rev) in a 50 µL reaction volume on a C 1000 Touch Thermal Cycler (Bio-Rad, USA). Each 50 µL reaction mixture contained ~200 ng of genomic DNA as template, 5X Phusion High Fidelity Buffer, 10 mM dNTPs, 0.4 pM each of the forward and reverse primers, and 100 U of high-fidelity Phusion polymerase. The optimized condition was an initial 5 minutes incubation at 98 ^{0}C for complete denaturation, followed by 35 cycles consisting of 98 ^{0}C for 10 seconds, 65 ^{0}C (varies with the primer pair) for 30 secs, 72 ^{0}C for 30 secs, and finally 72 ^{0}C for 8 minutes. The PCR products were run on a 1.2% agarose gel for electrophoresis to check amplification using a constant voltage of 100V and a 1X TAE (Tris Acetate EDTA) buffer system. The gel was stained with Ethidium bromide (EtBr) and viewed under a UV transilluminator system (Sambrook and Russell, 2001).

Statistical Analysis

Recorded data were subjected to and statistically analyzed using One-way analysis of variance (ANOVA) in MINITAB version 2017 to test for significant differences between treatments, and the differences in means were measured using Tukey Pairwise Comparisons Grouping Information method and 95% Confidence. The correlation (r) between the different parameters was also determined.

RESULTS AND DISCUSSION

Percentage (%) Germination under different conditions

Under anoxic conditions after seven days for the landraces, *Kamrum* had 100% germinations, *Bvofort* had 86.67% germination, *Fanjim* had 96.67% germination, *Danruwa* had 100% germination, while for the released FARO 44 had 26.67% germination, and FARO 66 had 20% germination, while FARO 67 had 06.67% germination (Table 1). The result of the percentage germination under anaerobic conditions shows that among the landraces, *Danruwa* had the highest percentage germination of 80.00%, followed by *Kamrum* with 76.67%, and *Fanjim* with 60.00%, while *Bvofort* had the lowest at 53.33%. Among the released accessions, FARO66 had the highest germination percentage of 36.67%, followed by FARO44 with 30.00%, while FARO67 had the lowest germination percentage of 13.33%.

Coleoptile length under different condition

The results of coleoptile length under anaerobic conditions for the different accessions are presented in the Table 2. Among the landraces**,** *Danruwa* had the highest coleoptile length (21.34mm), followed by *Kamrum* (17.99mm), *Fanjin* (17.39mm), while *Bvofort* had the least coleoptile length (11.09mm). The results from the

released accessions revealed that FARO 67 had the highest coleoptile length (8.10mm); FARO 66 had the second-highest (4.06mm), while FARO 44 had the lowest (2.87mm), as presented in Table 2.

Table 1: Seed Germination under different conditions

SN	Accessions	Aerobic (%)	Anoxic (%)	Anaerobic (%)
1	***Kamrum***	96.67±5.77^{a}	100.00±5.00^{a}	76.67± 23.10^{a}
2	***Bvofort***	100.00±0.00^{a}	86.67±01.00^{b}	53.33± 11.55^{ab}
3	***Fanjim***	96.67±5.77^{a}	96.67±01.00^{a}	60.00± 10.00^{ab}
4	***Danruwa***	100.00±0.00^{a}	100.00±05.00^{a}	80.00± 20.00^{a}
5	**FARO44**	76.67±5.77^{ab}	26.67±01.00^{c}	30.00±00.00^{bc}
6	**FARO66**	80.00±10.00^{ab}	20.00±02.00^{c}	36.67±05.77^{bc}
7	**FARO67**	66.67±20.80^{b}	06.67±01.00^{d}	13.33±05.77^{c}

Values are Mean± SD of biological triplicate. Means that do not share the same letter within a column are significantly different.

