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SSR ANALYSIS OF GENETIC CHANGES DURING ARTIFICIAL AGEING OF RICE SEEDS STORED UNDER GENE BANK MANAGEMENT

ABSTRACT

Two experiments were conducted to evaluate ageing-induced genetic changes and to establish physiological thresholds for loss of genetic integrity during ageing or storage of rice seeds. In the first experiment, seeds of 10 varieties of rice were subjected to artificial ageing in chambers conditioned to 55°C and 72±2% RH for 72 hours. In the second experiment, seeds of 4 varieties of rice stored in the NACGRAB gene bank, Nigeria in 2011 at 5± 4°C were compared with seeds of the same accessions freshly harvested in 2013. Data were collected on seed germination and seedling length to estimate the seed vigour index. Genetic changes during the ageing were evaluated by SSR markers using a Direct™ PCR kit. Genetic distance indices were computed using PAST™ software and percentage genetic integrity was estimated from the genetic distance matrices. At 72 hours of artificial ageing, seed germination percentage declined to 54.2% and vigour index 0.8 coinciding with the lowest estimate of genetic integrity of 99.5%. The decline in percentage genetic integrity during the artificial ageing indicated a systematic ageing-induced genetic alteration. SSR primer RM178 revealed variations that suggest losses of alleles in the course of ageing for 2 accessions at between 24 hours in WITA4 and 48 to 51 hours in CG14. In the gene bank storage trial, germination of seeds after one and two years of storage was above 80% and there were no significant differences among the accessions. SSR profiles for all the accessions were also similar. The result partly corroborates the artificial ageing data. This implies that seed viability benchmark of 54% is recommended for regeneration of stored rice seeds in order to maintain optimum genetic integrity during storage.

Key words: seed aging, seed storage and genetic integrity

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INTRODUCTION

Vast pool of genetic resources represents a treasure for crop improvement. Genetic resources are held in various genebank all over the world, for instance about 10,000 accessions of cereal crops' germplasm are being held at the Nigerian national gene bank (National Centre for Genetic Resources and Biotechnology) in Ibadan, as seeds in an *ex-situ* cold storage facility. The conservation of plant genetic resources is not limited to the acquisition and physical possession of the materials (i.e. collection and storage) but also the assurance that they will continue to exist in a viable condition, with their original genetic characteristics intact to breed new varieties. Seed deterioration in terms of viability and vigour has implications on erosion of variability in genetic resources especially in gene bank seed collections. Hence, maintaining the genetic integrity of seeds is one of the major challenges of *ex-situ* gene bank system. Deterioration process during ageing is inexorable in any living organism (seeds inclusive). Seed viability models had been established and implemented for predicting seed longevity of many crops gene bank management (Daniel *et al.,* 2011).

Similar to loss of genetic integrity induced by ageing, regeneration of plant genetic integrity may also induce genetic drift (Masel, 2011) leading to alterations in allelic frequency of the gene variant. Seed physiological integrity can be assessed using viability and germination assay. It is therefore logical to evaluate genetic integrity of seeds during the physiological ageing. Such data can be used to correlate physiological deterioration with genetic status of the materials maintained in the gene bank. The objectives of this research were to evaluate ageing-induced genetic changes and to establish physiological thresholds for loss of genetic integrity during ageing or storage of rice seeds.

MATERIALS AND METHODS

Two experiments were conducted for this study. In the first experiment, seeds of 10 varieties of African rice (Table 1) were subjected to artificial ageing in a chamber conditioned to 55°C and 72±2% RH for 72 hours. In the second experiment, seeds of 4 varieties of rice (Table 1) stored sealed in the NACGRAB gene bank, Nigeria in 2011 at 2±4°C were compared with seeds of the same accessions harvested in 2012. Data were collected on seed germination (%G) and seedling length. Seed vigour index was estimated as %G x seedling length. The percentage germination data were transformed using arc-sin formula. These data were subjected to Analysis of Variance (ANOVA) using a PROC GLM statement of SAS™. Genetic changes during the ageing course were evaluated by Phire Plant Direct PCR™ kits. Five highly polymorphic Simple Sequence Repeat (SSR) primers (Table 2) were sourced based on their Polymorphic Information Content (PIC) from literature (Doku *et al.*, 2013) and the sequences were prepared by Inquaba Pty., South-Africa. The SSR primers and digested

seeds were amplified with optimized PCR reactions. The PCR products was electrophoretically separated on 2% agarose gels after staining with GR-Green™ gel stain with 50 to 100 bp DNA ladder was used as molecular standard were visualized in Uvtech™ gel documentation system.