Table 2: Coleoptile length under different conditions

SN	Accessions	Aerobic (mm)	Anoxic (mm)	Anaerobic (mm)
1	***Kamrum***	13.23±0.44^{b}	5.46±0.46^{a}	17.99±2.84^{a}
2	***Bvofort***	14.12±1.10^{b}	6.28±1.10^{a}	11.09±1.66^{b}
3	***Fanjim***	12.17±1.06^{bc}	5.27±0.74^{a}	17.39±2.46^{a}
4	***Danruwa***	17.20±0.89^{a}	7.30±0.33^{a}	21.34±1.78^{a}
5	**FARO44**	7.85±0.96^{d}	2.13±0.44^{b}	2.87±0.88^{c}
6	**FARO66**	8.87±1.15^{d}	3.63±1.91^{b}	4.06±2.46^{c}
7	**FARO67**	10.02±1.27^{cd}	0.50±0.29^{b}	8.10±1.71^{bc}

Values are Mean± SD of biological triplicate. Means that do not share the same letter within a column are significantly different.

Table 3: Seed vigour index of selected rice accessions under Anoxic conditions

SN	Accessions	Germination (%)	Coleoptile(mm)	Vigour Index
1	***Kamrum***	100.00±5.00^{a}	54.60±0.46^{a}	5460.00±10.00^{b}
2	***Bvofort***	86.67±1.00^{b}	62.80±1.10^{a}	5442.88±10.00^{b}
3	***Fanjim***	96.67±1.00^{a}	52.70±0.74^{a}	5094.51±01.00^{c}
4	***Danruwa***	100.00±5.00^{a}	73.00±0.33^{a}	7300.00±100.00^{a}
5	**FARO44**	26.67±1.00^{c}	21.30±0.44^{b}	568.07±01.00^{e}
6	**FARO66**	20.00±2.00^{c}	36.30±1.91^{b}	726.00±01.000^{d}
7	**FARO67**	06.67±1.00^{d}	05.00±0.29^{b}	33.35±01.000^{f}

Values are Mean± SD of biological triplicate. Means that do not share the same letter within a row are significantly different.

Table 4: Seed vigour index of selected rice accessions under Anaerobic condition

SN	Accessions	Germination (%)	Coleoptile(mm)	Vigour Index
1	***Kamrum***	76.67 ±23.10^{a}	17.99±2.84^{a}	1379.2933±10.00^{b}
2	***Bvofort***	53.33±11.55^{ab}	11.09±1.66^{b}	591.4297±100.00^{d}
3	***Fanjim***	60.00±10.00^{ab}	17.39±2.46^{a}	1043.400±01.00^{c}
4	***Danruwa***	80.00± 20.00^{a}	21.34±1.78^{a}	1707.200±01.00^{a}
5	**FARO44**	30.00±0.00^{bc}	2.87±0.88^{c}	86.100±01.00^{e}
6	**FARO66**	36.67±5.77^{bc}	4.06±2.46^{c}	148.8802±10.00^{e}
7	**FARO67**	13.33±5.77^{c}	8.10±1.71^{bc}	107.973±01.004^{e}

Values are Mean± SD of biological triplicate. Means having different letter within a row are significantly different.

Table 5: Seed vigour index of selected rice accessions under Aerobic condition

SN	Accessions	Germination (%)	Coleoptile(mm)	Vigour Index
1	***Kamrum***	96.67±5.77^{a}	13.23±0.44^{b}	1278.944±10.00^{bc}
2	***Bvofort***	100.00±0.00^{a}	14.12±1.10^{b}	1412.000±10.00^{b}
3	***Fanjim***	96.67±5.77^{a}	12.17±1.06^{bc}	1176.434±10.00^{c}
4	***Danruwa***	100.00±0.00^{a}	17.20±0.89^{a}	1720.000±10.00^{a}
5	**FARO44**	76.67±5.77^{ab}	7.85±0.96^{d}	601.860±10.00^{d}
6	**FARO66**	80.00±10.00^{ab}	8.87±1.15^{d}	709.600±10.00^{d}
7	**FARO67**	66.67±20.80^{b}	10.02±1.27^{cd}	668.033±10.00^{d}

Values are Mean± SD of biological triplicate. Means that do not share the same letter within a column are significantly different.

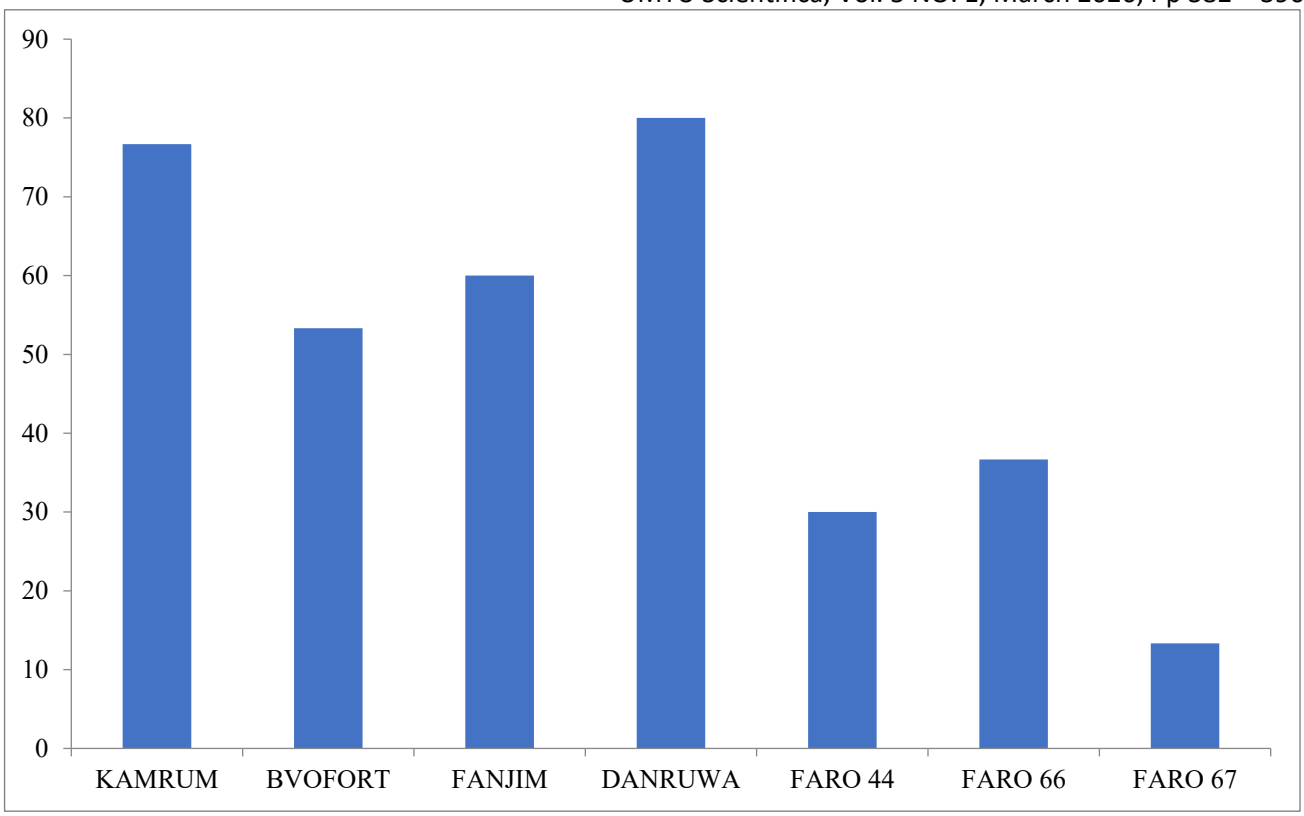

Figure 1: Survival Rate (%) of selected rice accessions under anaerobic germination