Table 1

List of the ten (10) varieties of rice used for the study

Accession	Accession name	Species	Experiment
1	TOg5681	*Oryza glaberrima*	
2	CG14	*Oryza glaberrima*	
3	IR64	*Oryza sativa*	
4	NERICA–L–34		WAS 161-B-6-3-FKR 1
5	NERICA2		WAB 450-11-1-P31-1-HB
6	NERICA8		WAB 450 1-BL1-136-HB
7	NERICA7		WAB 450 1-B-P-20-HB
8	NERICA–L–19		WAS 122-IDSA-1-WAS-6-1
9	NERICA1		WAB 450-1-B-P-38-HB
10	WITA4	*Oryza sativa*	

Table 2

List of the forward and reverse sequences of the five (5) SSR primers tested on the seed samples

	SSR Primers	Forward sequence	Reverse sequence
A	RM001	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCCTGAC
B	RM237	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC
C	RM178	TCGCGTGAAAGATAAGCGGCGC	GATCACCGTTCCCTCCGCCTGC
D	RM277	CGGTCAAATCATCATCACCTGAC	CAAGGCTTGCAAGGGAAG
E	RM005	TGCAACTTCTAGCTGCTCGA	GCATCCGATCTTGATGGG

Percentage genetic integrity was estimated from PAST™ software computed genetic linkage distances between 0 hour and each of the other hours of ageing. Average of this Percentage genetic integrity was calculated for each of the ageing hour and each rice accession according to Daniel *et al.* (2012):

$$\%Genetic\ integrity = 100 - \sum \frac{(X_1 \ldots X_n)}{n}$$

where: $X_1 \ldots X_n$= Values of genetic distances between seeds aged for 0 hours and each of the ageing hours estimated by PAST™ software; n=number of observations.

RESULTS AND DISCUSSIONS

Table 3

Effects of ageing on seed viability of ten accessions of rice seeds

Accessions	Germination over the ageing hours [%]						Std. Error
	0	3	24	48	51	72	
TOg5681	72.38	78.10	88.13	88.14	73.14	41.94	0.75
CG14	63.30	88.14	83.30	80.89	75.68	75.68	0.40
IR64	66.92	80.89	67.80	72.76	72.31	73.14	0.23
NERICA–L–34	85.72	83.18	88.14	78.10	78.10	13.28	1.15
NERICA2	88.14	85.72	70.47	75.68	83.30	80.76	0.26
NERICA8	75.56	83.13	64.76	62.47	70.60	73.14	0.32
NERICA7	80.76	85.72	83.30	78.35	83.18	71.76	0.21
NERICA–L–19	78.35	26.25	19.66	9.95	16.48	0.00	1.16
NERICA1	78.22	59.68	80.51	70.60	72.76	64.76	0.33
WITA4	59.68	47.78	40.90	65.14	46.71	48.00	0.44
Average	74.903	71.859	68.697	68.208	67.226	54.246	0.525

Table 4

Effects of ageing period on the seedling Vigour Index

Accessions	Vigour Index (VI) over the aging hours						Std.Error
	0	3	24	48	51	72	
TOg5681	4.14	4.69	3.45	4.82	3.24	1.04	0.25
CG14	4.02	3.59	5.14	3.36	3.78	1.40	0.23
IR64	2.47	2.61	2.77	2.26	2.56	1.30	0.13
NERICA–L–34	2.73	2.26	3.17	2.44	1.80	0.04	0.25
NERICA2	2.41	1.75	1.55	2.45	2.75	1.23	0.14
NERICA8	1.95	2.46	1.91	1.26	1.96	1.15	0.13
NERICA7	1.87	0.20	1.69	2.13	1.96	0.53	0.22
NERICA–L–19	3.74	0.52	0.09	0.11	0.17	0.00	0.28
NERICA1	1.23	3.45	2.27	1.72	1.67	1.13	0.29
WITA4	1.16	1.55	1.87	2.05	1.50	0.61	0.18
Average	2.572	2.308	2.391	2.26	2.139	0.843	0.21