Table 6: Consolidated parameters and Carbohydrate Quantification

Accessions	NSP	TCL(mm)	SVI	SR (%)	CQ (mg/g)
Kamrum	23	17.99±2.84^{a}	1379.2933±10.00^{b}	76.67 ±23.10^{a}	179±0.0208^{c}
Bvofort	16	11.09±1.66^{b}	591.4297±100.00^{d}	53.33±11.55^{ab}	424±0.1222^{a}
Fanjim	18	17.39±2.46^{a}	1043.400±01.00^{c}	60.00±10.00^{ab}	205±0.1229^{b}
Danruwa	24	21.34±1.78^{a}	1707.200±01.00^{a}	80.00± 20.00^{a}	91±0.1041^{d}
FARO44	09	2.87±0.88^{c}	86.100±01.00^{e}	30.00±0.00^{bc}	59±0.0208^{e}
FARO66	11	4.06±2.46^{c}	148.8802±10.00^{e}	36.67±5.77^{bc}	52±0.0458^{f}
FARO67	04	8.10±1.71^{bc}	107.973±01.004^{e}	13.33±5.77^{c}	45±0.0557^{g}
LR	21.67	18.22±2.11^{a}	1377.00±332.00^{a}	72.22 ±10.72^{a}	269±2.69^{a}
RA	08.00	5.01±2.74^{b}	114.00±32.00^{b}	26.67±12.02^{b}	52.35±0.14^{b}
P-Value	0.008	0.002	0.003	0.008	0.049
r (LR)	-------	1.0	----------	0.70	TCL-SR-CQ
r (RA)	------	1.0	----------	0.89	TCL-SR-CQ

Values are Mean± SD of biological triplicate. Means that do not share the same letter within a column are significantly different.

NSP= Number of survived plants out of 30 seeds under anaerobic conditions, TCL=Total coleoptiles length, SR=Survival rate, CQ=Carbohydrate quantification, LR=Landraces, RA=Release accession, **r**=correlation. The values for r under TCL and SR show the correlation between Carbohydrate status, survival rate, and total coleoptile length.

Seed Vigour Index under different conditions

The seed vigour index (SVI) of the accessions shows that *Danruwa* had the highest SVI, followed by *Bvofort* and *Kamrum*, while *Fanjim* had the lowest SVI among the landraces. FARO66 had the highest seed vigour among the released accessions, followed by FARO67, while FARO44 had the lowest seed vigour index, as presented in Tables 3, 4, and 5.

Survival Evaluation of Anaerobic Germination

The landraces had a higher survival rate than the released accessions. Regarding survival percentage, the released accessions showed poor survival under both anaerobic and anoxic conditions, while the landraces displayed a higher survival rate. Among the landraces *Danruwa* survival rate of (80%), *Kamrum* **(**76.67%), *Fanjim* **(**60%) and *Bvofort* had (53.33%) while among the released accessions FARO 66 had a survival rate of (36.67%), FARO44 had (30.0%) and FARO67 had the survival percentage of (13.33%). These findings are in agreement with those of Alam *et al.* (2020), who showed that the percentage of seedling survival of the pyramided genotype (57%) was statistically distinguishable from IR64 (76%), IR64 (AG1) (85%), and IR64 (SUB1) (91%). The survivability of the rice accession under anaerobic conditions after 14 days is presented in Fig 1.

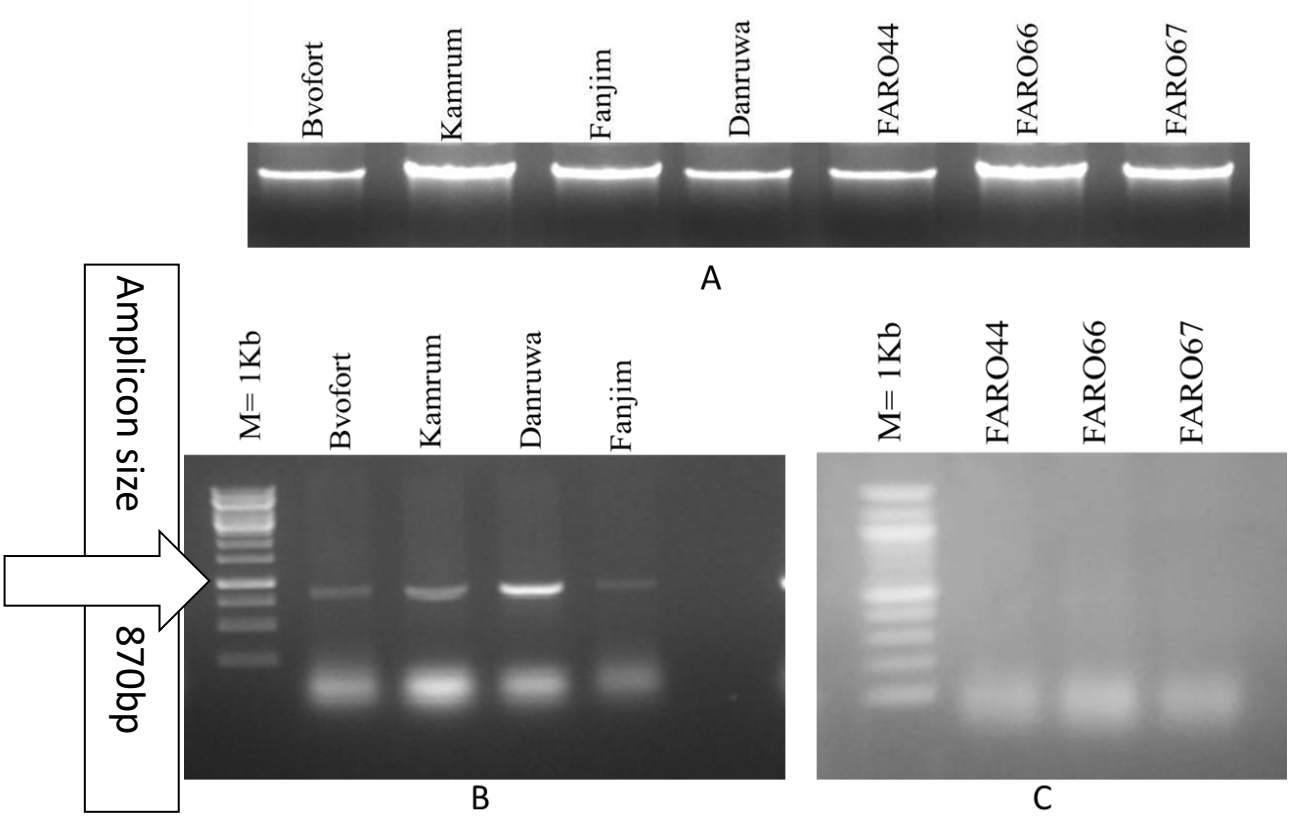

Figure 2: The amplified bands from the studied accessions

Carbohydrate Quantification

The total carbohydrate content from the endosperm and shoot tissues of the selected rice accessions among the landraces was recorded. *Bvofort* had the highest total carbohydrate content of 8.47mg/ml, followed by *Fanjim* with 4.10mg/ml, and *Kamrum* had 3.57mg/ml, while *Danruwa* had the least carbohydrate content of 1.82mg/ml. Among the released accessions, FARO 44 had the highest carbohydrate content of 1.18mg/ml, followed by FARO 66 with 1.05mg/ml, while FARO 67 had the lowest carbohydrate content of 0.91mg/ml, as presented in Table 6.

Amplification of the AG1 Gene

The isolated genomic DNA showed the presence of plant DNA under a UV transilluminator using 1.5% agarose gel electrophoresis in all 7 selected accessions (Figure 2A). The designed AG1 gene-specific primers (forward and reverse) showed the presence of the AG1 gene in the landraces among the 7 accessions after PCR using genomic DNA from each accession as a template (Figure 2B), whereas it was absent in the release accessions (Figure 2C). The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis to determine their sizes. Three amplified bands from the studied accessions, corresponding to sizes in base pairs, were identified using a 1kb ladder, as shown in Figure 2.

Anaerobic, Anoxic, Aerobic germination, Coleoptiles length, Survival rate, seed vigor, carbohydrate contents and gene amplification of the selected rice accessions

In this study, rice seed germination from different accessions was assessed under anaerobic, anoxic, and aerobic conditions. The landraces showed higher germination indices, seed vigour, germination rate, and coleoptile length than the released accessions. In terms of percentage germination, the landraces *Bvofort* and *Danruwa* performed better than all other accessions. Nevertheless, the least germination was recorded in the release accession FARO67. However, on the seed vigour assessment and germination rate, all the landraces performed better than the released accession. This shows their potential as an alternative seed for anaerobic tolerance. Based on the germination rate, otherwise known as the speed of germination, the landraces had a better germination speed than the released accession. The highest germination rate was recorded in both *Bvofort* and *Danruwa*, followed by *Kamrum* and *Fanjim*. The released accession FARO 66 had the highest germination rate, followed by FARO 44, and FARO 67 had the lowest germination rate. Anaerobically and anoxically germinated landrace rice accessions namely *Kamrum, Bvofort*, *Fanjim, Danruwa* showed rapid coleoptiles elongation compared to the released accessions namely FARO44, FARO66, FARO67and coleoptiles lengths differed significantly after 14 days demonstrating that carbohydrate availability was a limitation for the release accessions and consequently lacks the AG1 gene, OsTPP7 since highest carbohydrate content was found in the landrace *Bvofort* while the least was found in FARO67.