At 72 hours of artificial ageing, the accessions on the average recorded seed germination percentage declined to 54.2% and vigor index 0.8. NERICA2 and NERICA8 recorded some variation in the seed germination percentage and vigour index values during the ageing course (Tables 3 and 4). However, the de-

cline observed is not a coincidence or a random event since it is progressive and could have continued if the ageing period is extended beyond 72 hours. The variation observed is well understandable the genotype of seeds plays a major role in the rate of deterioration. Adebisi *et al.* (2003) while working on tropical soybean concluded that such variation gives the possibilities of selecting genotypes with superior seed quality and longevity performance. Seed scientists have employed seeds viability and seedling vigor index to assess seed quality and the decline in the parameter has been associated with seed deterioration (Kehinde *et al.*, 2013). Hence, from this study, it can be said that seed deterioration has occurred and it's maximal at 72 hours of ageing.

Genetic alterations during ageing of rice seeds were analyzed using SSR markers. SSR primer RM178 revealed variations which suggests losses of alleles in the course of ageing for 2 varieties at between 24 hours in WITA4 and 48 to 51 hours in CG14 (Fig 1and 2). Average Percentage genetic integrity calculated for each of the ageing hour and each rice accession (Table 5) showed a progressive decline in the percentage genetic integrity and the minimum value (99.45) was recorded at 72 hours of artificial ageing period. IR64 recorded the highest value of 100% while NERICA1 has the lowest value of 99.46. The decline in percentage genetic integrity during the artificial ageing indicated a systematic ageing-induced genetic alteration (Sun *et al.,* 2007).

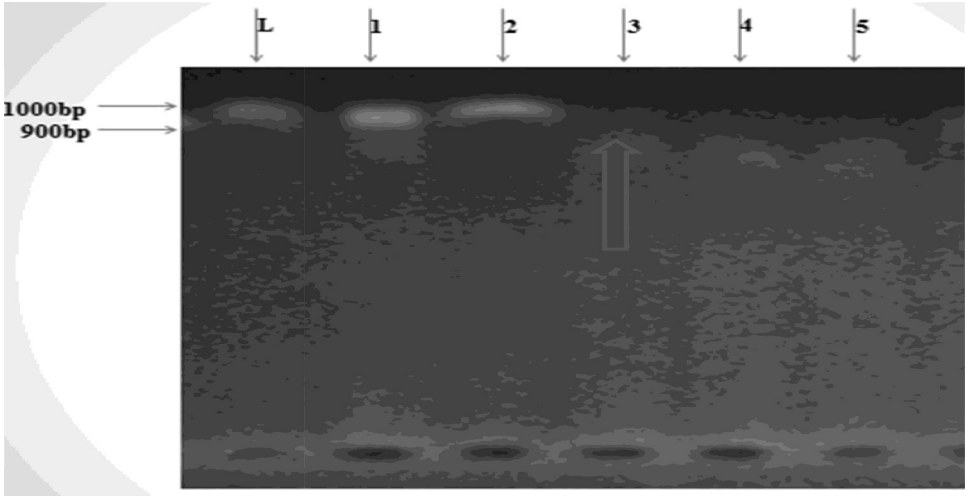

Fig. 1. SSR profiles of artificially aged seeds of CG14. (L=Ladder, lane 1= 0 hours, ….. lane 6= 72 hours)

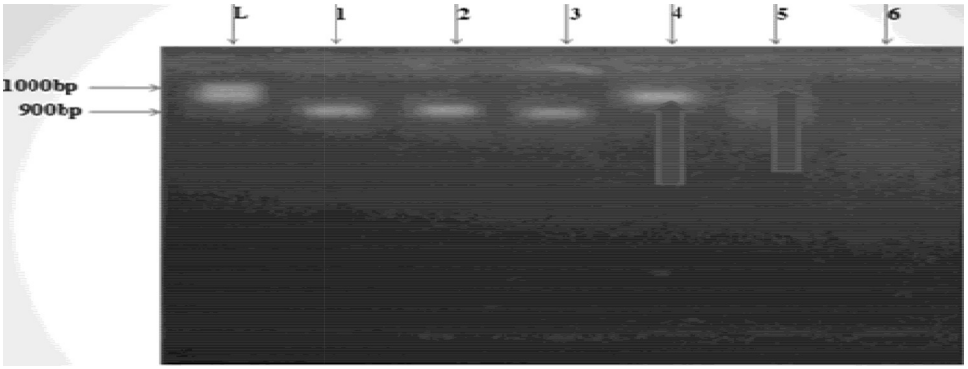

Fig. 2 SSR profiles of artificially aged seeds of WITA4. (L=Ladder, lane 1= 0 hours, ….. lane 6= 72 hours)