The coleoptile length, Survival rate, and carbohydrate content of the selected rice accessions were assessed under aerobic, anaerobic, and anoxic conditions. The germinated landraces, namely *Kamrum, Bvofort*, *Fanjim, and Danruwa,* showed rapid coleoptiles elongation compared to the released accessions, namely FARO 44, FARO 66, FARO 67, and coleoptiles lengths differed significantly after 14 days, demonstrating that carbohydrate availability is a limiting factor for the release accessions and consequently lack of the AG1 Gene; Tetrahalose-6-phosphate phosphotase (OsTPP7). This agrees with the report by Kretzschmar *et al.* (2015), which shows that anaerobically germinated rice species, KHO and NIL-AG1, displayed enhanced coleoptile elongation compared to IR64. This enhanced coleoptile elongation could be due to the presence of OsTPP7 in the AG1 gene, while the anaerobic germination susceptibility of IR64 was rescued by the supply of exogenous sucrose. This suggests that rapid mobilization of starch reserves could promote early vigour. These results are consistent with those reported by Adachi *et al.* (2015), who showed that IR06F459, an AG line with a long coleoptile, has high *a*-amylase activity and high sucrose and glucose concentrations in germinating seeds. These attributes partly explain its vigorous germination and coleoptile growth under hypoxic conditions.

According to Kretzschmar *et al.* (2015), the poor anaerobic germination of the high-yielding *indica* IR64 was attributed to a chromosomal deletion that includes TPP7. The Quantitative Trait Loci (QTL), Anaerobic Germination (AG1) was identified from the *japonica* landrace KhoaHlan and defined as Tetrahalose-6-Phosphate Phosphatase (TPP7) (Angaji *et al.,* 2009; Angaji *et al.,* 2010) encoding the enzymes that catalyzes the conversion of the low-abundance metabolite Tetrahalose-6-phosphates (T6P), which controls catabolic carbon metabolism allocating carbon from source to sink tissues (Yadav *et al.,* 2014; Kretzschmar *et al.,* 2015; Alam *et al.,* 2020).

Similarly, Anoxically germinated landraces namely *Kamrum, Bvofort*, *Fanjim, Danruwa* showed rapid coleoptiles elongation compared to the released accessions namely FARO44, FARO66, FARO67 and coleoptiles lengths differed significantly after 7 days of growth in the dark demonstrating that accelerated growth of coleoptiles is independent of oxygen environment (BOD) despite the fact that the landraces performed better than the released accessions but they both germinated under anoxic condition while under aerobic condition, landraces showed rapid coleoptiles elongation compared to the released accessions and coleoptiles lengths differed significantly after 10 days. According to Nghi *et al.* (2019), in japonica rice cultivars possessing TPP7, additional genes contribute to coleoptile elongation during anaerobic germination.

The survival percentage of the released accessions was poor under anaerobic and anoxic conditions, while the landraces displayed a higher survival rate. Among the landraces, *Danruwa* had a survival rate of (80%), *Kamrum* (76.67%), *Fanjim* (60%) and *Bvofort* had (53.33%) while among the released accessions, FARO66 had a survival rate of (36.67%), FARO44 (30.0%), and FARO67 had a survival percentage of (13.33%). This finding is in agreement with that of Alam *et al.* (2020), who showed that the percentage of seedling survival of the pyramided genotype (57%) was statistically distinguishable from IR64 (76%), IR64 (AG1) (85%), and IR64 (SUB1) (91%). Nonetheless, according to the report by Ghosal *et al.* (2019), the average survival for Kalarata is 5.1% to 10.3% for NSIC Rc238 and 0.7% to 8.6% for NSIC Rc222 under anaerobic conditions, and is not consistent with our findings, perhaps due to differences in days of submergence. The normal germination of Kalarata ranged from 77.6% to 89.7%; that for NSIC Rc238 was 82.2%, and that for NSIC Rc222 was 90.6%, which is consistent with our findings.

The results from the Carbohydrate Quantification revealed a significant difference in carbohydrate content between the landraces *Kamrum, Bvofort, Fanjim,* and *Danruwa*, and the released accessions FARO44, FARO66, and FARO67. The carbohydrate content in the landraces was relatively higher than in the released accessions, which is a pointer to enhanced coleoptile elongation in the landraces under anaerobic and anoxic conditions, since Carbohydrates in the form of starch are needed for mobilization in the endosperm for survival and coleoptile elongation under anaerobic conditions. This agrees with the finding of Das *et al.* (2005), who opined that differences in tolerance are not necessarily associated with initial carbohydrate status before submergence, but rather with the ability to sustain energy levels during submergence. This is also sufficiently reasonable to deduce that it is the major factor behind the higher survival rate of the landraces than the released accessions, and it corroborate with the findings of (Sarkar *et al.*, 1996; Huang *et al.*, 2005) which suggest that non-structural carbohydrates are utilized during submergence to supply the required energy for growth and maintenance metabolism. Similarly, Kretzschmar *et al.*(2015) suggest that the introduction of an ectopically-expressed TPP7 transgene into IR64 is sufficient to elevate T6P in coleoptiles under water for 4 days, thereby enhancing the activation of α-*AMYLASE(AMY)* genes associated with endospermic starch catabolism, early elongation and anaerobic germination tolerance. Therefore, based on the findings of this study, it can be deduced that high carbohydrate status before submergence and the extent of turnover and consumption during submergence are the key factor that determines the ability of a rice plant to withstand submergence stress and, as such plays a significant role in terms of survivability under anaerobic conditions. We also find that carbohydrate status is strongly positively correlated with coleoptile length (r=1) in both landraces and the release accession, and positively correlated with survival rate in the landraces (r=0.70) and in the release accession (r=0.89), as shown in Table 6. This agrees with the finding of Ismail *et al.* (2009), who revealed that Amylase activity correlated positively with elongation, both in shoot length (r=0.85) and root length (r=0.83), and with survival rate (r=0.92). However, other accessions with moderate carbohydrate content may still

show AG tolerance due to epigenetic factors and high moisture content.

The molecular characterization showed the presence of the AG1 gene in all accessions in the crude DNA product. However, it failed to clearly amplify the gene from the PCR amplicons, especially in the released accessions. In contrast, the landraces showed fairly strong AG1 amplification in three out of the four accessions. The putative presence of the amplified AG1 gene in the landraces suggests their performance in anoxic germination, anaerobic germination, coleoptile length, and Carbohydrate quantification, and points to AG tolerance in the landraces than in the released accessions.

CONCLUSION

The results of this study suggest that the landraces performed better than the released accession in all the agronomic traits measured. The combined phenotypic and molecular results indicate that all the landraces *Bvofort*, *Kamrum*, *Fanjim* and *Danruwa* possess strong anaerobic germination tolerance, as supported by higher germination rates, higher survival rates, and longer coleoptiles under flooded conditions. Landraces showed superior AG1 phenotypes and higher carbohydrate status; PCR screening suggests the putative presence of the AG1 gene in all landraces, but requires confirmatory sequencing and comparison with NIL-AG1 controls to authenticate and validate the claim.

RECOMMENDATIONS

From the findings of the research work, the following recommendations were made

- ✓ The indigenous landraces should be conserved because they are a potential sources of anaerobic germination (AG) tolerance and, as such, could serve as donor accessions for introgression into the genomic DNA of other AG-intolerant cultivars.
- ✓ Farmers are advised to use our local landraces for better rice production, especially in rain-fed lowlands or other flooded areas, due to their high survivability under flooded conditions.
- ✓ Gene expression and sequencing analysis/profiling should be carried out to confirm and authenticate the presence of the AG1.

CONFLICT OF INTEREST

The authors publicly declare that there are no conflicts of interest.

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