Table 5

Comparison of genetic distance indices (computed by PAST^{TM}) between 0 hour and other hours of ageing and the calculated percentage genetic integrity

Accession	Linkage distances between hours of ageing						$\sum X_1/n$	**100 - $\sum X_1/n$**
	0 hr	0&3hrs	0&24hrs	0&48hrs	0&51hrs	0&72hrs		
TOg5681	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00
CG14	0.00	0.00	0.00	0.00	0.32	1.73	0.41	99.59
IR64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	100.00
NERICA–L–34	0.00	0.24	0.00	0.00	0.00	1.00	0.25	99.75
NERICA2	0.00	0.41	0.41	0.41	0.41	0.00	0.33	99.67
NERICA8	0.00	0.32	0.71	0.73	0.32	0.32	0.48	99.52
NERICA7	0.00	0.00	0.00	0.32	0.00	0.32	0.13	99.87
NERICA–L–19	0.00	0.32	0.00	0.32	0.32	0.00	0.19	99.81
NERICA1	0.00	0.27	0.27	0.59	0.59	1.00	0.54	99.46
WITA4	0.00	0.24	0.27	0.27	0.27	0.59	0.33	99.67
$\sum X1/n$	0.00	0.20	0.18	0.29	0.25	0.55	0.30	99.70
100 - $\sum X1/n$	100.00	99.80	99.82	99.71	99.75	99.45	99.70	

Loss of germination capacity has being established as an indication of ageing and physiological deterioration in seeds (Demir and Mavis, 2008). Therefore, correlating physiological deterioration with the estimated genetic integrity showed (Table 6) that at 72 hours of ageing, genetic changes has occurred leading to alteration in the genetic integrity of rice. This implies that seed viability value of 54% is needed as the minimum value for regeneration of stored rice seeds in order to maintain optimum genetic integrity during storage. This study further established the increasing loss of DNA integrity as an important feature of loss of seed viability (Elder and Osborne 1993; Leprince *et al.*, 1996; Tuteja

et al., 2001). Loss of seed viability has been correlated with chromosomal aberration (Roberts, 1972) indicating a certain level of DNA damage (McDonald, 1999; Radha, 2014). Hussein *et al.* (2011) inferred that the ageing induced loss of seed vigour in sunflower was due to loss of membrane integrity, DNA degradation, impaired transcription and faulty enzyme synthesis.

Table 6

Summary of changes in seed viability, vigour index and genetic integrity during artificial ageing

Parameter	Time of artificial ageing (hours)					
	0	3	24	48	51	72
Seed viability (%)	74.9	71.9	68.7	68.2	67.2	54.2
Vigour index	2.6	2.3	2.4	2.3	2.1	0.8
Genetic integrity (%)	100.0	99.8	99.8	99.7	99.8	99.5

In the gene bank storage trial, germination of seeds after one and two years of storage was above 80% (Fig. 3) and there were no significant differences among the accessions. SSR profiles for all the accessions were also similar (Fig. 4).

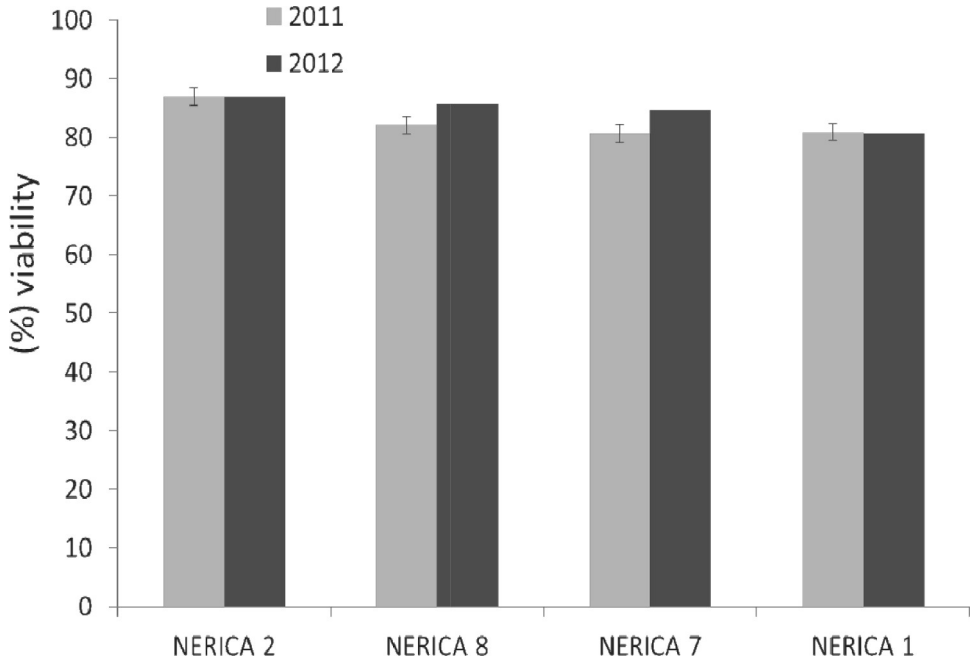

Fig. 3. Seed viability of four accessions of rice after 1 year (2012 seed lot) and 2 years (2011 seed lot) of storage in the gene bank

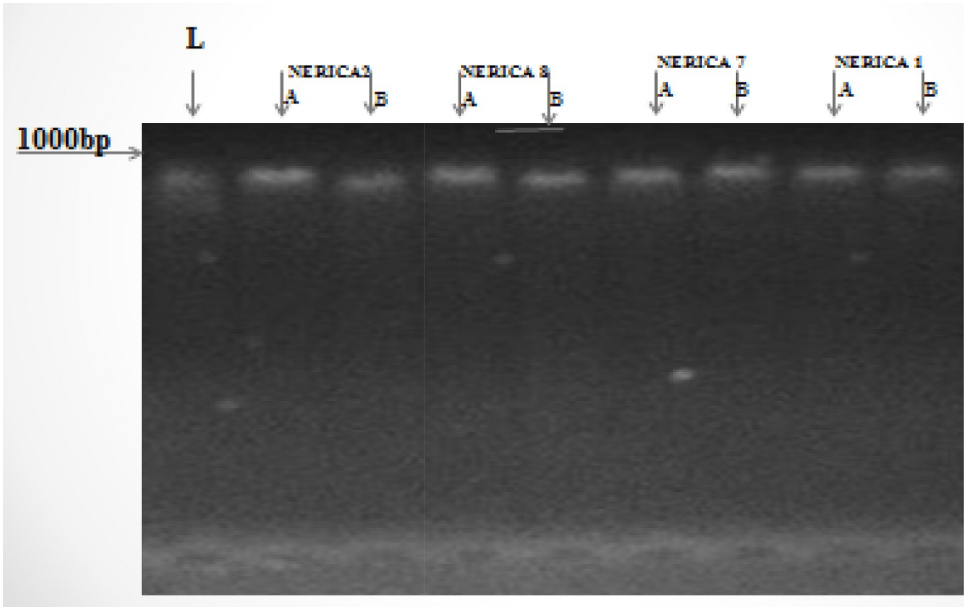

Fig. 4. SSR profile of rice seeds in gene bank storage for 2 years. A = 2011, B = 2012 lots

CONCLUSION

Seed germination value of 54% coincided with the lowest estimate of percentage genetic integrity. This implies that seed viability benchmark of 54% is recommended for regeneration of stored rice seeds in order to maintain optimum genetic integrity during storage. Also, from this study, 2 years of storage at gene bank conditions maintained physiological quality of the seeds above the benchmark germination values for estimating genetic integrity of rice seeds after two years of storage. The result partly corroborates the artificial ageing data,. However, more data on ageing seeds at longer gene bank storage periods are needed to validate the estimated physiological threshold for managing rice seeds in gene bank collections and to determine correlates of time during gene bank storage for genetic alterations observed in artificial ageing. Phenotypic analysis for the expressions of DNA alterations during ageing of rice seeds and/or gene bank storage should be incorporated in further studies. And also, the use of estimates of average genetic distances can be applied to evaluate genetic changes during seed storage.

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